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RESEARCH ARTICLE

Zebrafish Nedd8 facilitates ovarian development and the maintenance of female secondary sexual characteristics via suppression of androgen receptor activity

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ABSTRACT

Nedd8 is a ubiquitin-like protein that covalently conjugates to target proteins through neddylation. In addition to cullin-RING ligases, neddylation also modifies non-cullin proteins to regulate protein activity, stability and localization. However, the roles of *NEDD8* remain largely unknown *in vivo*. Here, we found that loss of *nedd8* in female zebrafish led to defects in oogenesis, disrupted oocyte maturation and stimulated growth of the breeding tubercles (BTs) on the pectoral fins. The BTs are normally present in males, not females. However, the loss of one copy of *ar* can partially rescue the phenotypes displayed by *nedd8*-null female zebrafish. Further assays indicated that Nedd8 conjugates to Ar and Ar is neddylated at lysine 475 and lysine 862. Moreover, Nedd8 conjugation efficiently suppressed Ar transcriptional activity. Lysine 862 (K862) of Ar is the key site modified by neddylation to modulate Ar transcriptional activity. Thus, our results not only demonstrated that Nedd8 modulates ovarian maturation and the maintenance of female secondary sexual characteristics of female zebrafish *in vivo*, but also indicated that androgen signaling is strictly regulated by *nedd8*.

KEY WORDS: Nedd8, Zebrafish, Androgen receptor, Ovary, Testis, Neddylation

INTRODUCTION

Reversible post-translational protein modifications modulate protein activity in diverse cellular processes (Menzies et al., 2016; Oh et al., 2018; Rape, 2018). Neural precursor cell expressed developmentally downregulated protein 8 (Nedd8), as a ubiquitin-like protein (UBL), is covalently conjugated to the lysine residues of target substrates resulting in protein neddylation, a post-translational modification (Enchev et al., 2015). Like ubiquitylation, neddylation involves E1 (activating), E2 (conjugating) and E3 (catalyzing) enzymes. Neddylation is a reversible modification; protein deneddylation is performed by deneddylase, such as DEN1/SENP8 (Gan-Erdene et al., 2003; Wu et al., 2003). The cullin-RING ligases (CRLs), in the E3 ubiquitin ligase family, are the principal substrates of neddylation (Enchev et al., 2015). Recently, neddylation has also been shown on non-cullin targets, regulating substrate protein activity, stability and subcellular localization (Abidi and Xirodimas, 2015; Enchev et al., 2015; Vogl et al., 2015; Watson et al., 2011; Zuo et al., 2013). Functionally, neddylation is crucial for gene regulation, cell survival, organ development and the stress response (Vogl et al., 2015; Xirodimas et al., 2004; Zou et al., 2018; Zuo et al., 2013). Dysregulation of neddylation is associated with disease pathogenesis (Soucy et al., 2009; Xirodimas et al., 2004). For example, an E1 inhibitor of neddylation (MLN4924) was shown to restrict tumor growth and is currently in clinical trials as a cancer treatment (Shah et al., 2016; Soucy et al., 2009).

To investigate the function of the NEDD system *in vivo*, various animal models have been developed (Chan et al., 2008; Tateishi et al., 2001; Vogl et al., 2015; Zou et al., 2018). Mice deficient in the *Uba3* gene that encodes a catalytic subunit of NEDD8-activating enzyme die *in utero* at the preimplantation stage, suggesting that the NEDD8 system is essential for cell cycle progression and the morphogenetic pathway (Tateishi et al., 2001). The cardiomyocyte-specific knockout of *Nae1*, a subunit of the E1 activating enzyme, led to myocardial hypoplasia, ventricular noncompaction and heart failure in mice late in gestation, resulting in perinatal lethality (Zou et al., 2018). In addition, studies of *Drosophila* DEN1-null mutants indicated that DEN1 deneddylates many cellular proteins in addition to cullin proteins (Chan et al., 2008). However, the functions of other components of the NEDD system have not been illustrated by animal models *in vivo*. In particular, the function of *nedd8 in vivo* remains largely unknown.

The zebrafish (*Danio rerio*) is a model organism that has been widely used for studies of gene function *in vivo*. To further investigate the role of the NEDD system, we used CRISPR/Cas9 to knock out *nedd8* in zebrafish. We found that loss of *nedd8* caused defects in ovarian development, and led to the growth of the breeding tubercles (BTs) on the pectoral fins of female zebrafish. The BTs are keratinized multicellular epidermal structures that normally present on the dorsal surface of the anterior rays of zebrafish male pectoral fins, not females (Kang et al., 2013; McMillan et al., 2013; Nachtrab et al., 2011). Moreover, we showed that Nedd8 conjugates to Androgen receptor (Ar), inhibiting its transactivity.

RESULTS

Loss of *nedd8* in zebrafish reduced female ratio, ovulation rate, fertilization rate and successful oocyte maturation

The *nedd8* gene is evolutionarily conserved across human, mice and zebrafish (Fig. S1A). During embryogenesis, *nedd8* was detected at a very early stage (two-cell) and was ubiquitously expressed up to 16 h post fertilization (hpf) (Fig. S1B). After 16 hpf, *nedd8* was

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specifically expressed in the brain, notochord and tail, with particularly high levels of expression detected in the notochord (Fig. S1B). In adults (≥4 months post fertilization, mpf), *nedd8* was highly expressed in the brain, testis, ovaries and muscles (Fig. S1C). Immunohistochemical staining showed that the Nedd8 protein was also expressed in the germ cells in testes and ovaries during gonad development (Fig. S1D). These data suggest that *nedd8* might play a role in gonadal development.

To investigate the function of *nedd8 in vivo*, we generated *nedd8*-null zebrafish (*nedd8*$^{ihb1227/ihb1227}$) using CRISPR/Cas9 (Yu et al., 2019). Mutant zebrafish (*nedd8*$^{-/-}$) were phenotypically identical to their wild-type siblings (*nedd8*$^{+/+}$). The heterozygous zebrafish (*nedd8*$^{+/-}$) were indistinguishable from their wild-type siblings (*nedd8*$^{+/+}$). Notably, the BTs were frequently observed on the surface of adult female *nedd8*$^{-/-}$ pectoral fins (~83% of all *nedd8*$^{-/-}$ females), but were hardly observed on the fins of wild-type females (~0%). BTs are male secondary sexual characteristics, which are typically present on the pectoral fins of adult males (Fig. S2) (Kang et al., 2013; McMillan et al., 2013). Notably, *ar*-knockout male zebrafish (*ar*$^{-/-}$) do not develop BTs, but do exhibit some female secondary sexual characteristics (Yu et al., 2018). Therefore, we hypothesized that *nedd8* might impact *ar* gene function. Next, we aimed to further characterize the phenotype of *nedd8*$^{-/-}$ females to clarify the relationship between *nedd8* and *ar*.

When we crossed *nedd8*$^{+/-}$ (♀) zebrafish with *nedd8*$^{+/-}$ (♂) zebrafish, the *nedd8*$^{-/-}$ offspring exhibited a male sex bias and very low fecundity of *nedd8*$^{-/-}$ female offspring while *nedd8*$^{-/-}$ male offspring exhibited normal fecundity. Subsequently, in order to obtain more *nedd8*$^{-/-}$ zebrafish from each mating, we mated *nedd8*$^{+/-}$ (♀) zebrafish with *nedd8*$^{-/-}$ (♂) zebrafish to get *nedd8*$^{+/-}$ and *nedd8*$^{-/-}$ offspring. The female:male ratio of the *nedd8*$^{-/-}$ offspring was substantially lower than the female:male ratio of *nedd8*$^{+/-}$ offspring (Fig. 1A). The ovulation rates of *nedd8*$^{-/-}$ females was also lower than those of the *nedd8*$^{+/+}$ females (Fig. 1B). Furthermore, the *nedd8*$^{-/-}$ eggs were fertilized at a significantly lower rate than the *nedd8*$^{+/+}$ eggs (Fig. 1C). Compared with *nedd8*$^{+/+}$ adult females, *nedd8*$^{-/-}$ adult females had elongated bodies, transparent ovaries and degenerated eggs (Fig. 1D). In addition, the mean gonadosomatic index (GSI) of the *nedd8*$^{-/-}$ adult females was lower than that of the *nedd8*$^{+/+}$ adult females (Fig. 1E). Histological analysis of the adult ovaries showed that *nedd8*$^{-/-}$ ovaries contained more oocytes at the primary growth stage (PG) and the previtellogenic stage (PV), but fewer oocytes at the early vitellogenic stage (EV), the midvitellogenic stage (MV) and the full-grown stage (FG), compared with *nedd8*$^{+/+}$ ovaries (Fig. 1F,G).

The crucial period of zebrafish sexual differentiation is 17-35 days post fertilization (dpf); during this time, zebrafish develop 'juvenile ovaries' containing gonocytes (Sun et al., 2013). After 40 dpf, juvenile ovaries pass the transitional period of sex determination and develop into an immature ovary and testis (Sun et al., 2013). To identify the stage at which the deletion of *nedd8* started to affect gonadal development and sexual differentiation, we examined the gonads of zebrafish from juvenile (24 dpf) to 2 mpf. At 24 dpf, the *nedd8*$^{-/-}$ gonads contained more gonocytes (GO, indicated by white arrows in Fig. S3A) and degenerated perinucleolar oocytes (indicated by red arrows in Fig. S3A) compared with their wild-type siblings (Fig. S3A,B). At 40 dpf, the *nedd8*$^{+/+}$ ovaries contained vitellogenic stage oocytes, but the *nedd8*$^{-/-}$ ovaries only contained early development stage PG and PV oocytes. Interestingly, the *nedd8*$^{-/-}$ testes developed even faster than those of their wild-type siblings: when *nedd8*$^{+/+}$ testes contained spermatogonia (SG) and spermatocytes (SC) only, spermatids (ST) were already present in the *nedd8*$^{-/-}$ testes (Fig. S3C,E). In the *nedd8*$^{-/-}$ ovaries at 2 mpf, most oocytes were arrested at the early development stage, but the *nedd8*$^{+/+}$ ovaries were filled with oocytes at different developmental stages (Fig. S3F,G). However, no obvious difference was observed between the *nedd8*$^{+/+}$ and the *nedd8*$^{-/-}$ testes (Fig. S3F,H). These results suggest that *nedd8* disruption leads to defects in ovarian development in female zebrafish, but did not substantially affect male zebrafish.

To get deeper insight into the effects of *nedd8* in ovarian development, we examined expression of Vasa (also known as Ddx4), Zili (Piwil2) and Ziwi (Piwil1) in *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ ovaries – three key proteins required for zebrafish germ cell differentiation and maintenance (Dranow et al., 2016; Hartung et al., 2014; Houwing et al., 2008, 2007; Zhu et al., 2019). As marked by Vasa, in juvenile ovaries at 24 dpf, more primordial germ cells (PGCs) and more primary oocytes (Zili and Ziwi expressed around nuclei) were detected in *nedd8*$^{-/-}$ compared with *nedd8*$^{+/+}$. In *nedd8*$^{-/-}$ ovaries at 2 mpf, the granulosa cells and theca cells marked by Zili and Ziwi were fewer than those in *nedd8*$^{+/+}$ ovaries (Fig. S4A,B). These observations suggested that loss of *nedd8* resulted in oocytes arrested at early developmental stages, with few developed granulosa cells and theca cells.

Intriguingly, *nedd8*$^{-/-}$ males displayed deeper yellow pigmentation on the anal fins compared with the *nedd8*$^{+/+}$ males (Fig. S5A). As *nedd8*$^{-/-}$ females exhibited some male secondary sexual characteristics, and as hyperactive behaviors are affected by androgen signaling (Kinch et al., 2015), we compared the behaviors of *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ males. The *nedd8*$^{-/-}$ males were not only more sexually aggressive (chasing females) than *nedd8*$^{+/+}$ males, but were also more active when chasing food (Movies 1 and 2). Our movement tracing records showed that the locomotor activity of *nedd8*$^{-/-}$ males was also greater than that of *nedd8*$^{+/+}$ males (n=6 per group, over a 10 min period; Fig. S5B,C).

We also measured levels of serum 11-ketotestosterone (11-KT) and estradiol (E2) in *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ zebrafish. At 3 mpf, serum 11-KT and estradiol were similar between *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ males, but serum 11-KT levels were higher and serum estradiol levels were lower in *nedd8*$^{-/-}$ females compared with *nedd8*$^{+/+}$ females (Fig. S6A,B). At 6 mpf, serum 11-KT and estradiol levels were higher in both male and female *nedd8*$^{-/-}$ zebrafish compared with *nedd8*$^{+/+}$ males and females (Fig. S6C,D). Subsequently, we examined two female determination genes [*cyp19a1a* and *foxl2* (*foxl2a*)] and two male determination genes (*amh* and *dmrt1*) in ovaries and testes. At 3 mpf, the expression of *cyp19a1a* and *foxl2* was downregulated, and that of *amh* and *dmrt1* was upregulated in *nedd8*$^{-/-}$ ovaries (Fig. S6E) (Lau et al., 2016; Yang et al., 2017; Yin et al., 2017), but the expression levels of *amh* and *dmrt1* were upregulated and the expression levels of *cyp19a1a* and *foxl2* were not altered significantly in *nedd8*$^{-/-}$ testes (Fig. S6F). At 6 mpf, the expression level of *cyp19a1a* was upregulated in *nedd8*$^{-/-}$ ovaries and testes, but the expression level of *amh* was downregulated in *nedd8*$^{-/-}$ ovaries (Fig. S6E,F). No significant change was observed for other genes at this stage (Fig. S6E,F). In fact, the levels of serum estradiol (E2) reflected the *cyp19a1a* expression level in *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ zebrafish.

Loss of *ar* rescues *nedd8*$^{-/-}$ ovarian development

We crossed *ar*$^{-/-}$ (♀) and *nedd8*$^{-/-}$ (♂) zebrafish to generate double knockout offspring. We previously showed that *ar*$^{+/-}$*nedd8*$^{+/+}$ ovaries develop normally, similar to those of wild-type (*ar*$^{+/+}$*nedd8*$^{+/+}$) females, but *ar*$^{-/-}$*nedd8*$^{+/+}$ ovaries exhibit premature ovarian failure during growth (Yu et al., 2018). This

Development (2020) 147, dev194886. doi:10.1242/dev.194886

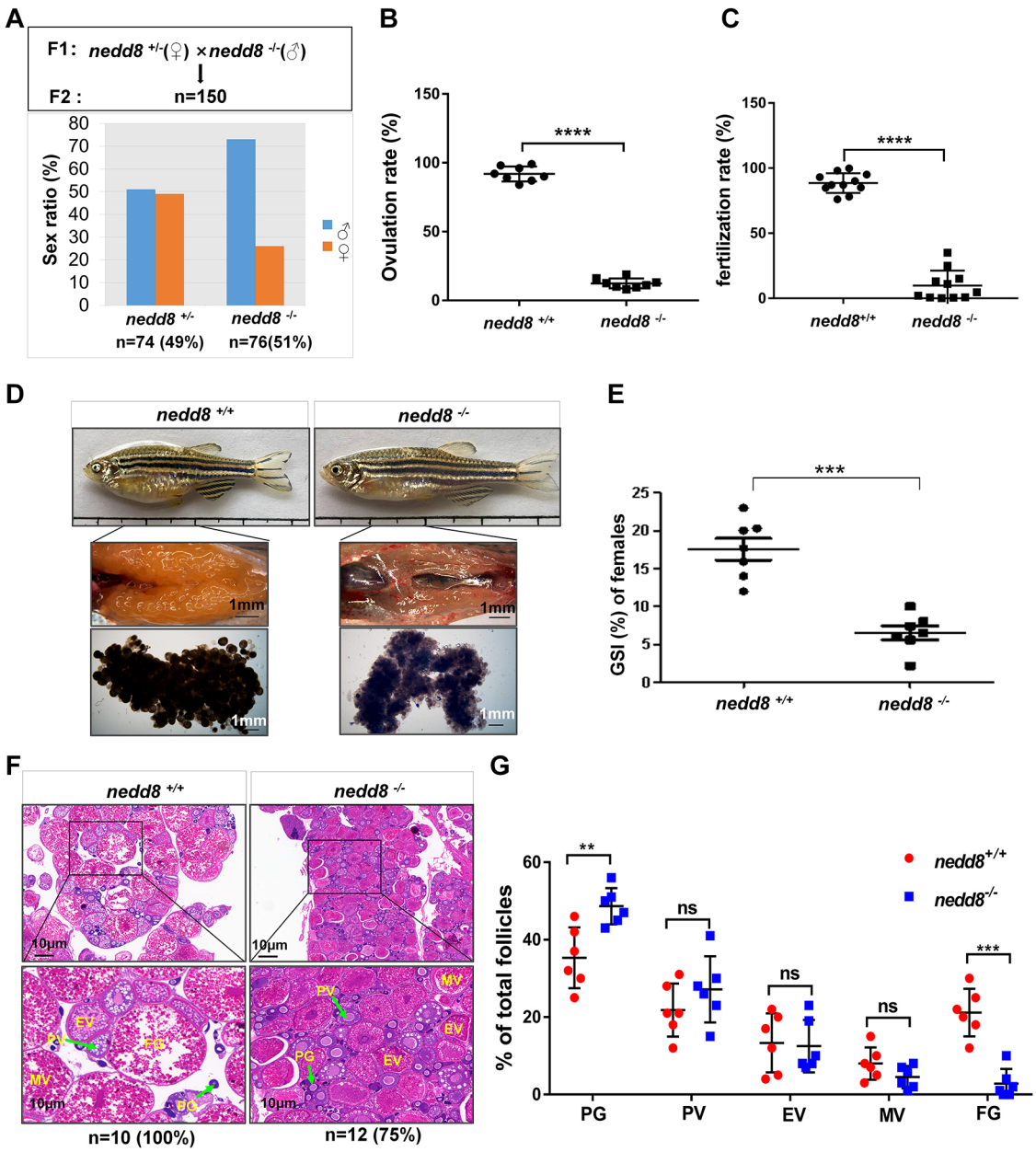

Fig. 1. Loss of *nedd8* in zebrafish causes defects in ovarian maturation and reduces female ratio, ovulation rate and fertilization rate. (A) The sex ratios of *nedd8*$^{+/-}$ and *nedd8*$^{-/-}$ progeny generated by *nedd8*$^{+/-}$ (♀)×*nedd8*$^{-/-}$ (♂) matings. Histograms show percentages of females and males in 150 crossbreed offspring. (B) The ovulation rates (*n*=8; number of spawned females/the total number of females tested×100) of the *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ female zebrafish mated with *nedd8*$^{+/+}$ male zebrafish (*n*=11). (C) The fertilization rates of *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ eggs. The fertilization rates were determined by mating *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ female zebrafish with *nedd8*$^{+/+}$ male zebrafish. (D) Bodies, ovaries and dissected eggs from a *nedd8*-null female zebrafish (*nedd8*$^{-/-}$) and her wild-type sibling (*nedd8*$^{+/+}$) at 4 mpf. Images show the elongated body, transparent ovaries and degenerated eggs of the *nedd8*$^{-/-}$ adult female, and the rounded body, egg-filled ovaries and mature eggs of the *nedd8*$^{+/+}$ adult female. (E) The GSI of the *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ females at 4 mpf (*n*=7). (F) H&E staining of the ovaries of wild-type zebrafish (*nedd8*$^{+/+}$) (*n*=10; 100%) and *nedd8*-knockout zebrafish (*nedd8*$^{-/-}$) (*n*=12; 75%) at 4 mpf. (G) The percentage of oocytes at different stages of development in wild-type zebrafish (*nedd8*$^{+/+}$) and *nedd8*-null zebrafish (*nedd8*$^{-/-}$) at 4 mpf (three sections/fish; six fish/genotype). Each dot represents average number of oocytes in one section. PG, primary growth stage; PV, previtellogenic stage (7-140 µm in diameter); EV, early vitellogenic stage (140-340 µm in diameter); MV, midvitellogenic stage (340-369 µm in diameter); FG, full-grown stage (0.69-0.70 mm in diameter). Data are mean±s.e.m. ***P*<0.01, ****P*<0.001, *****P*<0.0001 (one way ANOVA test). ns, not significant.

