# Knock-in of a *3' UTR* Stop Cassette into the *Wnt4* locus increases mRNA expression and leads to ovarian cyst formation

NSREIN ALI[1,*], QI XU[1], RENATA PRUNSKAITE-HYYRYLÄINEN[1],
JINGDONG SHAN[1], SEPPO J. VAINIO[1,2,*]

*[1]Faculty of Biochemistry and Molecular Medicine, Disease Networks, Laboratory of Developmental Biology,
University of Oulu, Oulu, Finland, [2]Kvantum Institute, University of Oulu, Finland*

**ABSTRACT** **Wnt4 signaling is critical for mammalian female sex determination, in female reproductive organ development, in follicular and oocyte maturation, and in steroid hormone production. When Wnt4 function is impaired, female embryos undergo partial female to male sex-reversal. This phenotype is associated with the expression of a set of somatic genes that are typical for the male differentiation pathways such as those of the Leydig cells. Given the roles of the 3`untranslated region (*3`UTR*) in control of gene expression, we addressed whether a knock-in of a stop cassette to 3`END of the *Wnt4* gene would impact female reproductive system development or function. The *3`UTR^stop* cassette indeed affected *Wnt4* gene expression *in vivo* so that the respective mRNA was upregulated in the ovaries of a three month-old female. The homozygous *Wnt4 3`UTR^stop* mice were noted to be leaner than their wild type (WT) littermate controls. Analysis of the ovarian follicular count at the age of three months revealed increased pre-antral but reduced ovarian corpus luteum follicular counts. Furthermore, two out of five of the homozygous female *Wnt4 3`UTR^stop* mice had ovarian cysts, not noted in WT controls. RT-qPCR and *in situ* hybridization analysis depicted changes in the expression of a panel of genes which encode enzymes that mediate the synthesis of female steroid hormones or their receptors due to the *Wnt4 3`UTR^stop* knock-in. Thus, female mice which had the homozygous construct exhibited elevated ovarian *Wnt4* mRNA expression and the corresponding knock-in was associated with changes in ovarian development and folliculogenesis. Our data reinforce the conclusion that deregulated *Wnt4* expression impacts female sex organogenesis, ovary development and function, and that the *Wnt4 3`UTR^stop* knock-in mouse provides a model to explore in more detail the roles of Wnt4 signaling in the process.**

**KEYWORDS:** *female fertility, folliculogenesis, steroid hormone synthesis, anovulatory follicle, germ line, meiosis*

## Introduction

The signals in the Wnt family are involved in the ontogenesis of many organs during embryogenesis, but they exhibit also functions in the adult, such as control of tissue regeneration via stem cells involved in the maintenance of homeostasis (Liu *et al.*, 2022; Nusse and Clevers, 2017). As secreted signaling type of molecules, the Frizzled receptor bound Wnts trigger in part beta-catenin driven downstream signal transduction cascades, which is involved in many processes such as cell differentiation, cell proliferation, cell polarity, and tissue regeneration (Zhang *et al.*, 2021). Deregulated Wnts or their downstream signal transduction components associate with a variety of diseases such as melanoma, leukemia,

and breast cancer (Zhan *et al.*, 2017), lung (Guo *et al.*, 2015; Aros *et al.*, 2021), cardiovascular (Foulquier *et al.*, 2018; Akoumianakis *et al.*, 2022; Gay and Towler, 2017), and bone diseases (Baron and Kneissel, 2013; Diegel *et al.*, 2023). These phenotypes point roles for Wnt signaling in control of stemness, metastasis, and inflammation in these diseases.

Of the Wnt family members, Wnt4 has been studied extensively and shown to be functional in several organ systems (Zhang *et al.*, 2021). Wnt4 the first identified signal that was connected to the mammalian female sex determination process (Vainio *et al.*, 1999). In a female indifferent gonad, *Wnt4* contributes to femaleness by promoting differentiation of the ovarian estrogen producing female follicular cells and development of the Müllerian duct coordinated by anti- Müllerian hormone signaling. Such evidence on how Wnt4

regulates female ovarian development was based on analyzing the female embryos that were deficient for Wnt4 function in a knockout mouse model. The data showed that the female germ cells, and consequently the oocytes underwent apoptosis later in female sexual development (Vainio *et al.*, 1999). In addition to the germ line cell differentiation, Wnt4 regulated polarity of the follicular cells, basement membrane dynamics, anti-Müllerian hormone expression, and meiosis together with the Wnt5a signaling (Prunskaite-Hyyrylainen *et al.*, 2014; Naillat *et al.*, 2010). Compromised Wnt4 signaling impairs follicle development and leads to premature ovarian failure (Naillat *et al.*, 2010).

Developmental gene expression control involves the untranslated regions (UTRs) in the respective mRNA species. The UTR differ in their sequence length but typically they contain binding sites for the miRNA regulatory elements (Bartel, 2009; Hoffman *et al.*, 2016; Mayr, 2017). Due to this reason, the UTR sequences can contribute to stability of mRNA and impact translation (Berkovits and Mayr, 2015; Ma and Mayr, 2018). The UTRs are connected to such processes as cell proliferation (Sandberg *et al.*, 2008; Gruber *et al.*, 2014), inflammation (Bergant *et al.*, 2023; Jia *et al.*, 2017), and organogenesis (Chekulaeva *et al.*, 2006; Freimer *et al.*, 2018; Yang *et al.*, 2017; Marshall *et al.*, 2021).

