1 **Supplementary Information for**

2 Hyperandrogenism in POMCa-deficieny zebrafish enhances somatic 3 growth without increasing adiposity

4 **Running title:** Hyperandrogenism's roles in POMCa deficient zebrafish

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Supplementary Materials and methods

Experimental animals

The AB strain of zebrafish adopted in this study were raised and bred at 28.5°C with a 14 hour light and 10 hour dark cycle. The developmental stages were determined according to days postfertilization (dpf) or morphological features as described previously (Kimmel et al., 1995). The transgenic fish of Tg (-1.0*pomca*:GFP) (CZ31) and Tg (-1.2ins:EGFP) (CZ15) and the *cyp17a1* mutant fish (ihb144) were obtained from the China zebrafish resource center (CZRC). The procedures of this research were approved by the Animal Research and Ethics Committee of the Institute of Hydrobiology of the Chinese Academy of Sciences (Approval ID: IHB 2013724).

Generation of pomca mutant lines via TALENs.

The sequence-specific TALENs effector pairs targeting the zebrafish *pomca* gene (NC_007128.6, NCBI) were designed according to a previous report (Cermak et al., 2011) and assembled using the Golden Gate TALEN kit (Addgene, 1000000024). For depletion of all of peptides derived from the POMCA preprotein, left arms and right arms of TALENs were designed in the region of exon 2 of *pomca*. Two independent *pomca* mutant 1 lines (M1L1 and M1L2) were screened by genotyping by enzyme digested with Eco31I (Fermentas) and DNA sequencing. Most of the M1 zebrafish assays were performed with the zebrafish of the M1L1 zebrafish unless specifically indicated. To obtain the mutant lines with disrupted functional α-MSH and ACTH only, a 3-aa deletion *pomca* mutant line 2 (M2) was generated. To obtain the mutant lines that were missing only β-MSH and β-END, two independent *pomca* mutant 3 lines (M3L1 and M3L2) were generated. The detailed information for TALENs is shown in Figure 5A and 5B. The PCR primers for genotyping are listed in Table S1. To avoid the sexual differences in metabolism, male fish were chosen in this study unless specifically indicated.

Whole-mount in situ hybridization (WISH)

WISH was carried out as described previously (Thisse and Thisse, 2008). The cDNA of zebrafish *pomca* and *hsd3b1* were amplified by RT-PCR with total RNA from 6 dpf larvae zebrafish. Primers were designed based on the sequences in GenBank for *pomca* and *hsd3b1* and are described in Figure S1.

Immunofluorescence and western blot

36 Adult zebrafish pituitaries glands were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered
37 saline (PBS) and incubated overnight at 4°C. The immunofluorescence labeling protocol was carried
38 out as described previously (Jaffe et al., 2010). The primary antibody of sheep anti-a-MSH (Millipore,
39 AB5087) was diluted 1:10000, and the secondary antibody of donkey anti-sheep conjugated with
40 DyLight 594 (ImmunoReagents, IMR-DkxSh-003-D594NHSX) was diluted 1:200.
41 For western blotting, SDS-PAGE was performed as previously described (Lou et al., 2012). The
42 protein samples were collected from the skeletal muscle tissue of adult zebrafish. The primary
43 antibodies of anti-GAPDH (Proteintech Group, 0004-1-Ig), anti-Akt (Cell Signaling Technology,
44 9272s), anti-phospho-Akt (S473) (Cell Signaling Technology, 4060s) and anti-phospho-s6 ribosomal
45 protein (S240/244) (Cell Signaling Technology, 2215s) were diluted 1:1000 in Can Get Signal Solution
46 for primary antibody dilution (Toyobo, NKB-101). The signal was detected by a CCD camera-based
47 imager (ImageQuant LAS 4000 mini, GE). The intensity of the bands was quantitated using ImageJ
48 version 1.49V software.