was consistent with results seen here (Fig. 2A,B). Surprisingly, although the gross shapes and tissue structure of *ar*$^{+/-}$*nedd8*$^{-/-}$ ovaries were similar to those of wild-type ovaries (*ar*$^{+/+}$*nedd8*$^{+/+}$) with fully-grown oocytes, the *ar*$^{+/+}$*nedd8*$^{-/-}$ ovaries mainly contained PG, PV and EV oocytes (Fig. 2A,B; Fig. 1D,F; Fig. S7). Owing to complete loss of *ar*, the *ar*$^{-/-}$*nedd8*$^{-/-}$ ovaries exhibited atretic and degenerated follicles (Yu et al., 2018) (Fig. 2A,B; Fig. S7). These results suggest that loss of one copy of *ar* at least partially rescued the defects of *nedd8*$^{-/-}$ ovary maturation. However, the defects of the *ar*$^{-/-}$*nedd8*$^{-/-}$ oogenesis were even more serious than those of *ar*$^{-/-}$*nedd8*$^{+/+}$ and *ar*$^{+/+}$*nedd8*$^{-/-}$ oogenesis (Fig. 2A,B; Fig. 1D,F; Fig. S7). This suggests that *ar* functions downstream of *nedd8*, as mutants lacking both copies of *ar* showed ovarian development traits characteristic of *ar*

Development (2020) 147, dev194886. doi:10.1242/dev.194886

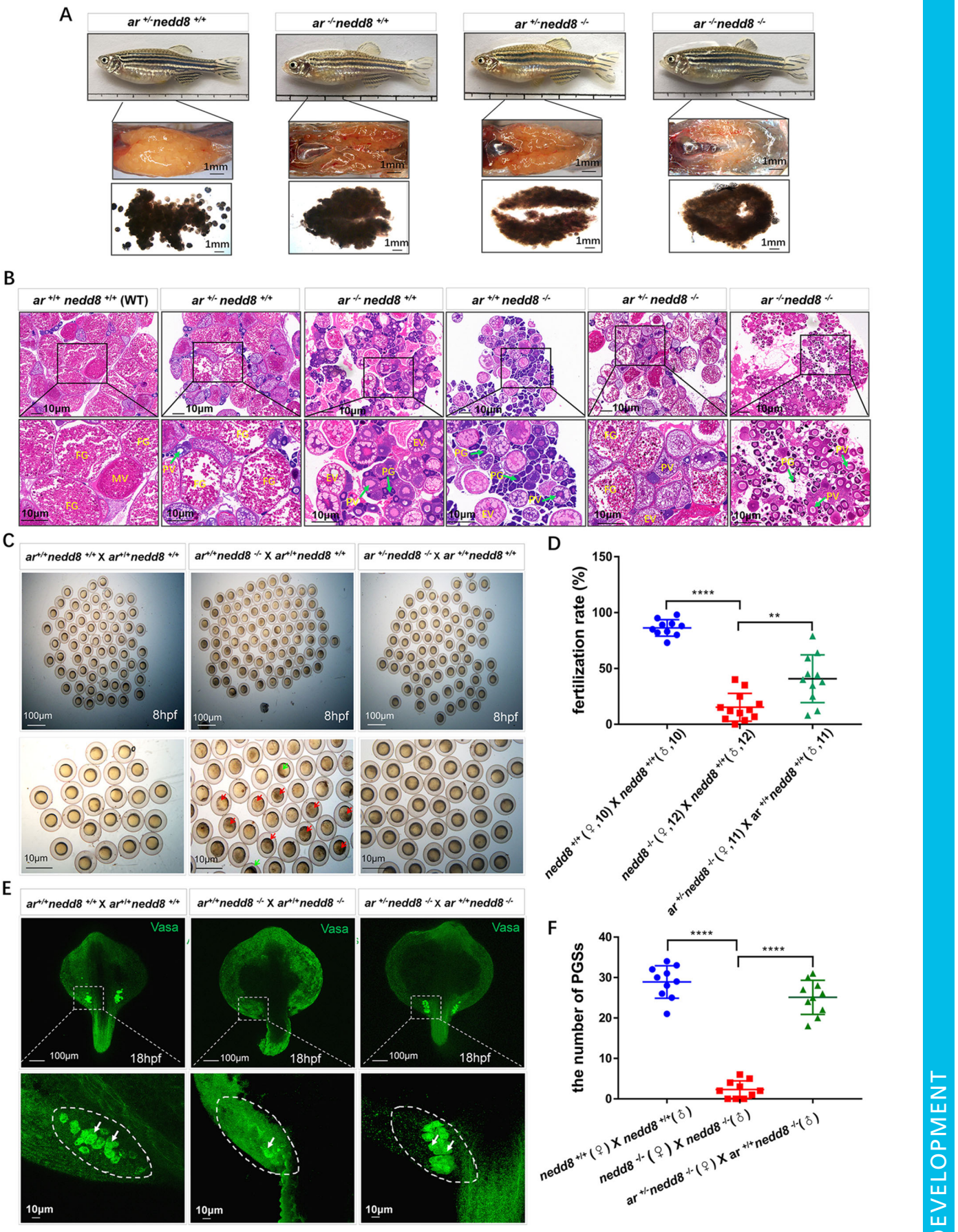

Fig. 2. See next page for legend.

Development (2020) 147, dev194886. doi:10.1242/dev.194886

Fig. 2. Loss of *ar* partially rescues *nedd8*$^{-/-}$ ovarian function. (A) Morphological comparison of ovaries and eggs from *ar*$^{+/-}$*nedd8*$^{+/+}$, *ar*$^{-/-}$*nedd8*$^{+/+}$, *ar*$^{+/-}$*nedd8*$^{-/-}$ and *ar*$^{-/-}$*nedd8*$^{-/-}$ female zebrafish at 4 mpf. Compared with the ovaries of the *ar*$^{+/+}$*nedd8*$^{-/-}$ zebrafish (i.e. *nedd8*$^{-/-}$; Fig. 1D), the ovaries of the *ar*$^{+/-}$*nedd8*$^{-/-}$ zebrafish were filled with more oocytes in the midvitellogenic stage. (B) Histological analyses of the ovaries from female zebrafish with different genotypes at 4 mpf. Compared with oogenesis in the *ar*$^{+/+}$*nedd8*$^{-/-}$ ovaries, oogenesis in *ar*$^{+/-}$*nedd8*$^{-/-}$ ovaries was relatively normal. Green arrows indicate oocytes in different developmental stages. Bottom panels show magnification of the boxed areas in top panels. (C) Whole-mount images of embryos produced by different matings: *ar*$^{+/+}$*nedd8*$^{+/+}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂); *ar*$^{+/+}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂); and *ar*$^{+/-}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂). The fertilization rate (number of fertilized eggs/total eggs laid×100) of the *ar*$^{+/+}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) eggs was significantly lower than that of the *ar*$^{+/-}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) eggs. Red arrows indicate degenerated embryos; green arrows indicate normal embryos. (D) Quantitation of the fertilization rates of different matings: *ar*$^{+/+}$*nedd8*$^{+/+}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) (*n*=10); *ar*$^{+/+}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) (*n*=12); and *ar*$^{+/+}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) (*n*=11). (E) Immunofluorescent staining of Vasa in PGCs of embryos (*n*=40) at 18 hpf from different matings: *ar*$^{+/+}$*nedd8*$^{+/+}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂); *ar*$^{+/+}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{-/-}$ (♂); and *ar*$^{+/-}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{-/-}$ (♂). (F) The scatterplots represent PGC numbers in *ar*$^{+/+}$*nedd8*$^{+/+}$, *ar*$^{+/+}$*nedd8*$^{-/-}$ and *ar*$^{+/+}$*nedd8*$^{-/-}$×*ar*$^{+/-}$ *nedd8*$^{-/-}$ embryos at 18 hpf. The numbers of PGCs were counted using a Leica dissection microscope based on the immunofluorescent density. EV, early vitellogenic stage; FG, full-grown stage; MV, midvitellogenic stage; PG, primary growth stage; PV, previtellogenic stage. Data are mean ±s.e.m. ***P*<0.01, *****P*<0.0001 (unpaired Student's *t*-test).

disruption (Yu et al., 2018). Therefore, we hypothesized that *nedd8* might affect folliculogenesis by modulating *ar* function, and that *nedd8* acted upstream of *ar*. This might explain why the loss of one copy of *ar* (but not two) rescued *nedd8*$^{-/-}$ oogenesis.

The lower fertilization rate of *ar*$^{+/+}$*nedd8*$^{-/-}$ eggs suggest that loss of *nedd8* not only disrupts ovarian development, but also affects egg quality. To determine whether loss of one copy of *ar* also rescued the low fertilization rate of *ar*$^{+/+}$*nedd8*$^{-/-}$ eggs, we conducted further fertilization rate assays. Different groups of wild-type males (*ar*$^{+/+}$*nedd8*$^{+/+}$) were separately mated with female zebrafish having one of three genotypes: *ar*$^{+/+}$*nedd8*$^{+/+}$, *ar*$^{+/+}$*nedd8*$^{-/-}$, or *ar*$^{+/-}$*nedd8*$^{-/-}$. The fertilization rate of the *ar*$^{+/+}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) cross was significantly lower than that of the *ar*$^{+/-}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂) cross, although this rate was still lower than that of the wild-type cross [*ar*$^{+/+}$*nedd8*$^{+/+}$ (♀)×*ar*$^{+/+}$*nedd8*$^{+/+}$ (♂); Fig. 2C,D]. These results suggest that loss of one copy of *ar* partially rescued the low fertilization rate of *ar*$^{+/+}$*nedd8*$^{-/-}$ eggs. Moreover, by mating *ar*$^{+/-}$*nedd8*$^{-/-}$ (♀)×*ar*$^{+/-}$*nedd8*$^{-/-}$ (♂) and subsequently examining sex ratio in their offspring, we found that the sex bias towards male phenotype characteristics of *nedd8* mutants was rescued in *ar*$^{+/+}$*nedd8*$^{-/-}$ zebrafish (Fig. S8).

It is evident that the germ cell number of zebrafish determines sexual differentiation (Dranow et al., 2016). To determine whether rescuing of sex bias in *nedd8*-null zebrafish by loss of one copy of *ar* resulted from rescuing germ cell number at early embryogenesis, we examined germ cell numbers by immunofluorescent staining using anti-Vasa antibody. As expected, loss of one copy *ar* indeed increased germ cell number in *nedd8*$^{-/-}$ background embryos (Fig. 2E,F). Interestingly, the disorganization, including lack of seminiferous tubule structure, in *ar*$^{-/-}$*nedd8*$^{-/-}$ testes was quite similar to that of the *ar*$^{-/-}$*nedd8*$^{+/+}$ testes (Fig. S9).

Loss of *ar* and/or treatment with the androgen antagonist flutamide eliminated BTs on the pectoral fins of *nedd8*$^{-/-}$ female zebrafish

To determine whether BTs developed on the pectoral fins of *nedd8*$^{-/-}$ female zebrafish because *nedd8* modulated *ar*, we compared BTs between adult female *ar*$^{+/+}$*nedd8*$^{-/-}$ and *ar*$^{+/-}$*nedd8*$^{-/-}$ zebrafish at 4 mpf. Although *ar*$^{+/-}$*nedd8*$^{-/-}$ females retained some BTs, these females had far fewer BTs than *ar*$^{+/+}$*nedd8*$^{-/-}$ females, both with respect to the total number of BTs on the pectoral fins (i.e. the dorsal surface from the second to the fourth pectoral fin rays) and with respect to the average number of BTs per pectoral fin segment (Fig. 3A-C) (Kang et al., 2013). When *ar*$^{+/+}$*nedd8*$^{-/-}$ and *ar*$^{+/-}$*nedd8*$^{-/-}$ female zebrafish were treated with flutamide (an androgen antagonist) (Martinovic-Weigelt et al., 2011), all BTs disappeared in both mutants, indicating that androgen signaling plays an essential role in BT formation in *nedd8*-null females (Fig. 3A,C) (McMillan et al., 2013). Furthermore, *flk* (*kdrl*) expression was increased in *nedd8*$^{-/-}$ females compared with *nedd8*$^{+/+}$ females (Fig. 3D); *flk* may be associated with androgen-induced BTs formation (McMillan et al., 2013). As expected, flutamide treatment reduced *flk* expression compared with untreated controls (Fig. 3D).

Importantly, BTs did not develop on the pectoral fins of adult male and female *ar*$^{-/-}$*nedd8*$^{-/-}$ zebrafish (Fig. S10), further suggesting that androgen signaling is essential for BT formation, and that *nedd8* acts upstream of *ar*.

Loss of *nedd8* upregulated the androgen responsive gene *kitlga* and the male determination genes *amh* and *dmrt1* in zebrafish ovaries, but loss of one copy of *ar* counteracted this effect

To determine how *nedd8* affects *ar* function and ovarian maturation, we quantified the expression level of *kitlga* (Yao and Ge, 2015) and two male determination genes, *amh* and *dmrt1* (Lin et al., 2017) in female zebrafish with different genetic backgrounds. To confirm that *kitlga* was indeed an androgen responsive gene, we measured its expression in zebrafish after androgen injection. Injection with dihydrotestosterone (DHT) induced *kitlga* expression in female zebrafish (Fig. S11A); *kitlga* was more strongly upregulated in *nedd8*$^{-/-}$ females than in *nedd8*$^{+/+}$ females (Fig. 4A), but *kitlga* gene expression in *ar*$^{+/-}$*nedd8*$^{-/-}$ females was substantially lower than *kitlga* gene expression in *ar*$^{+/+}$*nedd8*$^{-/-}$ females. When both copies of *ar* were deleted in female zebrafish (i.e. *ar*$^{-/-}$*nedd8*$^{-/-}$ mutants), *kitlga* expression was barely detectable (Fig. 4A). Notably, flutamide treatment dramatically reduced *kitlga* expression in *ar*$^{+/+}$*nedd8*$^{+/+}$ (wild type), *ar*$^{+/+}$*nedd8*$^{-/-}$, and *ar*$^{+/-}$*nedd8*$^{-/-}$ females (Fig. 4A), indicating that androgen signaling strictly controls *kitlga* gene expression.

Similarly, the expression levels of *amh* and *dmrt1* were also greater in *nedd8*$^{-/-}$ females than in *nedd8*$^{+/+}$ females, but loss of one copy of *ar* reversed this effect (Fig. 4B,C). Notably, in the *nedd8*-null background, the expression level of *ar* was highest in *ar*$^{+/+}$ ovaries, but moderate in *ar*$^{+/-}$ ovaries and undetectable in *ar*$^{-/-}$ ovaries (Fig. S11B). Our results thus suggest that the loss of *nedd8* enhances androgen signaling and disrupts ovarian maturation by upregulating male determination genes and downregulating female determination genes (Fig. S6E,F; Fig. 4B,C). Therefore, *nedd8* might modulate androgen signaling.

