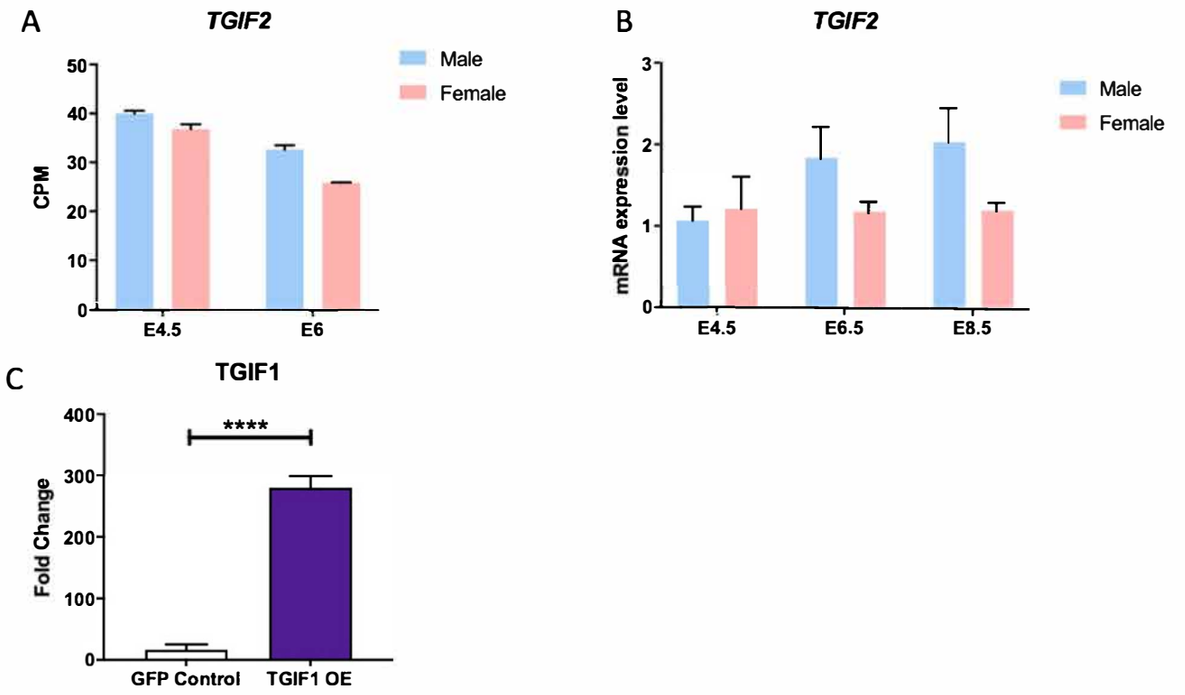


Fig. S1. *TGIF2* expression is not sexually dimorphic in chicken gonads. (A) *TGIF2* gonadal RNA-seq mRNA expression levels in count per million (CPM) at blastoderm stage, before (E4.5) and on the onset (E6) of sex determination. (B) *TGIF2* gonadal mRNA was quantified by qRT-PCR. Expression level is relative to β-actin and normalized to E4.5 male. (C) *TGIF1* mRNA was quantified by qRT-PCR in DF1 cells transfected with TGIF1 overexpression or GFP control plasmids. Expression level is relative to β-actin and normalized to GFP control. Bars represent Mean ± SEM. Unpaired two-tailed t-test. **** = p value <0.0001.

