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Supplemental Information

Function of Arl4aa in the Initiation of Hematopoiesis in Zebrafish by Maintaining Golgi Complex Integrity in Hemogenic Endothelium

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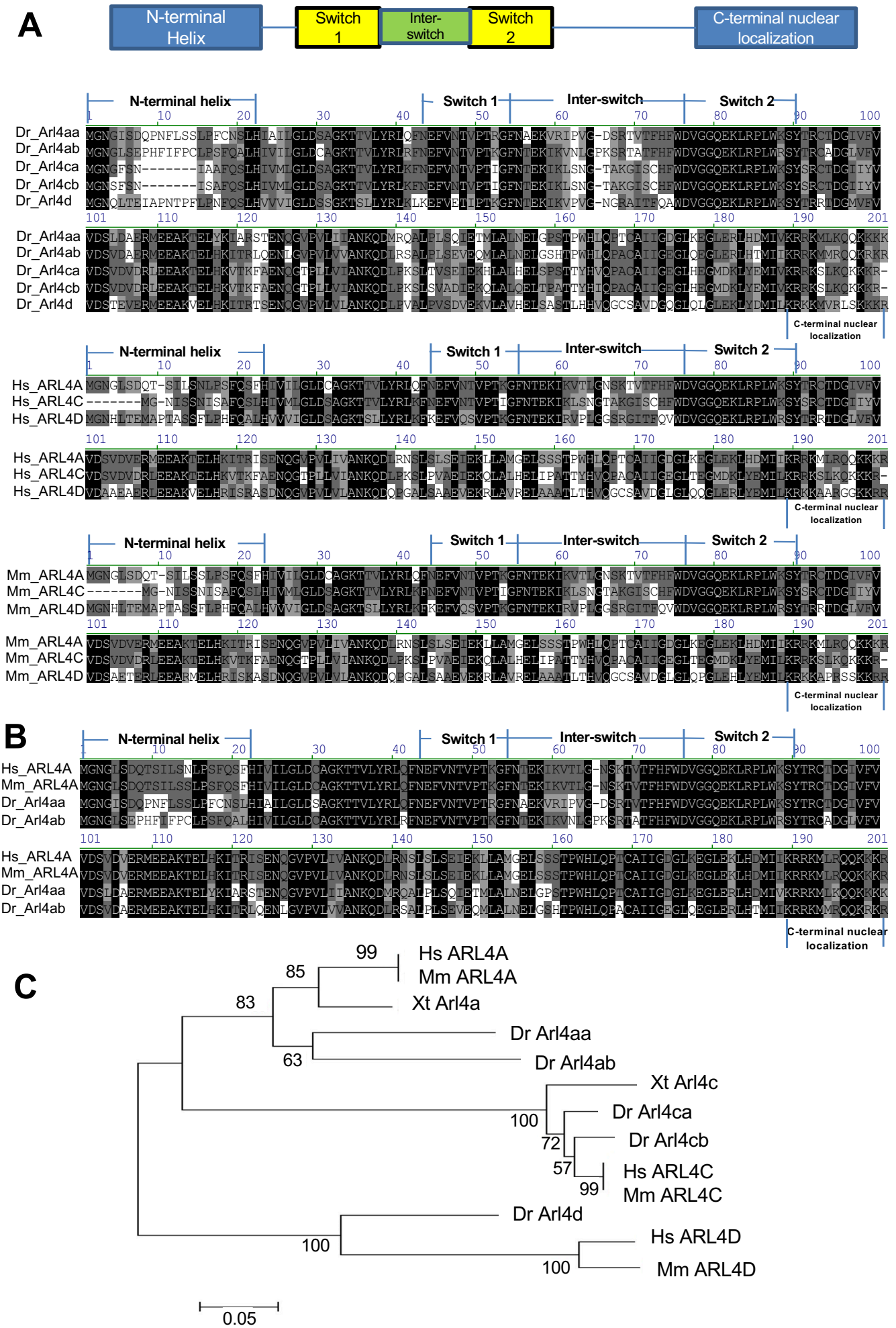

A
N-terminal Helix
Switch 1
Inter-switch
Switch 2
C-terminal nuclear localization
N-terminal helix
Switch 1
Inter-switch
Switch 2
Dr_Arl4aa
Dr_Arl4ab
Dr_Arl4ca
Dr_Arl4cb
Dr_Arl4d
Hs_ARL4A
Hs_ARL4C
Hs_ARL4D
Mm_ARL4A
Mm_ARL4C
Mm_ARL4D
C-terminal nuclear localization
B
C
Hs ARL4A
Mm ARL4A
Xt Arl4a
Dr Arl4aa
Dr Arl4ab
Xt Arl4c
Dr Arl4ca
Dr Arl4cb
Hs ARL4C
Mm ARL4C
Dr Arl4d
Hs ARL4D
Mm ARL4D
99
85
83
63
100
72
57
99
100
100
0.05

Figure S1. Amino acid sequence alignment and Phylogenetic analysis of ARL4. (A) Amino acid sequence alignment of ARL4 family members in zebrafish (upper), human (middle) and mouse (lower). (B) Amino acid sequence alignment of human, mouse ARL4A as well as zebrafish Arl4aa and Arl4ab. (C) Phylogenetic analysis of ARL4. Values at each inner node represented bootstrap values that were the percentages of replicates that reconstruct the selected node. Dr: Danio rerio; Hs: Homo sapiens; Mm: Mus musculus; Xt: Xenopus tropicalis.