Neddylation inhibited *ar* transcriptional activity

To investigate whether *nedd8* directly regulates *ar*, we initially used promoter assays to test how *nedd8* expression affected *ar* transcriptional activity. Promoter assays using probasin (*Pbsn*), a well-described androgen-responsive gene in rats, has been widely used to monitor androgen signaling (Greenberg et al., 1994; Wang et al., 2014; Yan et al., 1997). Therefore, we used a *Pbsn* promoter luciferase reporter to monitor zebrafish *ar* activity. In the presence

Development (2020) 147, dev194886. doi:10.1242/dev.194886

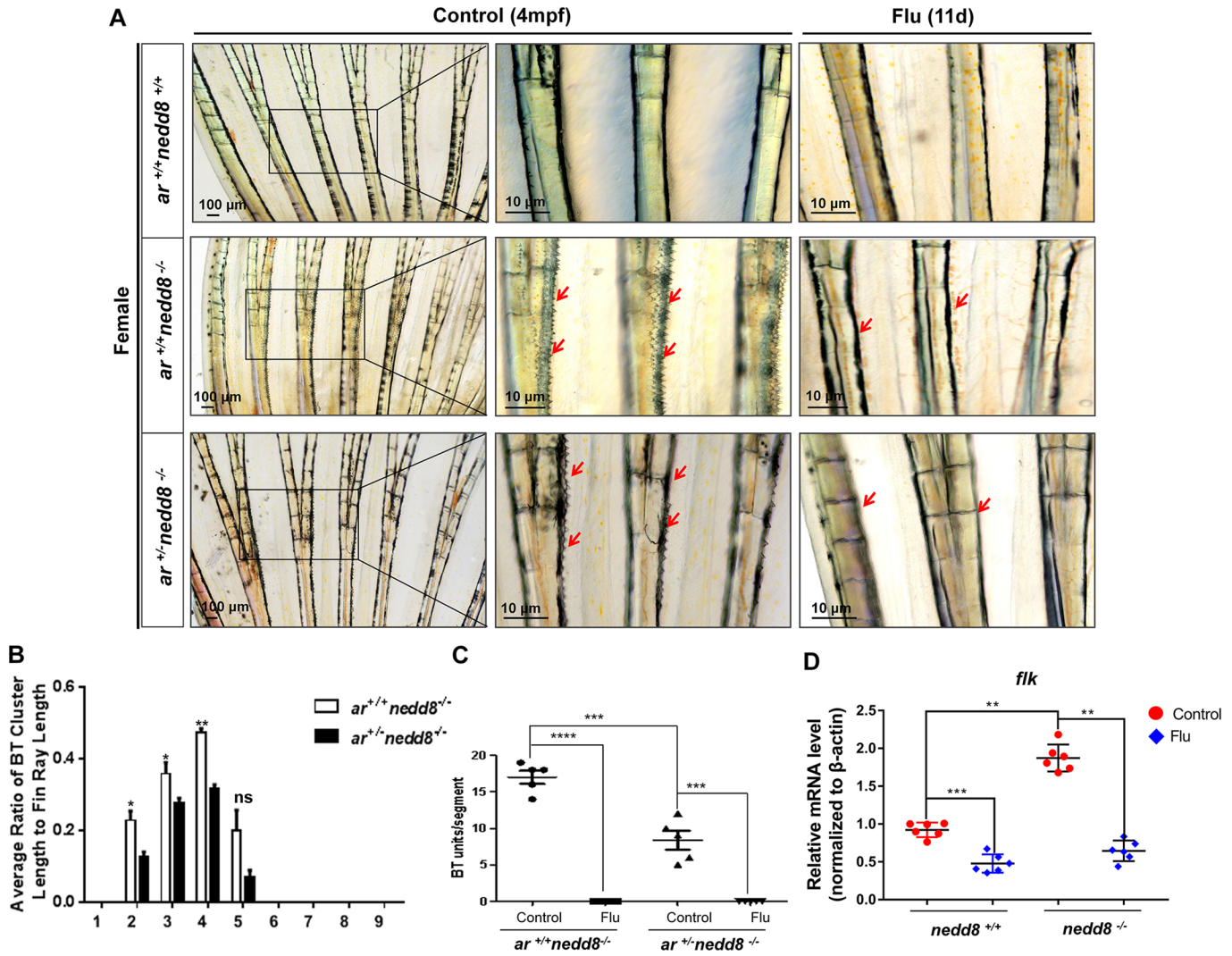

Fig. 3. Loss of *ar* and/or treatment with the androgen antagonist flutamide eliminates BTs on the pectoral fins of *nedd8*$^{-/-}$ female zebrafish. (A) Images showing the effects of flutamide (Flu) treatment or *ar* knockout on the *nedd8*-knockout-induced BTs on the pectoral fins of female *ar*$^{+/+}$*nedd8*$^{-/-}$ and *ar*$^{+/-}$*nedd8*$^{-/-}$ zebrafish at 4 mpf. The red arrows indicate BTs. Panels on right show magnification of areas indicated by boxed areas on left. (B) Ratio of BT cluster length to pectoral fin ray length in *ar*$^{+/+}$*nedd8*$^{-/-}$ (*n*=6) and *ar*$^{+/-}$*nedd8*$^{-/-}$ (*n*=6) female zebrafish (4 mpf), plotted against pectoral fin ray number. (C) BTs/segment in *ar*$^{+/+}$*nedd8*$^{-/-}$ (*n*=6) and *ar*$^{+/-}$*nedd8*$^{-/-}$ (*n*=6) female zebrafish (4 mpf), treated with 2 mg/l flutamide for 11 days or untreated (control). (D) *flk* gene expression in the pectoral fins of *nedd8*$^{+/+}$ (*n*=6) and *nedd8*$^{-/-}$ (*n*=6) female zebrafish (4 mpf) treated with 2 mg/l flutamide for 11 days or untreated (control). Gene expression was quantified using quantitative real-time PCR (qPCR). Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 (unpaired Student's *t*-test).

of DHT, the overexpression of the *ar* gene in epithelioma papulosum cyprinid cells (EPC), a fish cell line established from carp (*Cyprinus carpio*) (Fijan et al., 1983), activated the *Pbsn* promoter (Fig. 5A). However, when the *nedd8* gene was overexpressed, *Pbsn* promoter activity decreased (Fig. 5A), suggesting that Nedd8 might conjugate to Ar and inhibit *ar* transcriptional activity. The overexpression of genes encoding the *nedd8* enzyme E1 (*uba3*) and E2 (*ubc12*; *ube2m*) reversed the increase in *Pbsn* promoter activity induced by *ar* overexpression in the presence of DHT (Fig. 5B). This suggested that *nedd8* might inhibit Ar transcriptional activity through nedd8-conjugation. Furthermore, the overexpression of a mutated form of *nedd8* (nedd8ΔGG, a conjugation-defective mutant generated by Gly-75/76 deletion) (Ryu et al., 2011) did not suppress the *Pbsn* promoter activity by *ar* overexpression in the presence of DHT – instead, the overexpression of *nedd8*-ΔGG increased *ar* transcription (Fig. 5C). On the contrary, the overexpression of the deneddylase *senp8*, enhanced the *Pbsn* promoter activity induced by *ar* overexpression in the presence of DHT (Fig. 5D), further implying that neddylation may be responsible for the inhibition of Ar activity. These data suggested that *nedd8* might inhibit *ar* transcriptional activity through neddylation modification.

ar was modified by neddylation at lysine 475 and lysine 862

To determine whether zebrafish *ar* was indeed modified by neddylation, we initially performed *in vitro* neddylation assays using Ni-NTA-agarose beads. Overexpression of wild-type *nedd8* caused clear nedd8-conjugated Ar bands, but no band was detected when *nedd8* was absent (Fig. 6A). By contrast, overexpression of the *nedd8* mutant (nedd8ΔGG) did not cause Ar modification (Fig. S12A) (Vogl et al., 2015). Moreover, treatment with the E1 inhibitor MLN4924 reduced nedd8-conjugation with Ar, but overexpression of E1 (*uba3*) and E2 (*ubc12*) enhanced nedd8-conjugation with Ar (Fig. 6B).

Development (2020) 147, dev194886. doi:10.1242/dev.194886

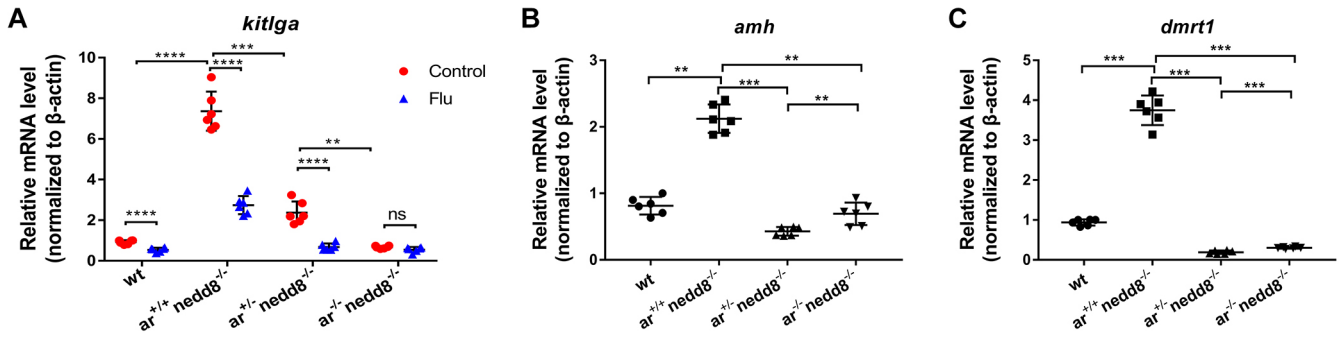

Fig. 4. Activation of the androgen signaling pathway in the ovaries of *nedd8*-null female zebrafish. (A) *kitlga* mRNA expression in the ovaries of female zebrafish with various genetic backgrounds at 4 mpf, treated with 2 mg/l flutamide or untreated (control) (n=6 per group): $ar^{+/+}nedd8^{+/+}$ (wt), $ar^{+/+}nedd8^{-/-}$, $ar^{+/-}nedd8^{-/-}$ and $ar^{-/-}nedd8^{-/-}$. (B) *amh* mRNA expression in the ovaries of female zebrafish with various genetic backgrounds at 4 mpf (n=6 per group): $ar^{+/+}nedd8^{+/+}$ (wt), $ar^{+/+}nedd8^{-/-}$, $ar^{+/-}nedd8^{-/-}$ and $ar^{-/-}nedd8^{-/-}$. (C) *dmrt1* mRNA expression in the ovaries of female zebrafish with various genetic backgrounds at 4 mpf (n=6 per group): $ar^{+/+}nedd8^{+/+}$ (wt), $ar^{+/+}nedd8^{-/-}$, $ar^{+/-}nedd8^{-/-}$ and $ar^{-/-}nedd8^{-/-}$. Data are mean±s.e.m. **P<0.01, ***P<0.001, ****P<0.0001 (unpaired Student's t-test). ns, not significant.

It has been shown that *nedd8* overexpression triggers unphysiological neddylation pathways (Enchev et al., 2015). To further confirm that Ar was indeed modified by Nedd8, we sought to conduct *in vivo* neddylation assays. A monoclonal antibody against zebrafish Ar was developed and its specificity was validated using zebrafish testes (Fig. S13). The *in vivo* neddylation assay indicated that endogenous Ar was modified by Nedd8 in $nedd8^{+/+}$ zebrafish testes, but not in $nedd8^{-/-}$ zebrafish testes (Fig. 6C).

To determine which residue(s) in Ar was (were) modified by neddylation, we performed mutant screening by taking advantage of *in vitro* neddylation assays. We mutated all lysine residues in Ar to arginine and made a series of mutants (Fig. 6D). Through *in vitro*

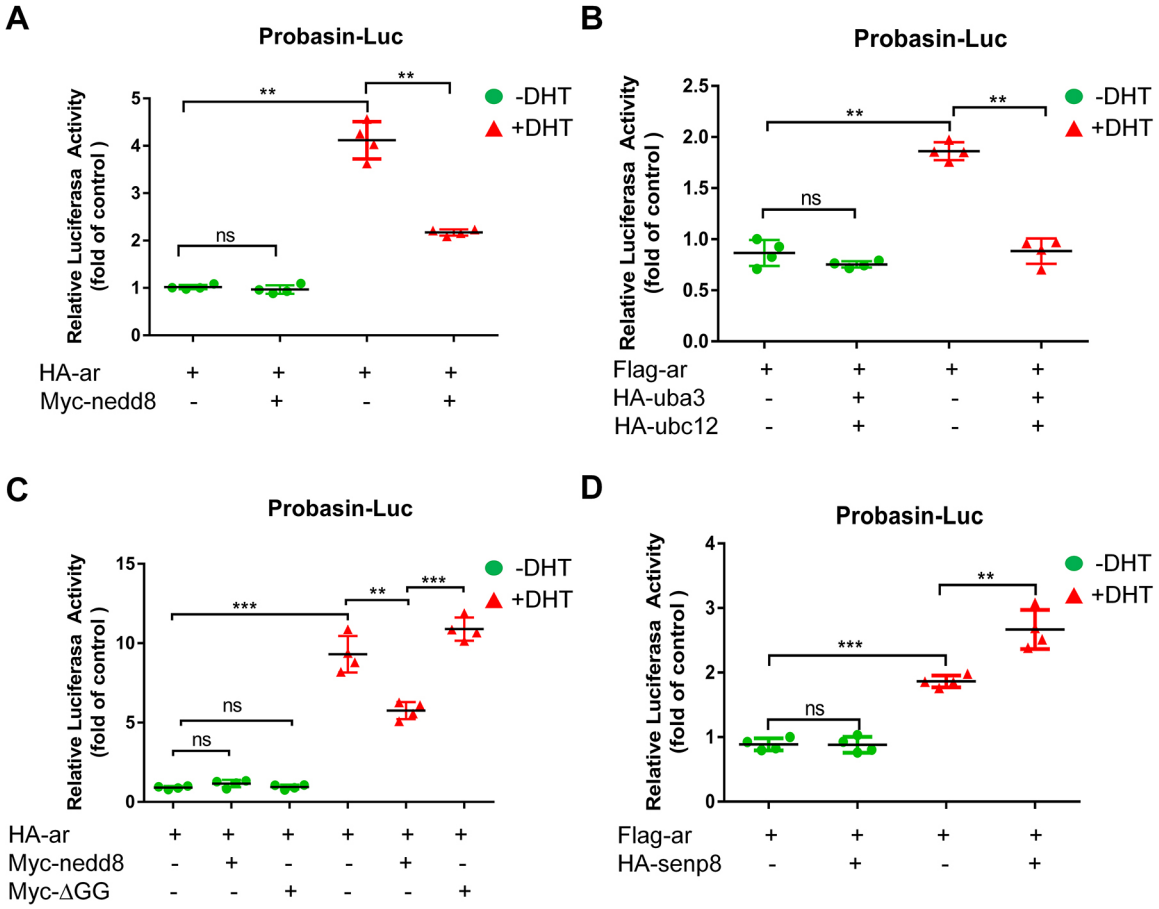

Fig. 5. *nedd8* inhibits *ar* transcriptional activity. (A) Relative luciferase activity of the *Pbsn* promoter in response to *nedd8* overexpression in EPCs co-transfected with HA-ar-expressing plasmid, with and without treatment with 20 nM DHT. (B) Relative luciferase activity of the *Pbsn* promoter in response to *uba3* and *ubc12* overexpression in EPCs expressing *ar*, with and without treatment with 20 nM DHT. (C) Relative luciferase activity of the *Pbsn* promoter in response to overexpression of *nedd8* and the *nedd8* mutant (*nedd8*-ΔGG) in EPCs expressing *ar*, with and without treatment with 20 nM DHT. (D) Relative luciferase activity of the *Pbsn* promoter in response to overexpression of *senp8* in EPCs expressing *ar*, in the presence and absence of 20 nM DHT. Four independent experiments were performed and each measurement was conducted in quadruplicate. Data are mean±s.e.m. **P<0.01, ***P<0.001 (one way ANOVA). ns, not significant.