49 *Food intake and oxygen consumption measurement*

50 The food intake protocol for larval fish was carried out as described previously (Shimada et al., 2012).
51 Larval fish were fed with 4-Di-10-ASP-labeled (Invitrogen, D291) paramecia in darkness at 6 dpf.
52 Fluorescence-integrated intensity was measured using ImageJ 1.49 V software. For adult fish food
53 intake, fish were selected randomly from the *pomca* mutant and control fish. The fish were placed
54 individually in 1-L tanks and starved for four days. Each fish was weighed, and an excess of newly
55 hatched brine shrimp were added to the tank. After 2 hours of feeding, the fish was weighed again. The
56 oxygen consumption measurements were performed as we previously described (Li et al., 2014).
57 Briefly, 3 adult fish were transferred to separate respiration chambers with 1000 ml of fresh water at
58 28°C and then starved for 5 days The oxygen concentrations within the chamber were measured using
59 a SevenGo pro-SG6 oximeter (Mettler-Toledo AG; Analytical; CH-8603) after 6 hours of chamber
60 enclosure. The oxygen concentration in a respiration chamber without any fish during the same period
61 was treated as the initial oxygen concentration. The oxygen consumption was calculated as milligrams
62 O_2/h/g body weight.

63 *Zebrafish behavioral assay*

64 The dark-light emergence test is a relatively simple and suitable behavioral assay for juvenile zebrafish.
65 Locomotor activity was monitored using a ZebraBox system (ViewPoint Life Sciences, Inc., Montreal,

66 Canada), following previously described methods (Peng et al., 2016). Swimming behavior was
67 monitored in response to dark-to-light transitions using larvae at 4 dpf. Since the HPI axis is under
68 circadian regulation, the behavioral assay was restricted to the afternoon (15:30). The light-driving
69 procedure was in accordance with a normal light/dark cycle from 15:30 to the next day at 8:00,
70 followed by a 10 min light/10 min dark cycle (repeated 6 times) and terminated with 2 hours of light.
71 The data were collected every 20 s. Locomotor behavior was monitored in 96-well flat-bottom plates,
72 and 48 larval were selected randomly from the *pomca* mutant and control fish.
73 For the adult zebrafish behavioral assay, each fish at the 150 dpf stage was placed into a 25×15 cm tank.
74 The locomotor trajectory was monitored using a ZebraTower system (ViewPoint Life Sciences).
75 Movements were recorded every 10 s over 10 min.

76 *Dark-induced melanosome dispersal assay*

77 A dark-induced melanosome dispersal assay of larval zebrafish was carried out following a previous
78 description (Wagle et al., 2011). Briefly, Petri dishes containing 4 dpf larval zebrafish were transferred
79 to an enclosed compartment for 30 min at night. After dark treatment, larval zebrafish were
80 anesthetized with cold water and fixed with 4% PFA/PBS. Images were obtained by an Olympus
81 SZX16 FL stereomicroscope. The melanocyte-covered areas and numbers were measured using ImageJ
82 1.49 version software.

83 *Overfeeding assay and body composition measurement*

84 Different genotypic male zebrafish at the 120 dpf stage were randomly divided into two tanks for
85 normal feeding and overfeeding. The normal feeding protocol was three meals of newly hatched brine
86 shrimp in circulatory water system. For overfeeding, the zebrafish were fed with excess brine shrimp
87 six times a day from approximately 8:30 AM until approximately 9:30 PM. The overfeeding assay
88 lasted for 15 days. Total lipid contents in whole zebrafish were extracted following homogenization in
89 chloroform/methanol (2:1, v/v) according to the Folch method (Folch et al., 1957).

90 *Drug treatment*

91 The mTORC1 inhibitors rapamycin and AZD8055 were purchased from Selleck Chemicals and
92 dissolved in DMSO (Sigma). M1 fish and control fish were pooled into 3 tanks and treated with DMSO,
93 rapamycin or AZD8055 respectively for 15 days. Each concentration of rapamycin and AZD8055 was
94 5 μM.

95 *qRT-PCR analysis*

96 Total RNA was extracted using the TRIzol^{TM} Reagent (Thermo Fisher, 15596026). Complementary
97 DNA was synthesized using the HiScript Ⅱ Q Select RT SuperMix (Vazyme, R233-01). qRT-PCR
98 analysis was performed using the SYBR Green Real-time PCR Master Mix (Toyobo, QPK-201) in a
99 real-time detection system (Bio-Rad). The actb1 gene was used as the reference gene. The primers used
100 are listed in Table S1.

