

Supplemental Fig. S1

A

```

sp|Q91YT7|YTHD2_MOUSE  MSASSLLEQ--RPKGGGNKVVQNGSVHQKDGILNDDDFEPYLSFQARPNNAATAMSDSYLPS  58
sp|P59326|YTHD1_MOUSE  MSATSV-DPQ-RTKGQDNKVVQNGSLHQDAVDHNDFFEYLSGQSNFNSYFMSDPYLSL  58
sp|Q8BYK6|YTHD3_MOUSE  MSATSV-DQPKKGGGNKVVQNGSVHQKDAVNDFFEPYLSQTNQNNYPPMSDPYMS  59
*****: : : !* : *****: : :*****: * : !* * * * *

sp|Q91YT7|YTHD2_MOUSE  YYPSIGFYSYLSGEAAWSTGGDAMPYLYSYGQLSNGEPHFLDAMFGPFGALGST--PFL  117
sp|P59326|YTHD1_MOUSE  YYPPSIGFPYLSLEAPWSTAGDPPIPIYLYTYGQLSNGDHHFMDAVFGPFGGLGNN---I  115
sp|Q8BYK6|YTHD3_MOUSE  YYAPSIGFPYLSLEAAWSTAGDPMPHYLYTYGQMSNGEHHYIPDCVFSOPGALGNTFPFL  119
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

sp|Q91YT7|YTHD2_MOUSE  GQHGPNFFPFGIDFSAMGNSSQGGSTQSSGYSSNYAYAPSSLGGMIDGQSAFANETLN  177
sp|P59326|YTHD1_MOUSE  YQHRFNFFPENPAFSAWGTSGSQGQQTQSSAYGSSYTYPPSSLGGTVVDGDTGFHSDSLN  175
sp|Q8BYK6|YTHD3_MOUSE  GQHGPNFFPFGADFSWTGTSQGGSTQSSAYGSSYTYPPSSLGTRAITDGAQFGNDTLLS  179
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

sp|Q91YT7|YTHD2_MOUSE  KAPGMNTIDQGMALKLSTEVASSVPKVGSVAVGSGSITSNIVASSLPPATIAAPPKA  237
sp|P59326|YTHD1_MOUSE  KAPGMNSLEQGMVGLKIGDVTTS-A-VKTVGSVVNSVALTG-VLSGNGGTNNVMPVSKPT  232
sp|Q8BYK6|YTHD3_MOUSE  KVPGISSEIQGNTLKIIGDLTA-AVTKTVGTALSSSGMTS-IAT-NNVPPVSSAAPKPT  236
* . * : : : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

sp|Q91YT7|YTHD2_MOUSE  SWADIASKPAKQPKLKTNG--IAGSSLPPPIKHNMDIGTWNKGPVAKAPSQALVQN  295
sp|P59326|YTHD1_MOUSE  SWAAIASKPAKQPKMKTRSGPIV--GCALPPPIKHNMDIGTWNKGPAPKASAPQPTFS  291
sp|Q8BYK6|YTHD3_MOUSE  SWAAIARKPAKQPKLKPKNVIGSVPVPPPIKHNMDIGTWNKGSVVKAPPTQPVLP  296
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

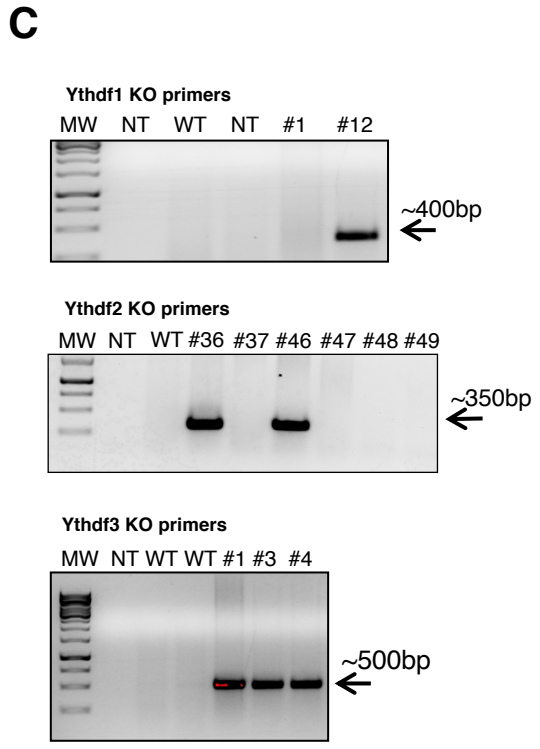