Development (2020) 147, dev194886. doi:10.1242/dev.194886

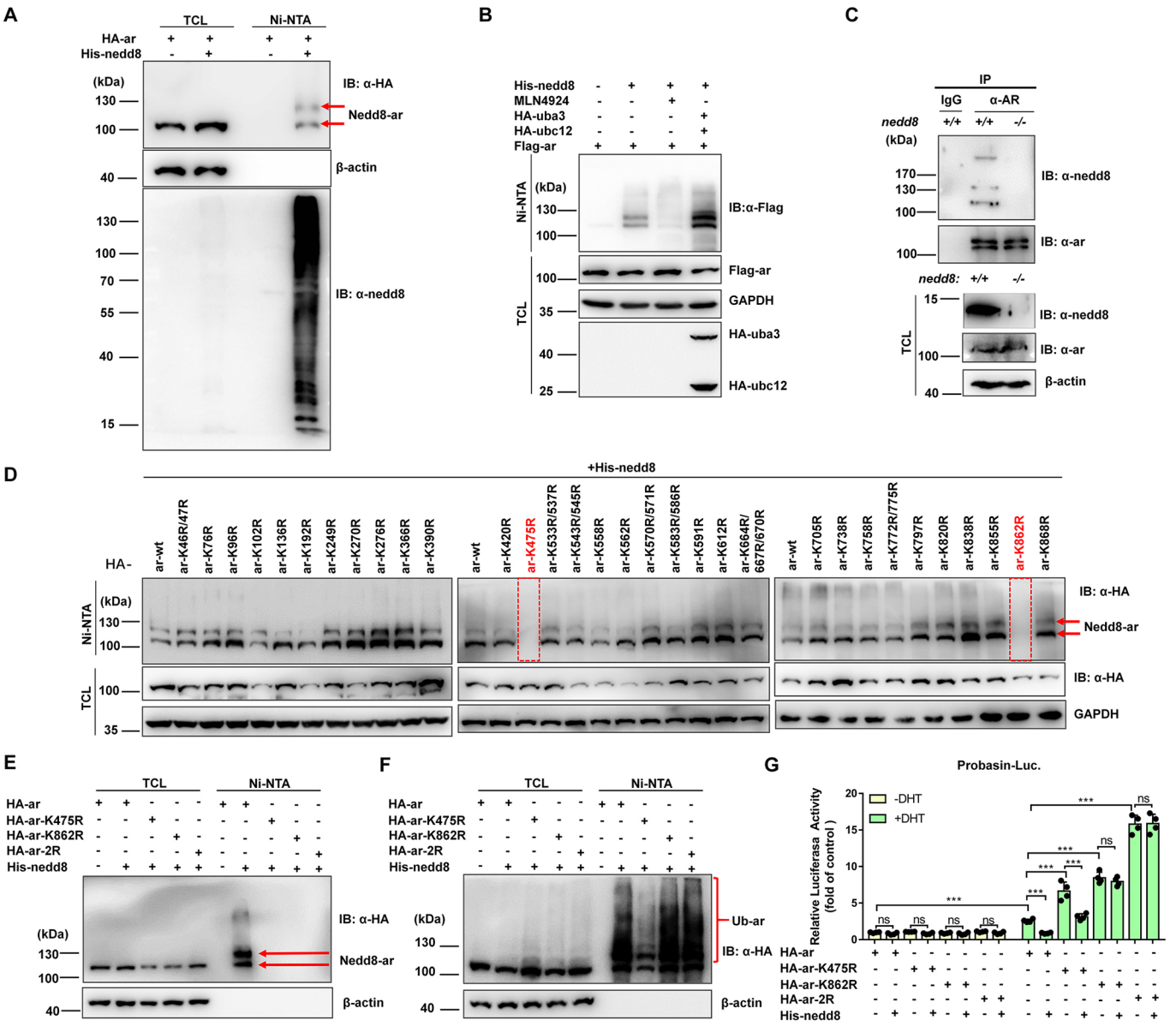

Fig. 6. *ar* is modified by neddylation. (A) Western blot showing the neddylation of *ar* in HEK293 T cells in response to *nedd8* overexpression. HEK293 T cells were transfected with the indicated plasmids. After 36 h, the cells were lysed in guanidinium chloride, and purified with Ni-NTA agarose. (B) Western blot showing that the addition of MLN4924 prevented *ar* neddylation, whereas the overexpression of *uba3* and *ubc12* enhanced *ar* neddylation. (C) *In vivo* neddylation assay showing that endogenous Ar was neddylated in the wild-type zebrafish (*nedd8*$^{+/+}$) but not in *nedd8*-null zebrafish (*nedd8*$^{-/-}$). The protein lysates from *nedd8*$^{+/+}$ or *nedd8*$^{-/-}$ zebrafish testes were immunoprecipitated with mouse IgG (control) or anti-zebrafish Ar antibody, respectively; and Co-IP was detected by anti-nedd8 antibody under partially denaturing conditions. (D) The potential neddylated lysine residues in zebrafish Ar were screened by mutagenesis and *in vitro* neddylation assays. HA-tagged wild-type Ar (HA-ar) and its all lysine-to-arginine mutants were transfected into HEK293 T cells together with His-Nedd8, respectively. Ni-NTA agarose beads were used to purify His-Nedd8 and neddylation was detected by anti-HA antibody. The red dashed rectangle marks the mutant without detected neddylation. (E) No neddylation in ar-K475R, ar-K862R and ar-2R (K475R/K862/R) mutants was further confirmed by neddylation assay. (F) Ubiquitylation assay showing that ubiquitylation was not detected in ar-K475R, but it was in ar-K862R and ar-2R (K475R/K862/R) mutants. Ni-NTA agarose beads were used to purify His-ubiquitin and ubiquitylation was detected by anti-HA antibody. (G) The relative luciferase activity of the *Pbsn* promoter in EPC cells expressing the wild-type Ar (400 ng/well), and the Ar mutants (400 ng/well), K475R and K862R together with or without His-Nedd8, in the presence and absence of 20 mM DHT. The red arrows indicate neddylated Ar bands. IB, immunoblotting; IP, immunoprecipitation; TCL, total cell lysate. Data are mean±s.e.m. ***P<0.001 (one way ANOVA). ns, no significance.

neddylation assays, we found that ar-K475R and ar-K862R completely lost neddylation by Nedd8 overexpression (Fig. 6D). Therefore, lysine 475 and lysine 862 might be the target sites of neddylation, which was further confirmed by the double mutant (Ar-2R, in which K475 and K862 were mutated into arginine simultaneously) (Fig. 6E). Of note, these two sites are evolutionarily conserved (Fig. S12B).

By *in vitro* ubiquitylation assays, we found that Ar-K475R could not be ubiquitylated, but wild type (Ar), Ar-K862R and Ar-2R (K475R/K862R) could still be ubiquitylated, indicating that K475 in Ar is not only one neddylated site, but also one ubiquitylated site (Fig. 6F). Thus, K862 in Ar might be targeted by neddylation specifically. Subsequent *Pbsn* promoter luciferase reporter assays showed that the activity of Ar-K475R was still suppressed by *nedd8*

Development (2020) 147, dev194886. doi:10.1242/dev.194886

overexpression, but the activity of Ar-K862R and Ar-2R (K475R/K862R) was not suppressed by *nedd8* overexpression (Fig. 6G), suggesting that K862 is the key neddylated site accounting for the modulation of Ar activity in response to neddylation modification. Intriguingly, in the presence of DHT, the activity of Ar-K475R, Ar-K862R and Ar-2R (K475R/K862R) was higher than that of wild-type Ar, implying that the modification of these two lysine residues, either ubiquitylation (K475) or neddylation (K862), was crucial for the inhibition of Ar transcriptional activity (Fig. 6G). Taken together, these data suggest that neddylation might occur at K475 and K862 of Ar, and that neddylation on K862 of Ar effectively repressed *ar* transactivity.

It has been reported that the SPOP-CUL3-RBX1 ubiquitin ligase complex targets mammalian AR for degradation (An et al., 2014). Given that the SPOP protein is highly evolutionarily conserved between zebrafish and mammals [97% amino acid (aa) match] and *nedd8* is well known to activate Cullin-based ubiquitin ligases (Petroski and Deshaies, 2005), we speculated whether *nedd8* could inhibit Ar through the activation of the SPOP-CUL3-RBX1 ubiquitin ligase. Initially, we examined Ar protein levels between $nedd8^{+/+}$ and $nedd8^{-/-}$ zebrafish testes and we found no difference in Ar protein level between $nedd8^{+/+}$ and $nedd8^{-/-}$ (Fig. S14A). Further *in vivo* ubiquitylation assays showed that Ar ubiquitylation in $nedd8^{-/-}$ zebrafish was similar to that in $nedd8^{+/+}$ zebrafish (Fig. S14B). Moreover, overexpression of neddylation components (*nedd8*, *uba3* and *ubc12*) did not enhance the Spop-induced suppressive effect on the activity of the *Pbsn* reporter (Fig. S14C). Consistently, knockdown of *spop* in EPC cells also had no effect on the activity of the *Pbsn* reporter when Ar and Nedd8 were overexpressed with or without DHT treatment (Fig. S14D,E). Therefore, the inhibition of Ar by *nedd8* was not mediated by affecting the activity of SPOP-CUL3-RBX1 ubiquitin ligase.

Based on these observations, we proposed a working model of the regulatory effects of *nedd8* on *ar* activity and gonadogenesis (Fig. 7). When *nedd8* is intact, the transcriptional activity of *ar* is strictly controlled by neddylation, and Nedd8 conjugation appears to serve as a suppressor of *ar* activity. Under these conditions, zebrafish gonads develop normally, and differentiate into testes or ovaries at various time points. If *nedd8* is disrupted, however, *ar* loses control by neddylation and then *ar* activity increases substantially after binding to DHT. As a result, in female zebrafish, the male determination genes (e.g. *amh* and *dmrt1*) are upregulated, and the female determination genes (e.g. *cyp19a1a* and *foxl2*) are inhibited. Conversely, in male zebrafish, the male determination genes (e.g. *amh* and *dmrt1*) are upregulated, but the female determination genes (e.g. *cyp19a1a* and *foxl2*) are not

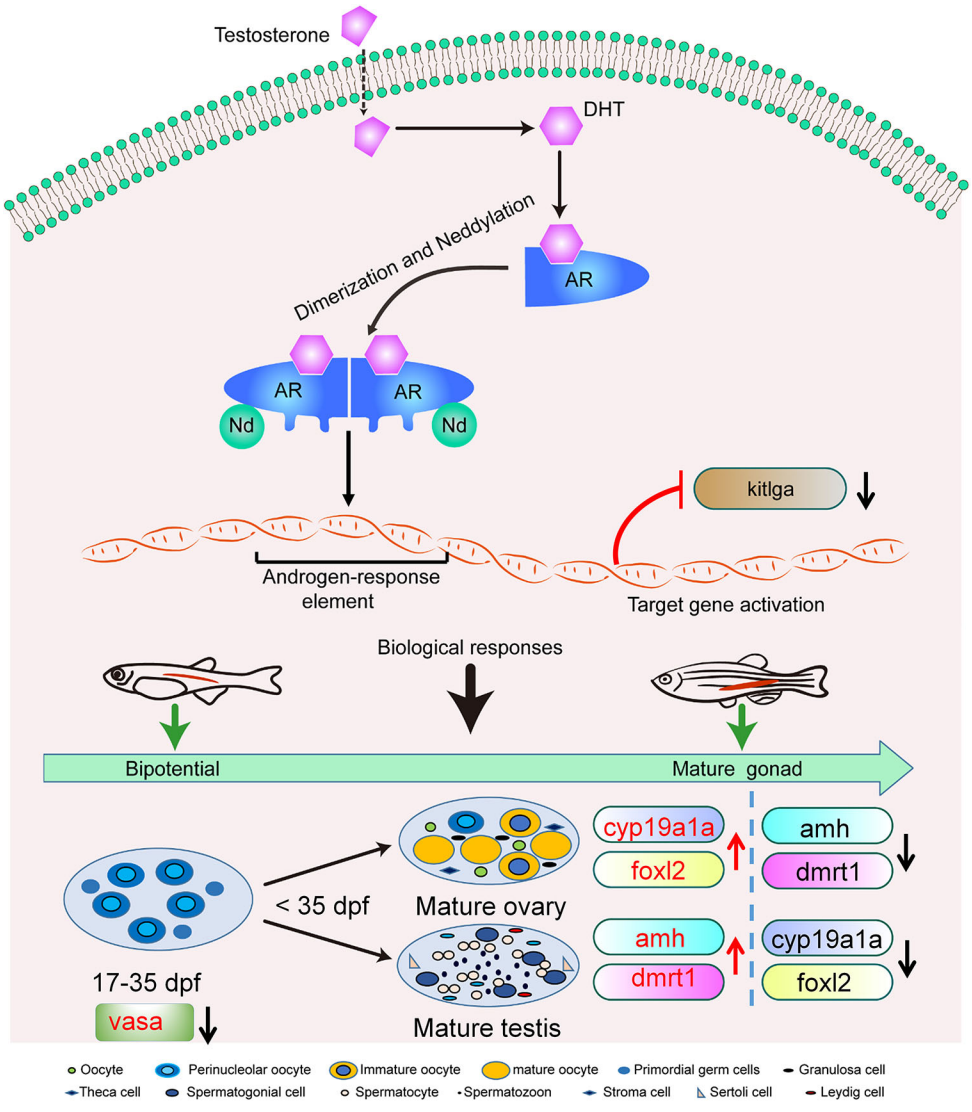

Fig. 7. A working model of the relationship between *ar* neddylation and zebrafish gonadogenesis. When *nedd8* is intact, the transcriptional activity of *ar* is strictly controlled by neddylation. Nedd8-conjugation appears to serve as a brake for controlling *ar* activity. The female determination markers, such as *cyp19a1a* and *foxl2*, and early male determination markers, such as *amh* and *dmrt1*, facilitate juvenile gonads to differentiate into ovaries or testes at different developmental stages. Therefore, zebrafish gonads develop normally. If *nedd8* is deleted, *ar* loses the brake control of neddylation and then *ar* activity increases substantially after binding to DHT. As a result, PGCs in *nedd8*-null zebrafish are reduced during early embryogenesis and the majority of juvenile gonads develop into testes and produce more males. Accordingly, in females, the male determination genes (e.g. *amh* and *dmrt1*) are upregulated and the female determination genes (e.g. *cyp19a1a* and *foxl2*) are inhibited. Conversely, in male zebrafish, the male determination genes are upregulated, but the female determination genes are not altered at 3 mpf. Consequently, oogenesis is disrupted, resulting in defects in folliculogenesis, and the expression of masculinized secondary sexual characteristics. In addition, *nedd8*-null males exhibit normal fecundity and become super-activated owing to the loss of androgen signaling inhibition.

Development (2020) 147, dev194886. doi:10.1242/dev.194886

altered at 3 mpf. Consequently, oogenesis is disrupted, resulting in defects in folliculogenesis and the expression of masculinized secondary sexual characteristics. In addition, *nedd8*-null males exhibit normal fecundity and become super-activated due to the loss of androgen signaling inhibition.

DISCUSSION

Neddylation is essential in all model organisms except *Saccharomyces cerevisiae* (Enchev et al., 2015). Disruption of some components of the NEDD system affects early embryogenesis dramatically, leading to embryonic lethality (Tateishi et al., 2001; Zou et al., 2018). In this study, we could generate viable Nedd8-null zebrafish, which can be used for a series of genotype analysis. Thus, our work provides a practical vertebrate model for further revealing the physiological function of neddylation *in vivo*.

In addition to CRLs, other proteins are modified by neddylation (Enchev et al., 2015; Ryu et al., 2011; Vogl et al., 2015; Xirodimas et al., 2004; Zou et al., 2018; Zuo et al., 2013). The importance of protein neddylation is clear (Enchev et al., 2015). Of note, *uba3*, the catalytic subunit of the activating enzyme of the Nedd8 conjugation pathway, could inhibit steroid receptor function, linking neddylation to the suppression of steroid receptor function (Fan et al., 2002). Uba3 has also been shown to bind to the ligand binding domain (LBD) of AR (Fan et al., 2002; Nadal et al., 2017), suggesting that neddylation might occur in the LBD of AR. In this study, we identify that K862, which is located in LBD of Ar, is the key target residue of neddylation and its modification influences *ar* function dramatically, providing further evidence to support the fact that neddylation occurs in the LBD of Ar and has an important role in modulating Ar transactivity. Interestingly, K475 of Ar is not only modified by neddylation, but also modified by ubiquitylation. In addition, although Ar-K475R has higher activity than wild-type Ar, its activity is suppressed by overexpression of Nedd8. These observations suggest that K475 neddylation may not affect Ar function; in contrast, other modifications in K475, such as ubiquitylation, may account for the regulation of Ar function through modifying K475 of Ar. Further investigation of this phenomenon and the underlying mechanisms will expand our knowledge about the regulation of Ar function through post-translational modifications and the crosstalk between different modifications.

Neddylation has been shown to influence germ cell differentiation in *Drosophila* ovaries (Lu et al., 2015; Pan et al., 2014) and to regulate gene expression in stem cells in *Drosophila* testes (Qian et al., 2015). In the *Drosophila* ovary, if *Csn4*, a deneddylase-like gene, is present, Nedd8 promotes self-renewal. If *Csn4* is absent, Nedd8 promotes differentiation (Lu et al., 2015; Pan et al., 2014). Here, we find that loss of *nedd8* in zebrafish causes reduced PGCs at early embryogenesis, resulting in more males and immature oocytes versus mature oocytes compared with wild-type zebrafish. This observation appears to be consistent with *nedd8* function in the *Drosophila* ovary.

In this study, we used a zebrafish model to demonstrate that zebrafish *nedd8* facilitates ovarian maturation and the maintenance of female secondary sexual characteristics. However, the loss of *ar* partially rescues ovarian function in *nedd8*-null females. Thus, not only did our results demonstrate that neddylation is important for gonadogenesis, but our data also indicated that *ar* might be a novel target of neddylation.

In fish, the plasticity of gonadal sex differentiation in response to treatment with exogenous steroids is well known (Godwin, 2010): sex steroids play a key role in fish sex determination (Nakamura, 2010). Androgen treatment for early embryonic fish transitions ovaries to testes, producing physiological males; this technique is widely used to generate all-female populations in the aquaculture industry (Nakamura, 2010). Thus, it is clear that additional androgen affects sex determination. Of note, in *nedd8* mutants, as well as an increase in Ar activity, the production of androgen (11-KT) was also significantly increased, suggesting that *nedd8* might also suppress androgen production. Therefore, the defects of the *nedd8* mutant could be due to both increased androgen production and androgen receptor signaling. To further figure out the mechanisms underlying this phenomenon will help us to understand the role of *nedd8* in gonadal development more completely.

Studies of *ar*-knockout zebrafish have shown that androgen signaling plays an essential role in the maintenance of ovary function (Crowder et al., 2018; Tang et al., 2018; Yu et al., 2018). Here, the *nedd8*-knockout-induced overactivation of the androgen receptor also disrupted ovarian oogenesis and led to the production of more fertile males. However, the female:male ratio of the $nedd8^{-/-}$ offspring suggested that the *nedd8*-knockout had a less dramatic effect on androgen levels than did direct androgen treatment. Our results indicated that appropriate levels of androgen were necessary for normal ovarian development and function. Intriguingly, the over-masculinized behaviors exhibited by the $nedd8^{-/-}$ males suggested that androgen signaling also plays an important role in fish behavior.