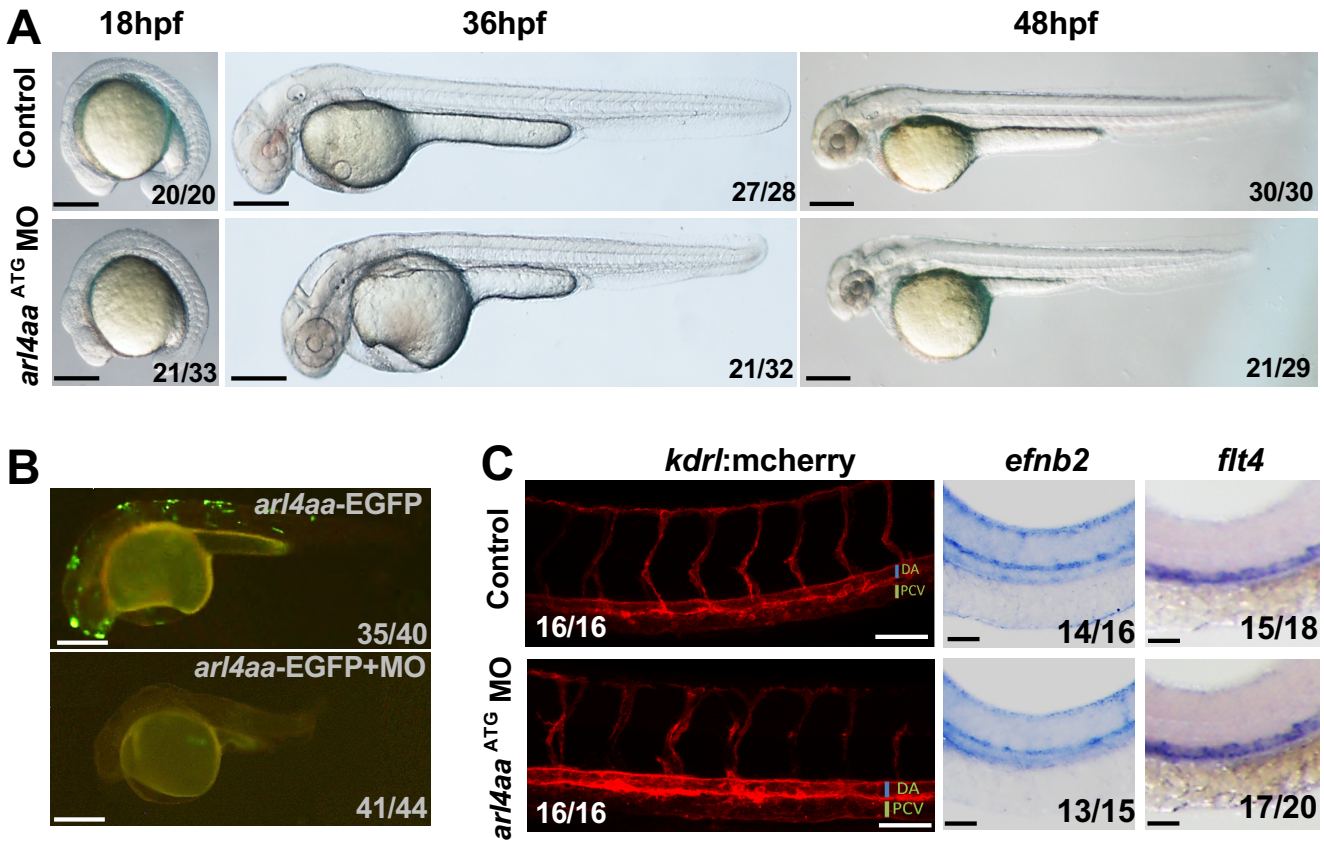


Figure S2. *arl4aa* knockdown with morpholino had no major effects on morphological and vascular development. Related to Figure 2. (A) Morphology of *arl4aa* morphant (MO) at 18, 36 and 48 hpf. (B) Mosaic fluorescent pattern induced by *arl4aa* 5'UTR-GFP fusion plasmid was abolished by *arl4aa* MO. The embryos were examined at 24 hpf. Scale bars (A and B): 250 μm. (C) Vasculogenesis was not affected by *arl4aa* MO as shown by confocal imaging of Tg(*kdrl*:mcherry) at 36 hpf and whole-mount in-situ hybridization (WISH) of *efnb2a* (arterial marker) and *flt4* (venous markers) at 30 hpf compared with control. DA: dorsal aorta, PCV: posterior cardinal vein. Scale bars (C): 100 μm. For each panel, the numbers of representative embryos over the total numbers of embryos observed were shown.