To target further the roles of Wnt4 in female sex organogenesis, we generated a novel allele by inserting a specific stop cassette into the gene locus. This cassette was expected to prevent the *3'UTR* function to control *Wnt4* gene expression. The results depicted that insertion of the *Wnt4 3'UTR* stop cassette was noted to result in an elevation in *Wnt4* mRNA expression in the ovarian follicular cells. Analysis of the *Wnt4 3'UTR* edited females depicted phenotypes in their sex organs. These data supported a regulatory role for *Wnt4* in female folliculogenesis. This seems to occur via control of a panel of genes that are involved in the synthesis of female sex hormones. We conclude that the *Wnt4 3'UTR*stop mouse may offer a novel model to investigate the impact of the gain of Wnt4 signaling for female fertility.

## Results

### The *Wnt4 3'UTR* genomic stop cassette insertion compromises post-natal female body weight

To test whether the inactivation of the *3'UTR* stop cassette in the *Wnt4* locus would deregulate the protein action, *Wnt4 3'UTR*stop transgenic mice were made, and bred as depicted in the methods section. The number of homozygous, heterozygous and wild-type (WT) mice obtained from the *Wnt4 3'UTR*stop and WT genetic crossings followed the Mendelian ratios (Table 1). The values were 24% for the *Wnt4 3'UTR*stop/*Wnt4 3'UTR*stop, 53% for *Wnt4 3'UTR*WT/stop, and 23% for *Wnt4 3'UTR*WT/WT in line with the reported roles of Wnt4 signaling function during embryogenesis (Zhang *et al.*, 2021; Bartel, 2009; Hoffman *et al.*, 2016; Mayr, 2017; Berkovits and Mayr, 2015; Ma and Mayr, 2018; Sandberg *et al.*, 2008).

We then assessed from the *Wnt4 3'UTR*stop intercrosses the overall weight of the mice and their organs. Selected organs such as the kidney, the testis, and the spleen were dissected, and their weight was measured. The overall body

TABLE 1

**MENDELIAN INHERITANCE TRAIL**

| | WT | *Wnt4 3'UTR*WT; *Wnt4 3'UTR*stop | *Wnt4 3'UTR*stop; *Wnt4 3'UTR*stop | Total |
|---|---|---|---|---|
| **Female** | 8 | 21 | 15 | 54 |
| **Male** | 8 | 40 | 15 | 63 |
| **Total** | 26 | 61 | 30 | 117 |

Data were collected from 17 litters. WT refers to wild type genotype, *Wnt4 3'UTR*WT;*Wnt4 3'UTR*stop refers to heterozygous genotype, and *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop refers to homozygous genotype.

weight of the *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop mice was reduced when compared to WT mice (26.18±2.34g versus 29.79±1.27g, respectively; n=8) (Fig. 1A). The spleen and the kidneys of the homozygous *Wnt4 3'UTR*stop mice were heavier than their normal WT controls being 88.50±6.50 mg / 73.05±7.61 mg and 230.34±20.76 mg / 193.50±30.23 mg, respectively (Fig. 1 B-D). No differences in the weight of the testis of the *Wnt4 3'UTR*stop and WT mice were noted (Fig. 1 E,F). We conclude that the weight of the homozygous *Wnt4*
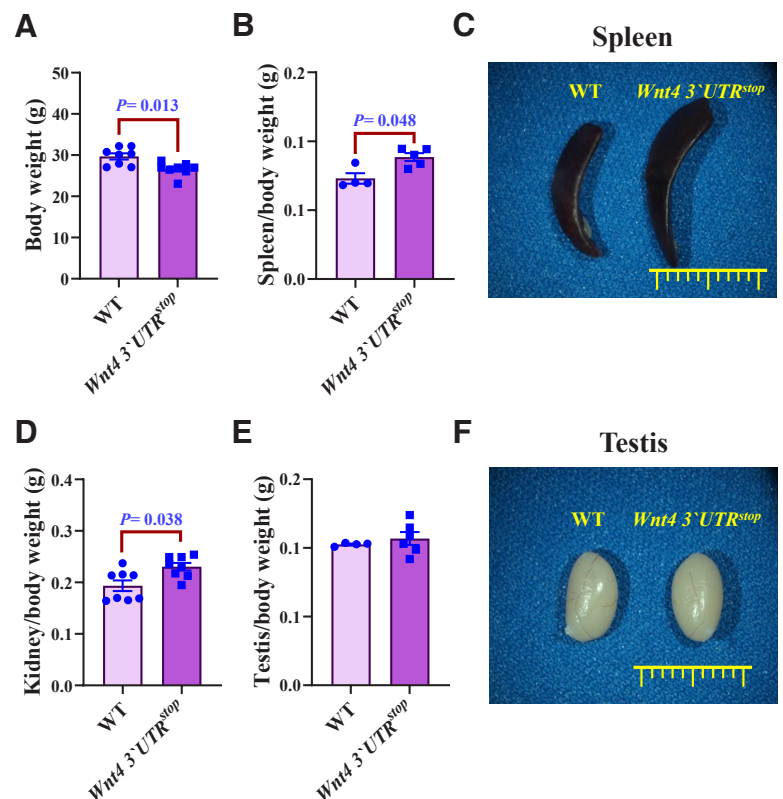
**Fig. 1. Genetic modification of *Wnt4 3'UTR* leads to morphological manifestations in organ development. (A)** Total body weight of *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop and WT mice. Data are presented as means ± SEM, n=8 (WT and *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop). **(B)** Spleen/body weight in *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop and WT mice. Data are presented as means ± SEM, n=4 (WT) and n=5 (*Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop). **(C)** Morphological examination of spleen size in *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop and WT mice. **(D)** Kidney/body weight in *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop compared to WT mice. Data are presented as means ± SEM, n=8 (WT and *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop). **(E)** Testis/body weight in *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop versus WT mice. Data are presented as means ± SEM, n=4 (WT) and n=6 (*Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop). **(F)** Morphological examination of kidney size in *Wnt4 3'UTR*stop;*Wnt4 3'UTR*stop and WT mice. The two-tailed Student test was used for statistical analysis in (A, B, D, and E). $P \leq 0.05$ was considered statistically significant.