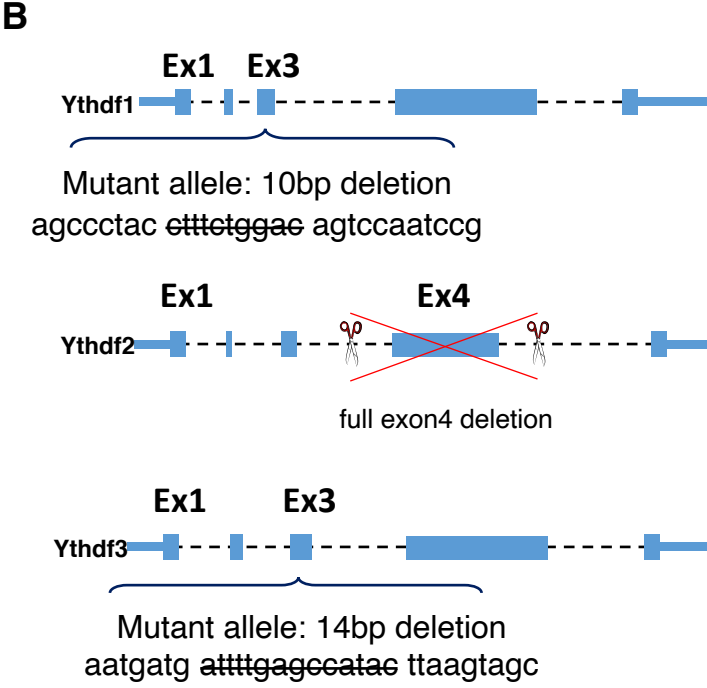
sp|Q91YT7|YTHD2_MOUSE  IQGPTQGSFQFVGGQAN-NSPPVAQASVGGQTPPLPPP-----PQAQLSVQ00A  345
sp|P59326|YTHD1_MOUSE  FQ-AA-POPQVQPLFVQPPLVQ-----PQYGS-PQQ  322
sp|Q8BYK6|YTHD3_MOUSE  FQ-TIQQPPLI-----QPPLVQGSLEQ-QQPQFPQ00GQFPQQA0PHVQGS-QQP  348
* * : : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

sp|Q91YT7|YTHD2_MOUSE  AQPTRWVAPNNGSGGCHNGV--DGNVGVQSAQSGGSTPSEHPVLEKLSINNYNPKD  402
sp|P59326|YTHD1_MOUSE  PLQPRWVAPNNAAFQSGGANSNSVGNQAP-TSAPVESHVPLEKLSAAHSYNPKK  381
sp|Q8BYK6|YTHD3_MOUSE  QLQNRWVAPNNGTGFNQNGTGSSENGLVVPSASPSSEVHPVLEKLSAANNYKPD  408
*****: : : !* * * * * * * * * * * * * * * * * * * * * * * * * * * *

sp|Q91YT7|YTHD2_MOUSE  FDWNLKNGRVFIIKSYSEDDIHRSIKYNLWCSTEHGKRLDAAYRSMNGKGPVYLLFSVN  462
sp|P59326|YTHD1_MOUSE  FDWNLKSGRVFIIKSYSEDDIHRSIKYSIWCSTEHGKRLDGAFRSMSSKGPVYLLFSVN  441
sp|Q8BYK6|YTHD3_MOUSE  FDWNLKNGRVFIIKSYSEDDIHRSIKYSIWCSTEHGKRLDAAYRSLNGKGPVYLLFSVN  468
*****: : : !* * * * * * * * * * * * * * * * * * * * * * * * * * * *

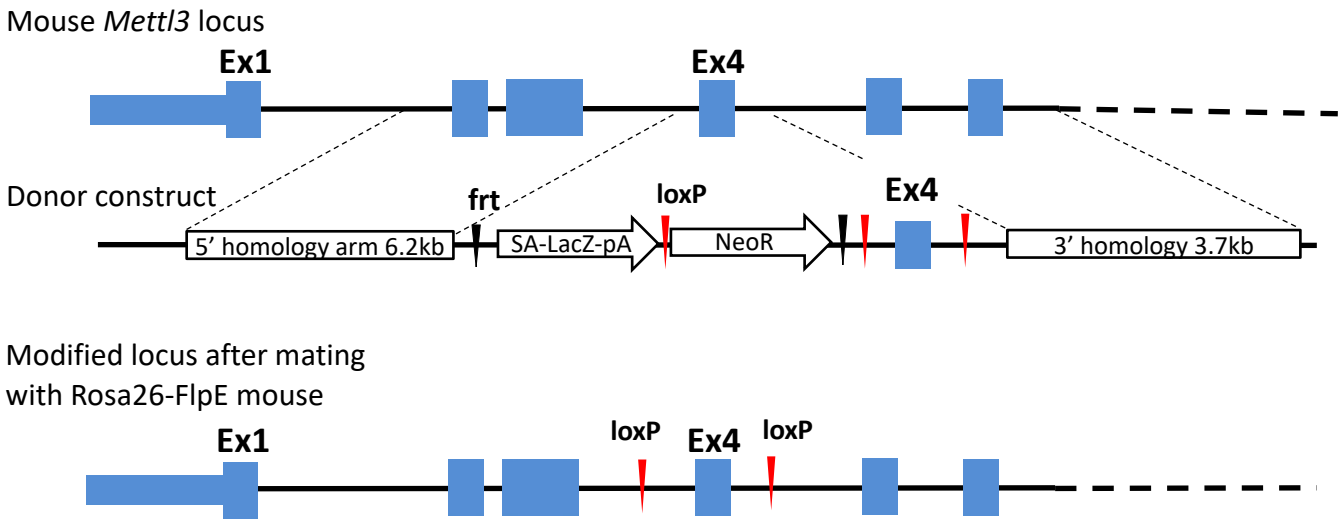
sp|Q91YT7|YTHD2_MOUSE  GSGHFCGVAEMHSVDYNTCAGVWSQDRWGRFDVRFVIFKDVFNSQLRHRILENNKPK  522
sp|P59326|YTHD1_MOUSE  GSGHFCGVAEMHSVDYNTSAGVWSQDRWGRFDVRFVIFKDVFNSQLRHRILENNKPK  501
sp|Q8BYK6|YTHD3_MOUSE  GSGHFCGVAEMHSVDYNTAYAGVWSQDRWGRFVRFVIFKDVFNSQLRHRILENNKPK  528
*****: : : !* * * * * * * * * * * * * * * * * * * * * * * * * * * *

sp|Q91YT7|YTHD2_MOUSE  VTNSRDQEVPLEKAKQVLKIIASVKHTTIFDDFSHYEKQEEESVKKERQGGK-  579
sp|P59326|YTHD1_MOUSE  VTNSRDQEVPLEKAKQVLKIIASVKHTTIFDDFSHYEKQEEEEVVRERQNNKQ-  559
sp|Q8BYK6|YTHD3_MOUSE  VTNSRDQEVPLEKAKQVLKIIATFKHTTIFDDFAHYEKQEEEAAMRRERNKQ-  585
*****: : : !* * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
```