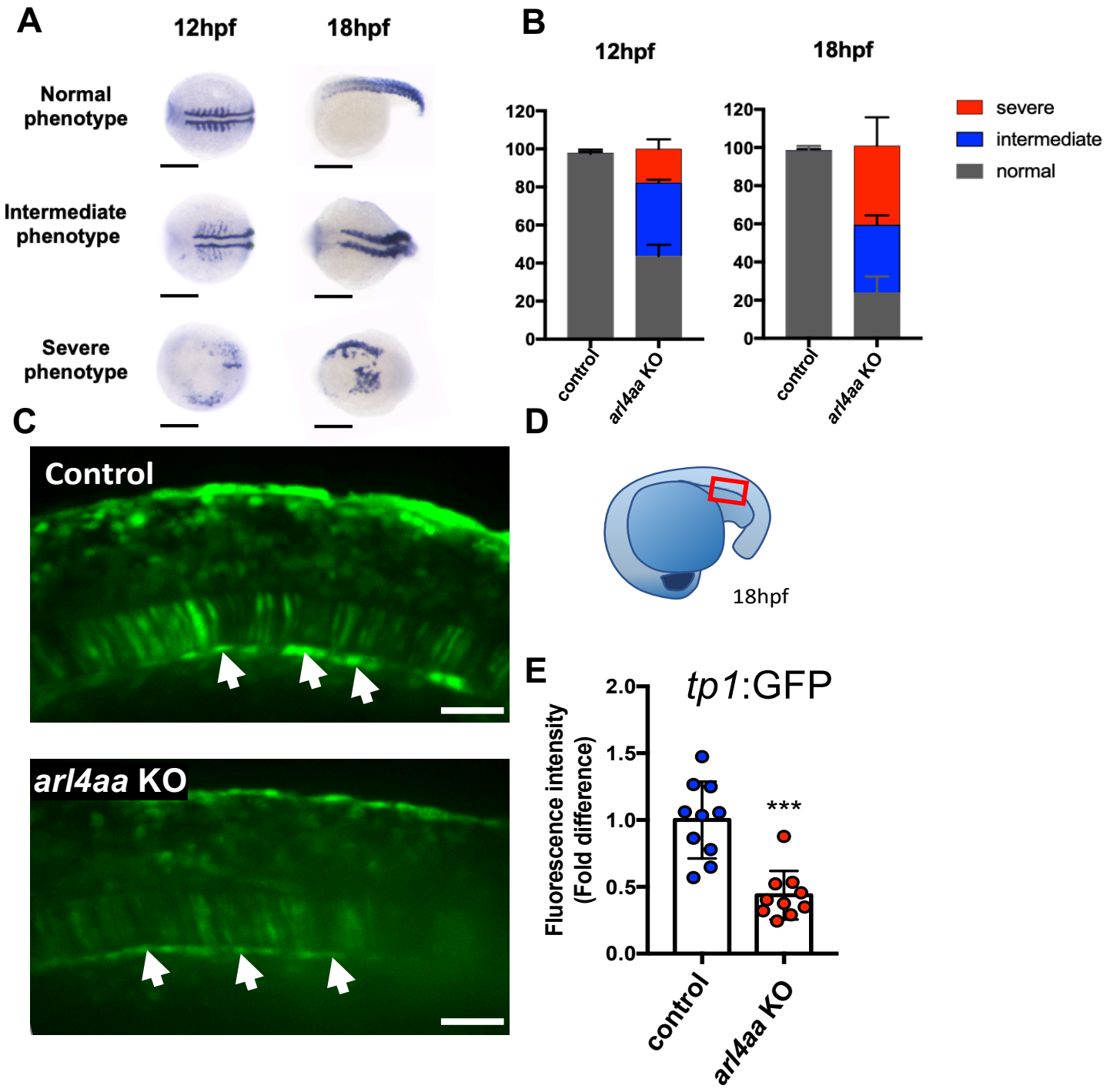
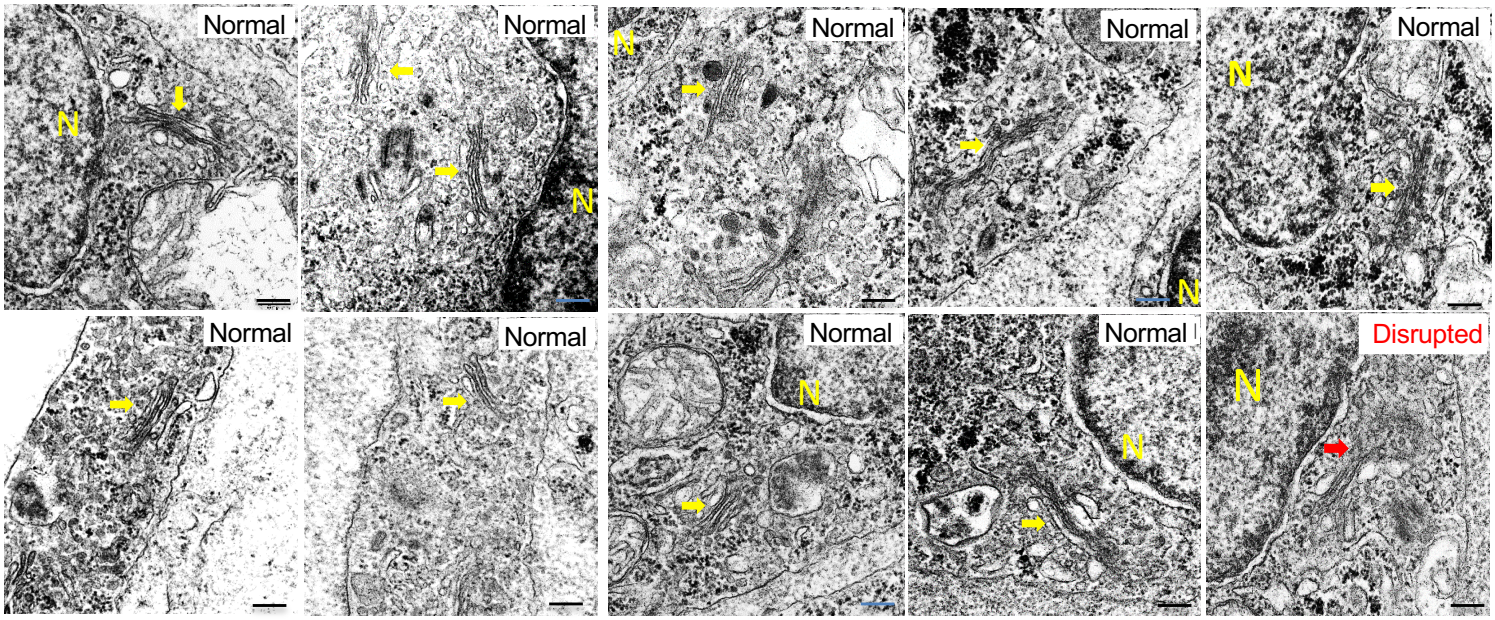
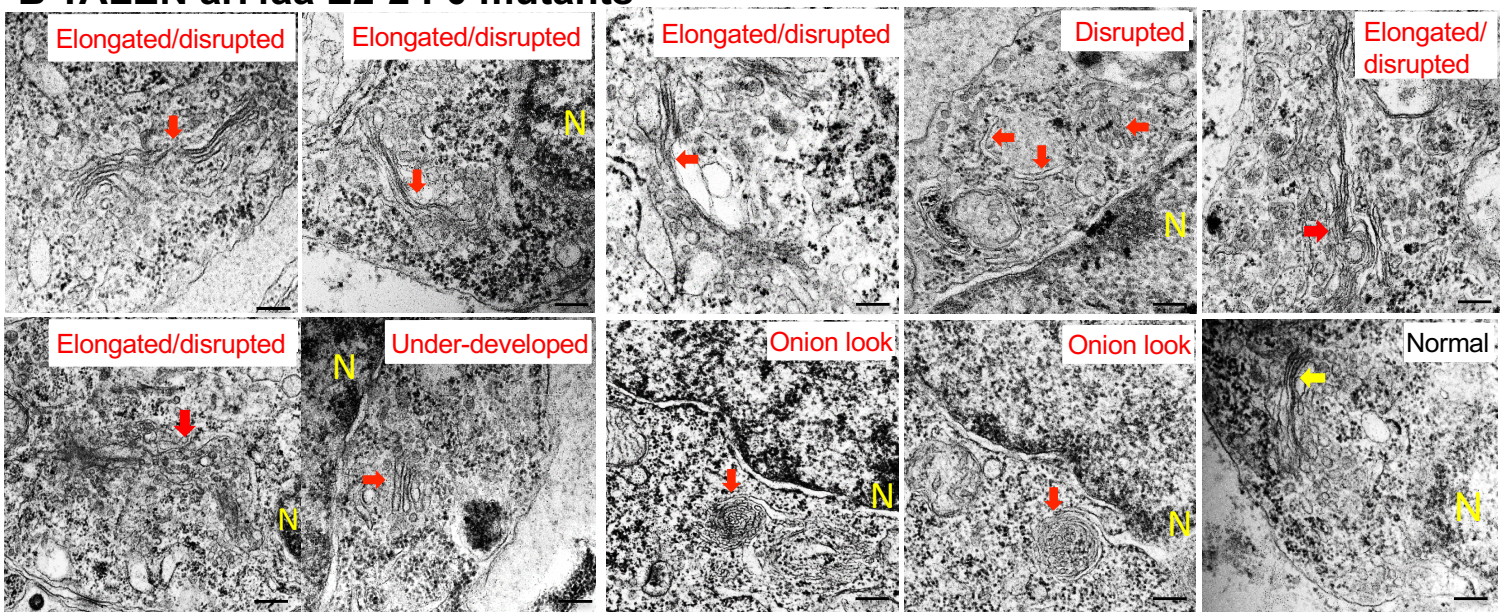