As it is difficult to ascertain the sex of, and collect blood from, early-stage zebrafish embryos, we were unable to determine whether the loss of *nedd8* affected serum hormone levels at the early stages of development. Based on our observations that the defects of oogenesis appeared in $nedd8^{-/-}$ ovaries at the early stage (24 dpf), the inhibitory role of *nedd8* on Ar activity should have a direct effect on oogenesis of $nedd8^{-/-}$ females. At 3 mpf, the serum levels of 11-KT and estradiol were similar in $nedd8^{+/+}$ and $nedd8^{-/-}$ males. However, we did observe the difference in serum hormone levels between 3 mpf wild-type and *nedd8*-null females, and between 6 mpf wild-type and *nedd8*-null males/females. Of note, expression of *cyp19a1a*, an aromatase which converts androgens to estrogens (Dranow et al., 2016), was downregulated in $nedd8^{-/-}$ ovaries at 3 mpf, but upregulated in $nedd8^{-/-}$ ovaries at 6 mpf. In fact, the serum level of estradiol coordinates with expression of *cyp19a1a*. The changes of serum hormones in $nedd8^{-/-}$ females might also influence oogenesis, oocyte maturation, BT growth and overactive behaviors secondarily. To further distinguish the direct and indirect effects of *nedd8* will help us to fully understand the role of *nedd8*.

Zebrafish sex determination and gonad differentiation are complicated, particularly with respect to the molecular control of these processes (Lau et al., 2016; Liew and Orban, 2014; Lin et al., 2017; Orban et al., 2009; Uchida et al., 2002; Yang et al., 2017). Recently, the roles of *ar* in sexual determination, ovarian development, and maintenance of secondary sexual characteristics have been well-characterized by analyzing *ar*-knockout zebrafish (Crowder et al., 2018; Tang et al., 2018; Yu et al., 2018). Loss of *ar* in zebrafish increases the proportion of female offspring, causes male infertility, leads to defects in oocyte maturation, reduces fecundity and produces males expressing female secondary sexual characteristics (Crowder et al., 2018; Tang et al., 2018; Yu et al., 2018). Here, our results indicated that zebrafish *nedd8* participates in sex determination, ovarian maturation and maintenance of secondary sexual characteristics by modulating *ar* activity, further demonstrating the importance of *ar* in zebrafish sex determination and gonad differentiation.

Importantly, the loss of one copy of *ar* did not completely rescue abnormalities observed in the $nedd8^{-/-}$ zebrafish (e.g. disrupted

Development (2020) 147, dev194886. doi:10.1242/dev.194886

ovarian maturation, reduced egg fertilization rate and the development of BTs on the female pectoral fins). It was possible that the loss of one copy of *ar* was not sufficient to counteract the *nedd8*-knockout-induced increase in *ar* activity. Alternatively, *nedd8* might affect folliculogenesis via mechanisms other than the modulation of *ar* activity only. Indeed, our finding that the defects in ovarian maturation were more severe in $ar^{-/-}nedd8^{-/-}$ females than in $ar^{-/-}nedd8^{+/+}$ females supported this second possibility.

Similar to ubiquitylation, neddylation also requires E3 ligases (Enchev et al., 2015). However, unlike the E3 ligases identified in ubiquitylation, few E3 neddylation ligases have been identified (Xirodimas et al., 2004; Zuo et al., 2013). The identification of the E3 ligases mediating *ar* neddylation is necessary to fully understand the role of neddylation in androgen signaling.

The roles of androgen signaling in prostate cancer pathogenesis has received much recent attention (Watson et al., 2015). Indeed, the post-translational modification of AR may be essential for the development of castration-resistant prostate cancer (CRPC) (Koryakina et al., 2014; Coffey and Robson, 2012; Gaughan et al., 2005; Gioeli and Paschal, 2012; Van der Steen et al., 2013). Here, we demonstrated that Ar was modified by neddylation; this represented a previously undescribed post-translational modification of AR. The association of neddylation with cancer initiation and progression has been widely explored (Abidi and Xirodimas, 2015; Zhou et al., 2018), and the E1 inhibitor of neddylation (MLN4924) has been used in clinical trials for various cancers (Soucy et al., 2010, 2009). Thus, a better understanding of the neddylation mechanisms associated with androgen signaling might inform the development of therapeutic treatments for prostate cancer.

MATERIALS AND METHODS

Zebrafish

Nedd8-null zebrafish and *ar*-null zebrafish have been described previously (Yu et al., 2018, 2019). Zebrafish were maintained in a re-circulating water system according to standard protocol. Fish were maintained at 28.5°C with a photoperiod of 14 h of light and 10 h of darkness, and fed regularly. All experiments with zebrafish were approved by the Institutional Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences under protocol number 2016-018.

Zebrafish drug treatment

Flutamide (Sigma, F9397) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. The final concentration of flutamide for zebrafish treatment was 2 mg/l. Zebrafish (4 mpf) were put into a 1 l tank with flutamide (2 mg/l) and water was changed every day.

DHT (Sigma-Aldrich) injection assay was performed as previously described (Yu et al., 2018). MLN-4924 (Merck) was dissolved in DMSO and used at a final concentration of 1 µM.

Histological analysis

After anesthesia with MS-222, the testes and ovaries of zebrafish were dissected and the GSI (gonad weight/body weight×100%) was obtained. The testes and ovaries were then fixed in 4% paraformaldehyde (PFA) overnight at 4°C. The samples were dehydrated and embedded in paraffin, and cut into 4 µm sections. Hematoxylin and Eosin (H&E) staining, immunohistochemical staining and immunofluorescent staining were performed as described previously (Yu et al., 2018; Zhu et al., 2019).

Fertility assessment

Adult female zebrafish with different genetic backgrounds: $ar^{+/+}nedd8^{+/+}$, $ar^{+/+}nedd8^{-/-}$, and $ar^{+/-}nedd8^{-/-}$ were transferred to breeding tanks respectively; adult wild-type male zebrafish ($ar^{+/+}nedd8^{+/+}$) were put into the same breeding tank at a female:male ratio of 1:1. The number of eggs ovulated and the ovulation rate (ovulation rate=number of spawned females/total number of females×100%) were assessed. If the female zebrafish did not spawn after mating with male zebrafish, the experiments were repeated 7 days later. All experiments were repeated at least three times. The female zebrafish were considered sterile if three attempts did not produce eggs, or the eggs could not be fertilized.

Whole-mount *in situ* hybridization

Whole mount *in situ* hybridization was performed as previously described (Liu et al., 2016). The probe for zebrafish *nedd8* was amplified from cDNA pools using the primers listed in Table S1, and the probe was synthesized using the Transcript Aid T7 High Yield Transcription Kit (Fermentas).

Serum hormone measurement

Blood samples were collected from 3- and 6-month-old zebrafish as described previously (Pedroso et al., 2012). For each zebrafish, 3-10 µl of blood could be collected. Blood collected from three individuals was used as one sample for measurement. The blood samples were centrifuged at 5000 ***g*** for 20 min at 4°C, and the supernatants were separated and purified according to the manufacturer's extraction protocol (Cayman Chemical). 11-KT and estradiol (E2) were measured by competitive enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical) following the manufacturer's instructions. All standards and samples were measured by three independent experiments performed in triplicate.

Cell lines and plasmid construction

EPC cells (Fijan et al., 1983; originally obtained from the American Type Culture Collection) were cultured in Medium 199 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), maintained at 28°C in a humidified incubator containing 5% CO_2. As a fish cell line, EPC cells should be suitable for investigating the regulation of zebrafish androgen receptor activity. HEK293 T cells, a human embryonic kidney cell line (originally obtained from the American Type Culture Collection), were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (HyClone), maintained at 37°C in a humidified incubator containing 5% CO_2. The HEK293 T cell line has extremely high efficiency for plasmid transfection, which is widely used for biochemical assays, such as co-immunoprecipitation assay, *in vitro* ubiquitylation assay, etc.

The *Pbsn*-luciferase reporter has been described previously (Wang et al., 2014). The zebrafish *ar* construct was provided by Dr Zhan Yin (Institute of Hydrobiology, Chinese Academy of Sciences) and was subcloned into pCMV-HA and pCMV-Flag vectors (Clontech Laboratories). All zebrafish *ar* mutants (lysine-to-arginine) were generated using PCR-based mutagenesis and subcloned into the pCMV-HA vector. Full-length cDNAs of zebrafish *nedd8* and mutant *nedd8* (*nedd8*-ΔGG) (1-73 aa) were PCR-amplified and sub-cloned into the pCI-his and pCMV-Myc vectors (Clontech Laboratories). Zebrafish *uba3*, *ubc12*, *senp8* and *spop* were PCR-amplified and subcloned into pCMV-HA or pCMV-Flag vectors. All constructs were verified by DNA sequencing.

Luciferase reporter assay

EPC cells were grown in 24-well plates and transfected with the indicated constructs by VigoFect (Vigorous Biotech), together with pRL-CMV as an internal control. After transfection for 12 h, DHT (40 nM; dissolved in ethanol) was added to the cells and incubated for 12 h. Then the cells were harvested for luciferase assays.

RNA interference for knocking down of *spop* in EPC cells

The total cDNA of EPC was used as a template and the full length of EPC *spop* was cloned by assembling exons and RACE products through overlap PCR. The small interfering RNAs (siRNAs) targeting for *spop* of EPC cells were designed based on the sequence of EPC *spop*. The siRNA sequences are: si-spop #1: 5′-GCCTGATGACAAATTGACA-3′; and si-spop #2: 5′-GTGGAAAACGCAGCAGAGATT-3′. The siRNAs for *spop* and the negative control siRNA (si-nc) were obtained from RiboBio. For knocking down *spop*, EPC cells were seeded in 6-well plates overnight and transfected with 100 nM siRNAs for *spop* or the negative control (si-nc) using X-treme

Development (2020) 147, dev194886. doi:10.1242/dev.194886

GENE HP DNA Transfection Reagent (Roche) following the protocol provided by the manufacturer.

In vitro neddylation and ubiquitylation assays

HEK293 T cells were transfected with the indicated constructs for 16-22 h, and then the cells were harvested. For MLN4924 treatments, after the cells were transfected for 12 h, MLN-4924 (1 μM) (Merck) was added into the medium and incubated for 12-14 h, then the cells were harvested. The cells were lysed using the lysis buffer [6 M guanidinium-HCl, 10 mM Tris-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0), 10 mM β-mercaptoethanol]. The lysates were mixed with Ni-NTA-agarose beads (Qiagen) pre-washed with lysis buffer and rotated at 4°C overnight. The beads were washed five times using washing buffer [8 M urea buffer (pH 8.0); 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl, 10 mM β-mercaptoethanol and 0.1% Triton X-100], and washed another five times using washing buffer II [8 M urea buffer (pH 6.3)]. Subsequently, the beads were eluted with the sample-loading buffer and analyzed by western blot.

In vivo neddylation and ubiquitylation assays

Zebrafish testes dissected from nearly 100 males were lysed with modified lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM Na_3VO_4, 1 mM DTT and 10 mM NaF] supplemented with a protease inhibitor cocktail. After incubation at 100°C for 10 min, the lysate was diluted 10 times with modified lysis buffer without SDS. The lysates were then incubated with the indicated antibody for 3 h at 4°C. Protein A/G-plus agarose beads (Santa Cruz Biotechnology) were added, and the lysates were rotated gently for 8 h at 4°C. The immunoprecipitates were washed at least three times in wash buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1 mM DTT and 10 mM NaF]. Proteins were recovered by boiling the beads in 2×SDS sample buffer and analyzed by western blot. Immunoblotting and co-immunoprecipitation (Co-IP) were performed using the indicated antibodies.

Western blot and immunoprecipitation assay

The following antibodies were used for western blot analysis: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, sc-47724; 1:2000), Flag (M2; Sigma, 1:5000), anti-HA (Covance, MMS-101R, 1:5000), anti-β-actin (Santa Cruz Biotechnology, sc-47778, 1:1000), anti-Nedd8 (Cell Signaling Technology, #2745, 1:1000), anti-Nedd8 (ABclone, #A1163, 1:1000), anti-ubiquitin (Cell Signaling Technology, P4D1, #3936, 1:1000), anti-Spop (Abcam, #137537, 1:1000). A monoclonal anti-zebrafish Ar antibody was raised against the synthesized peptide corresponding to aa 317 to 500 of zebrafish Ar (Dia-An Inc, 1:50). Anti-Vasa (GTX128306, 1:500), anti-Ziwi and anti-Zili antibodies were used as described previously (Zhu et al., 2019). HEK293 T cells were transfected with different combinations for 24 h, then the cells were lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA (pH 8.0), 150 mM NaCl, 1 mM NaF, 1 mM PMSF, 1 mM Na3VO4 and a 1:100 dilution of protease inhibitor mixture (Sigma-Aldrich), After incubation on ice for 1 h, lysates were collected and centrifuged at 10,000 ***g*** at 4°C for 15 min. The total cell lysates were boiled with 1×SDS sample loading buffer, separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Western blot was performed as described previously (Du et al., 2016). The Fujifilm LAS4000 mini-luminescent image analyzer was used to image the blots.

Quantitative real-time PCR analysis

Total RNA was extracted using RNAiso Plus (TaKaRa) following the protocol provided by the manufacturer. cDNAs were synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas). SYBR Green mix (Roche) was used for quantitative RT-PCR assays. The primers are listed in Table S1. *Actb1* (β-actin) was used as an internal control.

Behavior monitoring and observation

Male *nedd8*$^{+/+}$ (n=6 per group) and *nedd8*$^{-/-}$ (n=6 per group) zebrafish (4 mpf) were put into a tank filled with 1 l water respectively and their moving trace within 10 min was recorded respectively using View Point Behavior Technology (Zeb-view). The videos of male zebrafish chasing female zebrafish or chasing food were recorded directly (Movies 1 and 2).

Statistical analysis

Statistical analysis for sex ratio was performed using Microsoft Excel 2007. Other statistical analysis was performed using GraphPad Prism, v5 (GraphPad Software Inc). Significant differences between groups were determined using Student's *t*-test (paired or unpaired, as appropriate) or one-way ANOVA followed by Tukey's test for multiple group comparisons. Data are mean±s.e.m. of three independent experiments performed in triplicate. The difference was considered to be significant if the P<0.05 (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.X., G.Y., X.L.; Methodology: G.Y., X.L.; Validation: G.Y.; Formal analysis: W.X., G.Y.; Investigation: G.Y.; Resources: G.Y., X.L., D.Z., J.W., G.O., Z.C.; Data curation: G.Y.; Writing - original draft: W.X., G.Y.; Writing - review & editing: W.X.; Visualization: W.X., G.Y.; Supervision: W.X.; Funding acquisition: W.X.

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Supplementary information

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Table S1. The primer sequences

Primer	Sequence (5'-3')
nedd8$^{ihb1227/inb1227}$ identification	AATGTGAATCTCGTTCAGGTGG
	AGATGTACAGGAACACAACGTG
ar-RT-PCR	AGTGAAATGGGCCAAAGGAC
	ATCATTGAAGACCAGGTCTGG
amh-RT-PCR	CTCCTGTTCAGTGTCAATCCTG
	GGCCTGTTATCATCCATCGATG
beta-actin-RT-PCR	AGGTCCAATGTGATACCGC
	GCGCCATACAGAGCAGAA
cyp19a1a-RT-PCR	AGATGTCGAGTTAAAGATCCTGCA
	CGACCGGGTGAAAACGTAGA
foxl2-RT-PCR	AACAGCATCCGACACAAC
	AACATATCCTCGCATGCAG
dmrt1-RT-PCR	ACACTGACTGCACATCTG
	AGCTTCCAGACTCTGAAC
flk-RT-PCR	GGATCAACTGCACTGCAC
	GAGACGCAGATGAATCCT
kitlga-RT-PCR	AGAAGTGAGTGGCATGTGC
	CTTCACCTGCAGACGTCCAGCGTTT
nedd8-RT-PCR	CCACAGATAAGGTGGAGAG
	GTCCACCTCTTAGAGCAAG
nedd8-ΔGG identification	ATGCTAATTAAAGTCAAGACAC
	TTATAGAGCAAGAACCAG
nedd8-probe-F	ATGCTAATTAAAGTCAAGACAC
T7-*nedd8*-probe-R	GTAATACGACTCACTATATACATATTTAG GAGAAGCCG

Development • Supplementary information

Fig. S1

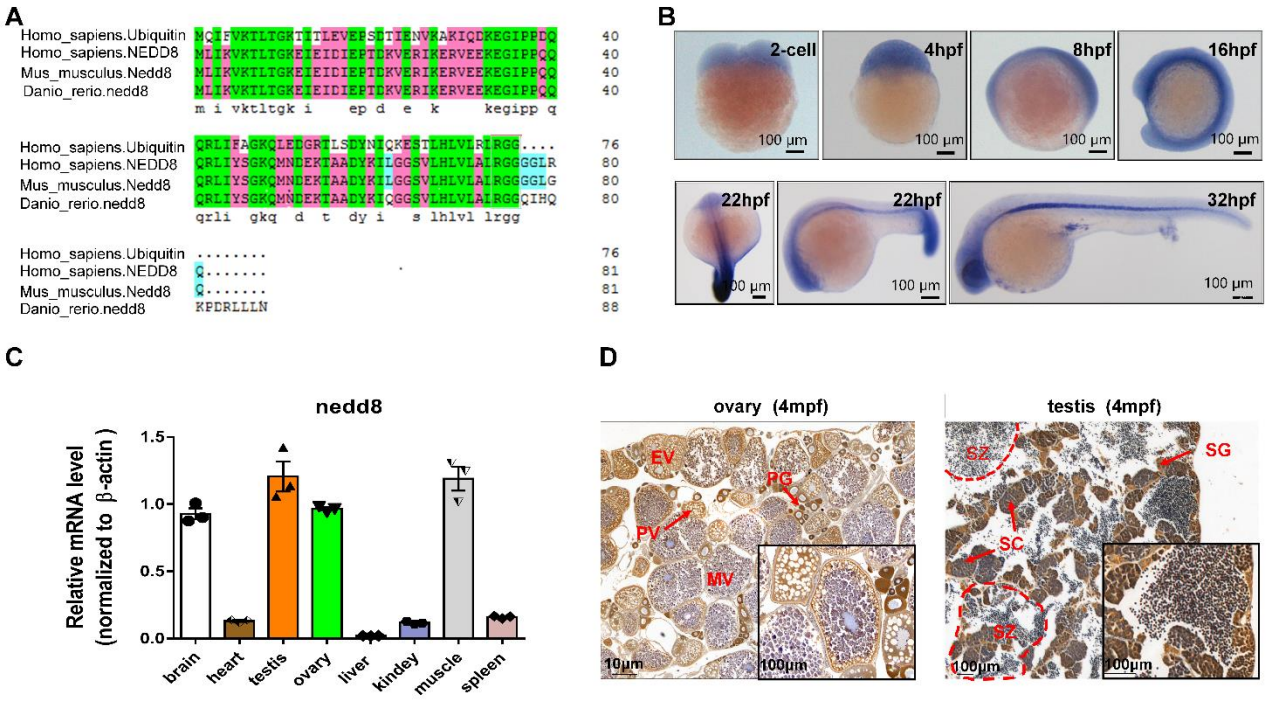


Figure S1. The gene expression patterns of *nedd8* in zebrafish embryo and adult tissues.