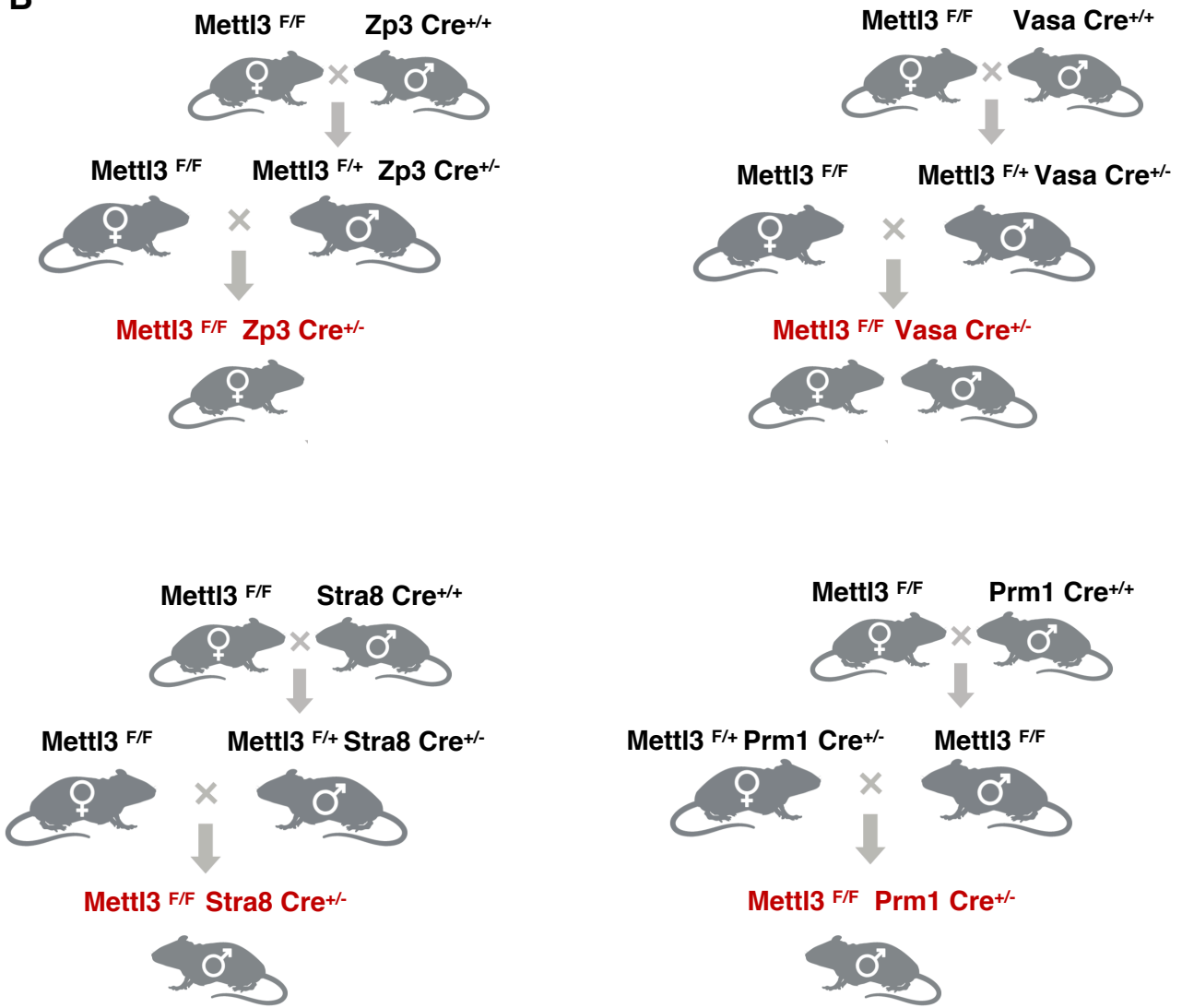


Supplemental Fig. S2

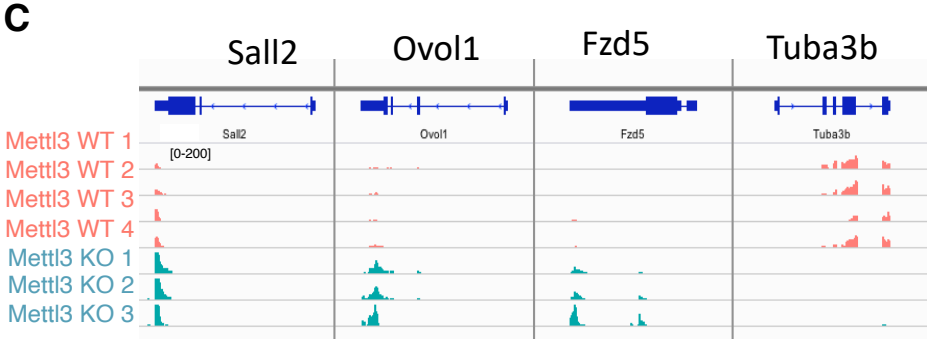
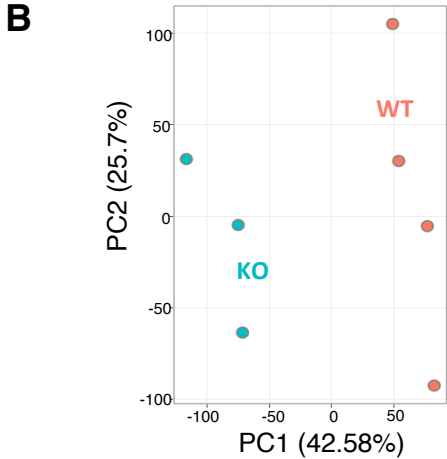
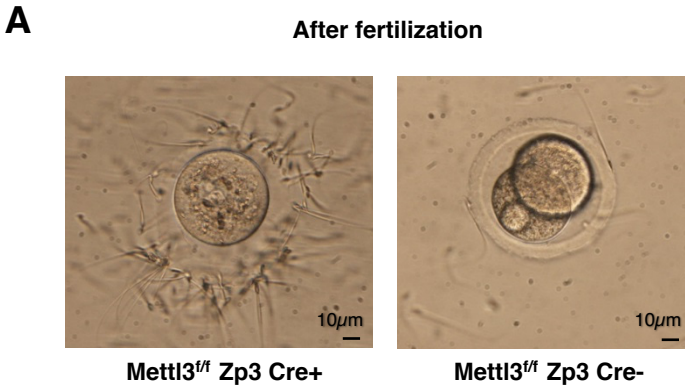
A