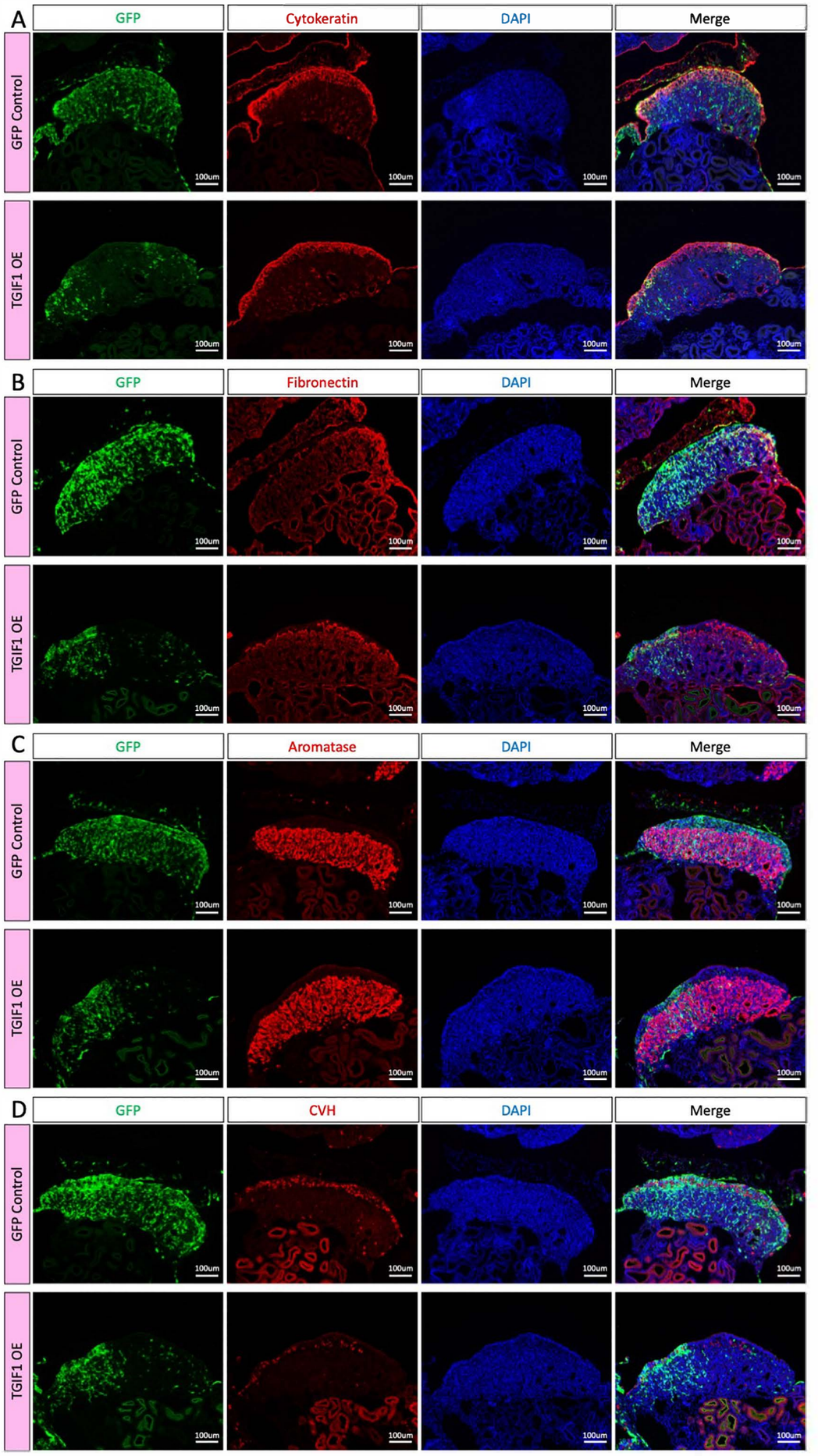


Fig. S2. TGIF1 overexpression in female left gonads has no effect. TOL2 TGIF1 overexpression (TGIF1 OE) or control (GFP OE) plasmids were electroporated in female left E2.5 coelomic epithelium. Gonads were examined at E8.5. Immunofluorescence against (A) cytokeratin (epithelial/cortical marker), (B) fibronectin (interstitial cell marker) and (C) aromatase (pre-granulosa cell marker) and (D) CVH (germ cell marker) were performed in transverse sections.