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102 # References

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Supplementary Table 1. Primers used in this study.

Test	Symbol	NCBI accession No.	Forward primers (5'-3')	Reverse primers (5'-3')	product length(bp)
Genotyping for *pomca*	M1	353221	ACTATTGGGTTATTTGATTC	CTAATTTTCTAATGGGAGTT	481
	M2 and M3		GGAATGCATACAATTATGC	GCATGATAAGAACCTCTC	622
WISH	*pomca*	NM_181438.3	GCAGGTCTGAACTTACAGAT	CCCACCTTCGTTTCTATGCA	622
	hsd3b1	XM_689112.7	GCAACAAAGCTGCTGCTTGA	GCCTCTCTTGTATCCCAAAGC	544
qRT-PCR	*lepa*	NM_001128576.1	CGCTGACAAACCCATCCAAG	CTTCAGTGTGCAGTCCATGC	157
	insa	NM_131056.1	GGTCGTGTCCAGTGTAAGCA	GGAAGGAAACCCAGAAGGGG	147
	gcga	NM_001271770.1	CACAAGACTTCGTTCAGTGGC	AGGTATGAGCTCACGTCGC	100
	pomca	NM_181438.3	TCTTGGCTCTGGCTGTTC	TCGGAGGGAGGCTGTAG	184
	gh1	NM_001020492.2	GCATCAGCGTGCTCATCAAG	TGAGACTGGTCTCCCCTACG	114
	fshb	NM_205624.1	AGAGCGAAGAATGTGGGAGC	GAATCAACCCCTGCAGGACA	178
	lhb	NM_205622.2	AGCTTGGTTTTTCCACGCTG	TACGTGCACACTGTCTGGTG	170
	tshb	NM_181494.2	AGGTTGCCGTGCCTATGTG	GGACCCACCAACTCCTTTATGT	145
	prl	NM_181437.3	CTCAGCACCTCACTCACCAAT	CAGAGACCGAGCCAATGACA	168
	cyp11a1	NM_152953.2	CAGTGTCCTTGCCTTCACCA	TGATGGCCCTCAGCTTTGAA	171
	Cyp17a1	NM_212806.3	CTCTTTGACCCAGGACGCTT	TTTGCAAAATCCACGCCAGG	154
	Cyp19a1a	NM_131154.3	GACTGGCTGCACAAAAAGCA	CAAGTTTCTCTGCGTGTGCC	106
	hsd17b3	NM_200364.1	ACGGCTGAGGAGTTTGTGAG	GTTTCACTCTGCAGGACCCA	131
	cyp11c1	NM_001080204.1	CCTGATGTGCAGGAGTGTGT	TGAACGGTGATTCCCACAGG	149
	cyp21a1	XM_021466882.1	CTTTTTGGAAGGTGGTGGCG	GTTCAGGCAGTGGCTCTTCT	170
	hsd3b1	XM_689112.7	TTGATGATTGCCGGGGAGAG	TTCACTGTATTCAACTGCTCCA	149
	actb1	NM_131031.1	ACTCAGGATGCGGAAACTGG	AGGGCAAAGTGGTAAACGCT	118
	Igf1a	NM_131825.2	GGCGCCTCGAGATGTATTGT	TGTTTCCTCGGCTCGAGTTC	165
	Igf1b	NM_001115050.1	GCGGTGGTCCTCGCTCTCG	TCTGCTAACTTCTGGTATCG	190
	socs1a	NM_001003467.1	CTGTGGAGGAAGCACACCTG	ACGGGACCGTTTTGTGCTTT	118
	socs2	NM_001114550.1	ACTCCACGGAAAGCATCGAG	TCCTTGGCTTCATTGGCTGT	148

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Supplementary Figures and Legend