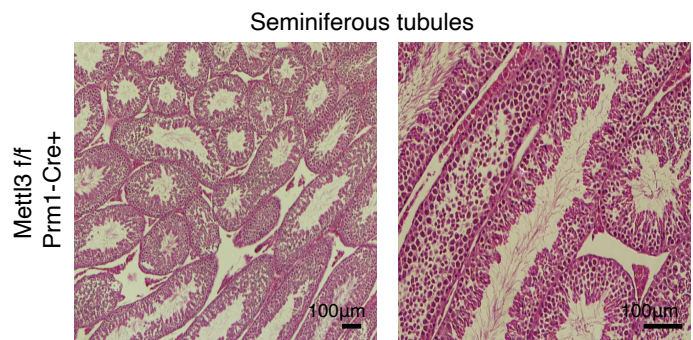
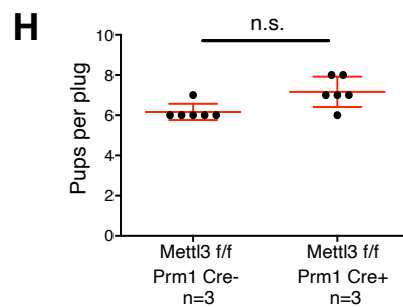
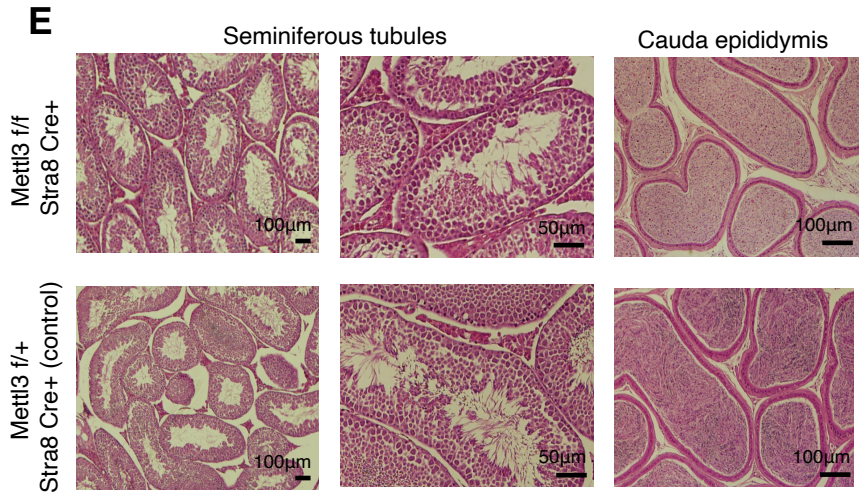
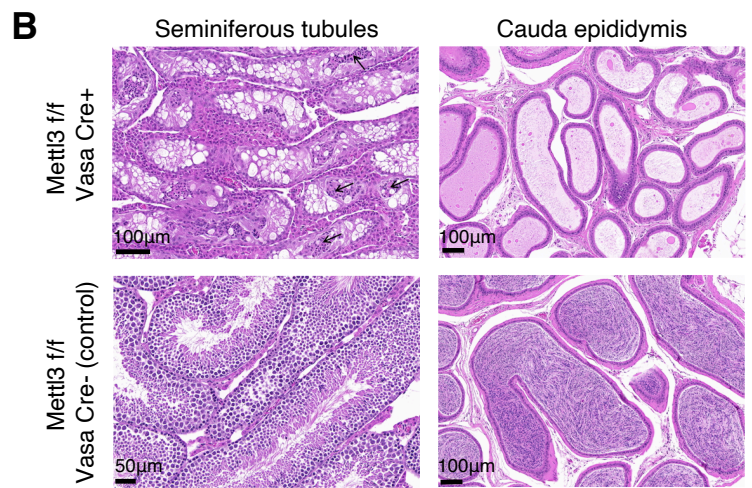
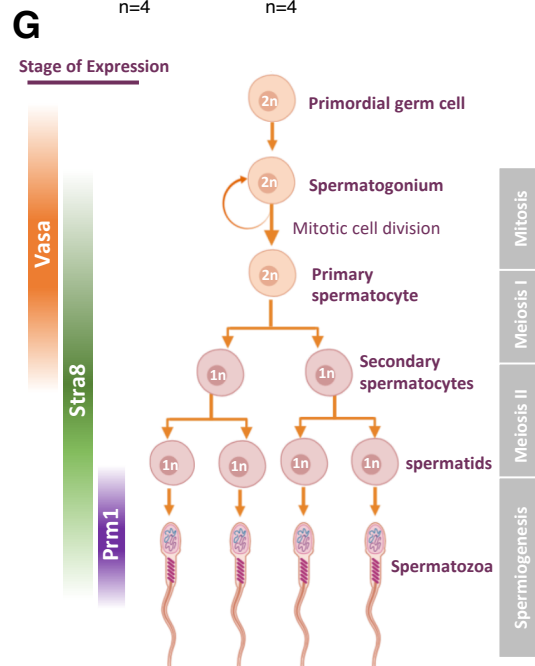
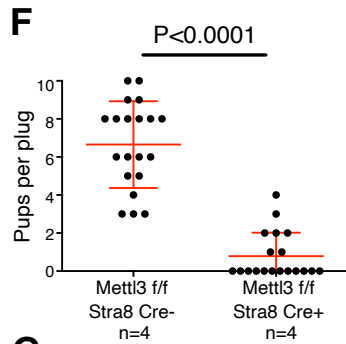
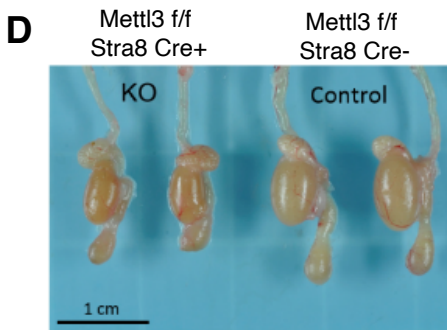
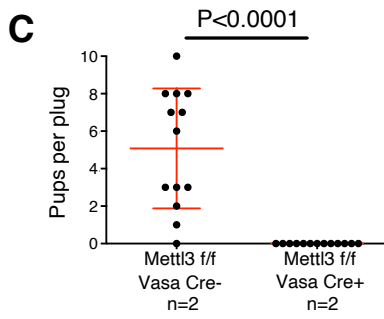
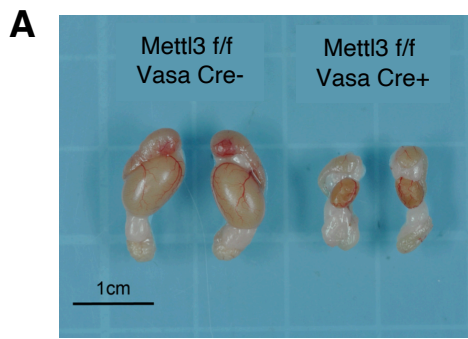
B