(A) Amino acid sequence alignment of Human *Ubiquitin* (ENST00000302182.7), Human *NEDD8* (ENSG00000129559), Mouse *Nedd8* (ENSMUSG00000010376), and Zebrafish *nedd8* (ENSDARG00000007989)**,** the conserved amino acids are highlighted with green background. (B) The gene expression patterns of *nedd8* during zebrafish embryogenesis. Scale bars =100μm. (C)The mRNA expression of *nedd8* in different tissues of adult zebrafish (4 mpf). (D) Immunohistochemical assay showing the gene expression patterns of *nedd8* in the ovaries and testes of adult zebrafish (4 mpf). PG, primary growth stage; PV, previtellogenic stage; EV, early vitellogenic stage; MV, midvitellogenic stage; FG, full-grown stage and lumen are indicated. Scale bars: 100 μm. SG, spermatogonia; SC, spermatocyte; SZ, spermatozoa. Scale bar = 100 μm.

Development • Supplementary information

Fig. S2

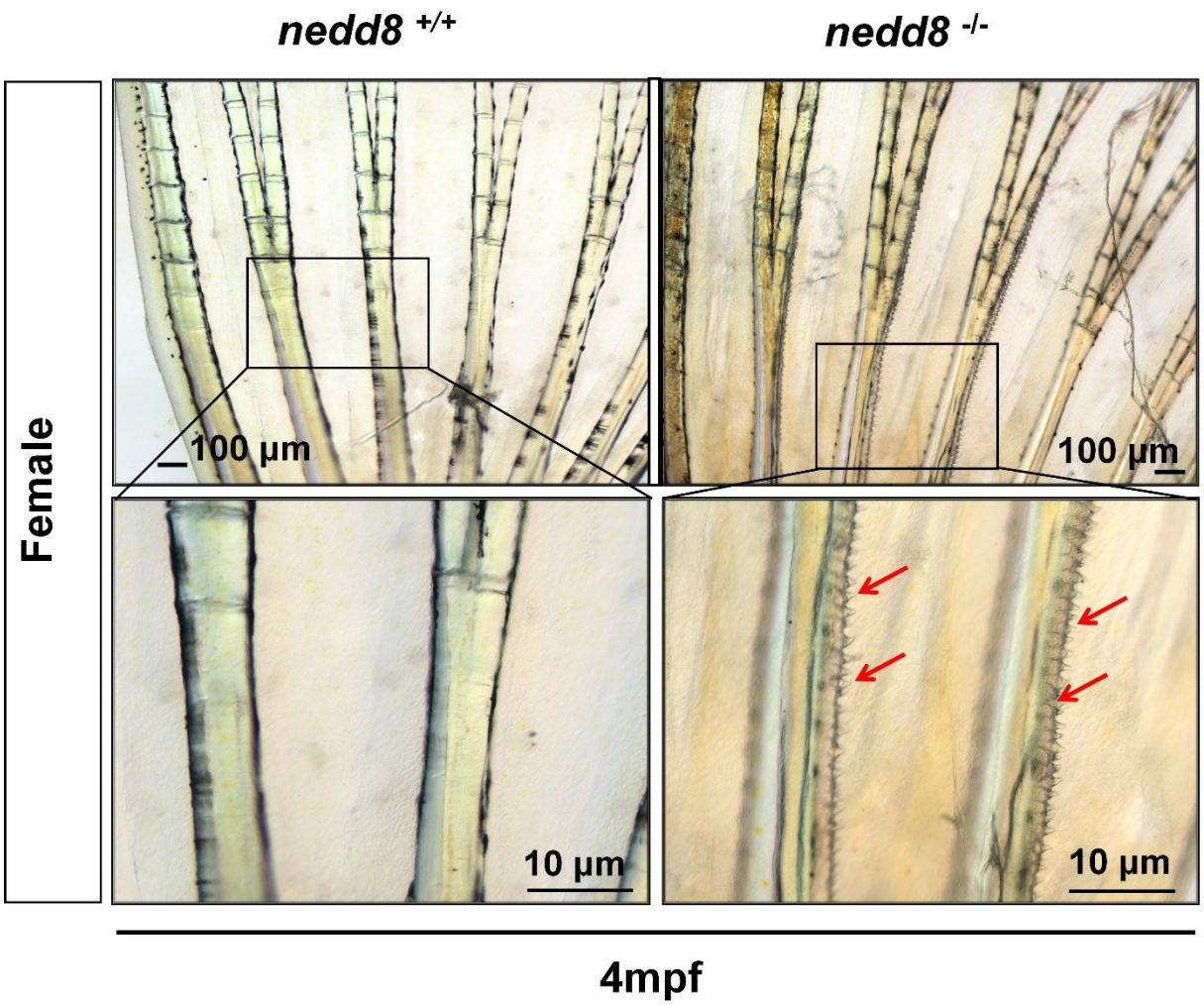


Figure S2. The BTs on the pectoral fins of the *nedd8*-null female zebrafish.

Whole-mount images of the pectoral fins of wildtype (*nedd8*$^{+/+}$) and *nedd8*-null female zebrafish (*nedd8*$^{-/-}$) at 4 mpf. BTs are indicated by red arrowheads. Mpf, months post fertilization. Scale bar = 100μm, 10 μm.

Development • Supplementary information

Fig. S3

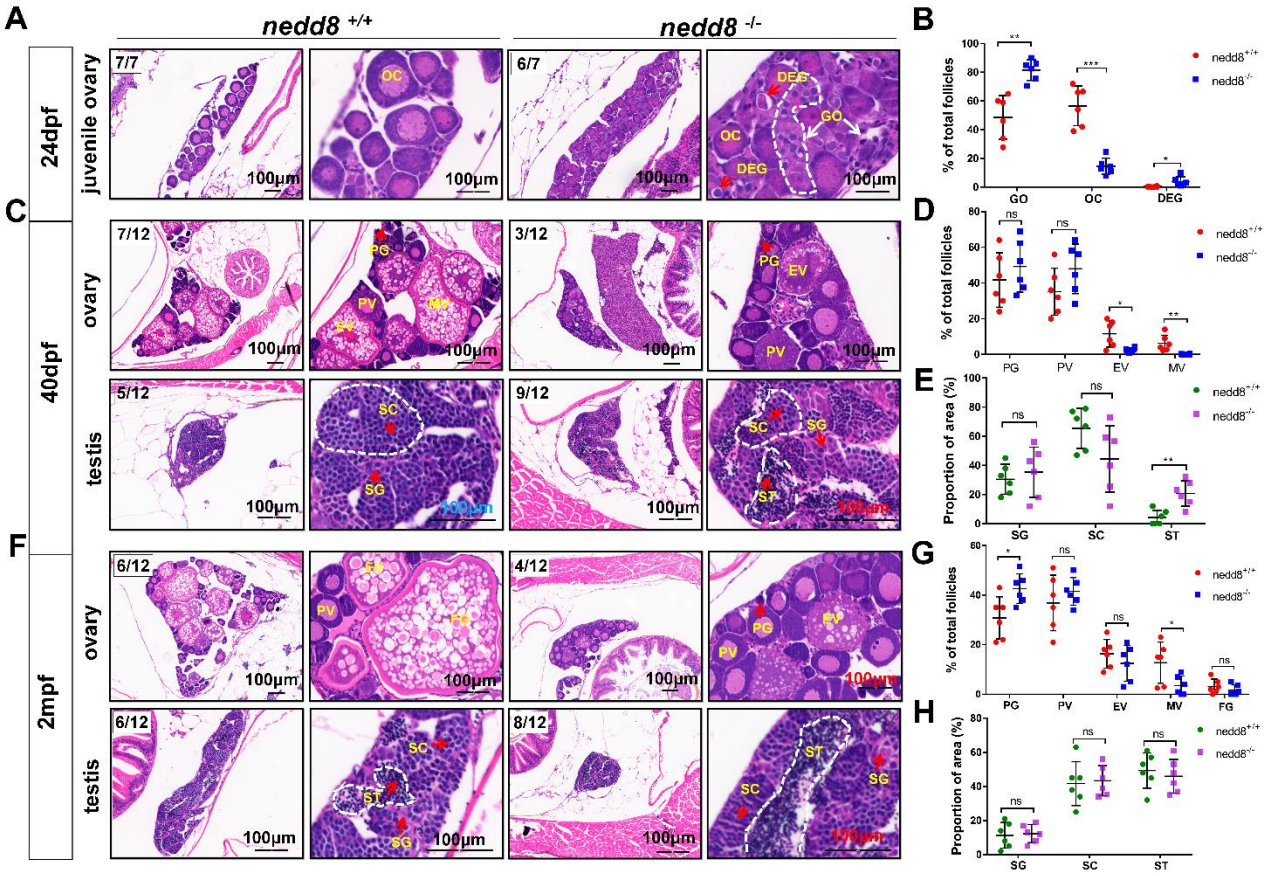


Figure S3. Loss of *nedd8* results in defects of ovarian maturation.

(A) Comparison of juvenile ovaries between *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ at 24dpf. OC, oocyte; GO, gonocyte (indicated by the white arrowheads); DEG, degeneration of perinucleolar oocytes (indicated by the red arrowheads). (B) The percentage of oocytes in different developmental stages in *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ zebrafish at 24 dpf. (C)Comparison of ovaries and testes between *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ at 40 dpf. (D) The percentage of oocytes in different developmental stages in *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ zebrafish at 40 dpf. (E) The proportion of different germ cells in *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish testes at 40 dpf.. (F) Comparison of ovaries and testes between *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ at 2 mpf. (G) The percentage of oocytes at different developmental stages in *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish at 2 mpf. (H) The proportion of different germ cells in *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish testes at 2 mpf. The oocyte number counting was based on 3 sections/per ovary and 6 fish/per genotype. PG, primary growth stage; PV, previtellogenic stage; EV, early vitellogenic stage; MV, midvitellogenic stage; FG, full-grown stage. Dpf, days post fertilization; Mpf, months post fertilization. SG, spermatogonia; SC, spermatocyte; ST, spermatid. Scale bars = 100 μm.

Development • Supplementary information

Fig. S4

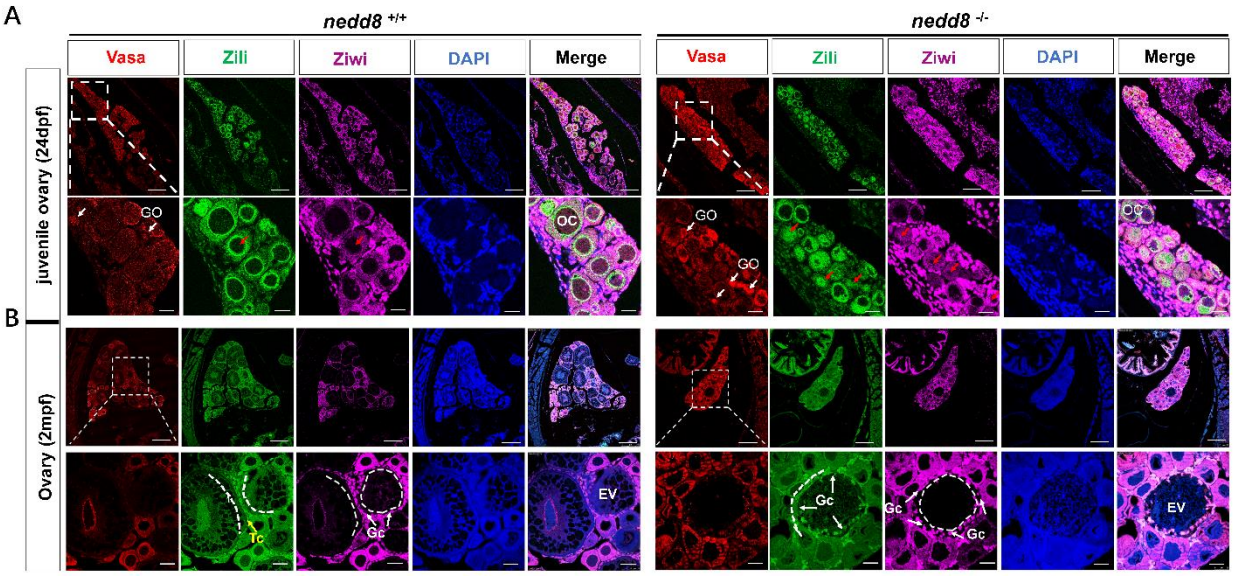


Figure S4. Loss of *nedd8* results in oocytes arrested at early developmental stages and few granulosa cells and theca cells developed.

(A) Immunofluorescent staining of Vasa (red), Zili (green), Ziwi (pink) and DNA (DAPI staining; blue) in *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish ovaries (n=6) at 24 dpf. OC, oocyte (indicated by the red arrowheads); GO, gonocyte (indicated by the white arrowheads). (B) Immunofluorescent staining of Vasa (red), Zili (green), Ziwi (pink) and DNA (DAPI staining; blue) in *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish ovaries (n=6) at 2 mpf. EV, early vitellogenic stage; Gc, granulosa cells (indicated by the white arrowheads); Tc, theca cells (indicated by the yellow arrowheads). Scale bars = 100μm, 25μm.

Development • Supplementary information

Fig. S5

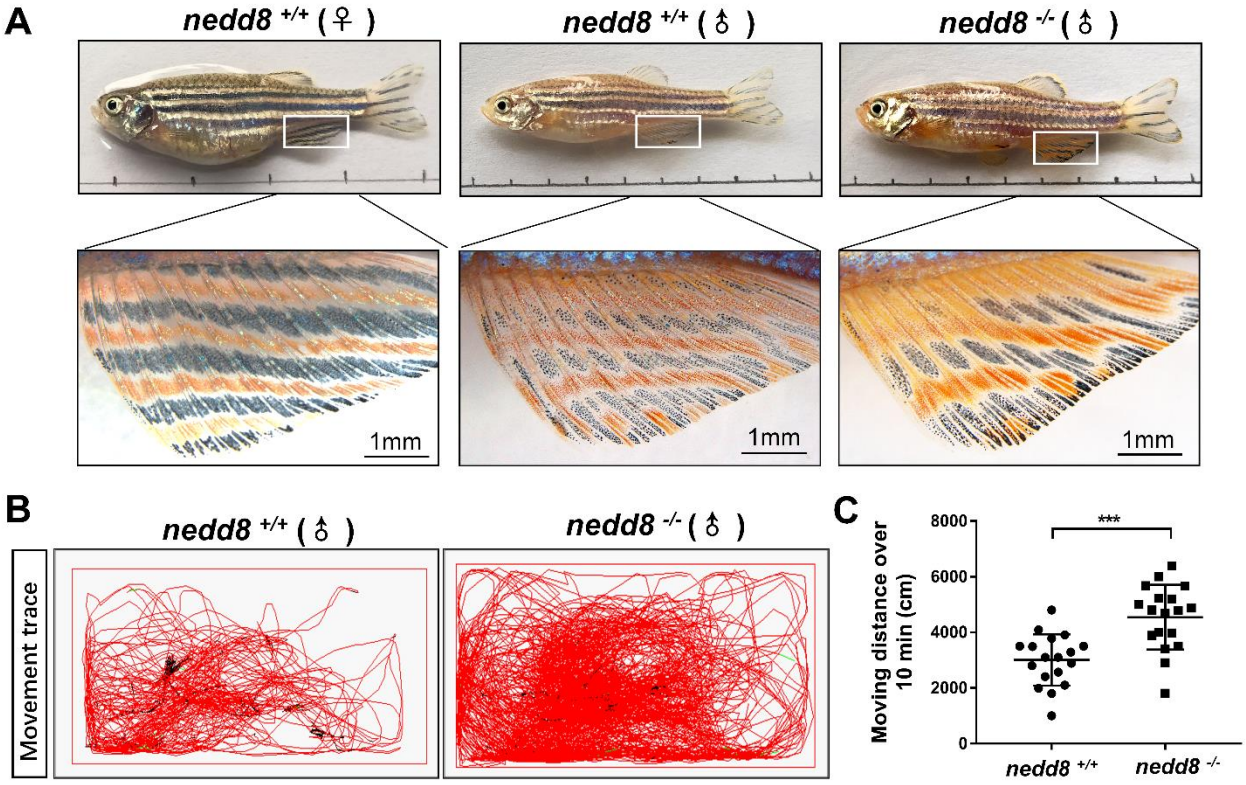


Figure S5. Male *nedd8*-null zebrafish exhibit hyperactive behaviors.

