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**Translin restricts the growth of pubertal mammary epithelial cells estrogen-independently in mice**

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**Abstract**

Translin, a ubiquitous RNA/DNA-binding protein that forms a hetero-octamer together with Translin-associated factor X (TRAX), possesses endoribonuclease activity and plays a physiological role in restricting the size and differentiation of mesenchymal precursor cells. However, the precise role of Translin in epithelial cells remains unclear. Here, we show evidence that Translin restricts the growth of pubertal mammary epithelial cells. The mammary epithelia of Translin-null females exhibited retarded growth before puberty, but highly enhanced growth and DNA synthesis with increased ramification after the onset of puberty. Primary cultures of Translin-null mammary epithelial cells showed augmented DNA synthesis in a ligand-independent and ligand-enhanced manner. Translin-null ovariectomized mice implanted with slow-release estrogen pellets showed enhanced length and ramification of the mammary glands. Mammary epithelial growth was also observed in ovariectomized Translin-null mice implanted with placebo pellets. Luciferase reporter assays using embryonic fibroblasts from Translin-null mice showed unaltered estrogen receptor α function. These results indicate that Translin plays a physiological role in restricting intrinsic growth, beyond mesenchymal cells, of pubertal mammary epithelial cells.

**Key words**

Translin/TRAX complex; Mammary epithelial cells; Puberty; Estrogen receptor α; DNA synthesis

## 1. Introduction

Translin, an RNA/DNA binding protein that is highly conserved from yeast to humans, forms a hetero-octamer together with Translin-associated factor X (TRAX) [1, reviewed in 2]. Translin was initially found in a pathological condition in lymphoma where it was recruited near the translocation sites [3] and subsequently implicated in DNA repair [4]. It was later documented, in a more physiological context, to possess endoribonuclease activities for microRNAs — in conjunction with RNA-induced silencing complex (RISC) activation [1,5,6] — and for tRNA [7]. As Translin was also shown to interact with the transcriptional repressor RP58/ZBTB18 [8] and coactivate the orphan nuclear receptor SF1/NR5A1 [9], it may be involved in some transcriptional controls.

Despite its high conservation across species, Translin does not appear to be essential to eukaryotic life and may in fact not even be needed for mammalian survival, as Translin-null mice survive. Nevertheless, the Translin/TRAX complex has a physiologically essential role in mammals. For example, it has been implicated in mediating the actions of the DISC1/GSK3β complex, which is involved in psychiatric disorders such as schizophrenia and autism [reviewed in 10].

Another line of study suggests the importance of Translin in mesenchymal cell physiology. Translin-null mice exhibit dwarfism [11] and obesity with disproportionate fat deposition in both visceral [11] and subcutaneous [12] fat depots. However, *in vitro*-fertilized Translin-null mice show normal stature, weight, and visceral adiposity, indicative of Translin's role in protecting epigenetic stability against intrauterine and nursing environments [13]. Furthermore, Translin-null mice have increased numbers of mesenchymal stem cells (MSCs) in the bone and bone marrow, and these MSCs intrinsically proliferate faster with heightened potencies of osteogenic and adipocytic differentiation. Translin-null subcutaneous fat-derived MSCs also show intrinsically enhanced adipocytic differentiation [13]. Translin-null mice reportedly show altered endochondral ossification, ectopic osteogenesis, adipogenesis in the bone marrow, and defective hematopoiesis that is potentially due to altered bone marrow microenvironment [14]. All these phenotypes suggest that Translin is involved in functions of mesenchymal cells, having an important role in restricting aberrant proliferation and differentiation of early mesenchymal progenitor cells. However, it is not known whether the role of Translin in limiting cell growth and differentiation is restricted to the mesenchyme.

During the process of reviewing the organs of Translin-null mice anatomically, we noticed an unexpected enhancement in the growth of mammary epithelial cells during puberty. The enhanced growth of the mammary epithelial cells in Translin-null mice was intrinsic, estrogen-independent, and boosted by estrogen. Estrogen receptor α (ERα) was functionally normal in Translin-null cells. These results suggest that the role of Translin in restricting aberrant cell growth is not limited to mesenchymal cells, but also extends to mammary epithelial cells.

## 2. Materials and methods

### *2-1. Mice*

*Translin* knockout mice [14], backcrossed at least ten times with C57BL6, were provided kindly by Dr. Masataka Kasai. Genotypes were determined as described [13]. Female offspring obtained by heterozygous crossings were used for the studies unless otherwise indicated. *In vitro*-fertilized mice were generated from *Translin*$^{-/-}$ sperms and *Translin*$^{+/-}$ ova as described [13].

To examine 17β-estradiol (E2)-stimulated mammary gland growth, 28-day-old virgin littermate females were ovariectomized and implanted with 60-day slow-release estradiol (E2) pellets (0.1 mg; Innovative Research of America) or placebo. After 60 days, the inguinal glands were examined following whole mount staining (described in the following section).

All mouse studies were performed according to institutional guidelines of Tokyo Medical University and Kobe BM Laboratory, Oriental Bioservice, Inc.

### *2-2. Whole mount staining and 5-bromo-2'-deoxyuridine (BrdU) staining of mammary*

*glands*

For whole mount staining, the inguinal mammary glands were excised, fixed with Carnoy's fixative for 2 hrs, and stained overnight in carmine alum. Samples were then dehydrated with ethanol, cleared in xylene overnight or longer until they were translucent, and mounted in MGK-S (Matsunami Glass) [15].

For BrdU staining, BrdU (0.1 mg/g of body weight) was administered intraperitoneally. After 2 hrs, the mice were sacrificed and perfusion-fixed with 4% paraformaldehyde in PBS. The inguinal mammary glands were isolated, post-fixed overnight at 4°C, and embedded in paraffin. Sections were then stained using the BrdU In-Situ Detection Kit (BD Pharmingen) to visualize the BrdU-positive cells, and counterstained with hematoxylin [15].