A
12hpf
18hpf
Normal phenotype
Intermediate phenotype
Severe phenotype
B
12hpf
18hpf
120
100
80
60
40
20
0
control
arl4aa KO
severe
intermediate
normal
C
Control
arl4aa KO
D
18hpf
E
tp1:GFP
Fluorescence intensity (Fold difference)
2.0
1.5
1.0
0.5
0.0
***
control
arl4aa KO

Figure S3. *arl4aa* KO affected somite development as indicated by *myoD* expression at 12hpf and 18hpf, and reduced notch signaling at 18hpf. (A) representative WISH images showing the effect of *arl4aa* KO on somites development and *myoD* expression in 12hpf and 18hpf embryos. Scale bars (A): 250 μm. (B) The proportion of embryos showing phenotype as indicated at 12hpf and 18hpf respectively. Error bars showing mean ± SEM. Data are representative of three independent experiments. (C) Representative confocal images showing dorsal aorta (DA) region of Tg(*tp1*:GFP) notch signaling reporter line. While arrows indicated cells at the floor bed of DA with active notch signaling. Scale bars (C): 100 μm. (D) cartoon showing the Tg(*tp1*:GFP) line at 18hpf with the red box indicating the DA region to be captured with confocal imaging. (E) Fold difference of the fluorescence intensity (FI) of the cells at the floor bed of DA with active notch signaling. Each dot represent a single Tg(*tp1*:GFP) embryo at 18hpf. (Error bars showing mean ± SEM; ***P<0.001). We selected only the DA (as indicated by the white arrows in panel C) for FI measurement and the latter was normalized with respect to the area selected for analysis in that embryo(the DA area included the same number somites both in the control and *arl4aa* KO group). To circumvent potential difference in focal depths between images due to the lateral curvature of embryos, we evaluated only DA area with clear focus.

A Control



B TALEN arl4aa E2-2 F0 mutants



C

Golgi cisternae morphology in VDA	TALEN *arl4aa* E2-2 F0 mutants	Control
Elongated/disrupted	**6/10**	**1/10**
Under-developed	**1/10**	**0**
Onion look	**2/10**	**0**
Normal	**1/10**	**9/10**
Chi-square test: P=0.0003		

Figure S4. Electron micrographs (EM) of Golgi morphology in ventral wall of dorsal aorta (VDA) in control (A) and F0 *arl4aa* mutant embryos injected with TALEN *arl4aa* E2-2 (B) at 30 hpf. Related to Figure 5. (C) Summary of Golgi morphology in Control and F0 mutant embryos. Number of Golgi complex abnormalities and normalities between the mutant and wildtype embryos was statistically evaluated by Chi-square test, p=0.0003, n=10 per group. N: Nucleus. Scale bars: 200 nm. Yellow arrows indicated normal Golgi morphology. Red arrows indicated abnormal Golgi morphology.