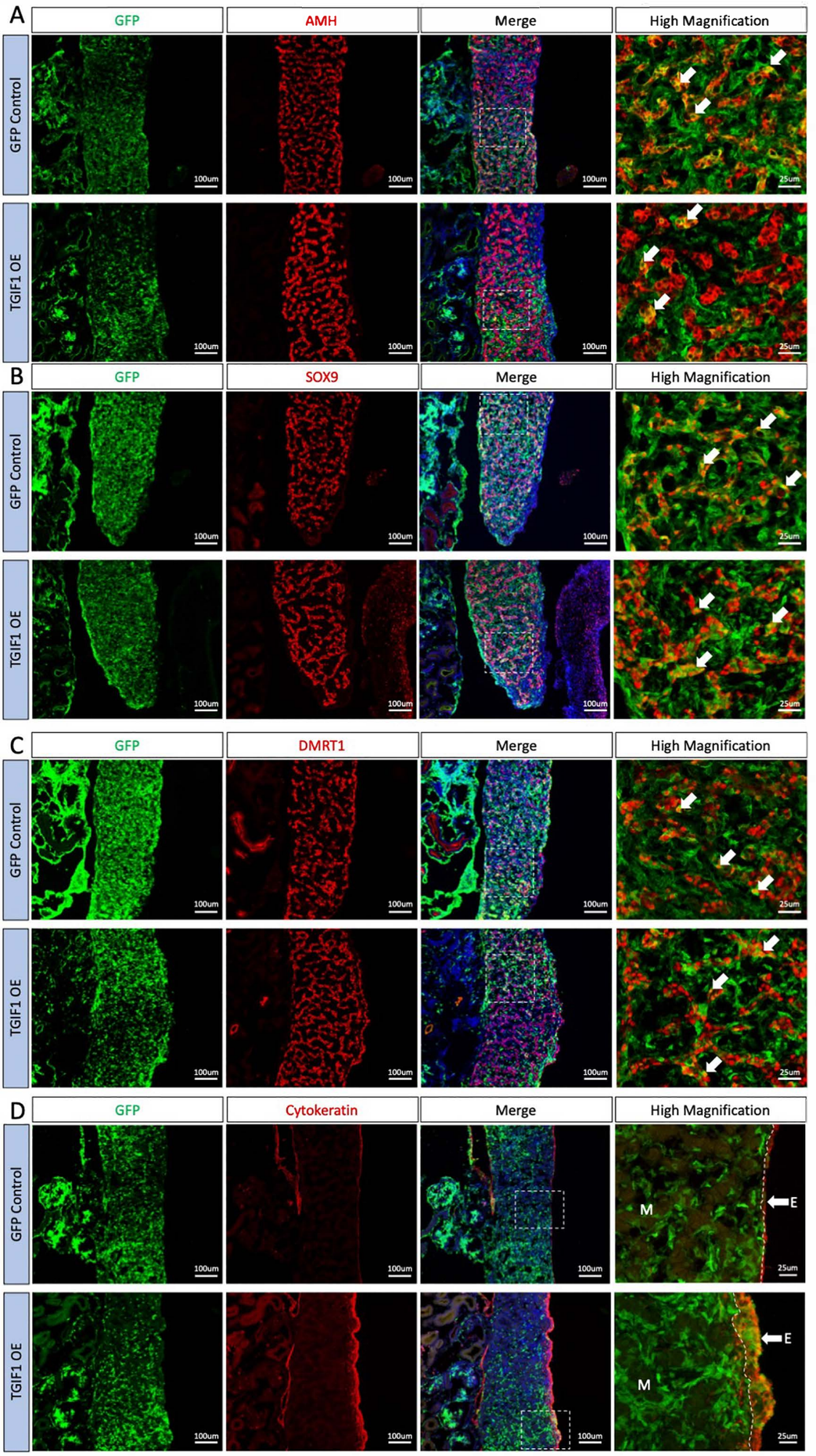


Fig. S3. **TGIF1 overexpression did not alter the supporting cell differentiation program.** RCAS(A) TGIF1 overexpression (TGIF1 OE) or control (GFP OE) plasmids were electroporated in male E2.5 left coelomic epithelium. Male gonads were examined at E8.5. Immunofluorescence against the supporting cell markers (A) AHM (B) SOX9 and (C) DMRT1. Dashed box indicates the magnified area. White arrows indicate colocalization of GFP (TGIF1) and Sertoli cell markers. (D) Immunofluorescence against the epithelial/cortical marker cytokeratin was performed in longitudinal male gonadal sections. Dashed box indicates the magnified area. Dashed line delineates the gonadal epithelium. White arrow indicates the epithelium (E). M indicates the medulla.