**Fig. 2. Modification of the *Wnt4 3`UTR*^stop allele results in activation of *Wnt4* expression in the ovary of two-three months old mice. (A-C)** Quantification of *Wnt4* mRNA by RT-QPCR in kidney **(A)**, testis **(B)**, and ovaries **(C)**. Data are shown as averages after normalization to the control ± SEM, n=4 (WT) and n=3 (*Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop); each sample was run in four biological replicates. **(D)** Western blot Wnt4 analysis of three month-old WT and *Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop ovaries. **(E)** Quantification of Wnt4 protein expression levels in three month-old WT and *Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop. The results are shown as averages after normalization to the control ± SEM, n=5 from both genotypes. The two-tailed Student test was used for statistical analysis in (A-E). $P \leq 0.05$ was considered statistically significant.



*3`UTR*^stop mice was altered in those organs where Wnt4 has been reported to be functional (Naillat *et al*., 2010; Vainio *et al*., 1999; Prunskaite-Hyyryläinen *et al*., 2016).

**The *Wnt4 3`UTR* genomic stop cassette insertion compromises *Wnt4* mRNA expression in ovary**

To assess the putative consequences of the inserted *3`UTR* stop cassette into the *Wnt4* locus, we examined possible changes in the *Wnt4* mRNA expression by the RT-QPCR. Messenger RNAs were extracted from the dissected kidney, testis, and ovaries that were prepared from two to three months old mice. Analysis of the *Wnt4* mRNA expression level in the kidney of the homozygous *Wnt4 3`UTR*^stop remained unchanged when compared to control (Fig. 2A), while there was a trend for increased *Wnt4* expression in the testis (Fig. 2B). A prominent difference in *Wnt4* mRNA expression was observed in the ovaries of the *Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop females compared to WT female mice samples (Fig. 2C).

To substantiate the obtained results, *in situ* hybridization approach was applied on the kidney, the testis, and the ovaries of

three months old mice. In line with the above RT-QPCR data, no differences in the *Wnt4* mRNA expression were observed in the kidney and the testis sections (*data not shown*), whereas increased *Wnt4* mRNA expression was evident in the ovary of the *Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop females when compared to WT female samples (Suppl. Fig. 2).
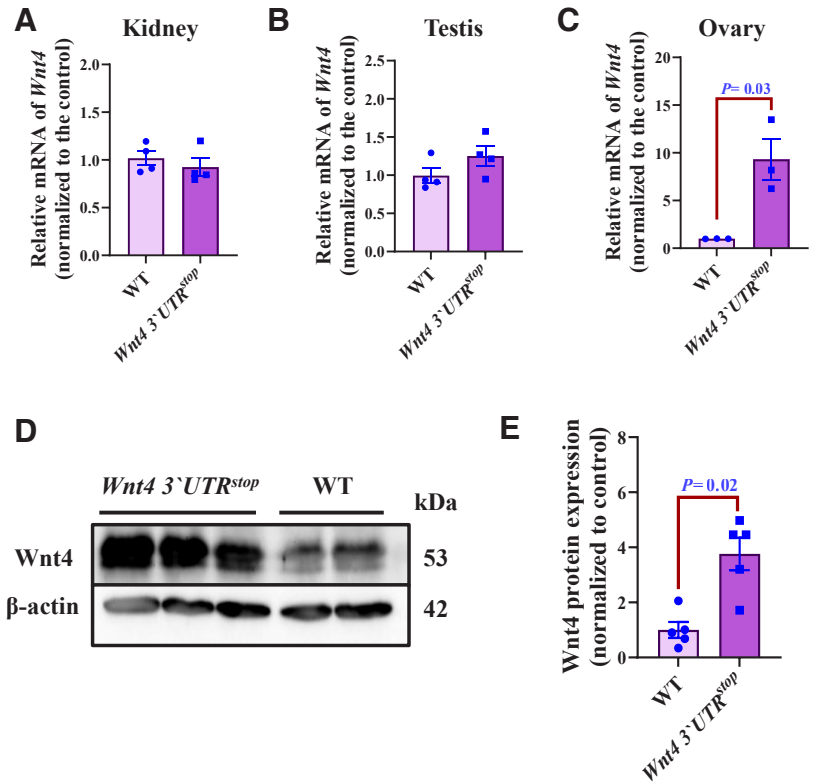