*2-3. Cell culture*

For primary culture of mammary epithelial cells, thoracic and inguinal mammary glands were resected from 11-week-old virgin females, minced, and incubated overnight at 37°C in phenol red-free Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12) containing 0.1% collagenase A (Roche), 100 U/mL hyaluronidase

(Sigma), and 100 U/mL penicillin/streptomycin (Thermo Fisher). Samples were centrifuged at 1,500 rpm for 10 min, and the pellets were washed with DMEM/F12 three times. The pellets were resuspended in CnT-Prime Epithelial Culture Medium (CELLnTEC) supplemented with 10 mg/ml insulin (Takara), 1 mg/ml hydrocortisone (Wako), 10 μl/ml EGF (R&D) and 100 U/mL penicillin/streptomycin, plated on fetuin (Millipore)-coated plates, and incubated at 37°C with 5% $CO_2$.

To measure DNA synthesis in the cultured mammary epithelial cells, 100 μM BrdU with or without $10^{-6}$ M E2 was added to 50% confluent cells plated in 12-well plates, and incubated for 24 hrs at 37°C. The incorporation of BrdU was then measured using Cell Proliferation ELISA, BrdU (chemiluminescence) (Roche) [15].

Mouse embryonic fibroblasts (MEFs) were obtained from E11.5 embryos derived from a heterozygous cross, and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C [15,16].

*2-4. Luciferase reporter assays*

MEFs were plated on 24-well plates with phenol red-free DMEM and 10%

charcoal-stripped FBS. The cDNA for activators (human (h) ERα, hPPARγ, p53 or Gal4-E1A) cloned into pcDNA3.1 (20 ng) (Thermo Fisher), and firefly luciferase reporter pGL3 (Promega) containing 3×ER-responsive elements (*ERE*), 3× PPAR-responsive elements (*PPRE*), *mdm2* promoter, or 5× Gal4-binding sites, were cotransfected with 5 ng *Renilla* control luciferase vector into cells using Lipofectamine 2000 (Thermo Fisher). After 24 hrs, reporter activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to control *Renilla* luciferase activity [17].

*2-5. Statistical analysis*

Numerical results are presented as mean ± SD. The significance of differences between the independent means was assessed by one-way ANOVA or Student's *t* test, and $P < 0.05$ was considered significant. $P < 0.05$ and $P < 0.01$ are represented by * and **, respectively. Reproducibility of each experiment was confirmed by repeated experiments.

## 3. Results

### *3-1. Translin promotes prepubertal and restricts pubertal mammary gland development*

Although we previously observed enhanced growth and differentiation of Translin-null mesenchymal precursor cells [13] and increased subcutaneous adiposity [12], we did not reproducibly observe the reported *Translin* knockout phenotypes such as visceral obesity and runting [11] in our rearing environment. Rather, most of the general development parameters of the naturally born Translin-null mice appeared to be normal, and thus the penetrance was incomplete. Their normality was even uniform in *in vitro*-fertilized *Translin*$^{-/-}$ and *Translin*$^{+/-}$ mice: their body weights [13] and weights of their various organs, including visceral white adipose and brown adipose tissues (supplementary Fig. 1), were comparable.

However, on further anatomical analyses of Translin-null organs, we incidentally noticed that the mammary glands of *Translin*$^{-/-}$ mice overgrew in thick fat pads. Therefore, we analyzed the pubertal mammary gland development in *Translin* knockout mice. At 4 weeks of age, the growing tips of the wild-type inguinal mammary glands reached the landmark lymph node. However, growth of the mutant mammary glands was retarded in a *Translin* dose-dependent manner: tips of the *Translin*$^{-/-}$ mammary

glands barely reached the node, and those of the *Translin*$^{+/-}$ glands only slightly exceeded the proximal end of the lymph node, thereafter at 6 weeks of age (Fig. 1A and B). However, elongation of the mutant glands gradually caught up by 8 weeks of age, and eventually exceeded the length of wild-type. At 16 weeks of age, the *Translin*$^{-/-}$ glands were most elongated, followed by the *Translin*$^{+/-}$, and the wild-type (Fig. 1A and B). The number of duct branches was also attenuated in the mutant mammary glands at 4 weeks of age. However, the ramification of mutant ducts caught up, and exceeded that of the wild-type after 8 weeks of age, and by 16 weeks of age the number of duct branches in *Translin*$^{-/-}$ was the highest, followed by *Translin*$^{+/-}$ and *Translin*$^{+/+}$ (Fig.1A and C).

We speculated that if E2 mediated responses are generally enhanced in Translin-null mice, then E2-influenced organ development might also be altered. Hence, we investigated uterine development in the Translin-null mice. We found that the uterus and the uterine adnexa of the *Translin*$^{-/-}$ females were grossly, and histologically comparable to those typically observed in 6-week-old wild-type littermates (supplementary Fig. 2), indicating that the enhanced growth was specific to Translin-null mammary glands. These results suggest that Translin-null mammary

glands grow in a retarded manner before puberty, and overgrow later during adolescence.

*3-2. Translin restricts E2-stimulated pubertal mammary ductal growth in vivo*

To investigate the E2-responses of Translin-null pubertal mammary glands *in vivo*, ovariectomized littermate females were implanted with slow-release E2 pellets. As expected, at 12 weeks of age, the *Translin*$^{+/-}$ mammary glands elongated more extensively than the wild-type glands, and the edges of *Translin*$^{-/-}$ mammary glands exceeded those of both the wild-type and *Translin*$^{+/-}$ glands by extending further massively towards the flanks (Fig. 2A and B). The number of *Translin*$^{-/-}$ mammary ductal branches was also increased compared to that of the other genotypes (Fig. 2A and C). These results further confirm the role of Translin in restricting E2-stimulated growth of pubertal mammary glands.

*3-3. Translin restricts steroid hormone-independent pubertal mammary ductal growth in vivo*

Pubertal mammary gland development depends strongly on ovarian steroid hormones. Therefore, the development of mammary glands in ovariectomized females generally

stops [reviewed in 18]. As expected, when 28-day-old littermate females were ovariectomized and implanted with placebo pellets, the wild-type mammary glands hardly elongated at 12 weeks of age (Fig. 2D and E). However, oddly, the *Translin*$^{+/-}$ mammary glands elongated to some extent, although not extensively, while the *Translin*$^{-/-}$ mammary glands extended even more, at 12 weeks of age (Fig. 2D and E). The number of branches in the *Translin*$^{-/-}$ mammary glands also exceeded those in the other genotypes (Fig. 2D and F). Given the delayed growth of the Translin-null mammary glands at 4 weeks of age (Fig. 1A-C), it is obvious that the Translin-null mammary glands grew without ovarian steroid hormone, although not extensively. These data suggest that Translin restricts ovarian steroid hormone-independent growth of mammary glands postnatally.