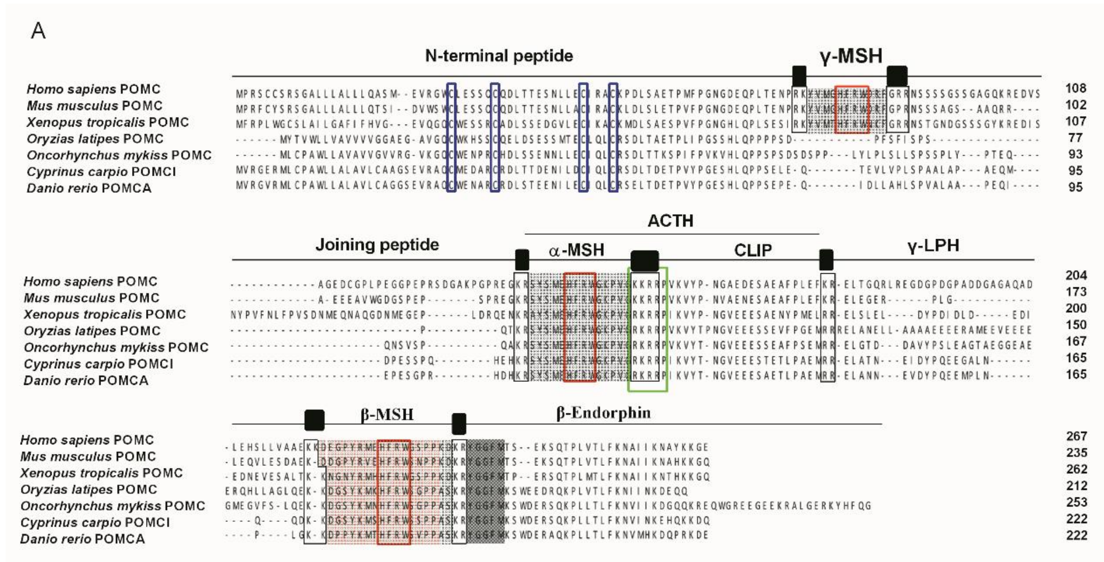

Figure S1. Multiple sequence alignment of POMC. (**A**) Alignment of the POMC sequences from human (*Homo sapiens*, NP_001306133.1), mice (*Mus musculus*, NP_001265512.1), Western clawed frogs (*Xenopus tropicalis*, NP_001011318.1), rainbow trout (*Oncorhynchus mykis*s, NP_001118190.1), medaka (*Oryzias latipes*, XP_004066504), carp (*Cyprinus carpio*, CAA74968.1) and zebrafish (*Danio rerio*, NP_852103.1). The blue boxes indicate conserved cysteine residues in all POMC sequences. Light and dark gray boxes show MSH peptides and endorphin core, respectively. The black boxes represent endoproteolytic cleavage sites of POMC. The red boxes and green boxes show the fully conserved HFRW motif and K/RKRRP motif in all selected species.

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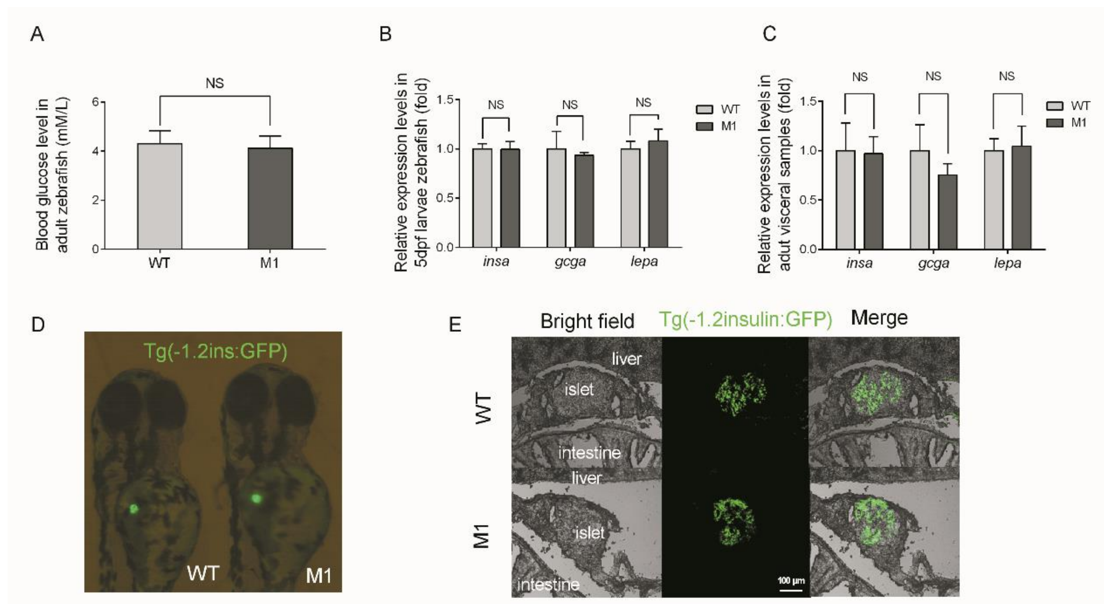