**Fig. 3. *Wnt4* gain of function compromises female fertility. (A)** Number of pups per litter. Data are presented as means ± SEM, n=12 (WT) and n=10 (*Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop). **(B,C)** Follicle counts in WT and *Wnt4 3`UTR*^stop;*Wnt4 3`UTR*^stop, n=4 from both genotypes. Data are presented as means ± SEM. **(D)** Quantification of *Fshr* mRNA by RT-QPCR. The results are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. **(E)** Quantification of *Lhcgr* mRNA by RT-qPCR. The results are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. The two-tailed Student test was used for statistical analysis in (A-E). $P \leq 0.05$ was considered statistically significant.
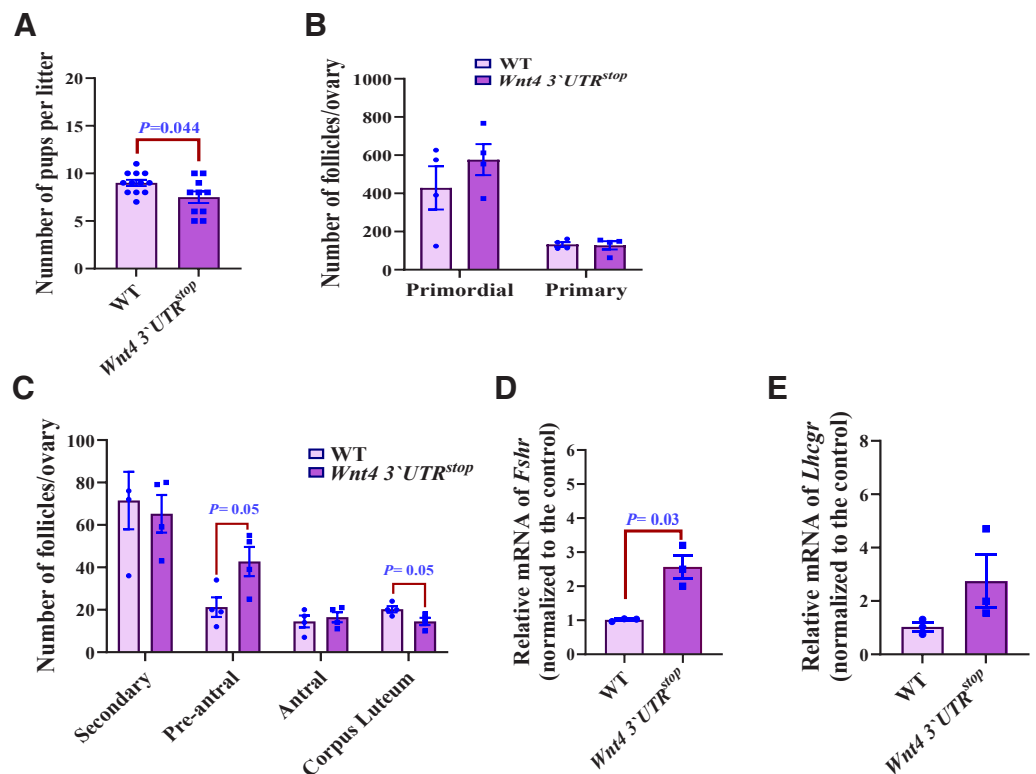
We explored a possible impact of the *Wnt4 3`UTR*stop knock-in on the Wnt4 protein expression in the ovaries of three month-old female mice in comparison to controls. Western blotting with an anti-Wnt4 antibody revealed, as was the case with the *Wnt4* mRNA expression, an increase in the protein level. This was evident also later in the ovaries of three months *Wnt4 3`UTR*stop knock-in females, compared to ovaries of WT control (Fig. 2 D,E). Together the results suggest that the *Wnt4 3`UTR*stop knock-in leads to elevated expression of *Wnt4* mRNA and protein in the ovary of two to three months old mice.
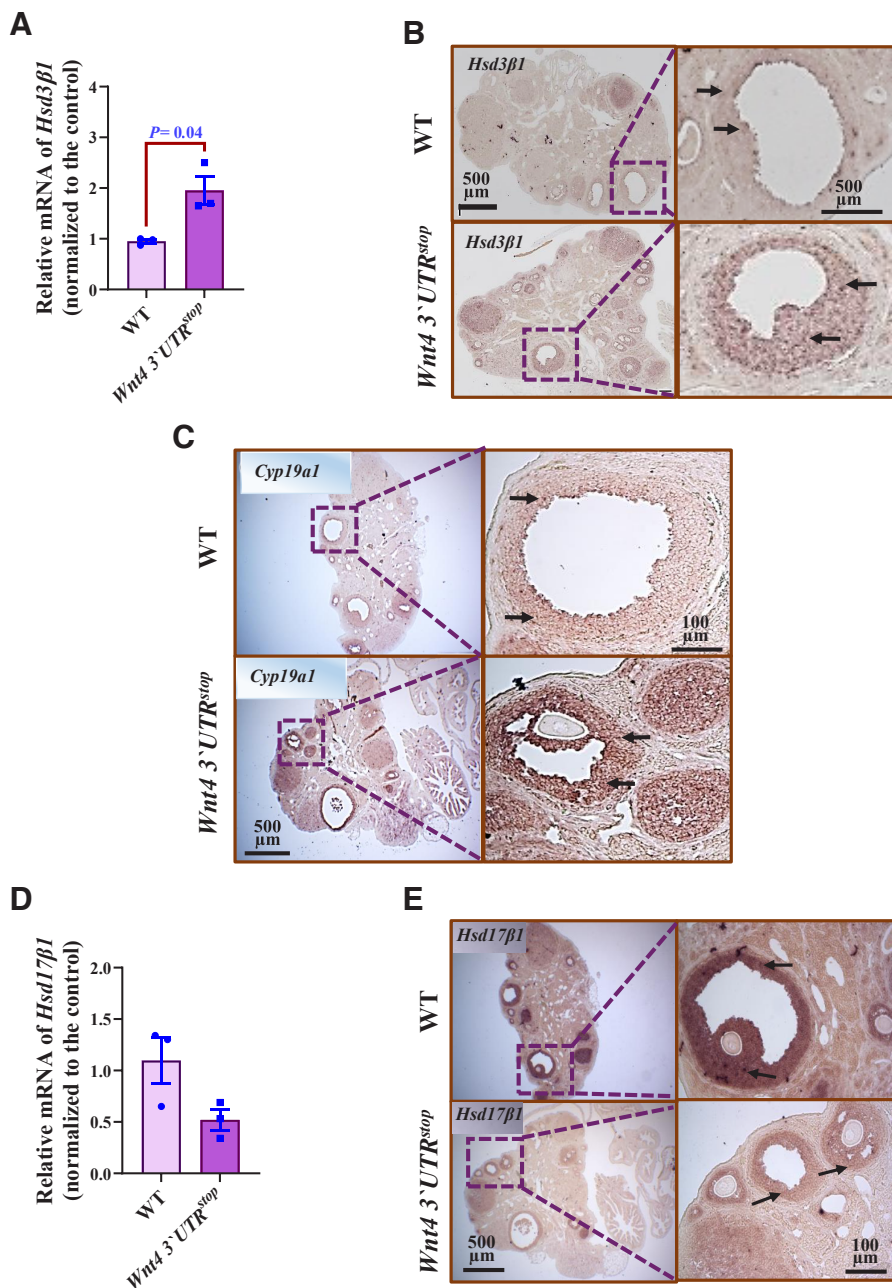
### The *Wnt4 3`UTR* genomic stop cassette insertion compromises ovarian follicle maturation and female fertility