*3-4. Translin intrinsically restricts growth of mammary epithelial cells*

To exclude the possibility that the enhanced Translin-null pubertal mammary gland development reflects an altered tissue microenvironment that supports mammary epithelial stem cells [reviewed in 19,20], we performed primary culture of mammary epithelial cells, and analyzed intrinsic DNA synthesis. When mammary epithelial cells were dissociated from the tissues, and subjected to two-dimensional culture with media

that selectively retains the epithelial cells in a proliferative state, the proliferating epithelial cells initially formed aggregates/colonies in plates. Intriguingly, the size of the aggregates formed from *Translin*$^{-/-}$ mammary epithelial cells was significantly larger than those of wild-type littermate epithelial cells (Fig. 3A and B). Cells from the next passage were then used for analyzing DNA synthesis. Incorporation of BrdU into the wild-type and *Translin*$^{+/-}$ mammary epithelial cells was enhanced in an E2-dependent manner, as expected. However, the E2-dependency of BrdU incorporation into the *Translin*$^{-/-}$ epithelial cells was not statistically significant due to enhanced E2-independent mechanism. Indeed, fold-increase of BrdU incorporation in the presence of E2 as against the baseline incorporation in the absence of E2 into Translin-null cells (*Translin*$^{+/-}$, 2.3-fold; and *Translin*$^{-/-}$, 1.8-fold) was not enhanced compared to that of wild-type cells (2.9-fold) (Fig. 3C). Notably, however, BrdU incorporation into the *Translin*$^{-/-}$ epithelial cells in the presence of E2 was increased 3-fold compared to that in wild-type cells (Fig. 3C). These results suggest that DNA synthesis and growth of *Translin*$^{-/-}$ mammary epithelial cells are intrinsically and E2-independently enhanced.

*3-5. Translin restricts DNA synthesis in mammary epithelial cells in vivo*

To confirm the enhancement of DNA synthesis in mammary epithelia *in vivo*, virgin littermate females of 12 weeks of age were purged with BrdU. We found that the positivity for BrdU in both luminal and basal cells of the wild-type mammary epithelia was low. In contrast, the positivity for BrdU in the *Translin*$^{+/-}$ mammary luminal cells was enhanced, and that in the *Translin*$^{-/-}$ luminal and basal cells was notably more than that in the wild-type cells (Fig. 3D and E). These results further confirm enhancement of *Translin*$^{-/-}$ mammary epithelial cell growth *in vivo*.

*3-6. Translin does not affect the transcription function of ERα*

While our canonical knowledge of mammary gland physiology indicates an indispensable role for ERα in postnatal mammogenesis [reviewed in 18], the abovementioned results indicate a role for Translin in restricting E2-independent mammary epithelial growth. To determine the E2-dependent and -independent function of ERα in Translin-null cells, we performed luciferase reporter assays using MEFs. As expected, ERα-induced transcription, assessed by 3×*ERE*-promoter reporter, was unaltered in *Translin*$^{+/-}$ and *Translin*$^{-/-}$ MEFs compared to the wild-type MEFs. The function of another nuclear receptor PPARγ, as well as that of other representative activators (p53, and adenoviral activator Gal-E1A) [16,21], was also unaffected in the

Translin-null cells. These results suggest that Translin does not affect ERα function, supporting the conclusion that Translin restricts sex hormone- or ERα-independent mammary epithelial growth.

## 4. Discussion

This study demonstrates that the growth-restricting role of Translin extends beyond mesenchymal cells to pubertal mammary epithelial cells. This study also challenges our current knowledge of the canonical physiology of postnatal mammogenesis, specifically that of its dependence on sex hormones. In contrast, our data show that, in the absence of Translin, mammary glands can extend even without ovarian sex hormones.

It is surprising that a phylogenetically conserved protein such as Translin is not essential for mammalian survival, *per se*. However, it appears that, without Translin, mice are sensitive to different environments, as the Translin-null mice do not show the reported phenotypes of runting or visceral adipose cell hypertrophy in our housing environment [11,13]. Therefore, Translin may be generally indispensable for mammalian life by offering protection from epigenetic alterations in fluctuating and/or harsh environmental conditions, through a hereto undefined mechanism. The mechanism of Translin-mediated growth-restriction, specifically in mesenchymal precursor and mammary epithelial cells, demonstrated in previous [13] and current studies also remains unclear. Translin's protective role against epigenetic fragility and aberrant cellular proliferation may be explained by its function of specifically regulating small

RNAs through endonuclease activities, and this mechanism remains to be elucidated in future.

While the development of rudimentary mammary glands during fetal stage does not require estrogen action, it is generally believed that postnatal mammogenesis is strongly dependent on estrogen [18]. Contrary to this, our study demonstrates for the first time that Translin-null pubertal mammary epithelial cells proliferate even without estrogen. Thus, our study raises an interesting hypothesis: mammary epithelial cells intrinsically possess weak proliferative ability, both prenatally and postnatally, and Translin inhibits their spontaneous growth postnatally. The growth-inhibitory effect of Translin may be overcome by the pubertal surge of sex hormones. An appropriate balance between the growth-promoting effect of sex hormones, and the inhibitory effect of Translin may be important, as mammary epithelial growth is prominently enhanced when Translin is absent or low (this study). Apart from Translin's role in adolescent growth of mammary glands, its role in other mammary epithelial changes such as during pregnancy, lactation, and regression is not known, because *Translin*$^{-/-}$ females seldom get pregnant. This issue needs to be clarified in the future using conditional knockout strategies.

In the context of Translin's role in inhibiting cell growth and protecting against epigenetic alteration, Translin-null females may have an increased risk for breast carcinogenesis and progression; it would be worth studying them further by crossing them with breast cancer-prone mice. Translin might also be attenuated in human breast carcinoma cells, and reverting Translin expression levels might lead to cancer regression. If so, modulating Translin might be a new therapeutic strategy for cancer in future.