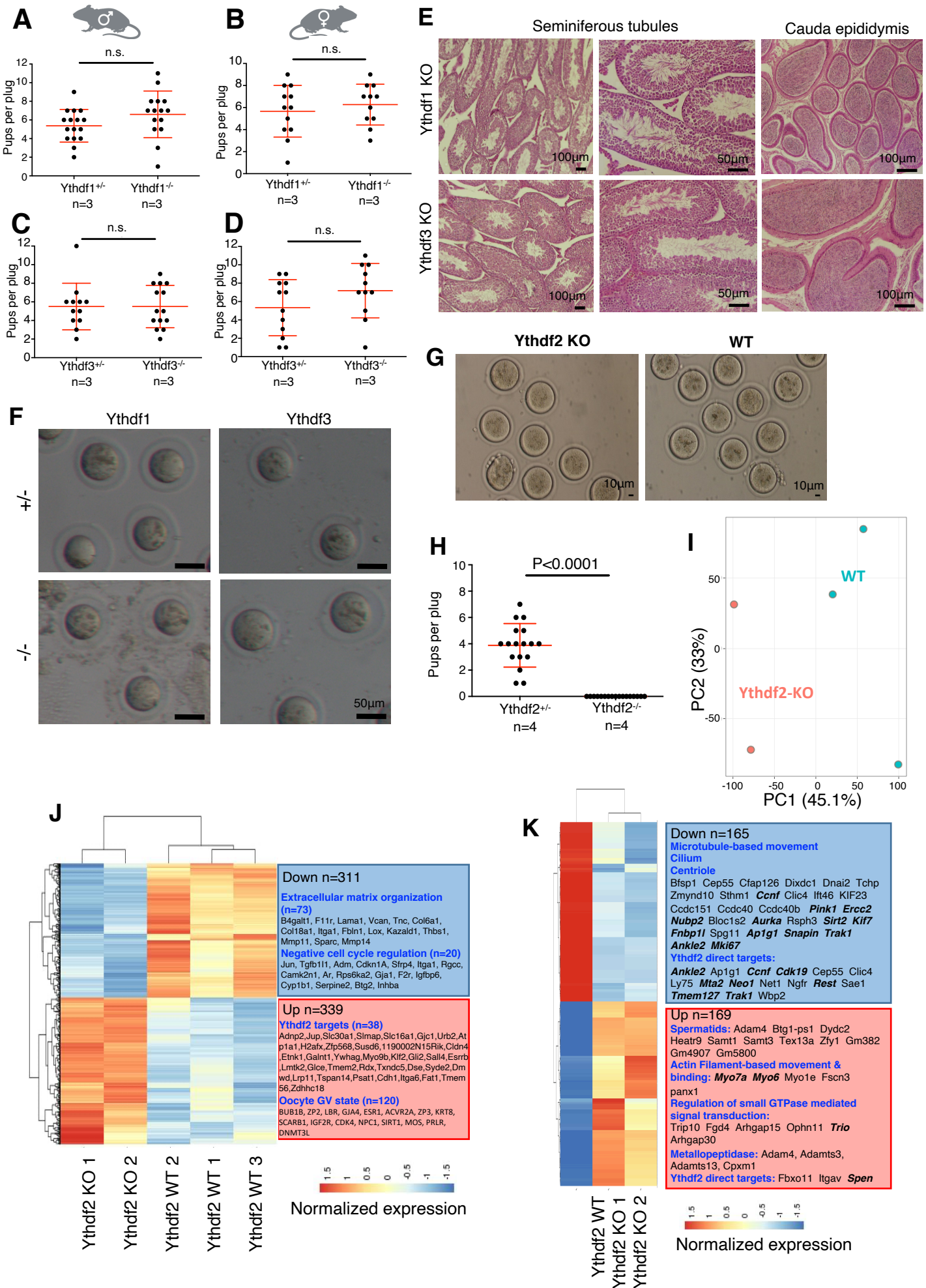
Supplemental Fig. S3



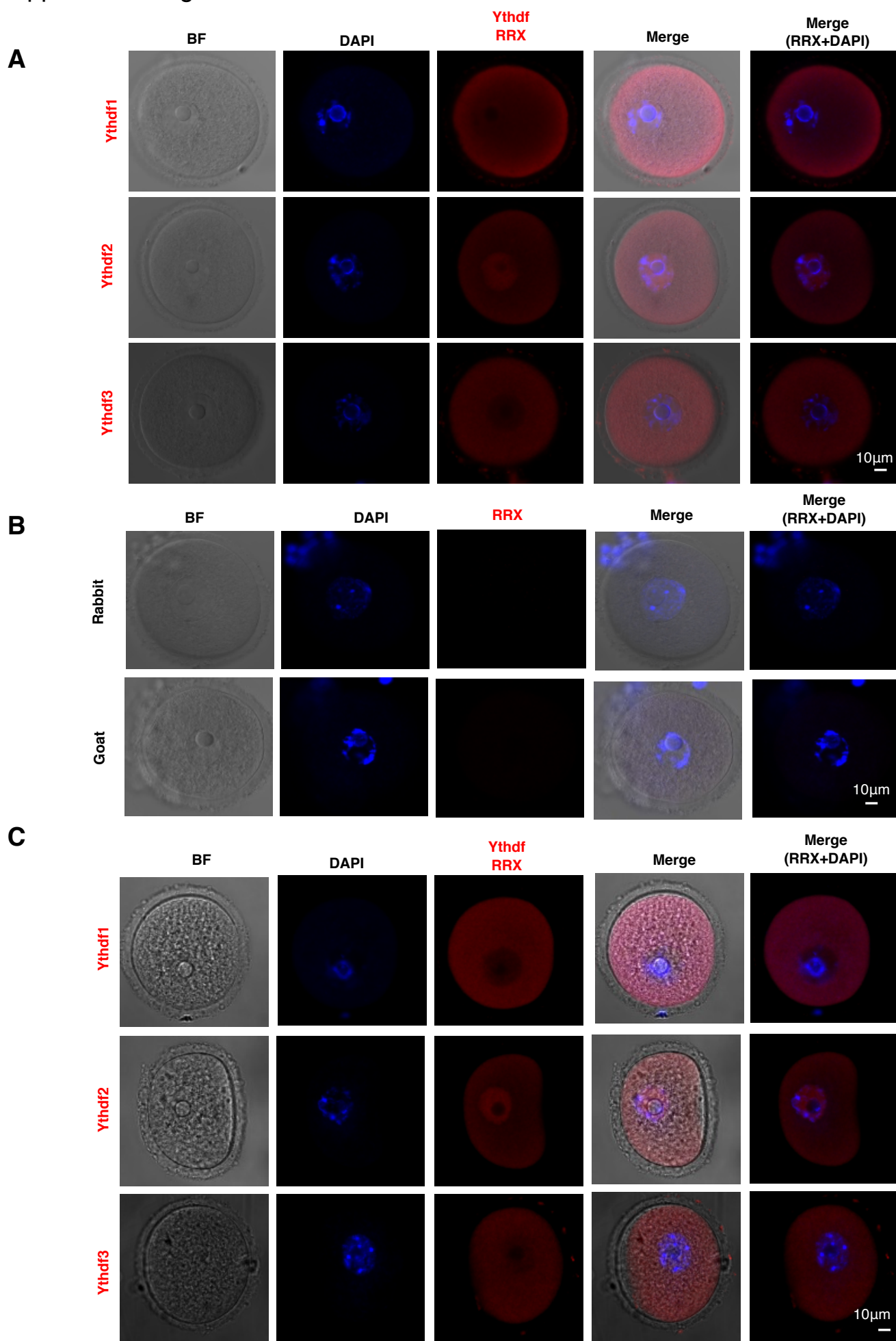
Supplemental Fig. S4



Supplemental Fig. S5

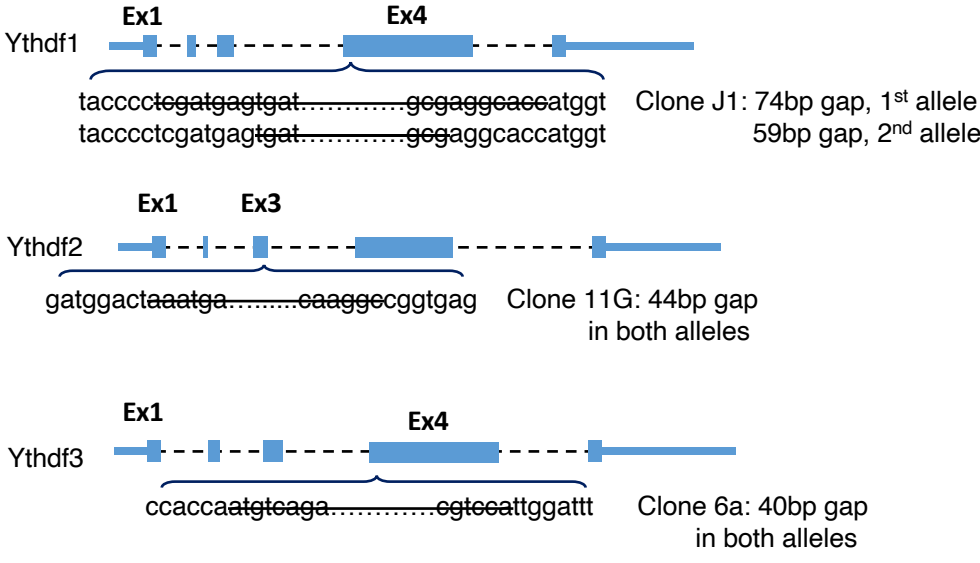


Supplemental Fig. S6



Supplemental Fig. S7

A

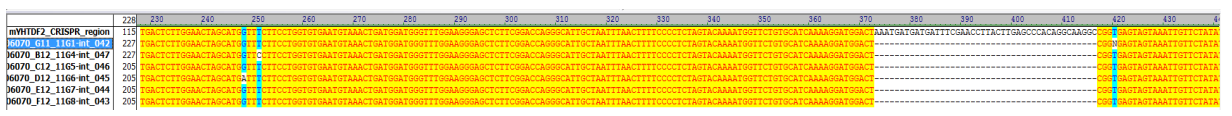


B

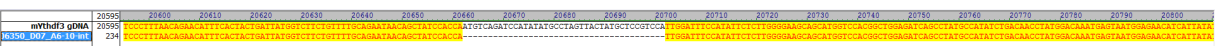
mESC Ythdf1 KO Clone J1 - 74bp gap 59bp gap