141 **Figure S2. Features of major signaling involved in glucose homeostasis in *pomca*-deficient**
142 **zebrafish**. (**A**) The blood glucose levels of *pomca* M1 fish and WT control siblings 30 minutes after
143 feeding at adult stage (n=9/group). (**B** and **C**) Relative expression level of *insa, gcga* and *lepa* in larval
144 zebrafish at 4 dpf (**B**) and visceral tissue at the adult stage (**C**) (n=3/group). (**D** and **E**) GFP (insulin:
145 GFP) expression pattern in the islet at 4dpf (**D**) and the adult stage (**E**) of *pomca* M1 fish and WT
146 control siblings. Data are presented as the means ± SD from independent student's t-test. 'NS' indicates
147 that there're no significant differences between two groups.

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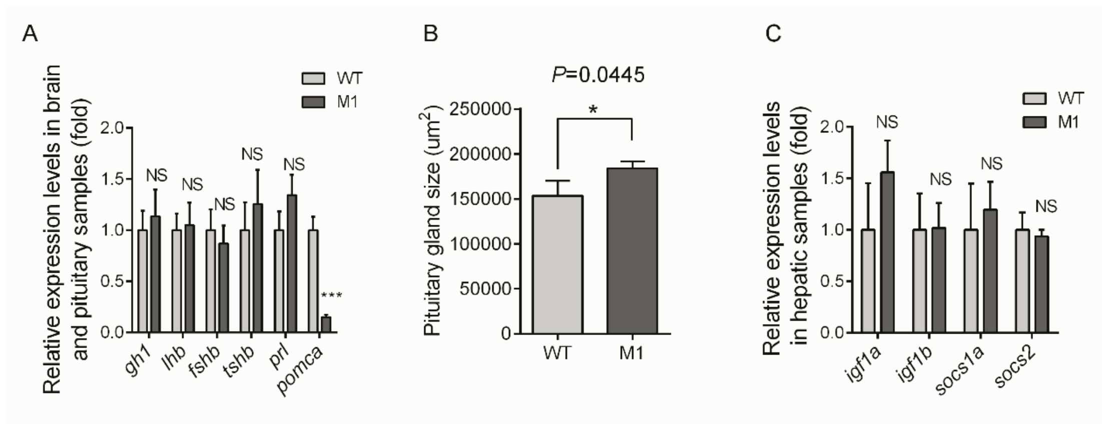
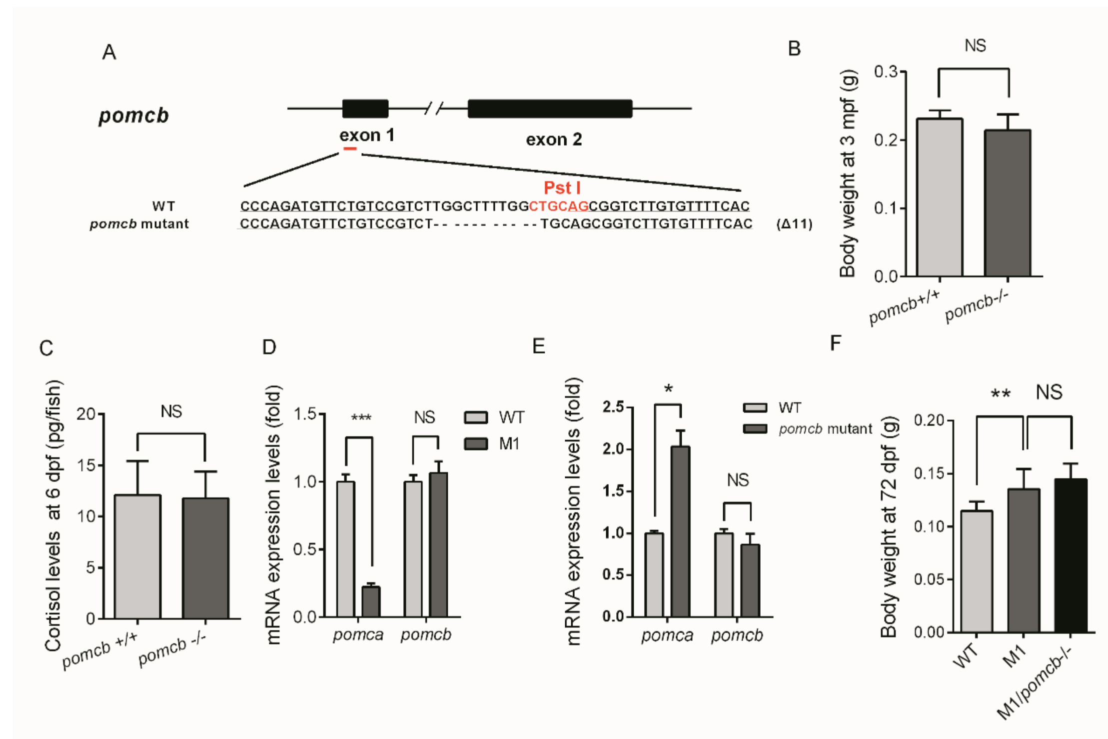
149 