Given our prior data demonstrating a key role for Wnt4 signaling in the development and function of the female reproductive organs (Vainio *et al*., 1999; Prunskaite-Hyyrylainen *et al*., 2014; Naillat *et al*., 2010; Prunskaite-Hyyryläinen *et al*., 2016; Veiga-Lopez *et al*., 2012), and that *Wnt4* mRNA expression becomes upregulated in oogenesis, we started to study if the *Wnt4 3`UTR*stop knock-in allele would impact female fertility.

The *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females and males were crossed with WT mice and the liter sizes were monitored and compared with WT mice breeding. The results revealed that while the average litter size of the bred WT females and males was 9.0±1.1 (n=12), the corresponding number of the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop female and WT males was 7.5 ± 1.9 (n=10) (Fig. 3A). As expected, no differences in the mean litter size were noted in the crosses of *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop males to WT females being 7.5 ± 1.9 (n=10) (Suppl. Fig. 3).

We then counted the number of ovarian follicles based on their maturation stage in the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females and WT ones. The follicular counts were the following: the primordial; 576.5±110.1/530.3±227, the primary; 138.6±24.14/127.75±5.5, the secondary; 65.255±17.7/83.3±27.1, and the antral; 16.5±4.7/17±5.6, suggesting no differences between the two genotypes (Fig. 3 B,C). A significant increase, however, in the pre-antral follicle counts 42.7±13.7/24.3±9.2 was accompanied by a reduction in the corpus luteum follicle count 14.5±3.4/20.3±2.8 was noted in *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop when compared to values of WT females (Fig. 3C). We conclude that the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop genotype impacts ovarian folliculogenesis.

### The *Wnt4 3`UTR* genomic stop cassette insertion compromises the expression of genes involved in ovarian steroid hormone synthesis

We next explored the molecular mechanisms behind the noted changes in ovarian folliculogenesis process. Given the roles of Wnt4 signaling in control of genes that encode for steroid hormone synthesizing enzymes, we

**Fig. 4. *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop alters gene expression related to hormone synthesis in the ovary. (A)** Quantification of *Hsd3b1* mRNA by RT-qPCR. Data are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. **(B)** *Hsd3b1* mRNA *in situ* hybridization (brown color, black arrows) of three month-old WT and *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop ovaries. Scale bars, 500 μm. **(C)** *Cyp19a1* mRNA *in situ* hybridization (brown color, black arrows) of three month-old WT and *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop ovaries. Scale bars, 500 and 100 μm. **(D)** Quantification of *Hsd17b1* mRNA by RT-QPCR. Data are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. **(E)** *Hsd17b1* mRNA *in situ* hybridization (brown color, black arrows) of three month-old WT and *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop ovaries. Scale bars, 500 and 100 μm. The two-tailed Student test was used for statistical analysis in (A,D). $P \leq 0.05$ was considered statistically significant.

assayed putative changes in their expression due to the *3`UTR*stop knock-in in the mice. We studied possible changes in the expression of the luteinizing hormone receptor (*Lhcgr*) and the follicle-stimulating hormone receptor (*Fshr*) genes by RT-QPCR.

The results revealed that expression of the *Fshr* mRNA was significantly increased in the ovary of the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop as compared to WT samples (Fig. 3D). Furthermore, a trend of increase in the expression of *Lhcgr* mRNA was observed (Fig. 3E).

Wnt4 signaling regulates genes that encode steroid hormone synthesis enzymes in the ovary as well (Vainio *et al*., 1999; Prunskaite-Hyyrylainen *et al*., 2014; Naillat *et al*., 2010). Based on this knowledge we conducted RT-QPCR and *in situ* hybridization to study if the *Wnt4 3`UTR* knock-in would be associated with changes in the expression of the 3-beta-hydroxysteroid dehydrogenase (*Hsd3b1*), aromatase (*Cyp19a1*), and hydroxysteroid 17-beta dehydrogenase-1 (*Hsd17b1*).

The RT-QPCR studies demonstrated an increase of the *Hsd3b1* mRNA expression in the ovaries of the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females when compared to control mice (Fig. 4A). Examining the *in situ* hybridization images, a stronger signal of *Hsd3b1* mRNA in the granulosa cells of the *Wnt4 3`UTR*stop homozygous mice in

comparison to that detected in control mice was observed (Fig. 4B). Similarly, *Cyp19a1* mRNA expression had changed due to the *Wnt4 3`UTR* knock-in and increased in the homozygous female samples (Fig. 4C).

Of the three analyzed hormone synthesis associated genes, the *Hsd17b1* was the only one whose expression appeared to be decreased in the ovaries of *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop mice when compared to WT control (Fig. 4D). This result was reinforced with the *in situ* hybridization analysis, which revealed a diminished signal of the *Hsd17b1* mRNA expression in the granulosa cells of the heterozygous compared to WT female samples (Fig. 4E).

Together, the data support a role of Wnt4 signaling in control of the genes that encode steroid hormone synthesis in the ovary, critical for maturation of the ovarian follicular complex.