In conclusion, Translin specifically inhibits the aberrant growth of pubertal mammary epithelia in an estrogen-independent manner. This effect of Translin is autonomous and intrinsic.

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**Figure legends**

Fig. 1. Translin-null mammary glands develop slowly before puberty and overgrow during puberty.

(A) Whole-mount staining of inguinal mammary glands. Glands prepared from littermate virgin females of different ages. Bar, 2.5 mm (4 weeks), 5 mm (6 and 8 weeks), or 10 mm (10 and 12 weeks).

(B) Mammary duct length. The distance of the glandular tips from the inner ends of the lymph nodes was measured.

(C) Number of duct branches per visual field. N = 8.

Fig. 2. Translin-null mammary glands overgrow both E2-dependently and sex hormone-independently.

(A-F) Mammary ductal growth with (A-C) or without (D-F) estrogen. Littermate 28-day-old females were ovariectomized and implanted with either E2 pellets (A-C) or placebos (D-F) for 60 days. Whole-mount staining (A, D), mammary duct length (B, E), and number of duct branches per visual field (C, F) are shown. Bar, 5 mm (A, D); N = 4 (C, F).

Fig. 3. DNA synthesis in mammary epithelial cells is enhanced *in vitro* and *in vivo*.

(A-C) Primary mammary epithelial cell culture obtained from littermate females. Photographs of representative cell aggregations (A), and diameters of aggregates (B), observed before passages (P0) are shown. DNA synthesis of the passaged (P1) epithelial cells in the absence or presence of E2 is shown (C). N = 6 (B) or 7 (C).

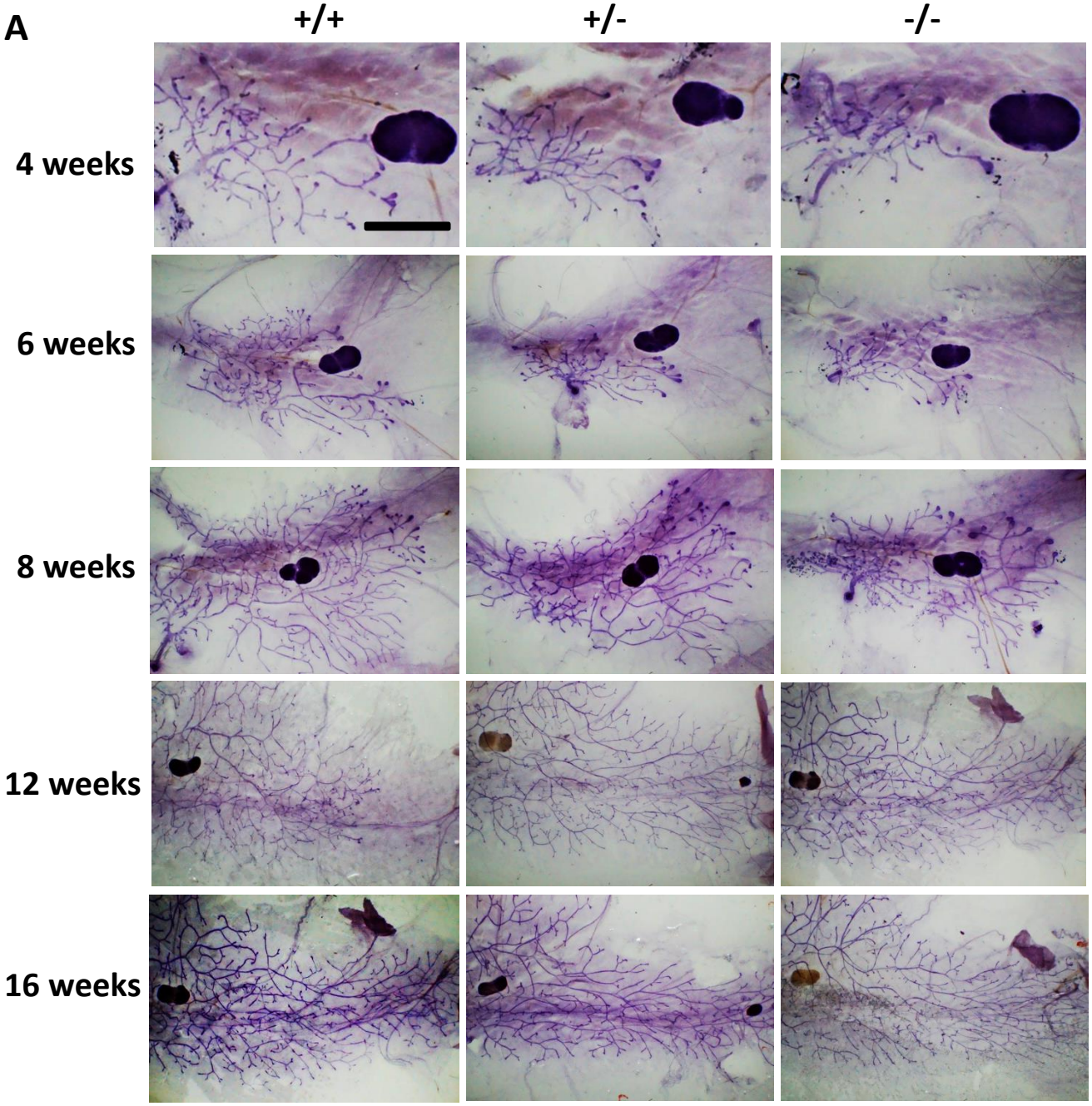
(D, E) DNA synthesis in mammary epithelia *in vivo*. Littermate females were purged with BrdU, and BrdU-positive cells were visualized. Representative sections are shown. Bar, 50 μm (D). Percentage of BrdU-positive luminal and basal cells are shown. N = 4 (E).

Fig. 4. ERα function is unaltered in Translin-null cells.

(A, B) Luciferase reporter assays of MEFs showing functions of ERα (A) and another representative nuclear receptor PPARγ (B). Assays were performed in the absence or presence of $10^{-6}$ M E2 (A) or $10^{-5}$ M 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (B).

(C, D) Luciferase reporter assays of MEFs showing functions of other representative activators, p53 (C) and Gal-E1A (D).