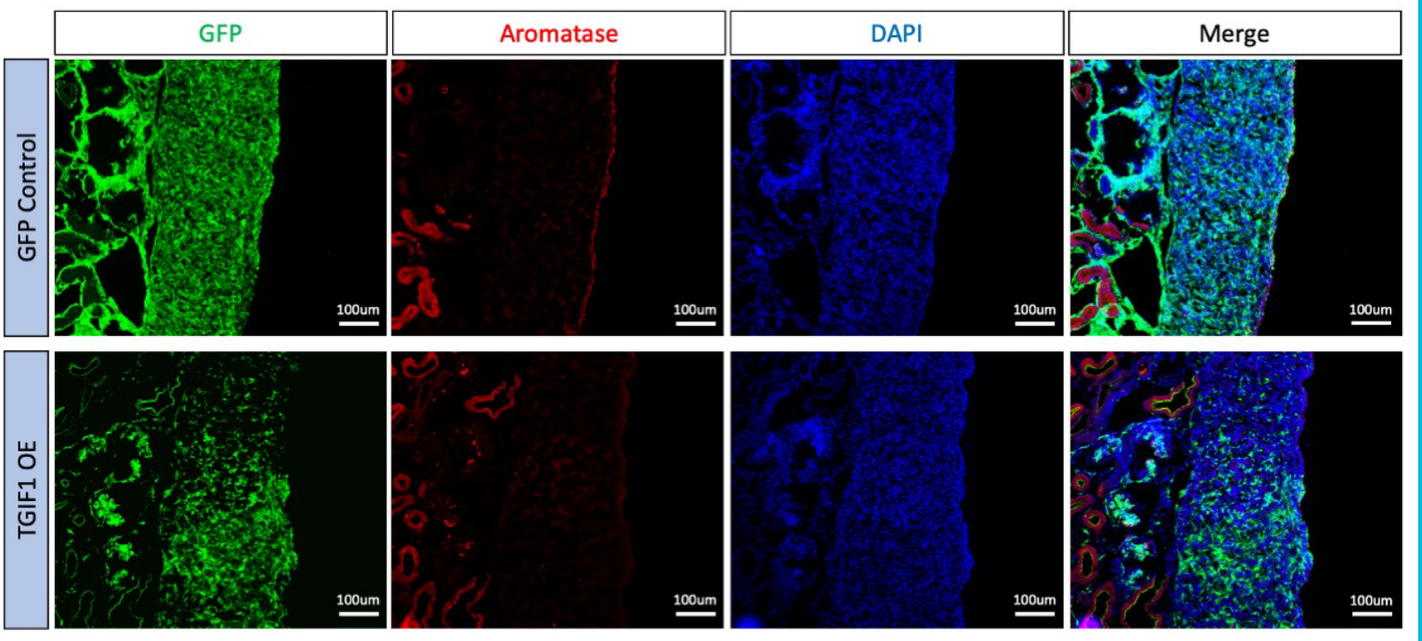
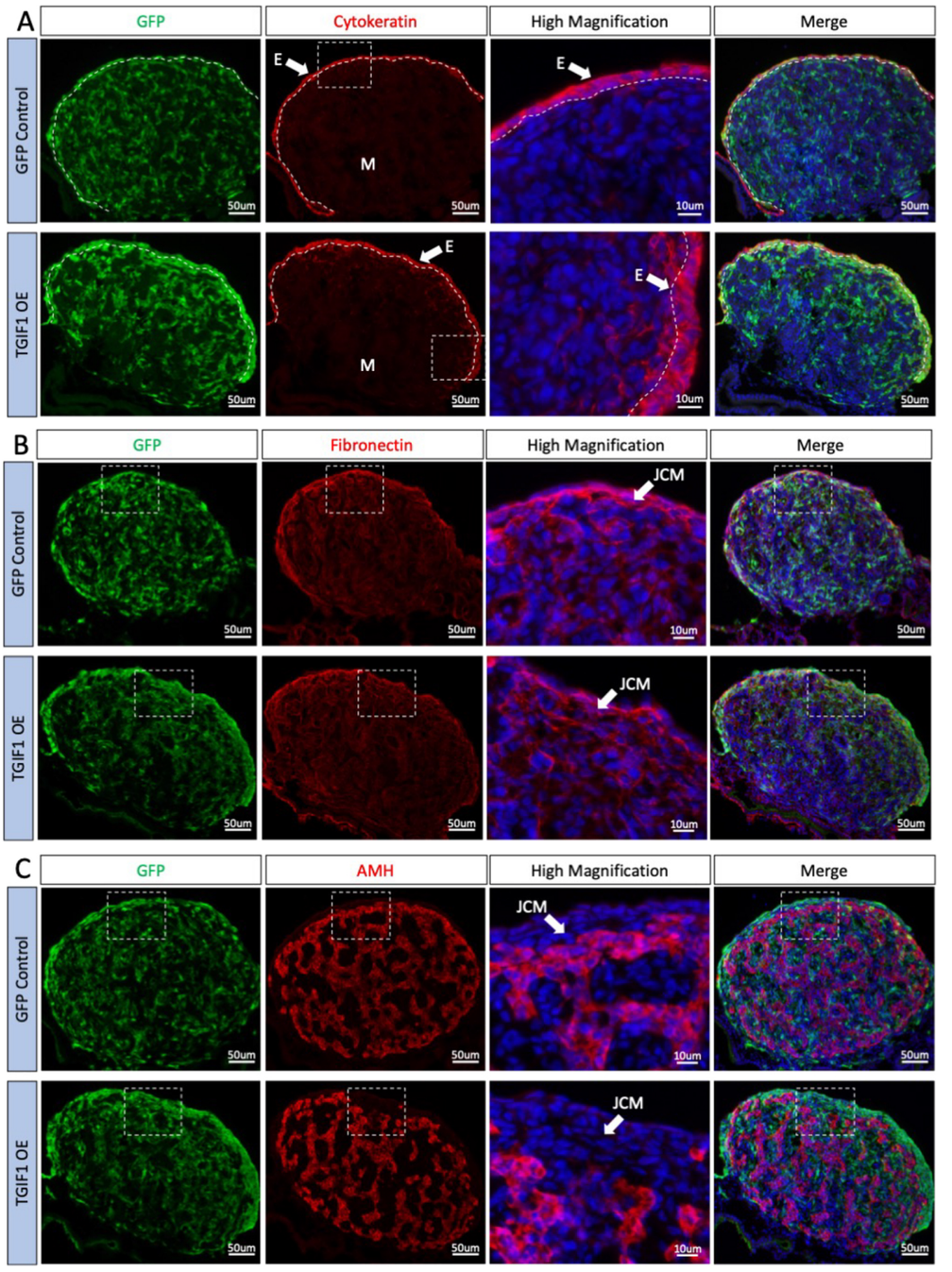


Fig. S4. **TGIF1 overexpression did not upregulate aromatase expression.** RCAS(A) TGIF1 overexpression (TGIF1 OE) or control (GFP OE) plasmids were electroporated in male E2.5 left coelomic epithelium. Male gonads were examined at E8.5. Immunofluorescence against the pre-granulosa markers aromatase was performed in longitudinal male gonadal sections.