**Impaired *Wnt4 3`UTR* signaling leads to ovarian cyst formation**

Given the observed ovarian changes brought by the knock-in expression of the *Wnt4* gene by the *3`UTR*stop cassette, we focused our attention on monitoring the ovary development of aged females. Histological examination of the ovaries dissected from the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females at the age of six months revealed anovulatory follicles containing two oocytes, not noted in control female mice (Fig. 5A). This was associated with a significant elevation of *Wnt4* mRNA expression in the females at the age of six months (Fig. 5B). In ovaries derived from one-year-old females 2/5 samples depicted enlarged cysts that were not noted in ovaries of control females (Fig. 5C). As was the case in the females at the age of three and six months, the RT-QPCR analysis demonstrated upregulated *Wnt4* mRNA expression at the age of one year when compared with WT female controls (Fig. 5D).

*Wnt4* knock-out has been reported to increase anti-Müllerian hormone levels and gene expression (Prunskaite-Hyyryläinen *et al*., 2016) in line with the anti-Müllerian hormone as a biomarker of ovary cyst formation (Butt *et al*., 2022; Oh *et al*., 2019; Sivanandy and Ha, 2023). Given this, we set out to investigate the putative association of the anti-Müllerian hormone gene (*Amh*) and the anti-Müllerian hormone receptor (*Amhr*) expression in the
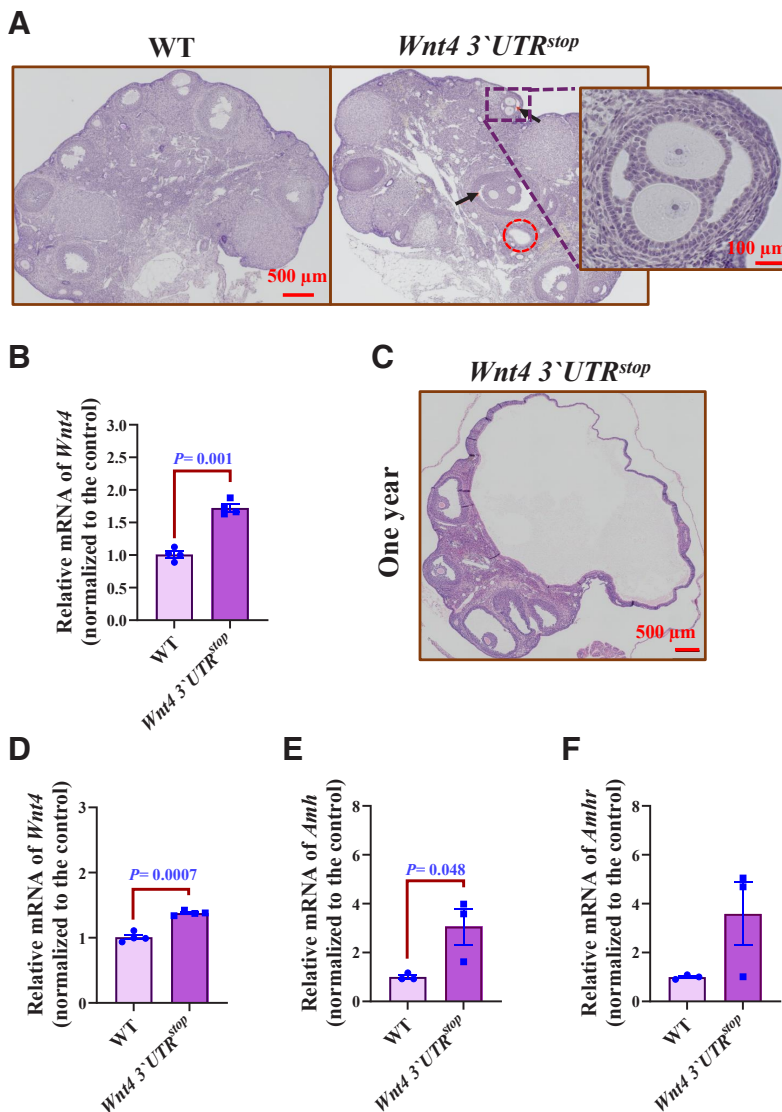


**Fig. 5. *Wnt4* gain of function enhances cyst formation in the ovary. (A)** Histological examination using eosin/hematoxylin staining demonstrating poly-ovular follicle formation (black arrows) and cyst formation (dotted red circle) in six month-old *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop mice compared with WT. Scale bars, 500 and 100 μm. **(B)** Quantification of *Wnt4* mRNA by RT-qPCR in ovaries of six month-old mice. Data are shown as averages after normalization to the control ± SEM, n=4 from both genotypes. **(C)** Histological examination using eosin/hematoxylin staining demonstrating cyst size in one year-old *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop ovary. Scale bars, 500 μm. **(D)** Quantification of *Wnt4* mRNA by RT-qPCR in ovaries of twelve month-old mice. Data are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. **(E)** Quantification of *Amh* mRNA by RT-qPCR in six month-old mice. Data are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. **(F)** Quantification of *Amhr* mRNA by RT-qPCR in six month-old mice. Data are shown as averages after normalization to the control ± SEM, n=3 from both genotypes. The two-tailed Student test was used for statistical analysis in (B) and (D-F). *P*≤ 0.05 was considered statistically significant.

aged mice using RT-QPCR approach. The results demonstrated an increase in expression of the *Amh* and *Amhr* genes in the ovaries of the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females at the age of six month when compared to controls (Fig. 5 E,F). Thus the anti-Müllerian hormone may be involved in formation of the noted ovarian cysts in the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females.

Together, the findings with the novel *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop mouse model provide further evidence for an important role of coordinated regulation of Wnt4 signaling in female sexual development also later in the female reproductive life cycle.