(A) Male *nedd8*$^{-/-}$ zebrafish (n=6 per group) displayed deeper yellow pigmentation on the anal fins compared with the female and male *nedd8* $^{+/+}$ zebrafish (n=6 per group) at 4 mpf. Scale bar = 1 mm. (B) The locomotor trajectories of male *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ adult zebrafish within 10 min recorded respectively with ViewPoint Behavior technology (Zeb-view, USA). (C) Quantitation of moved distance of male *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ adult zebrafish (n=6 per group). Data are presented as mean ± S.E.M; *** $P < 0.001$ (unpaired Student's *t* test).

Development • Supplementary information

Fig. S6

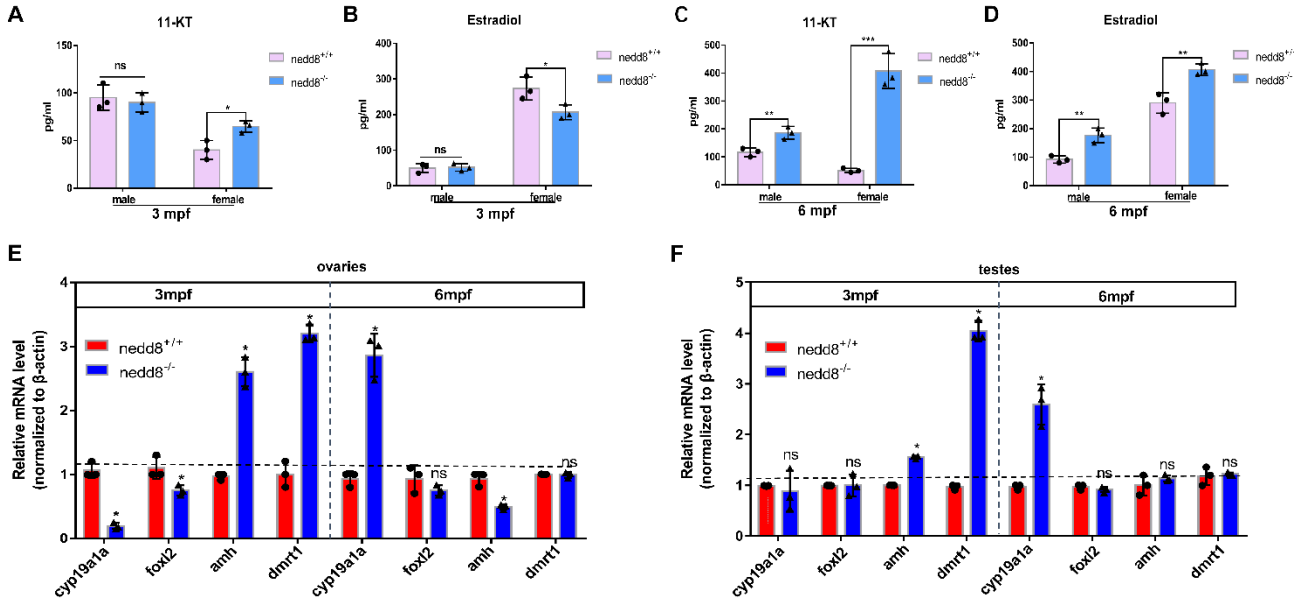


Figure S6. Serum sex hormone levels and expression of the sex determination genes in wildtype (*nedd8* $^{+/+}$) and *nedd8*-null (*nedd8* $^{-/-}$) zebrafish.

(A, B) Serum 11-KT and estradiol levels in wildtype and *nedd8*-null male and female zebrafish at 3 mpf. (C, D) Serum 11-KT and estradiol levels in wildtype and *nedd8*-null male and female zebrafish at 6 mpf. (E) mRNA levels of *cyp19a1a, foxl2*, *amh* and *dmrt1* in ovaries of *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish at 3 mpf and 6 mpf (n = 6 per group). (F) mRNA levels of *cyp19a1a, foxl2*, *amh* and *dmrt1* in testes of *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ (wildtype) zebrafish at 3 mpf and 6 mpf (n = 6 per group). Data are presented as mean ± S.E.M; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (unpaired Student's *t* test).

Development • Supplementary information

Fig. S7

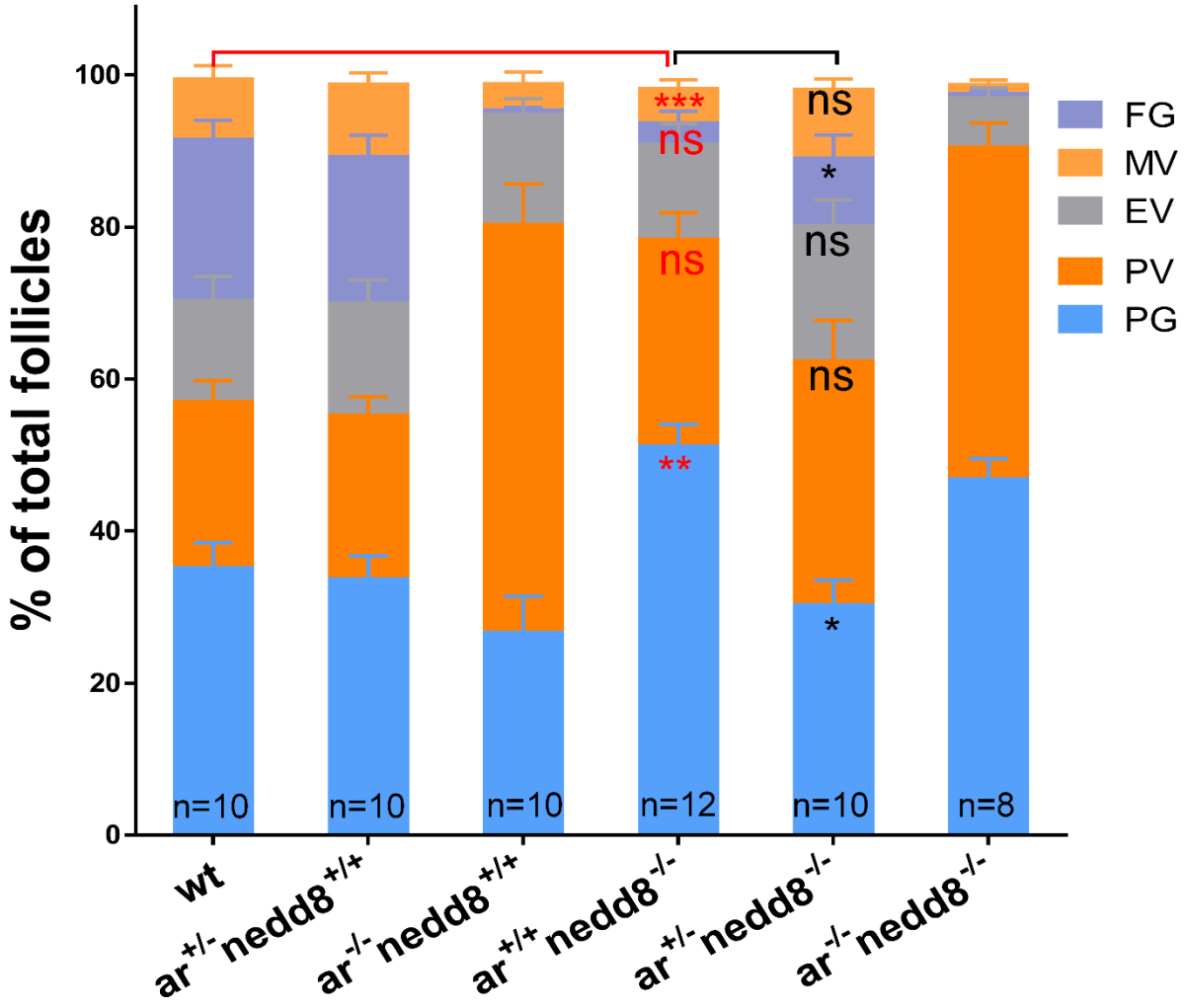


Figure S7. Quantitation of oocytes in zebrafish with different genetic background.

The percentage of oocytes in different developmental stages in wildtype, *ar*$^{+/-}$*nedd8*$^{+/+}$, *ar*$^{-/-}$*nedd8*$^{+/+}$, *ar*$^{+/+}$*nedd8*$^{-/-}$, *ar*$^{+/-}$*nedd8*$^{-/-}$, and *ar*$^{-/-}$*nedd8*$^{-/-}$ female zebrafish at 4 mpf.

The cell number counting was based on 3 sections/per fish and 6 fish/per genotype. PG, primary growth stage; PV, previtellogenic stage; EV, early vitellogenic stage; MV, midvitellogenic stage; FG, full-grown stage. Data are presented as mean ± S.E.M; * $P < 0.05$, ns, no significance (one way ANOVA).

Development • Supplementary information

Fig. S8

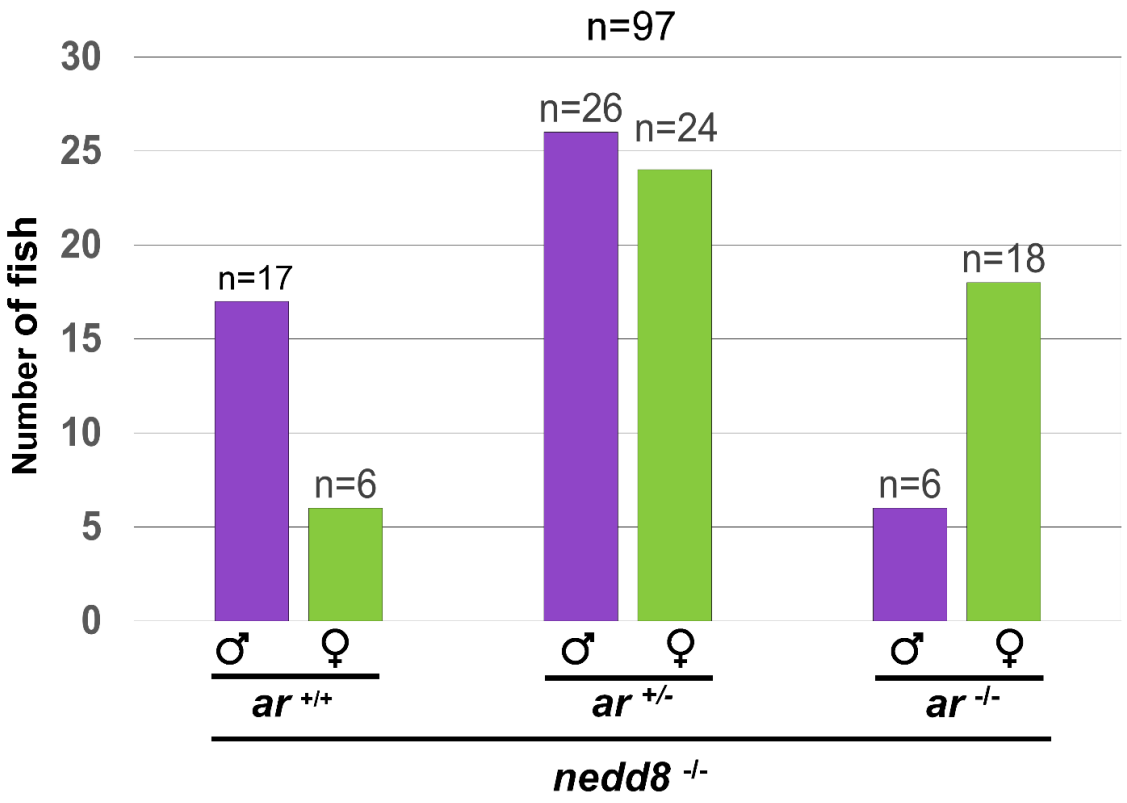


Figure S8. Loss of one copy of *ar* rescues sex bias in *nedd8*-null zebrafish.

Summary of male and female numbers in the offspring (n=97) by mating *ar* $^{+/-}$*nedd8* $^{-/-}$ (♀)× *ar* $^{+/-}$*nedd8* $^{-/-}$ (♂).

Fig. S9

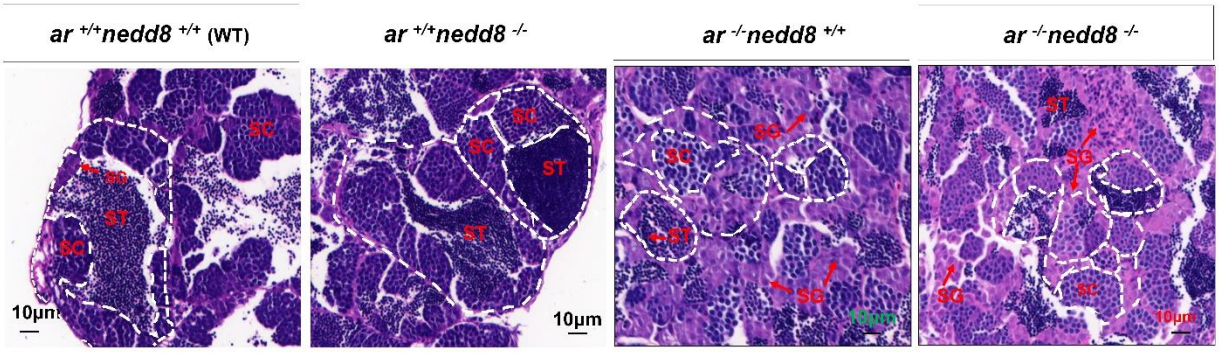


Figure S9. The testis organization of *ar* $^{-/-}$*nedd8* $^{-/-}$ males was similar to that of *ar* $^{-/-}$*nedd8* $^{+/+}$ males.

Representative images of H & E stained testes from *ar*$^{+/+}$*nedd8*$^{+/+}$ (wildtype), *ar*$^{+/+}$*nedd8*$^{-/-}$, *ar*$^{-/-}$ *nedd8*$^{+/+}$ and *ar*$^{-/-}$ *nedd8*$^{-/-}$ zebrafish at 4 mpf (n = 6 per group). Tubules are marked by white dashed cycles. Mpf, months post fertilization. SG, spermatogonia; SC, spermatocyte; ST, spermatid. Scale bar = 10 μm.

Development • Supplementary information

Fig. S10

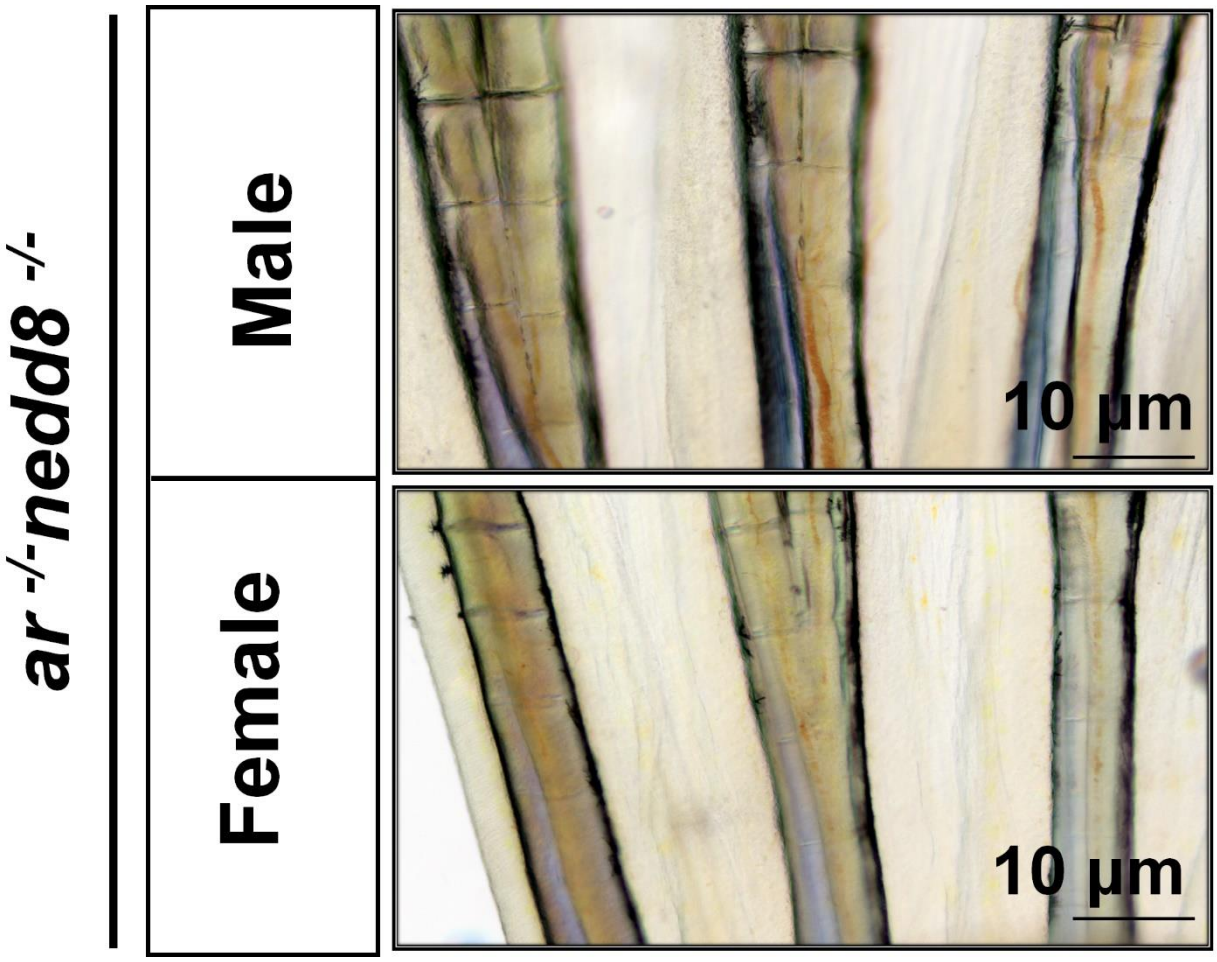


Figure S10. BTs do not develop on *ar*$^{-/-}$*nedd8*$^{-/-}$ male and female zebrafish.