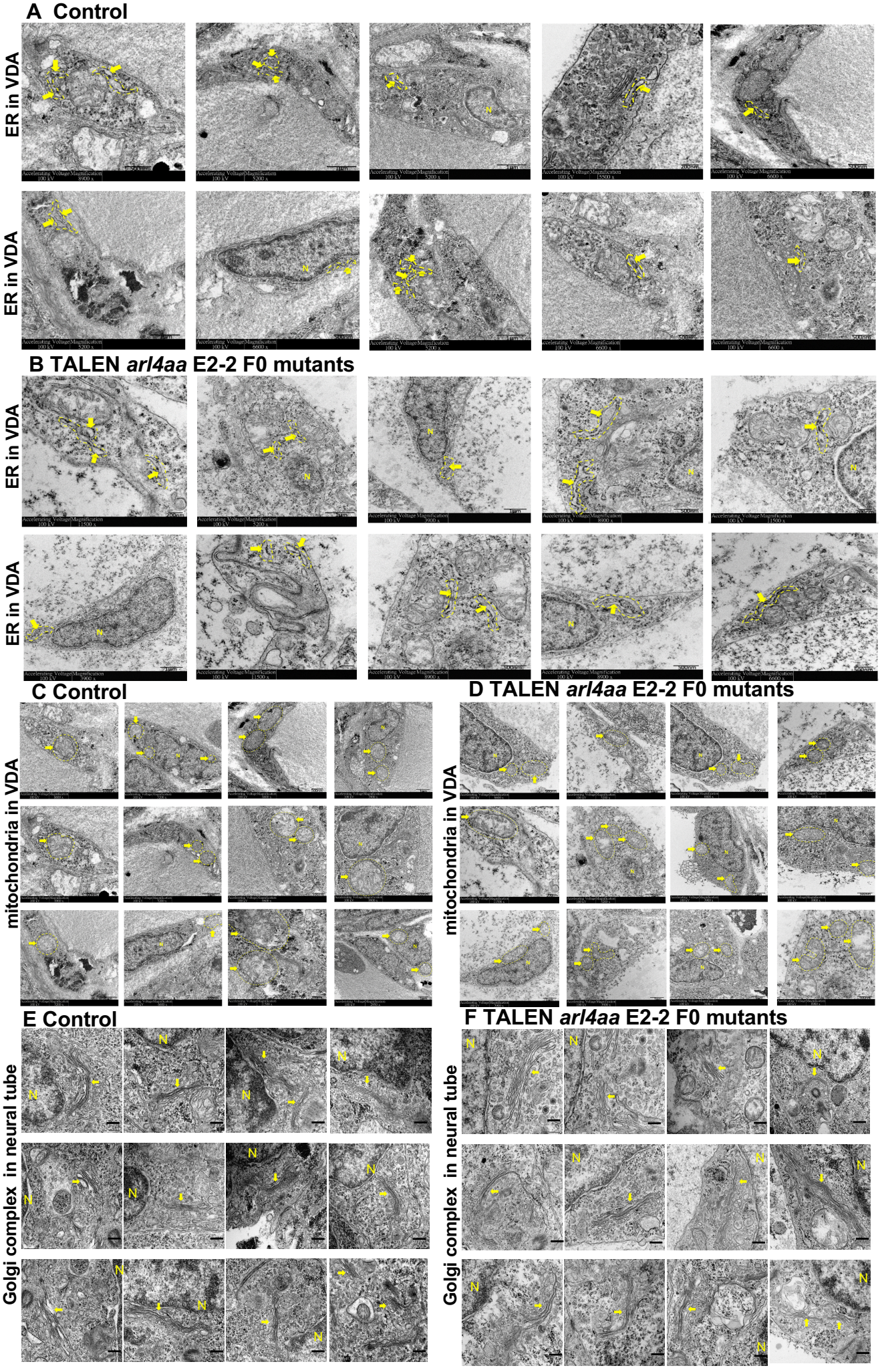

A Control
ER in VDA
ER in VDA
B TALEN arl4aa E2-2 F0 mutants
ER in VDA
ER in VDA
C Control
mitochondria in VDA
D TALEN arl4aa E2-2 F0 mutants
mitochondria in VDA
E Control
Golgi complex in neural tube
F TALEN arl4aa E2-2 F0 mutants
Golgi complex in neural tube

Fig S5. Electron micrographs (EM) of endoplasmic reticulum (ER) and mitochondria in cells of VDA and EM of neural tube in both wild-type and F0 *arl4aa* mutant embryos injected with TALEN *arl4aa* E2-2 at 30hpf. Related to Figure 5. (A and B) ER in cells of VDA in both wild-type and F0 *arl4aa* mutant embryos injected with TALEN *arl4aa* E2-2. Yellow arrows indicated ER and the morphology of ER is displayed in yellow dotted line. Scale bars are indicated in each micrographs. (C and D) mitochondria in cells of VDA in both wild-type and F0 *arl4aa* mutant embryos injected with TALEN *arl4aa* E2-2. Yellow arrows indicated mitochondria and the morphology of mitochondria is displayed in yellow dotted line. Scale bars are indicated in each photo. (E and F) Golgi in cells of neural tube in both wild-type and F0 *arl4aa* mutant embryos injected with TALEN *arl4aa* E2-2. Scale bars: 200 nm. Yellow arrows indicated normal Golgi morphology. N: Nucleus.

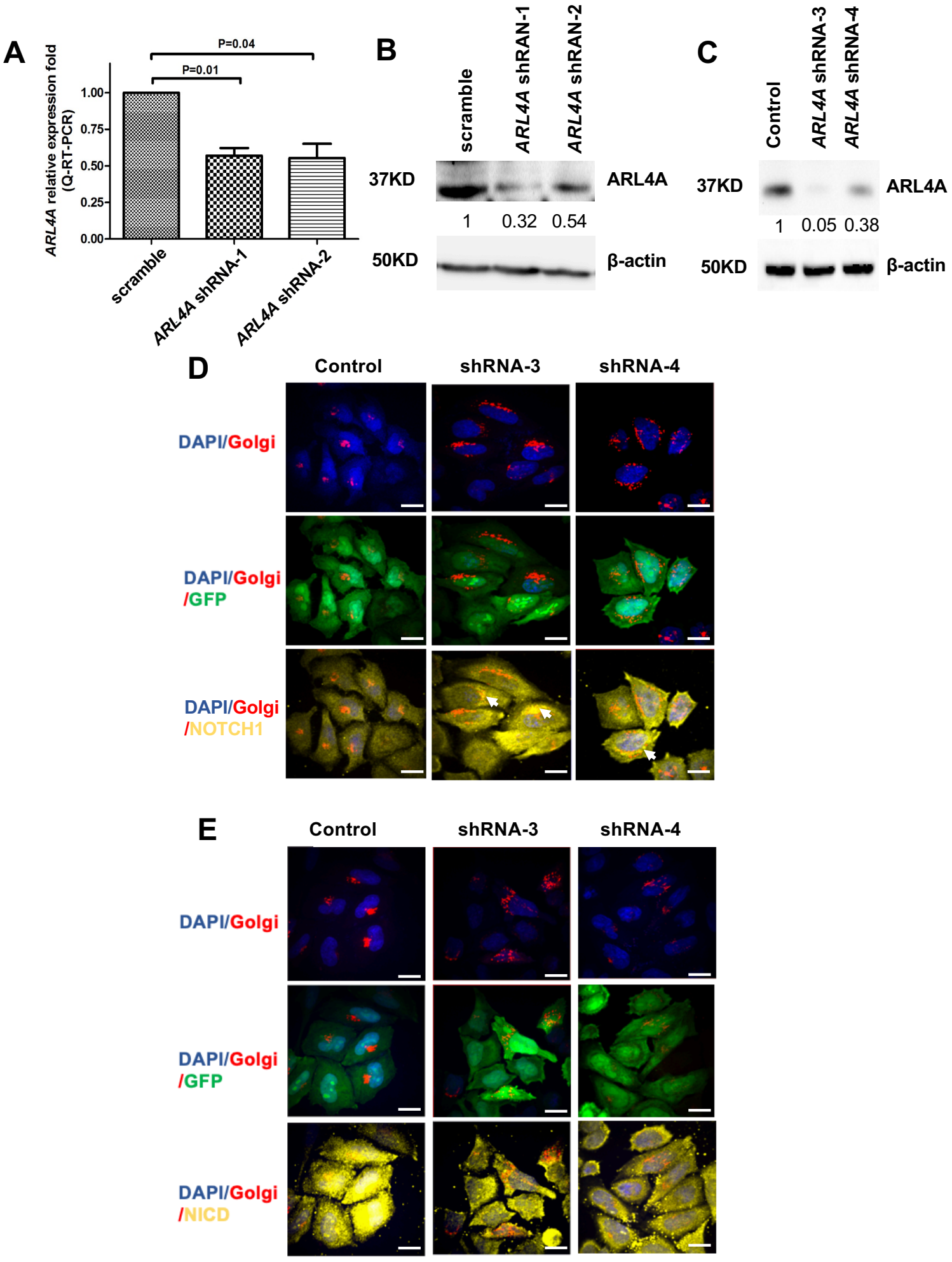

A
ARL4A relative expression fold (Q-RT-PCR)
P=0.04
P=0.01
1.00
0.75
0.50
0.25
0.00
scramble
ARL4A shRNA-1
ARL4A shRNA-2
B
scramble
ARL4A shRAN-1
ARL4A shRAN-2
37KD
ARL4A
1 0.32 0.54
50KD
β-actin
C
Control
ARL4A shRNA-3
ARL4A shRNA-4
37KD
ARL4A
1 0.05 0.38
50KD
β-actin
D
Control
shRNA-3
shRNA-4
DAPI/Golgi
DAPI/Golgi /GFP
DAPI/Golgi /NOTCH1
E
Control
shRNA-3
shRNA-4
DAPI/Golgi
DAPI/Golgi /GFP
DAPI/Golgi /NICD