## Discussion

In this study, we report a novel mouse model, in which the *Wnt4* gene was targeted by inserting a stop cassette in front of the *3`UTR*. Such a knock-in mouse model was generated successfully. Analysis of the *Wnt4 3`UTR*stop cassette knock-in females provided evidence that the level of *Wnt4* mRNA and protein expression had become elevated as consequence of the UTR stop cassette insertion into the locus. The examination of the *Wnt4 3`UTR*stop homozygous mice suggested that the females developed normally but they were smaller in size when compared to WT mice and exhibited increased spleen and kidney weight. Wnt4 serves as a key signal for nephrogenesis during kidney development (Shan *et al*., 2010; Zhao *et al*., 2016; DiRocco *et al*., 2013; He *et al*., 2018). In the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop mice, no notable kidney associated phenotypes were found when compared to samples derived from WT controls. This was also the case with the testis. Thus, the *3`UTR* mediated regulation on Wn4 expression may not be functional for these organs.

In the novel *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop mouse model, the ovarian folliculogenesis process was notably affected when compared with control females and correlated with upregulation of certain hormone receptors' expression levels, such as *Fshr* and *Lhcgr*. Furthermore, the noted increase in the ovarian *Wnt4* gene and protein expression, lead to changes in the expression of genes that are involved in control of estrogens. The *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females had also cysts in the ovary at six months of age and in line with the anomalies in the follicle maturation in these transgenic mice. Collectively, our data suggest that the established *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop mouse represents a new model to investigate further the roles of Wnt4 signaling in the ovarian function, via controlling the events illustrated in Fig. 6.

Previously, we have described the generation and analysis of the hypomorph *Wnt4*mCh/mCh mouse model, where the Wnt4 and the *mCherry* encoding sequences were fused together and inserted into the *Wnt4* locus to replace the endogenous Wnt4 protein expression. This genetic modification lead to reduced Wnt4 expression in the *Wnt4*mCh/mCh mice and decreased number of ovarian follicles (Prunskaite-Hyyrylainen *et al*., 2014). Moreover, the *Wnt4*mCh/mCh females exhibited few poly-ovular follicles. Now, in our novel *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop experimental allelic model, we noted the opposite, namely an increase in the Wnt4 expression and both at the protein and the mRNA level. This elevation was associated with induction in follicle development to the pre-antral stage. However, after this, a reduction in the amount of corpus luteum was noted. We hypothesize that these phenotypes may be caused in part by the changes in cell polarity and extracellular matrix controlled also Wnt4 signaling as show earlier (Prunskaite-Hyyrylainen *et al*., 2014).
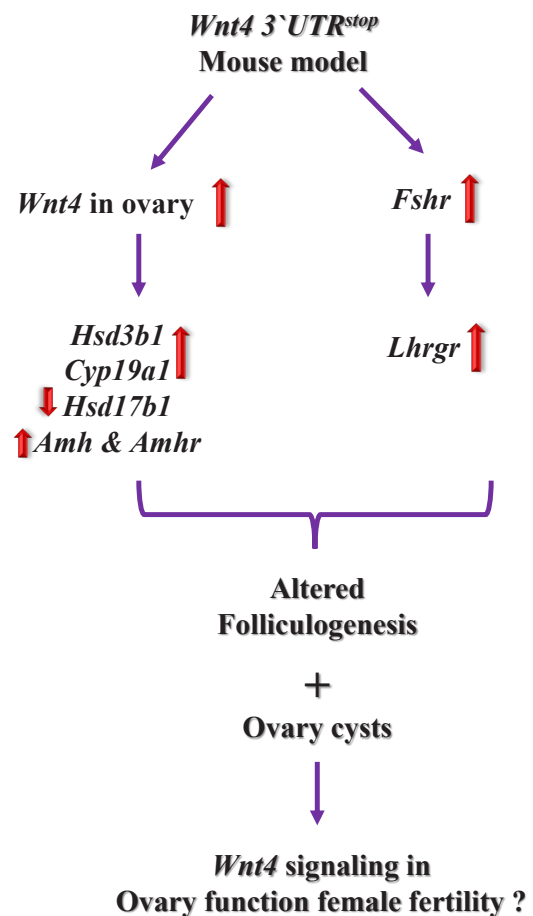


**Fig. 6. Proposed model of Wnt4 signaling in the female ovary.** Following Wnt4 signaling activation in the ovary of *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop, the expression of a set of genes associated with hormone synthesis is altered leading to changes in follicle counts. Consequently, the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop female exhibited reduced folliculogenesis which may be linked with cyst development and metabolic changes, namely lipid metabolism.

The FSH is critical for maturation of the pre-antral follicles (Hardy *et al*., 2017; Wang and Greenwald, 1993; François *et al*., 2017; Richards and Pangas, 2010). Here, FSH stimulates estradiol (E2) synthesis and associates with induction of the Cyp19b1 expression (François *et al*., 2017). These regulatory roles are in line with our findings in the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females where we observed an increase in the number of pre-antral follicles correlated with upregulation of *Fshr* and *Cyp19b1* expression. In agreement with earlier reports (Casarini *et al*., 2017; Ziecik *et al*., 2021; Richards and Pangas, 2010), the positive relationship between the *Hsd3b1* and the *Cyp19b1* gene expression and also the *Amh* and the *FSH* genes in the *3`UTR*stop;*Wnt4 3`UTR*stop females may be connected with the accumulation of pre-antral follicles.