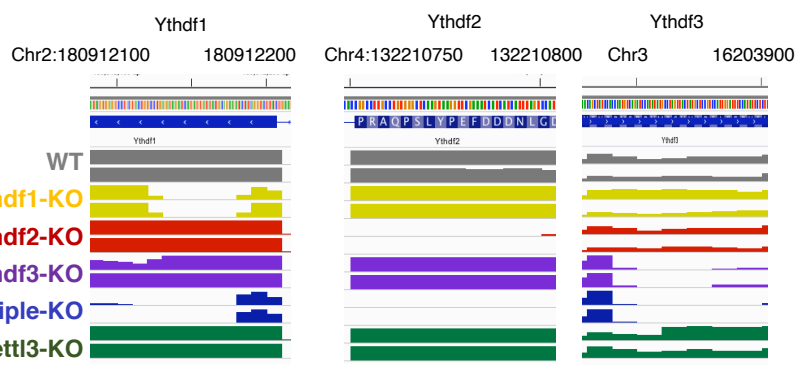
mESC Ythdf2 KO Clone 11G - 44bp gap



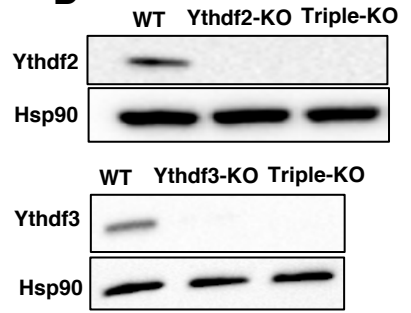
mESC Ythdf3 KO Clone 6A - 40bp gap



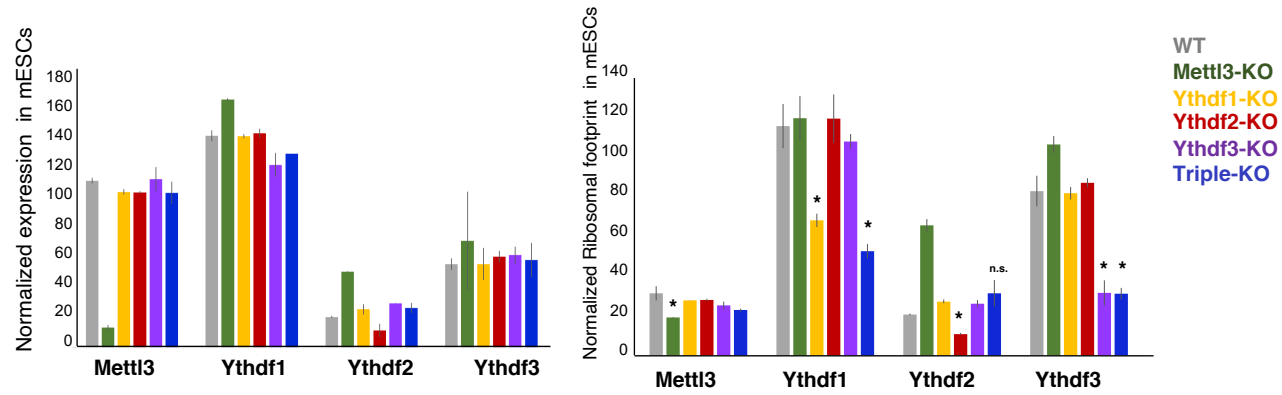