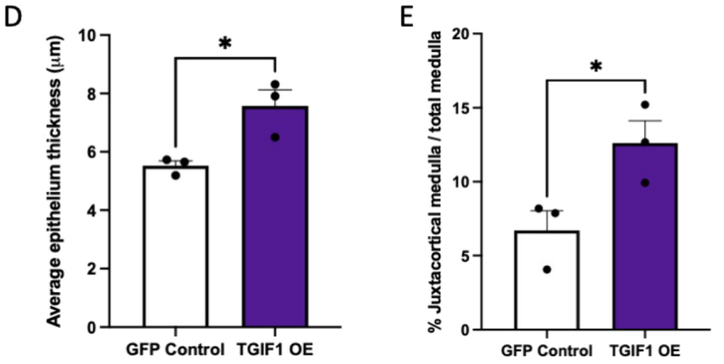


Fig. S5. **TGIF1 overexpression in right male gonads results in epithelial thickening and JCM formation.** TOL2 TGIF1 overexpression (TGIF1 OE) or control (GFP Control) plasmids were electroporated in male right E2.5 coelomic epithelium. Gonads were examined at E8.5. Immunofluorescence against (A) cytokeratin (epithelial/cortical marker), (B) fibronectin (interstitial cell marker) and (C) AMH (Sertoli cell marker) were performed in transverse sections. Dashed box indicates the magnified area. Dotted line delineates the gonadal epithelium. White arrow indicates the epithelium (E) or the juxtacortical medulla (JCM). (D) Quantification of the average epithelium thickness (in um) in control or TGIF1 overexpressing male gonads. (E) Quantification of the percentage of juxtacortical medulla area, related to the total medullar area. Bars represent Mean ± SEM, n=3. Unpaired two-tailed t-test. * = p value <0.05.