150 **Figure S3. Expression of major pituitary hormones and key factors involved in GH signaling**. (**A**)
151 Relative expression of pituitary gland hormone genes, *gh1, lhb, fshb, tshb, prl* and *pomca* in brain and
152 pituitary gland tissue (n=3/group). (**B**) Pituitary gland size was measured with image J software
153 (N=3/group). (**C**) Relative expression of *igf1a, igf1b, socs1a* and *socs2* in hepatic tissue (n=3/group).
154 Data are presented as the means ± SD from independent student's *t*-test. ***$P < 0.001$; 'NS' indicates
155 that there're no significant differences between two groups.



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157 **Figure S4. The HPI axis was intact in *pomcb* mutant fish and *pomcb* had no redundant roles in**
158 **M1 zebrafish.** (**A**) The schematic represents engineered TALENs in *pomcb.* The restriction enzyme
159 PstI was used to identify the genotypes. The underlined fonts indicate the sequences of the two
160 targeting arms of TALENs. (**B**) The body weights of *pomcb* mutant fish and wild-type control fish at 3
161 mpf. (n=10-12/group). (**C**) Whole-fish cortisol content in *pomcb* mutant fish and wild-type control fish
162 at 6 dpf. (n=10/group). (**D**) The relative expression levels of *pomca* and *pomcb* mRNA in M1 zebrafish
163 and WT zebrafish at 12 dpf (n=3/group). (**E**) The relative expression levels of *pomca* and *pomcb*
164 mRNA in *pomcb* mutant zebrafish and WT zebrafish at 12 dpf (n=3/group). (**F**) *pomca/pomcb* double
165 mutant fish and *pomca* M1 zebrafish body weights at 72dpf (n=5-8/group).Data are presented as the
166 means ± SD. 'NS' indicates that there're no significant differences between two groups.