Development • Supplementary information

Fig. S11

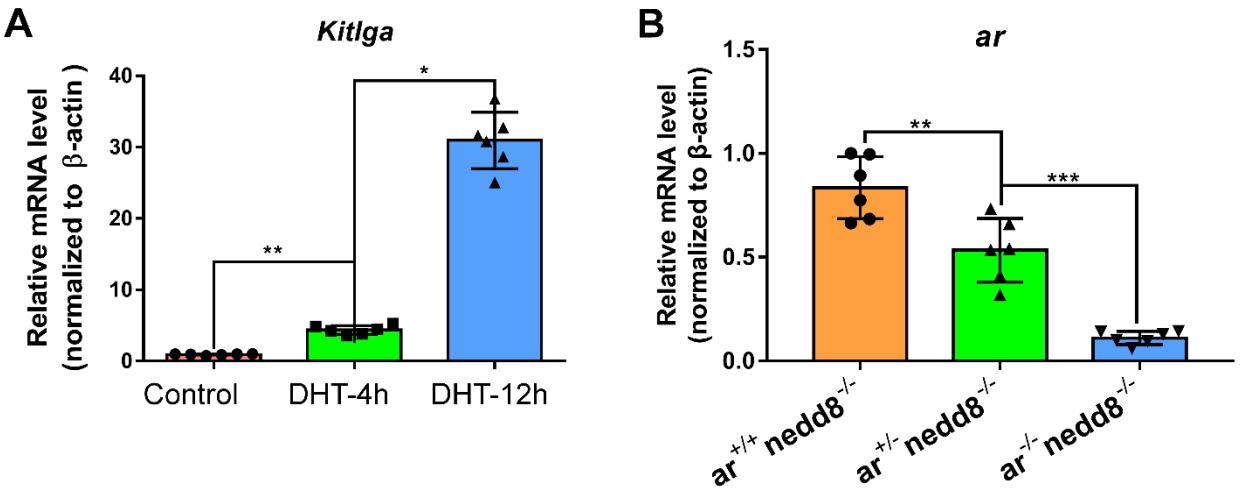


Figure S11. Induction of *kitlga* expression by DHT in zebrafish; *ar* mRNA levels in zebrafish with different genetic background.

(A) Injection with DHT (100 nM, 10µl, n=6) in zebrafish (4 mpf) after 4 h or 12 h induced *kitlga* expression in ovaries as revealed quantitative RT-PCR (qPCR) assays compared with injection with the same amount of vehicle (EtOH). (B) mRNA levels of *ar* in zebrafish ovaries ($ar^{+/+}nedd8^{-/-}$, $ar^{+/-}nedd8^{-/-}$, and $ar^{-/-}nedd8^{-/-}$) at 4 mpf (n=6 per group). Data are presented as mean ± S.E.M; * $P < 0.05$, ** $P < 0.01$ (unpaired Student's *t*-test).

Development • Supplementary information

Fig. S12

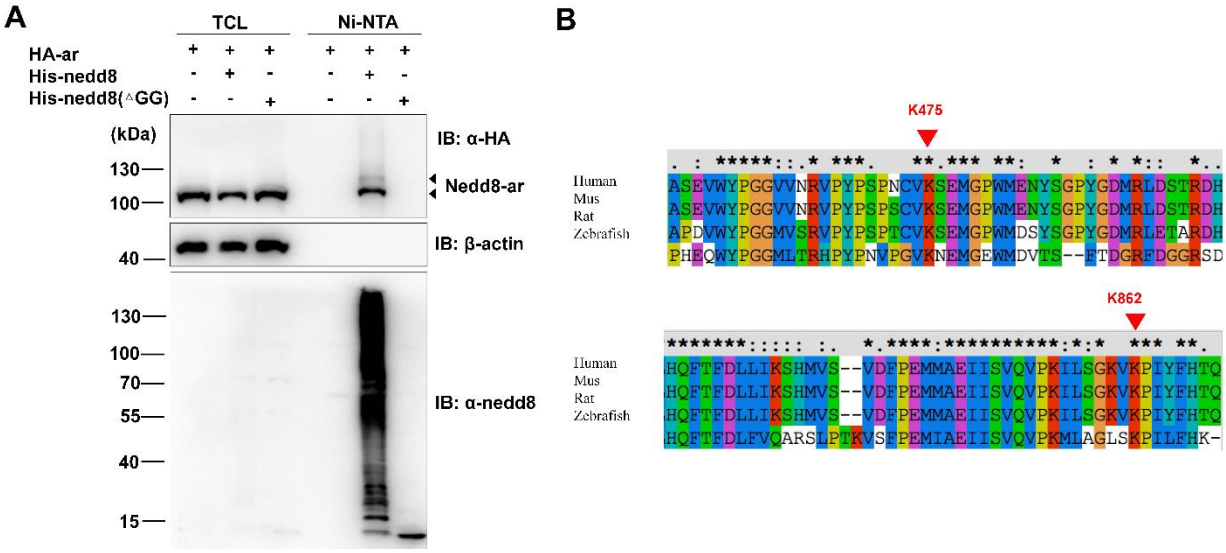


Figure S12. Validation of ar neddylation

(A) Western blot showing that *ar* was not neddylated in HEK293T cells in response to overexpression of *nedd8* mutant (*nedd8*-△GG). HEK 293T cells were transfected with the indicated plasmids together with His-nedd8 and *nedd8* mutant (*nedd8*-△GG) respectively (5 μg/each). After 36 hr, cells were lysed in guanidinium chloride, and His-nedd8 and the *nedd8* mutant (*nedd8*-△GG) was purified with Ni-NTA agarose. (B) Sequence alignment showing that K475 and K862 of ar are evolutionarily conserved. IB: immunoblotting; TCL, total cell lysates; IP, immunoprecipitation.

Development • Supplementary information

Figure 13

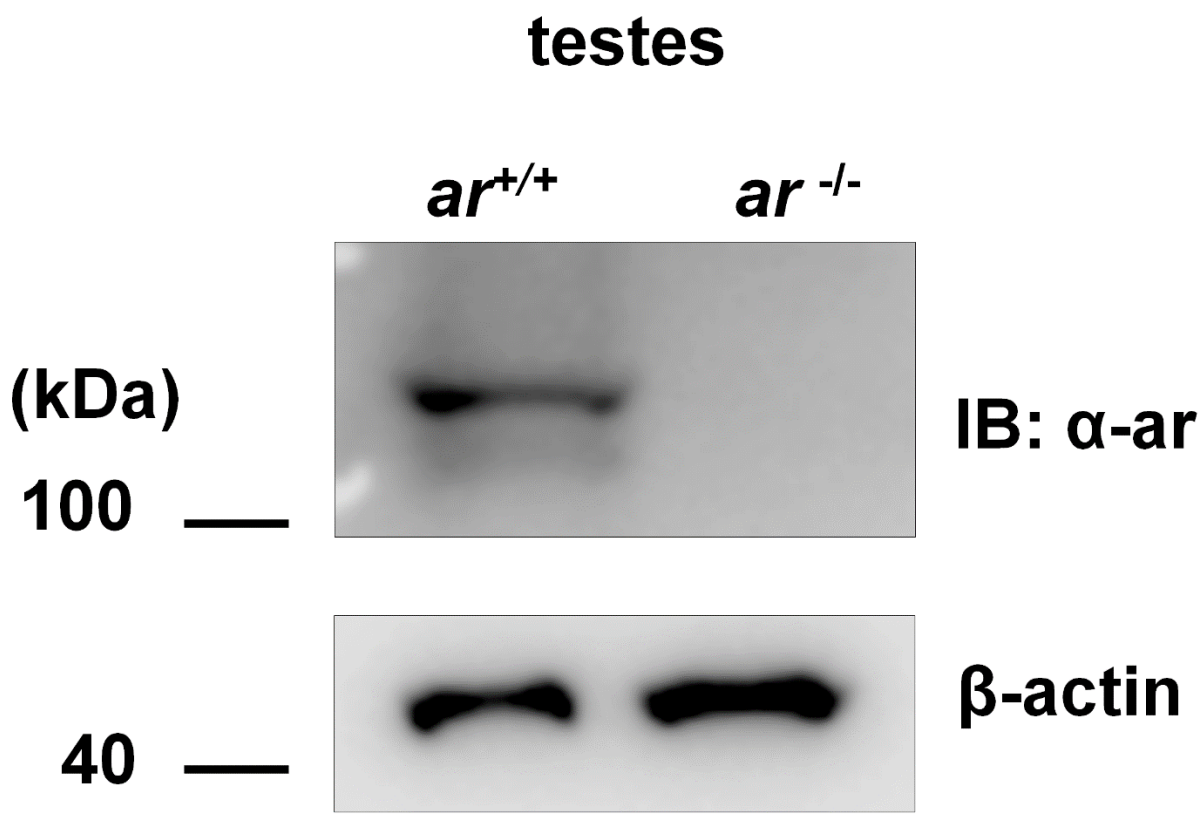


Figure S13. Validation of anti-zebrafish *ar* monoclonal antibody.

Development • Supplementary information

Fig. S14

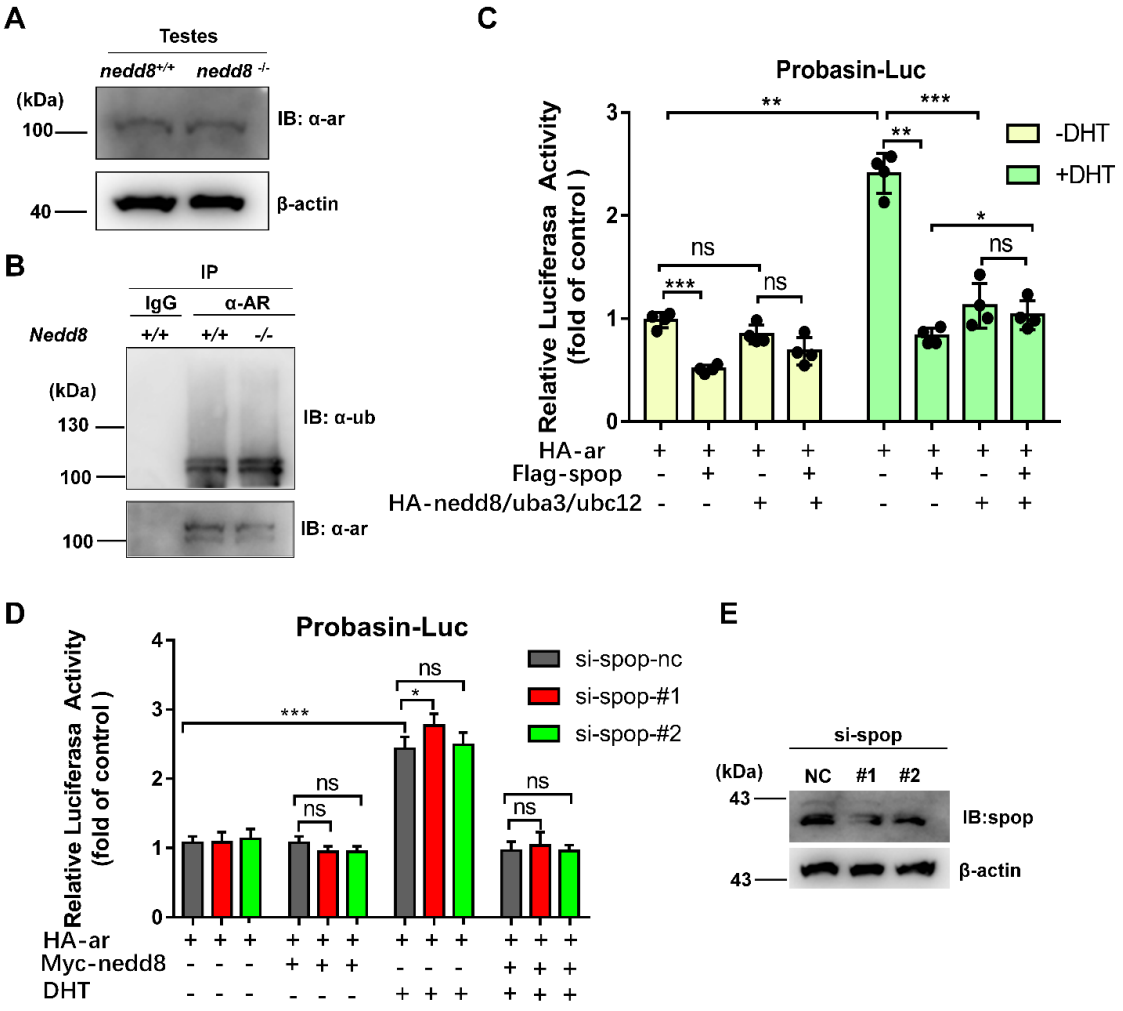
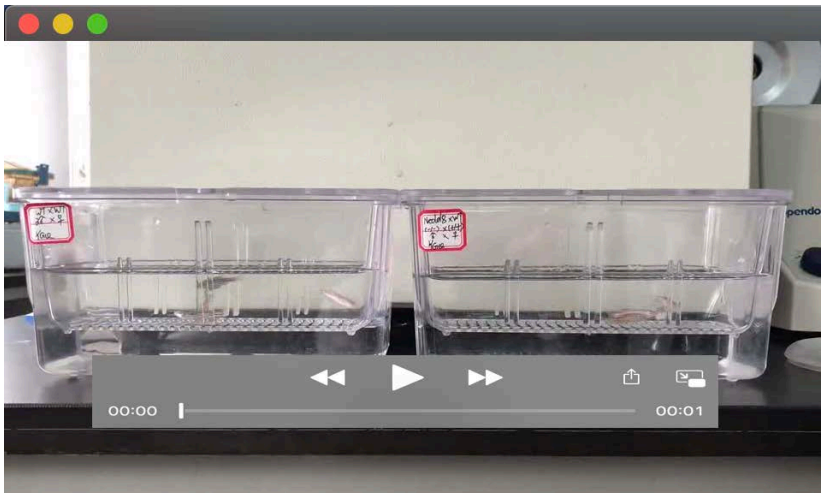


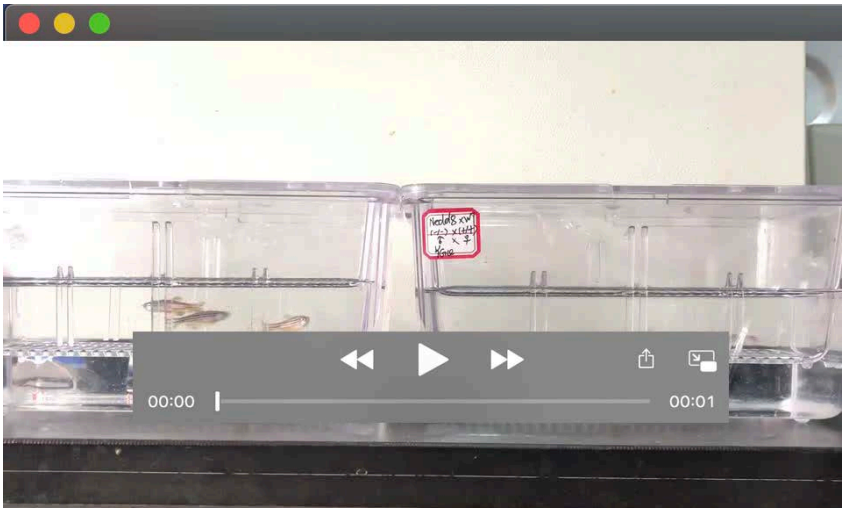
Figure S14. The inhibition of ar by *nedd8* may not be mediated by affecting the activity of SPOP-CUL3-RBX1 ubiquitin ligase

(A) The protein levels of ar in testes of *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish at 4 mpf (n=20). *in vivo* ubiquitination assay showing that endogenous ar ubiquitination was similar between *nedd8* $^{+/+}$ and *nedd8* $^{-/-}$ zebrafish. The protein lysates from testes of *nedd8*$^{+/+}$ and *nedd8*$^{-/-}$ male zebrafish were subjected to immunoprecipitated with mouse IgG or anti-zebrafish ar antibody, and subsequently detected by anti-ubiquitin antibody under partially denaturing conditions. (C) Relative luciferase activity of the *Probasin* promoter in response to spop, nedd8, *uba3* and *ubc12* overexpression in EPC cells expressing *ar* (400ng/per well), with and without treatment with 20 nM DHT. (D) Relative luciferase activity of the *Probasin* promoter in response to knock down of *spop* by RNA interference in EPCs expressing *ar*, in the presence or absence of nedd8, with or without 20 nM DHT addition. (E) The protein level of *spop* in EPC cells after knocked down by *spop* si-RNAs (#1 and #2) or the negative control siRNA (NC). IB: immunoblotting; IP: immunoprecipitation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$; ns, no significance (one way ANOVA).

Development • Supplementary information



Movie 1. Male *nedd8*$^{-/-}$ zebrafish (n=3; 4 mpf) are more active for chasing female zebrafish compared with male *nedd8*$^{+/+}$ zebrafish (n=3, 4 mpf). Left tank: *nedd8*$^{+/+}$ (3♂) and *nedd8*$^{+/+}$ (1♀); right tank: *nedd8*$^{-/-}$ (3♂) and *nedd8*$^{+/+}$ (1♀). Two female *nedd8*$^{+/+}$ zebrafish were put into the two tanks respectively at the same time.



Movie 2. Male *nedd8*$^{-/-}$ zebrafish (n=3; 4 mpf) are more active for chasing food compared with male *nedd8*$^{+/+}$ zebrafish (n=3; 4 mpf). Left tank: *nedd8*$^{+/+}$ (3♂); right tank: *nedd8*$^{-/-}$ (3♂). The same amount of food was added into the two tanks simultaneously.

Development • Supplementary information