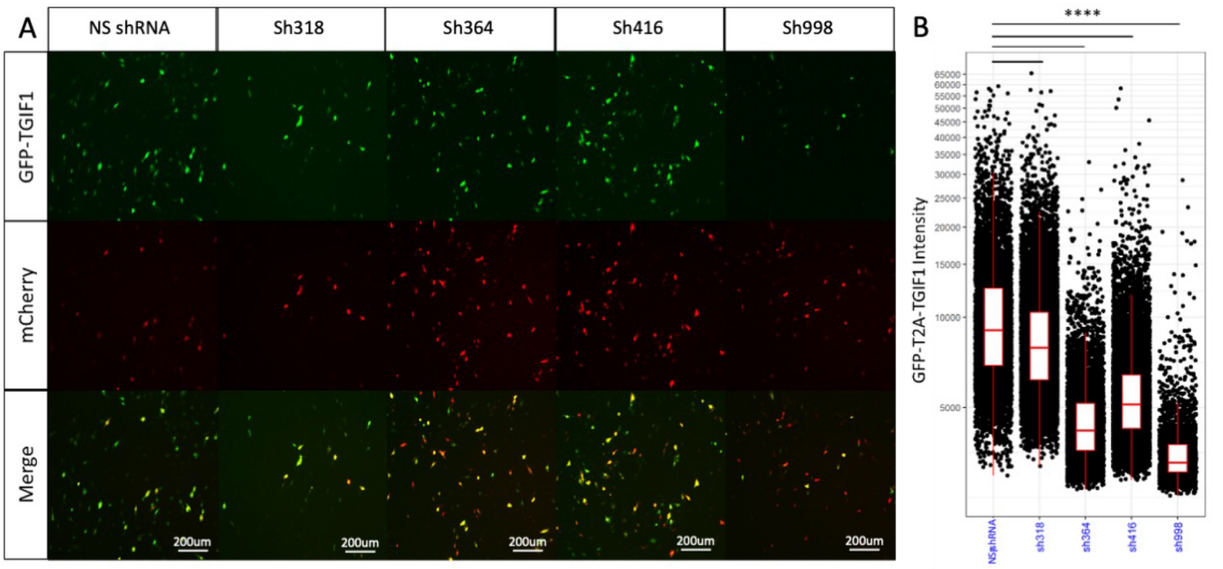


Fig. S6. **TGIF1 sh998 showed the higher repression of TGIF1 in vitro**. DF-1 cells were transfected with a self-replicative viral plasmid containing BFP-T2A and a non-specific shRNA (NS shRNA) or with 4 different putative shRNA designed for TGIF1 knockdown (sh318, sh364, sh416 or sh998). After all cells were BFP positive, they were transfected with the overexpression TOL2-GFP-T2A-TGIF1 plasmid and a plasmid expressing mCherry (as a transfection control). After 48 hours cells were fixed and analyzed under the microscope. (A) Representative fluorescence images of the outcomes. (B) Imaris analysis of the GFP-T2A-TGIF intensity in mCherry positive (transfected) cells. Box plots show each sample's median, interquartile ranges (IQR) and the whiskers extend to the highest/lowest value within 1.5 x IQR. Each dot represents an individual cell. t-test was performed using NS shRNA as a control condition. Dunnett's multiple comparisons test was used as posttest. **** = $p < 0.0001$.