The values were normalized against those of *Translin*$^{+/+}$ MEFs in the absence of exogenous ligands (A, B) or activators (C, D). N = 3.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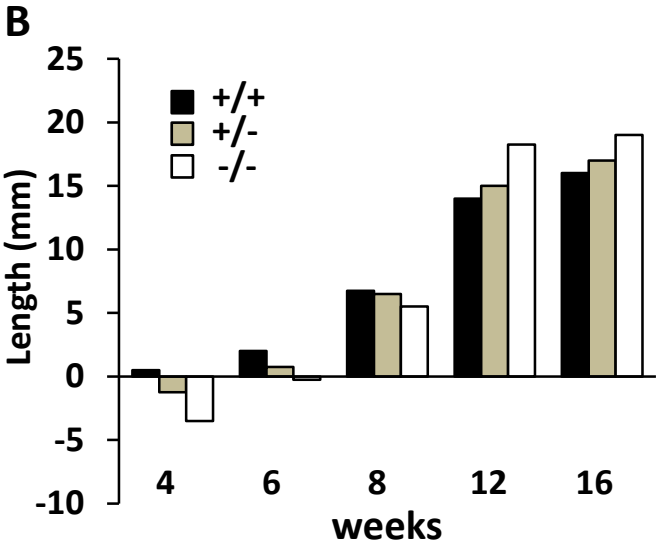
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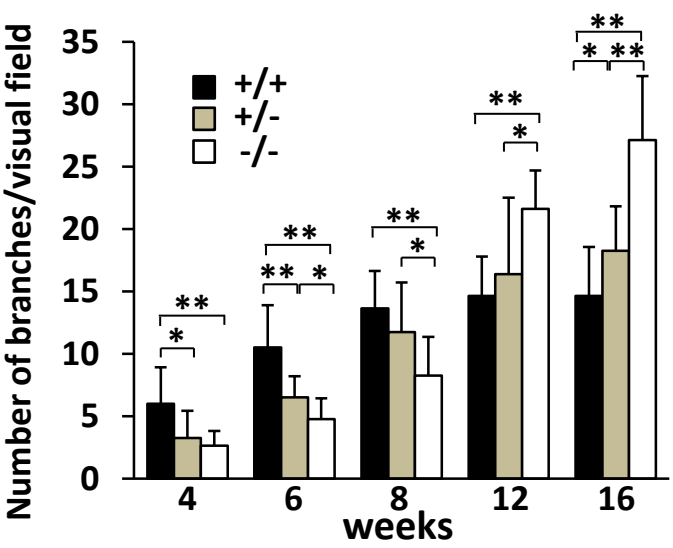
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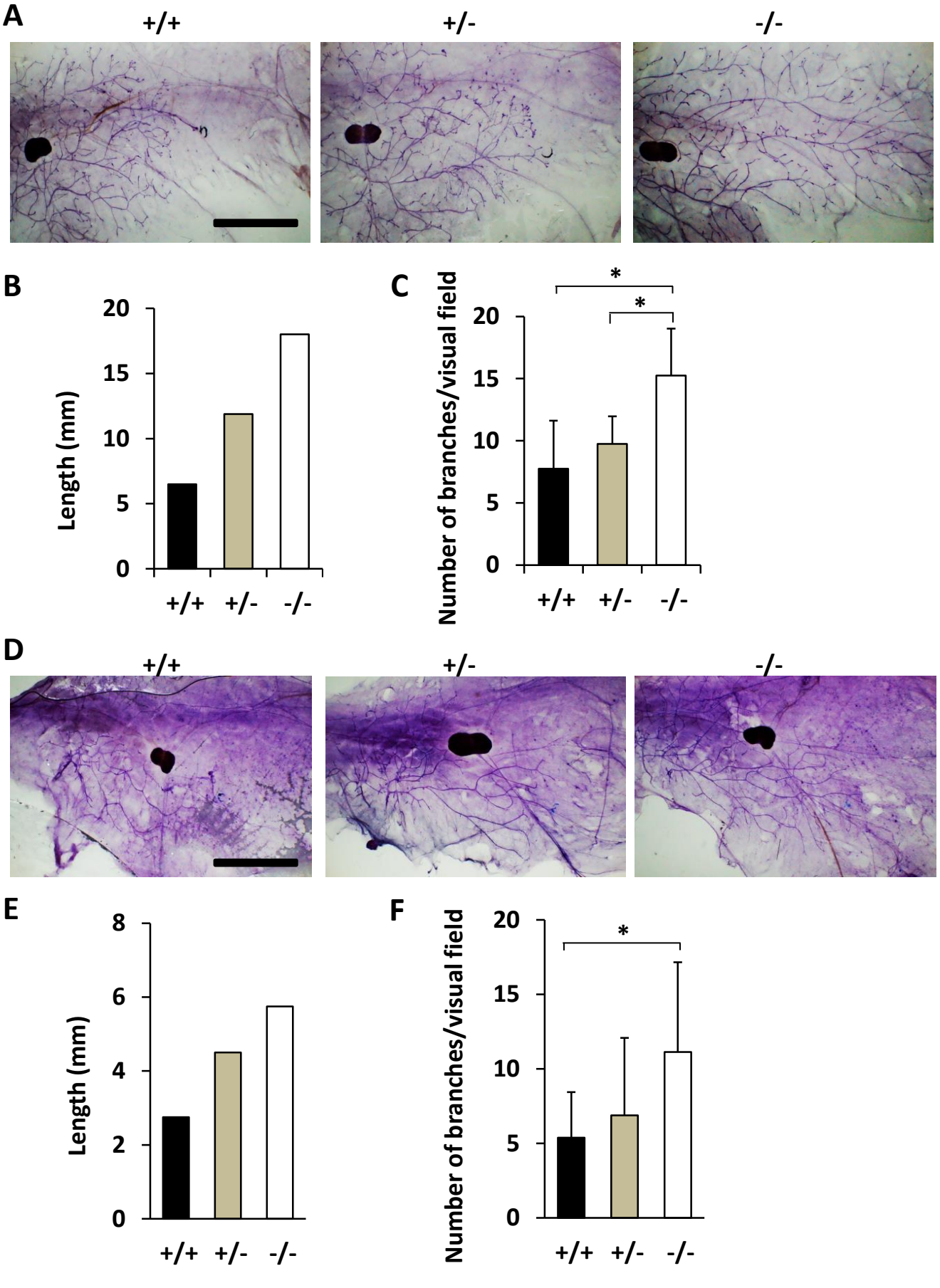
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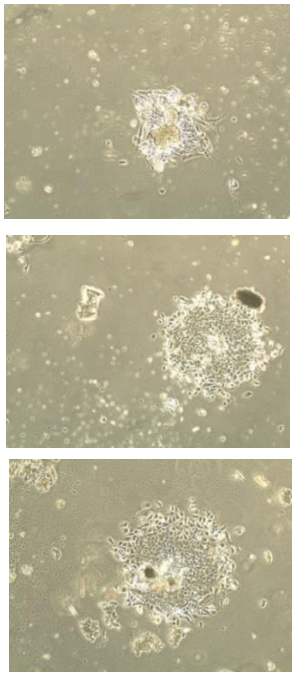
+/-
-/-
B
Length (mm)
20
15
10
5
0
+/+ +/- -/-
C
Number of branches/visual field
*
*
20
15
10
5
0
+/+ +/- -/-
D
+/+
+/-
-/-
E
Length (mm)
8
6
4
2
0
+/+ +/- -/-
F
Number of branches/visual field
*
20
15
10
5
0
+/+ +/- -/-