Figure S6. Knockdown of *ARL4A* in HeLa cells. Related to Figure 5. (A) *ARL4A* mRNA expression by quantitative RT-PCR at 48 hours after transfection in scramble sequence shRNA, *ARL4A* shRNA-1 and *ARL4A* shRNA-2 transfected HeLa cells. (B) ARL4A protein expression at 72 hours after transfection. (C) ARL4A protein expression after stable transfection with empty vector control, *ARL4A* shRNA-3 and *ARL4A* shRNA-4. (D and E) *ARL4A* knock-down induced an increase in NOTCH1 (D) and a decrease in NICD (E) in human Hela cells. Hela cells transfected with empty vector control, *ARL4A* shRNA-3 and *ARL4A* shRNA-4, successfully transfected cells were labeled with GFP. To visualize Golgi, cells were further transfected with RFP fused to human Golgi resident enzyme (N-acetylgalactosaminyltransferase). NOTCH1 and NICD were stained with anti-NOTCH1 antibody or anti-NICD antibody respectively followed by Alexa Fluor 647-conjugated secondary antibody and the nuclear outline was shown by DAPI staining. Arrows: perinuclear zone. Scale bars: 20 μm.

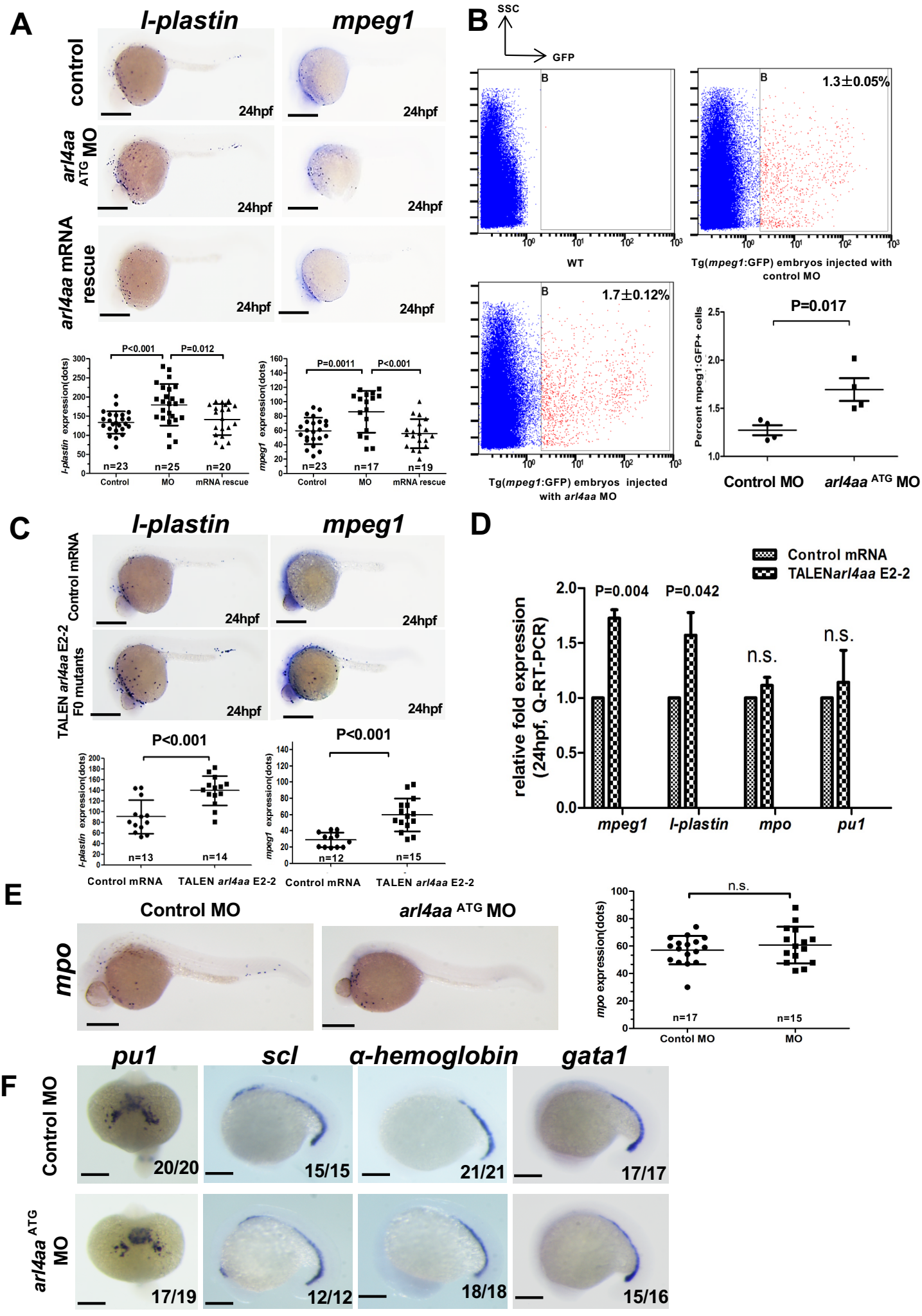

A
l-plastin
mpeg1
control
arl4aa ATG MO
arl4aa mRNA rescue
24hpf
P<0.001
P=0.012
P=0.0011
P<0.001
l-plastin expression(dots)
mpeg1 expression(dots)
n=23
n=25
n=20
n=23
n=17
n=19
Control
MO
mRNA rescue
B
SSC
GFP
WT
1.3±0.05%
Tg(mpeg1:GFP) embryos injected with control MO
1.7±0.12%
Tg(mpeg1:GFP) embryos injected with arl4aa MO
P=0.017
Percent mpeg1:GFP+ cells
Control MO
arl4aa ATG MO
C
l-plastin
mpeg1
Control mRNA
TALEN arl4aa E2-2 F0 mutants
24hpf
P<0.001
P<0.001
n=13
n=14
n=12
n=15
Control mRNA
TALEN arl4aa E2-2
D
Control mRNA
TALENarl4aa E2-2
relative fold expression (24hpf, Q-RT-PCR)
P=0.004
P=0.042
n.s.
n.s.
mpeg1
l-plastin
mpo
pu1
E
Control MO
arl4aa ATG MO
mpo
n.s.
mpo expression(dots)
n=17
n=15
Contol MO
MO
F
pu1
scl
α-hemoglobin
gata1
Control MO
arl4aa ATG MO
20/20
15/15
21/21
17/17
17/19
12/12
18/18
15/16