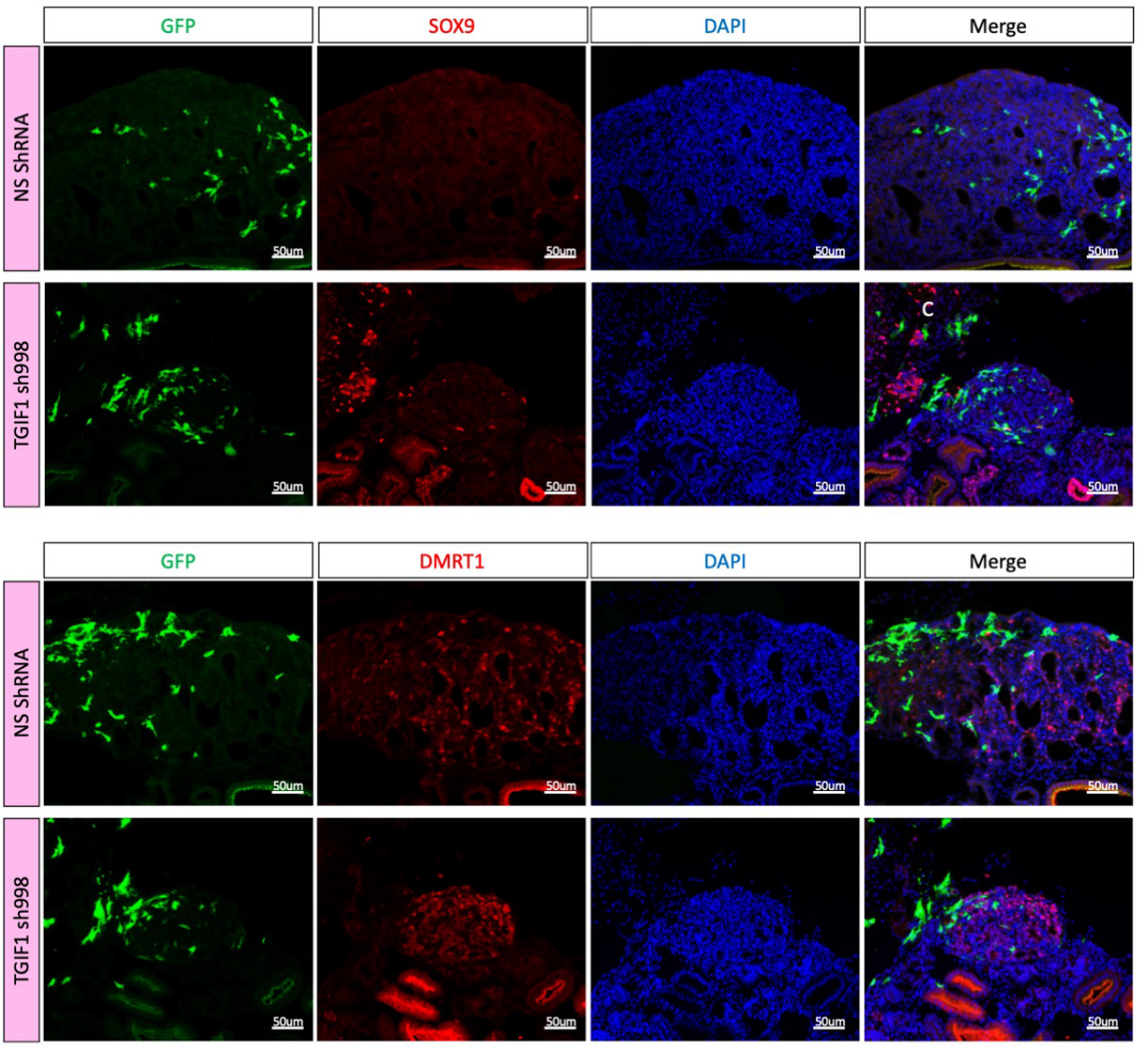


Fig. S7. TGIF1 knockdown does not induce Sertoli cell markers expression. TOL2 TGIF1 knockdown (TGIF1 sh998) or non-silencing control (NS shRNA) plasmids were co-electroporated with a GFP-expressing plasmid (reporter) in female left E2.5 coelomic epithelium. Gonads were examined at E8.5. Immunofluorescence against SOX9 and DMRT1 was performed on transverse sections.

Table S1. Primer list.

Gene and technique	Primer sequence
TGIF1 qPCR	Fw: ACACACCTCTCCACACTACAG Rv: GGTTTGGGTCTTTGCCGTCC
TGIF2 qPCR	Fw: CGCAAACTTTTCGGGGCAC Rv: GGCGCGGTCCTGCTTC
β-Actin qPCR	Fw: GCTACAGCTTCACCACCACA Rv: TCTCCTGCTCGAAATCCAGT
TGIF1 WISH	Fw: AGGAACAAAGGCGAAGCGAG Rv: TTTGGGTCTTTGCCGTCCTT
TGIF1 Overexpression	Fw:GAAAATCCCGGCCCCATGAAAAGTAAAAAAGGTGTAGTTGCAATATCAGG Rv: GTCGTCCTTGTAGTCACTTAGGCCATGAGTTTTGCCTGC
TGIF1 Sh318	Fw: GGCCAGTGAATTCGCGTACCTCCTTCTCGCAGGGC Rv:AGCTATGACGAATTCGCAAAAAACCACTAGATCTTTCCTCATTCTCTTGAAATGAGGAAAGATCTAGTGG AAACCCCAGTTGCTCTCGG
TGIF1 Sh364	Fw: GGCCAGTGAATTCGCGTACCTCCTTCTCGCAGGGC Rv:AGCTATGACGAATTCGCAAAAAAGTGGCAACCTACCCAAAGAGTTCTCTTGAAACTCTTTGGGTAGGTTG CCACAAACCCCAGTTGCTCTCGG
TGIF1 Sh416	Fw: GGCCAGTGAATTCGCGTACCTCCTTCTCGCAGGGC Rv:AGCTATGACGAATTCGCAAAAAAGCACCGATACAATGCTTATTCTCTTGAAATAAGCATTGTATCGGTGC AAACCCCAGTTGCTCTCGG
TGIF1 Sh998	Fw: GGCCAGTGAATTCGCGTACCTCCTTCTCGCAGGGC Rv:AGCTATGACGAATTCGCAAAAAAGGTGGATGTTGCACTCAAATCTCTTGAATTTGAGTGCAACATCCACC AAACCCCAGTTGCTCTCGG