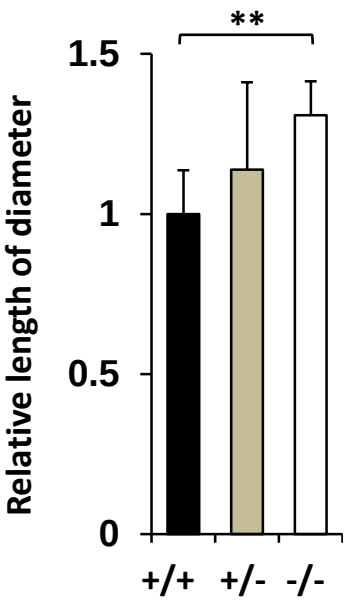
A

Typical cell aggregation (P0)



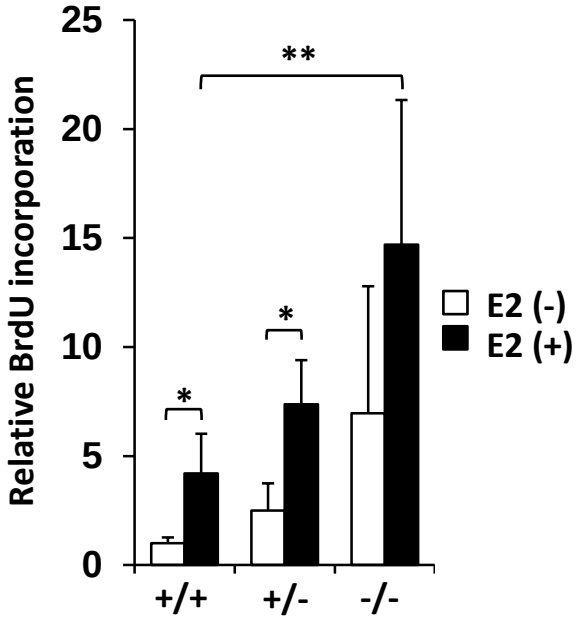

B

Size of cell aggregation (P0)

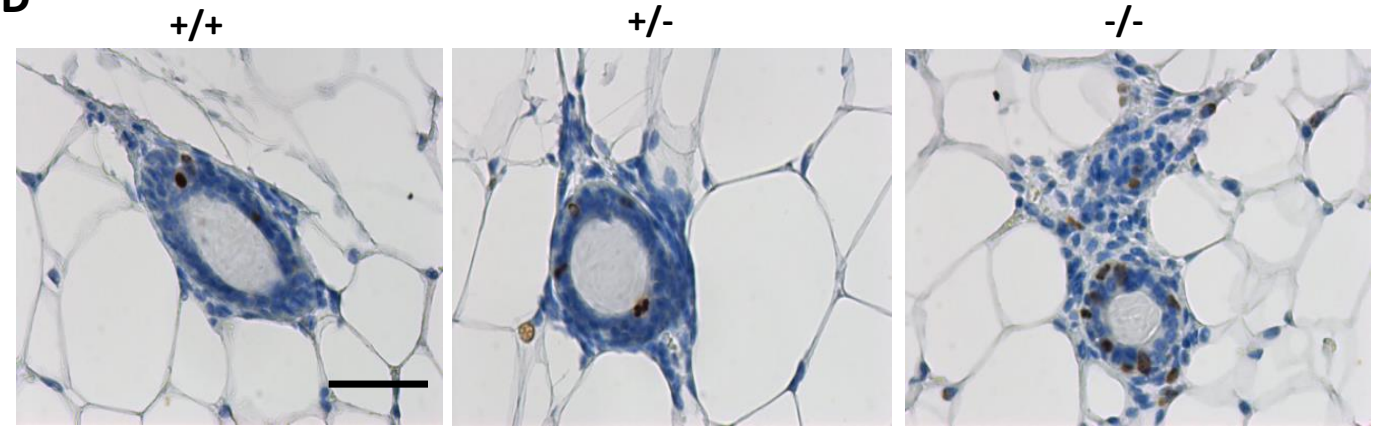



C

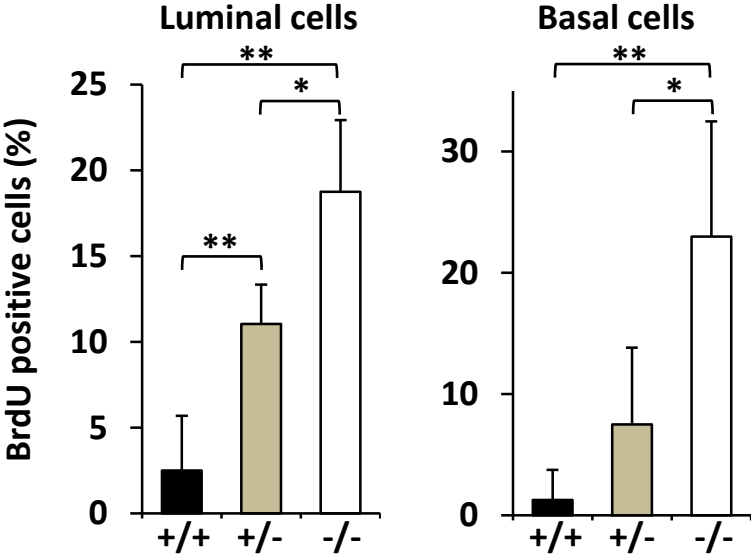
DNA synthesis (P1)