Figure S7. *arl4aa* knockdown and knockout induced increase in primitive macrophage development. (A, upper) Whole-mount in-situ hybridization (WISH) of *l-plastin* and *mpeg1* in control, *arl4aa* morpholino (MO) injected as well as *arl4aa* MO and *arl4aa* mRNA co-injected groups at 24 hpf. (A, lower) Statistical evaluation of *l-plastin* and *mpeg1* expression in the three groups. Data are shown as means ± SEM, P values and n are indicated in the panel A. (B) Flow cytometric analyses of dissociated Tg (*mpeg1*:GFP) embryos in control and *arl4aa* MO injected embryos at 24 hpf. Data are shown as means ± SEM (n=4, P=0.017). (C) WISH of *mpeg1* and *l-plastin* expression in control (left arm TALEN E2-2L) and TALEN *arl4aa* E2-2 injected embryos at 24 hpf. Data are shown as means ± SEM, P values and n are indicated in the panel C. (D) Quantitative RT-PCR of *mpeg1*, *l-plastin*, *mpo* and *pu.1* in control and TALEN *arl4aa* E2-2 injected embryos. Data are shown as means ± SEM with three independent experiments. (E) Whole-mount in-situ hybridization (WISH) of *mpo* in embryos injected with control and *arl4aa* MO. Right: Statistical evaluation of *mpo* expressing cells. Data are shown as means ± SEM , n was indicated in the panel E. There was no significant difference between the two groups. (F) WISH of *pu1*, *scl*, α-hemoglobin and *gata1*. There was no difference between the two groups in these gene expression. Scale bars: 250 μm.

Table S1. List of morpholinos and primers in this study. Relative to Main-text and Supplemental Experimental Procedures.

Name	Oligonucleotide Sequences
Morpholinos	
arl4aa MO	TAT CCC GTT CCC CAT TTC CTT CAG C
Control standard MO	CCT CTT ACC TCA GTT ACA ATT TAT A
Primers for the cloning of zebrafish *arl4aa* gene in riboprobe synthesis	
arl4aa-probe-F	GCA CGC AGA GCA ATG AAT GAC
arl4aa-probe-R	CAG CTA AAG TGC TTG CTG GCT C
Primers for the cloning of zebrafish *arl4aa* into pEGFP-N3 for testing *arl4aa*MO	
EcoRI-*arl4aa*-F	GCA TGA ATT CCA CGC AGA GCA ATG AA
BamHI-*arl4aa*-R	AGT CGG ATC CTG CAG AGT CCA GAC CC
Primers for the zebrafish *arl4aa* rescue experiment	
arl4aa-rescue-F	GCT GAA GGA AAT GGG AAA TGG AAT CTC A
arl4aa-rescue-R	CTA TTT TTT CTT TTT TTG CTG CTT GAG
Primers for zebrafish *arl4aa* TALEN genotyping	
*arl4aa*E2-talen-F	CCT GGA TAT GCT ATG CTA T
*arl4aa*E2-talen-R	CAC AGG CAC TCC TTG ATT
arl4aa-E1F1	CTC GGT CGG TGA GCA CAA G
arl4aa-E1R1	TGC TGA CGC AGC TTC TAT GG
arl4aa-E2F1	CCC TCT ACA CCG TGG CAT TT
arl4aa-E2R1	TCT GGC TAG AGT ACC CCT GA
Q-PCR Primers to detect zebrafish *arl4aa* knockout efficiency	
arl4aa-intron-GQF	GGA GCT ACT GTC GTA ACG AT
arl4aa-intron-GQR	AAC TAA GCC CAA AGT TCA GC
arl4ab intron-GQF	GGA GCT CAA AAT GAT AAC GTC TG
arl4ab intron-GQR	GAG ACC AGG TGT CGC CGC TG
Primers for zebrafish Q-RT-PCR	
β-actin-QF	TTC CTT CCT GGG TAT GGA ATC
β-actin-QR	GCA CTG TGT TGG CAT ACA GG
pu1-QF	GGG CAG TTT TAA CCA AAG ATC A
pu1-QR	CCC AAG AGT GAT CGT TCT GAC
l-plastin-QF	GAA GCT CTG ATC GCT CTG CT
l-plastin-QR	GTT GTT GAT TTT GGG GCA TC
mpo-QF	GGG GCA GAA GAA GAA AGT CC
mpo-QR	CCC TTG CTA AAC TCT CAT CTC G
mpeg1-QF	CCC ACC AAG TGA AAG AGG
mpeg1-QR	GTG TTT GAT TGT TTT CAA TGG
c-myb-QF	TTT CTA CCG AAT CGA ACA GAT G
c-myb-QR	CAA TCA CCC GTT GGT CTT CT
runx1-QF	CGT CTT CAC AAA CCC TCC TC
runx1-QR	TAC TGC TTC ATC CGG CTT CT
notch1-QF	GAA TGC ATC TTT TCT TCG TG