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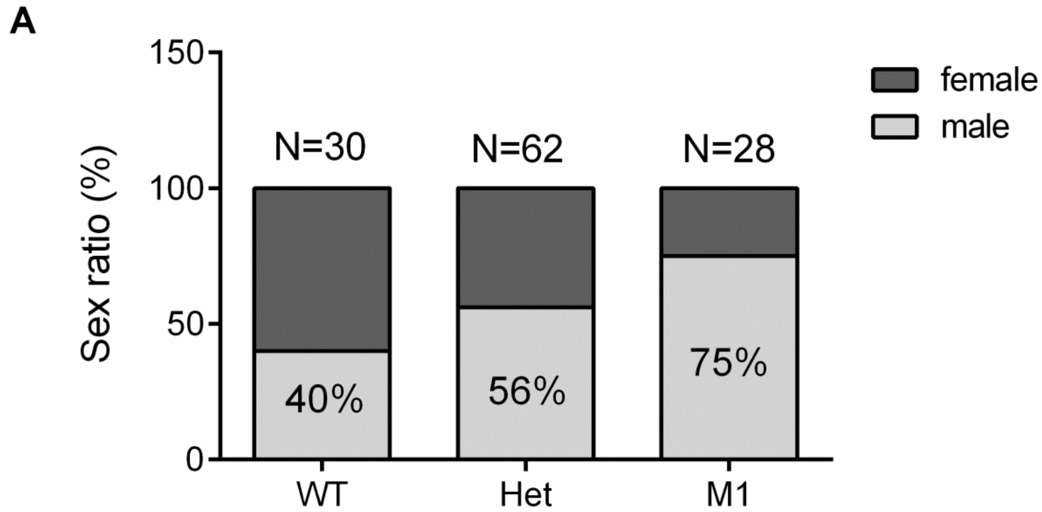