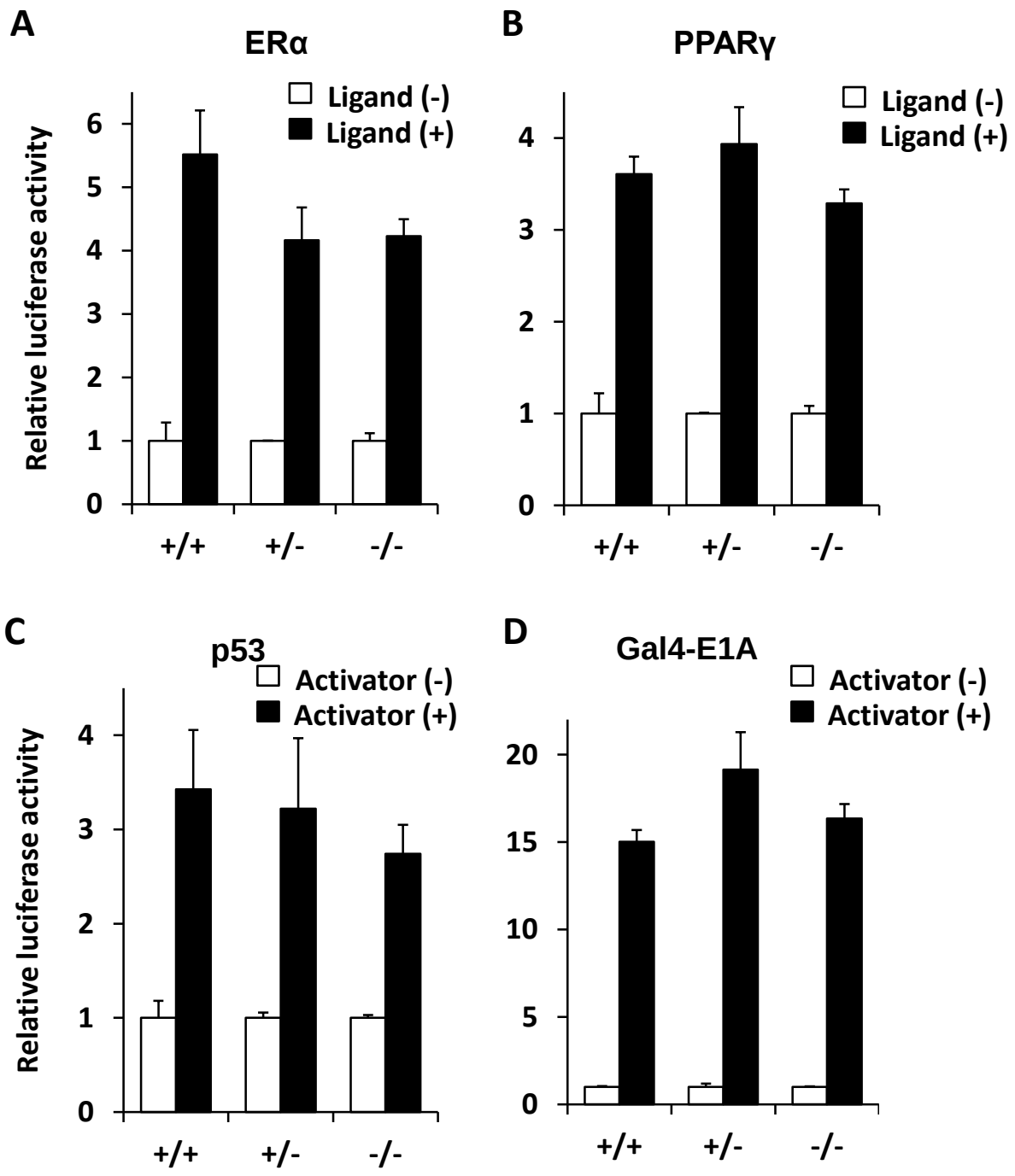


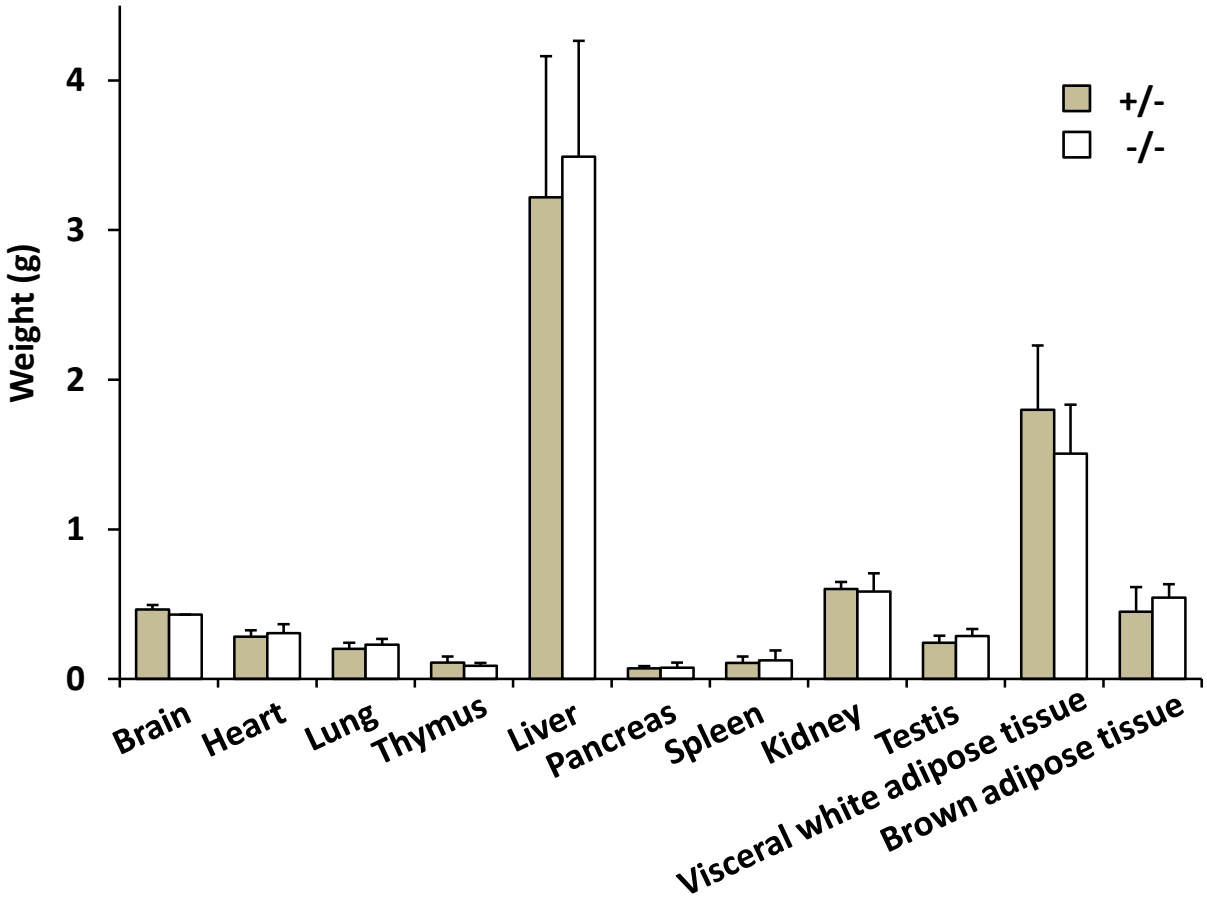

D



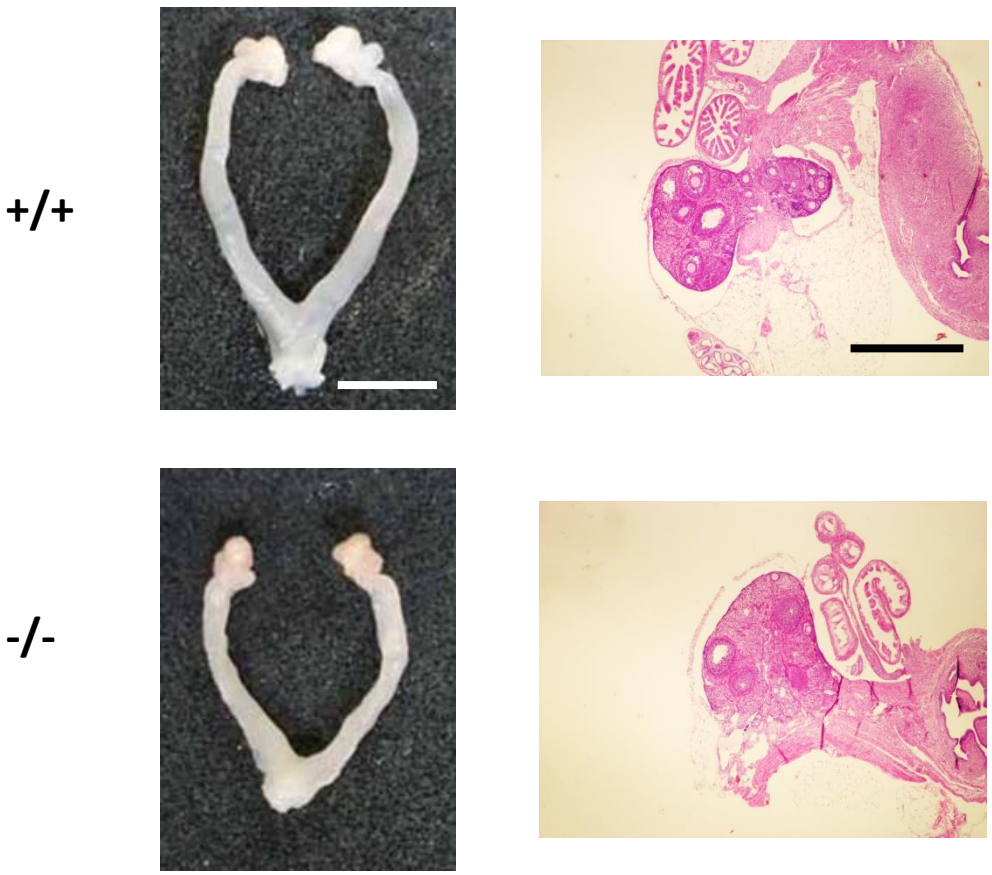

E




A
ERα
Ligand (-)
Ligand (+)
Relative luciferase activity
0
1
2
3
4
5
6
+/+
+/-
-/-
B
PPARγ
Ligand (-)
Ligand (+)
0
1
2
3
4
+/+
+/-
-/-
C
p53
Activator (-)
Activator (+)
Relative luciferase activity
0
1
2
3
4
+/+
+/-
-/-
D
Gal4-E1A
Activator (-)
Activator (+)
0
5
10
15
20
+/+
+/-
-/-




Supplementary Fig. 1. Weights of organs of *ex vivo*-fertilized *Translin*$^{-/-}$ males are not altered. C57BL6 *Translin*$^{-/-}$ sperms and *Translin*$^{+/-}$ ova were fertilized *ex vivo*, and pups were fostered by ICR females. Weights of various organs of *Translin*$^{-/-}$ and *Translin*$^{+/-}$ males (37-week-old) were comparable. N = 8 (*Translin*$^{+/-}$) or 4 (*Translin*$^{-/-}$).




Supplementary Fig. 2. *Translin*$^{-/-}$ uterus and uterine adnexa are apparently normal.
Gross appearance (left) and hematoxylin-eosin staining (right) of 6-week-old littermate wild-type and *Translin*$^{-/-}$ uterus and uterine adnexa are shown. Bar, 5 mm (left) or 1 mm (right).