notch1-QR	CAG ACA CTT GCA TTC TCC TC
notch3-QF	AAT GCA CAG GAT AAC ACA GG
notch3-QR	GCT TCA ACG TTA TTG ACT GC
hey1-QF	GAC CGT AGA TCA TTT AAA GAT GC
hey1-QR	GCT ATT GAG ATG TGA AAC CAG AC
hey2-QF	CAA GTT GGA GAA AGC GGA AAT
hey2-QR	TGA GAA ACC AGA CGG ACA CG
hey12-QF	TTC ACT TCA GCG ATA AGG AA
hey12-QR	GGA TCG TGA CTG TGG AAT T
her1-QF	TGG AAT TGG CTG TTG AGT ATA TTA GG
her1-QR	CCT TGG ACG AAG CTG TCT CG
her2-QF	CAA TGG CAC CAA CTG TCT
her2-QR	CTT GAA TAT CCG TCA GCA TAG
her5-QF	AGC CTC TCA TGG AGA AAA GGA GG
her5-QR	GTA GCT CTG ACG TTT GCA TGG TG
her6-QF	CGT TAA TCT TGG ATG CTC TG
her6-QR	CTT CAC ATG TGG ACA GGA AC
her7-QF	TGA AAC TTC TGC TCC TGC AAG G
her7-QR	CAC AGC ATG GAT GTG ACC GAG
her9-QF	CAG CCA CGG ACG GAC AGT TT
her9-QR	AAC GCC CGA GAA GGA GGT CA
gata2a-QF	ACT ACT CCA ACT CTC GGG CTA GAG
gata2a-QR	AAC TGC TGC TGC ACG GAT AAG
gata2b-QF	GGT CTG GGA CAT CAT AGT TCG
gata2b-QR	CAC ATT GAG CCA CCC GTA A
Genotyping of Tg(*hsp70l*:GAL4) and Tg(*UAS*:NICD-myc)	
Tg(hsp70l:GAL4)	
kca4-F	CGC TAC TCT CCC AAA ACC AAA AGG
kca4-R	TCT CTT CCG ATG ATG ATG TCG CAC
Tg(UAS: NICD-myc)	
kca3-F	CAT CGC GTC TCA GCC TCA C
kca3-R	CGG AAT CGT TTA TTG GTG TCG
Primers for human *ARL4A* Q-RT-PCR	
ARL4A-QF	CCA GCA CAG TCC AAA CCC
ARL4A-QR	GTA GAA ACG CCT GCC AAT G

Table S2. List of targeting sequence of TALEN pairs used in this study. Related to Main-text Experimental Procedures and Figure 3.

Name	Targeting site	targeting sequence	RE site
TALEN E1	Exon1	GAATGACGATTATAAACCAGGCTTTAACCGAGCATGACGTCTG	BstNI
TALEN E2	Exon2	GGATAGTTGTGAATTGTCATTGTTATGTGCCTAGAGTTGGTTATAG	MslI
TALEN E2-2	Exon2 translation start codon	GCTGAAGGAAATGGGGAACGGGATATCAGATCAACCCAACTTTCT	EcoRV

Supplemental Experimental Procedures

Human *ARL4A* shRNA construct, transfection in HeLa cell line and immunofluorescence staining

The following shRNA sequences were used: scramble shRNA: CCTAAGGTTAAGTCGCCCTCG[S(Lin et al., 2011)]; *ARL4A* shRNA-1: CAGTCTTTCCACATTG-TTATT[S(Lin et al., 2011)]; *ARL4A* shRNA-2: ACAAGATTTGAGGAACTCATT[S(Lin et al., 2011)]; *ARL4A shRNA-3:* CCGGCAGTCTTTCCACATTGTTATTCTCGAGAATAACAATGTGGAAAGACTGTTTTTG; *ARL4A* shRNA-4: CCGGACAAGATTTGAGGAACTCATTCTCGAGAATGAGTTCCTCAAATCTTGTTTTTTG. shRNA-1 and -2 were expressed in the pLVTHM vector (Addgene，Watertown, Massachusetts, USA), shRNA-3 and -4 were expressed in pLKO.1-puro-CMV-TurboGFP vector (Addgene). HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, in a humidified incubator with 5% CO_2 at 37˚C. For *ARL4A* shRNA-1 and -2, transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen). Cells were harvested 48 hours after transfection for immunofluorescence staining and Q-RT-PCR analysis. Immunofluorescence staining of Golgi complex has been described.[S1] Briefly, cells grown on coverslips were fixed in 4% formaldehyde for 15 minutes at room temperature, washed with PBS, incubated in blocking buffer (5% Lamb serum in 0.3% Triton X-100 in PBS) for 30 minutes at room temperature followed by rabbit anti-Giantin (ab24586, Abcam) at a dilution of 1:2000 in antibody buffer (1% BSA in 0.3% Triton X-100 in PBS) for 1 hour. Thereafter, the cells were incubated with goat anti-rabbit IgG Alexa Fluor 594-conjugated (A11037, Life Technologies) (1:500) and DAPI (0.2μg/mL) (abcam, ab104139) for 1 hour. After washing with PBS, the cells were mounted and examined by confocal LSM710 microscope (Carl Zeiss). Primers of *ARL4A* used in quantitative RT-PCR were listed in Table S1. Cells were harvested 72 hours after transfection for Western Blot. Anti-ARL4(B-10):sc-398352 (1:200, Santa Cruz Biotechnologies) and HRP-linked ECL antibodies (sheep anti-mouse-HRP antibody [1:5000, NA931V, Amersham Biosciences]) were used as primary and secondary antibodies. For *ARL4A* shRNA-3 and -4, stable transduction was performed using Lipofectamine 2000 reagent (Invitrogen). Successfully transduced cells were selected with 1μg/ml puromycin supplemented medium and is marked by GFP. To label Golgi, CellLight Golgi-RFP, BacMam (Invitrogen, Waltham) was added to cell medium and incubated overnight. 4% paraformaldehyde fixed cells were washed with PBS and incubated in blocking buffer (5% Lamb serum in 0.3% Triton X-100 in PBS) followed by anti-Notch1 (abcam, ab52627) or anti-NICD (abcam, ab8925) in antibody diluent (abcam, ab64211). Cells were incubated with Goat anti-rabbit Alexa Fluor 647 (Thermofisher, A-21245) and mounted in Fluoroshield Mounting Medium with DAPI (abcam, ab104139) and examined by UltraView VOX Spinning Disc Confocal (PerkinElmer，Massachusetts, USA).

Amino acid sequence alignment and construct of phylogenetic tree

Amino acid sequence of arl4 family members in human and zebrafish species were aligned using Align X software (a component of Vector Invitrogen NTI advance11.0 software, Invitrogen Corporation). The phylogenetic tree was built using the bootstrap neighbor-joining method of the MEGA6.0 software, the bootstrap was set to 2000 replicates.

Supplemental references:

S1. Lin, Y.C., Chiang, T.C., Liu, Y.T., Tsai, Y.T., Jang, L.T., and Lee, F.J. (2011). ARL4A acts with GCC185 to modulate Golgi complex organization. Journal of cell science *124*, 4014-4026.