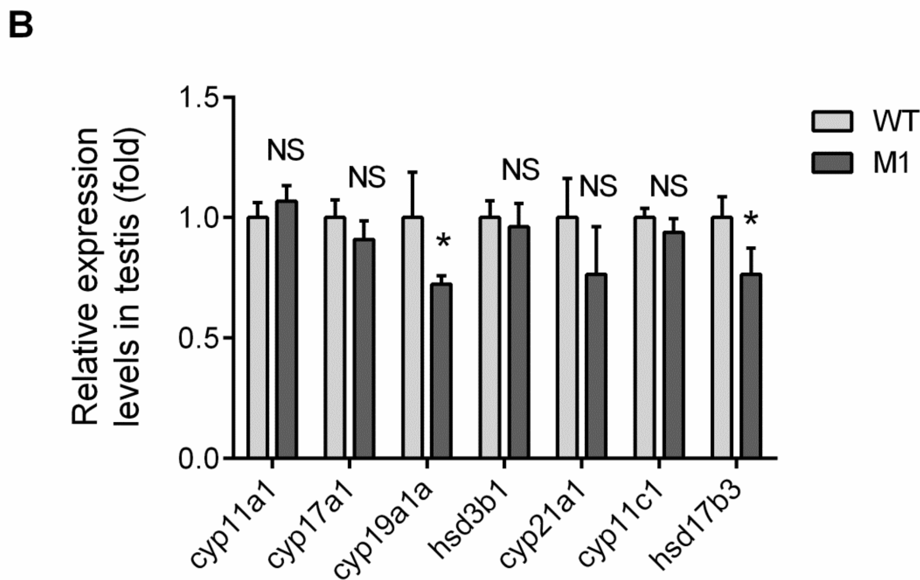

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169 **Figure S5.** Sex ratios and the expression levels of the relevant genes in the testis. (**A**) Sex ratios of
170 *pomca* M1 fish, heterozygotes and their wild-type siblings at 6 mpf. (**B**) The transcriptional levels of
171 key molecules, *cyp11a1, cyp17a1, cyp19a1a, hsd3b1, cyp21a1a, cyp11c1* and *hsd17b3*, involved in the
172 cortisol and testosterone synthesis in the testis (n=3/group). Data are presented as the means ± SD from
173 independent student's t-test. *P < 0.05; 'NS' indicates that there're no significant differences between
174 two groups.
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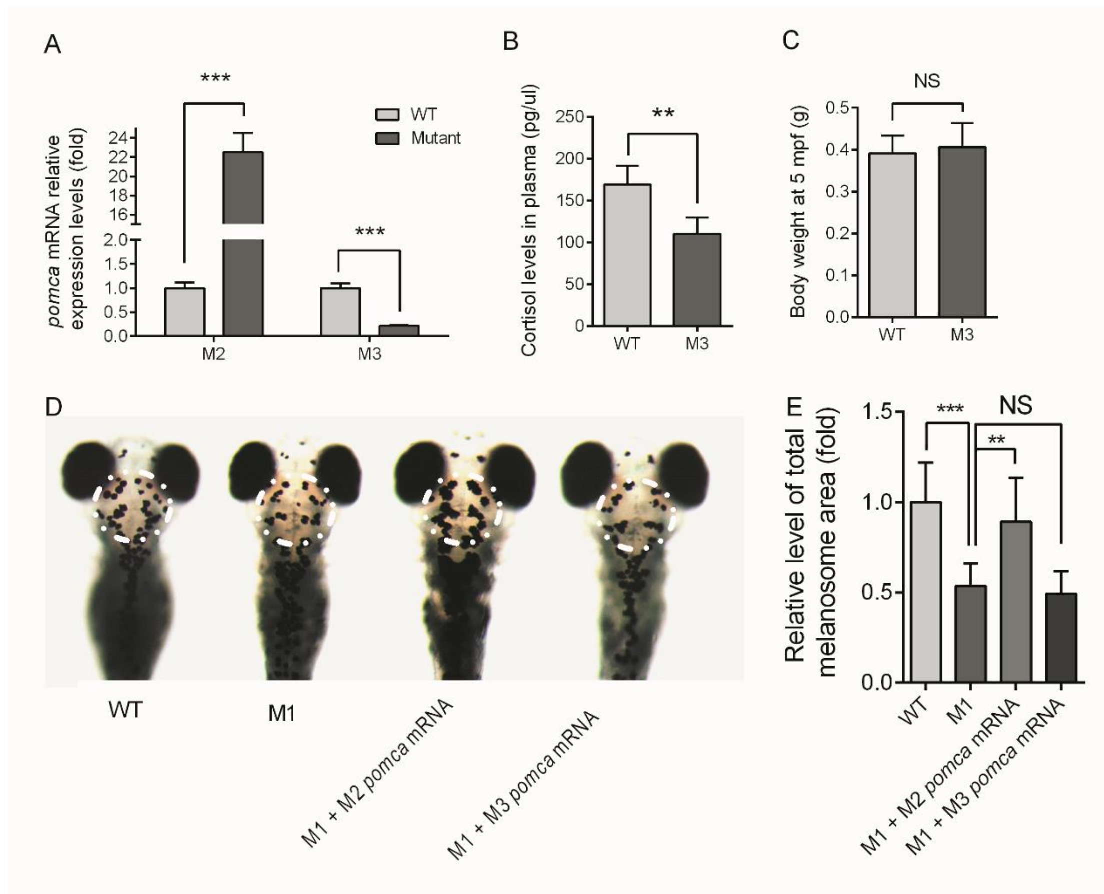

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177 **Figure S6**. Phenotypes observed in M3 zebrafish. (**A**) Relative expression levels of *pomca* mRNA in
178 M2 and M3 brain and pituitary gland tissue at 5 mpf (n=4/group). (**B**) Plasma cortisol levels in WT and
179 M3 zebrafish at 5mpf (n=4/group). (**C**) Body weight of WT and M3 zebrafish at 5 mpf
180 (n=15-18/group). (**D**) Image of 4 dpf zebrafish exposed in dark 30 min. (**E**) Relative levels of indicated
181 melanosome areas in WT (n=8), M1 (n=8) and M1 injected with M2 (n=5) or M3 *pomca* mRNA (n=8)
182 zebrafish. Data are presented as the means ± SD. 'NS' indicates that there're no significant differences
183 between two groups.

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