The Hsd17b1 function has been studied by making a *Hsd17b1* knockout mouse model. Here, the ovaries undergo rather normal folliculogenesis development, with some reduction in the corpus luteum count (Hakkarainen *et al*., 2015). In the ovaries of the *Wnt4 3`UTR*stop;*Wnt4 3`UTR*stop females, a decrease in the *Hsd17b1* expression in the granulosa cells when compared with WT mice was found. This discovered link between Wnt4 signaling is in line

with the earlier reports on the roles of Wnt4 to advance hormone synthesis such as the androstenedione conversion to testosterone (Hiltunen *et al*., 2019; Nokelainen *et al*., 1996), those of the corpus luteum ones (O'Shaughnessy *et al*., 2000), and luteinizing hormone (LH) (Hakkarainen *et al*., 2015; Shehu *et al*., 2011). Our herein data support the previous observation demonstrating that the Wnt4 signaling is involved in coordination of the steroidogenesis signaling to regulate the follicle complex, thus promoting oocyte maturation (Jiang *et al*., 2021; Lewis *et al*., 2019; Alexandre and Mueller, 2023).

Together, the data with the *Wnt4 3`UTR*[stop];*Wnt4 3`UTR*[stop] mouse, in which Wnt4 signaling was enhanced via UTR engineering is in line with earlier studies which had indicated important roles for Wnt4 in ovarian ontogenesis and follicle maturation. Thus, the model reported herein provides a new tool with which to study Wnt4 signaling in female reproduction.

## Materials and Methods

### Cloning of the *Wnt4 3`UTR*[stop] transgenic constructs and screening of the *Wnt4 3`UTR*[stop] targeted ES cells

The targeting construct was cloned in pBluescript II[SK-] vector containing a 5' homologous arm, targeting cassette and a 3' homologous arm (Suppl. Fig. 1A). An 8.8kb XbaI-SacI genomic fragment corresponding to the nucleotides 38289-47130 was subcloned from BAC AL645468 (Shan *et al*., 2010), which contains the exon3, 4 and 5 of the *Wnt4* gene. The 5' homologous arm corresponding to nucleotides 42352 (KpnI)-43290 (stop codon) covers a part of the intron 4 and the whole exon 5. The 3' homologous arm corresponding to nucleotides 43291-47130 (SacI) contains a part of the *3`UTR*. The targeting construct was linearized by ScaI digestion and electroporated into SV129 ES cells. After selection, 300 ES clones were screened by PCR using primer F1: 5'AGCC-GGGCACTCATGAATCTTC3' and R1: 5'GAAGGAGCCAAGCTGCTA3'. Six putative correct targeted clones were found (Suppl. Fig. 1B). The PCR conditions were a 10-min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 45 s at 60 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. Six expended ES clones were verified by Southern blot analysis (Suppl. Fig. 1C). A Genomic fragment XbaI-HindIII corresponding to nucleotides 38289-40137, was used as a probe.

### Generation of the *Wnt4 3`UTR*[stop] mouse model, genotyping, and breeding

Two out of six positive ES cells were used for blastocyst injection with the standard procedure. Both showed germ line transmission. To avoid any ectopic expression effect on *Wnt4* gene, caused by the presence of a PGK promoter in the selected cassette, the PGK-Neo sequence was removed from both lines by Flip-Frt system. The mice were genotyped by PCR analysis using DNA isolated from ear clips. The wild type allele was amplified with primer F2: 5'ATTGAC-GGCTGCGAGCTACT3' and R2: 5'TGGGGGTAGGTGGTGGGAGA3'. The targeted allele was amplified with primer F2: 5'ATTGACG-GCTGCGAGCTACT3' and R3: 5'GGTACTCTGTTCTCACCCTTC3'.

The animal care principles and experimental procedures followed here were in accordance with the National Animal Experiment Board (ELLA) for the use of laboratory animals, the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123), and EU Directive 86/609/EEC.

### Tissue sections and PAS-Staining

The ovaries were dissected in 1× Dulbecco's PBS and fixed in 4% paraformaldehyde (PFA). The ovaries were then washed, dehydrated, embedded in paraffin, and cut into 6μm sections. These sections were stained with hematoxylin and eosin, and then imaged under the Zeiss Axio Imager motorized histology microscope (Germany). A minimum of 3 ovary-derived sections for each genotype were examined.

### RNA extraction and RT-QPCR

The ovary samples were dissected from three- and six month-old *Wnt4 3`UTR*[stop];*Wnt4 3`UTR*[stop] and wild type mice, and total RNA was purified with an RNeasy mini kit (Qiagen). 1 μg RNA was converted into cDNA with a First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The cDNA (2 μL), 2.5 μM of the primers, and Brilliant II SYBR® Green QPCR master mix (5 μl; Agilent Technologies) set to a total volume of 10 μl. The primers used are described in Suppl. Table 1. The real-time qPCR program consisted of 40 cycles at 95°C for 30 s and at 60°C for 1 min in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). GAPDH was used for normalization. The real-time qPCR results are presented as means ± SEM (4/4 mice).

### Follicular count and fertility

Follicular count and maturation were estimated as described previously (Veiga-Lopez *et al*., 2012; Myers *et al*., 2009). To assess the fertility of *Wnt4 3`UTR*[stop];*Wnt4 3`UTR*[stop] mice, two month-old female and male mice were housed with WT partners and monitored daily for litters for 3 months (*Wnt4 3`UTR*[stop];*Wnt4 3`UTR*[stop] female vs WT male & *Wnt4 3`UTR*[stop];*Wnt4 3`UTR*[stop] male vs WT female). Litter size was recorded at birth and the experiment ended 20 days after the removal of the males to allow for a potential final litter. Fertility capacity was judged by litter number and sizes.

### Western blotting

The ovaries were collected from two month-old mice and stored at -80 °C until they were used for protein purification. Western blot was conducted as previously described (Veikkolainen *et al*., 2020). Membranes were incubated with either anti Wnt4 (R&D system # AF475, 1:300) or anti-b-actin (Sigma #5441, 1:3000) antibodies.

### Statistics

Statistical analysis was performed with the unpaired, two-tailed Student's test. Values of *P*≤ 0.05 were considered statistically significant.

Conflicts of interest

The authors declare no conflict of interest.

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