C



D

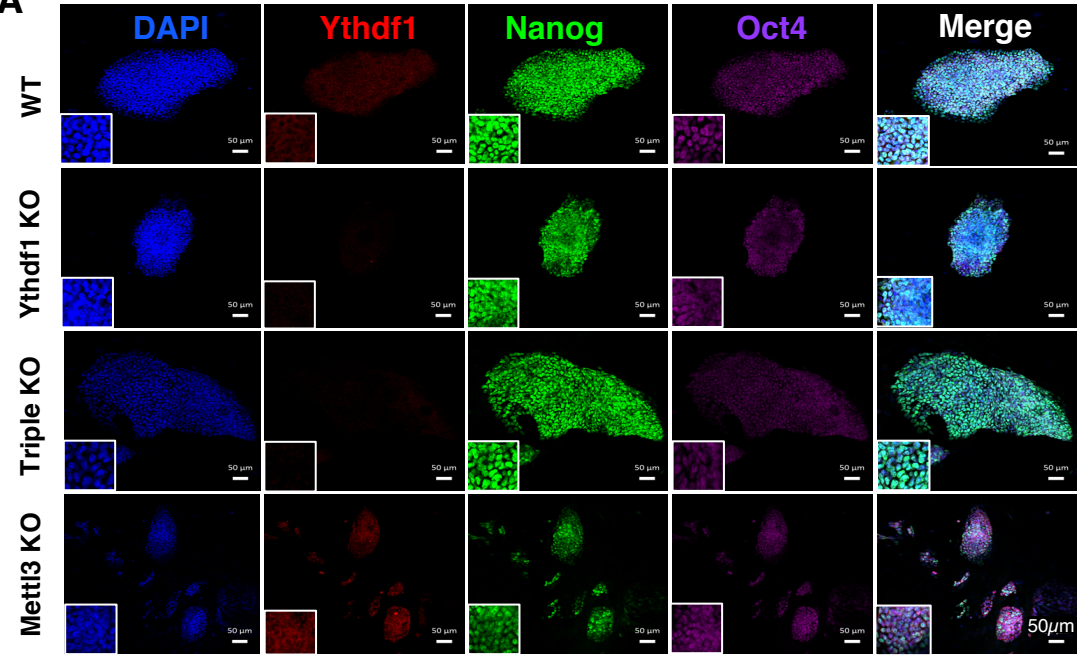


E

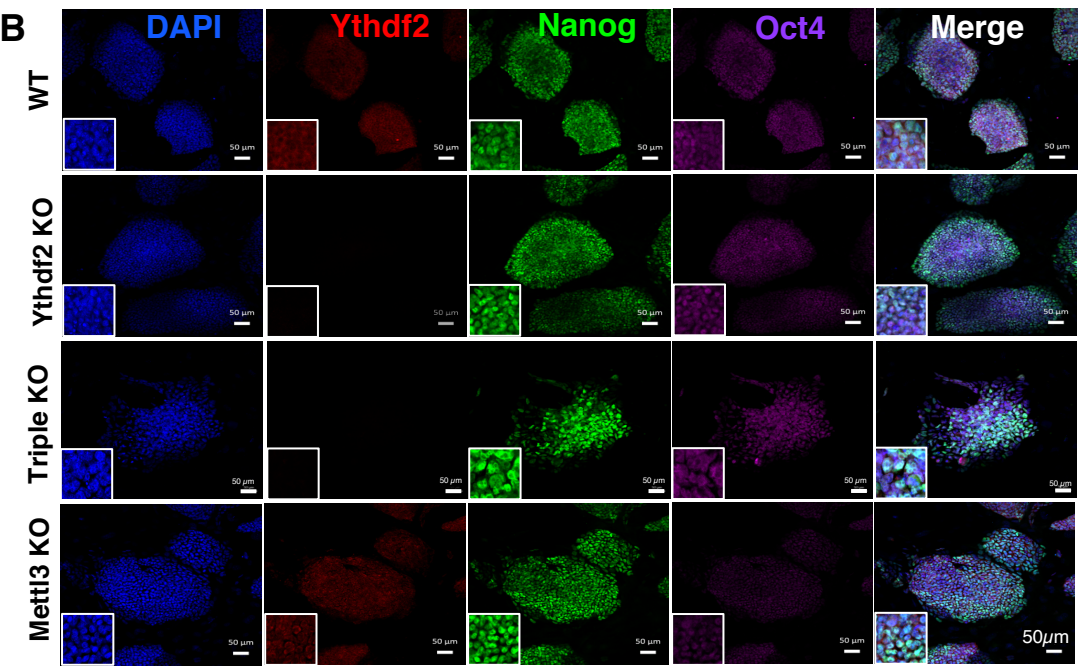


Supplemental Fig. S8

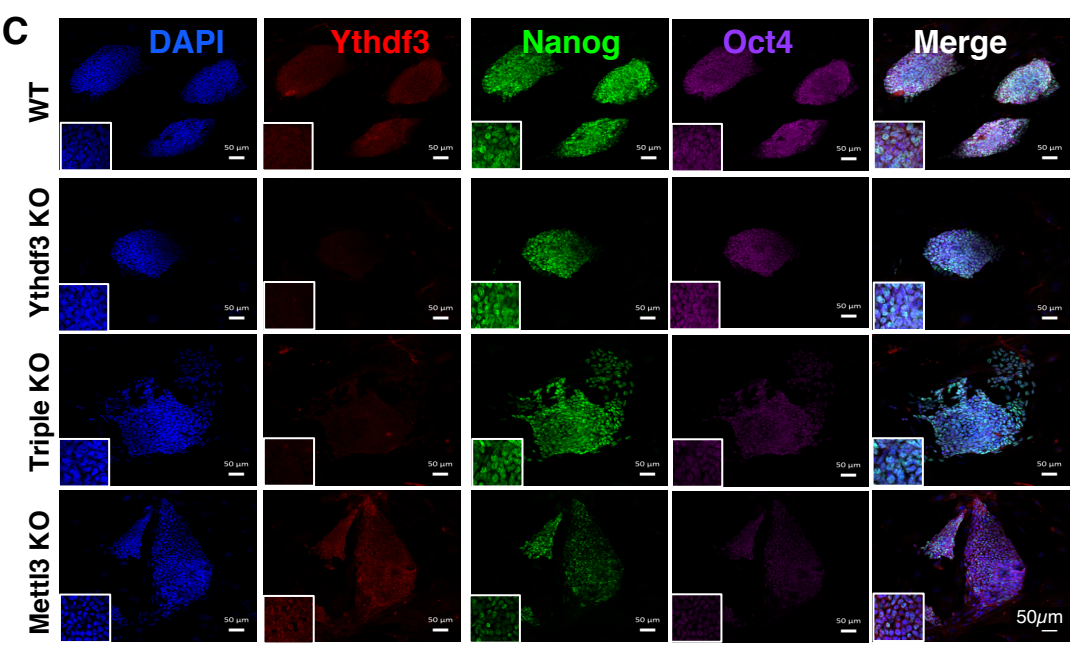
A



B

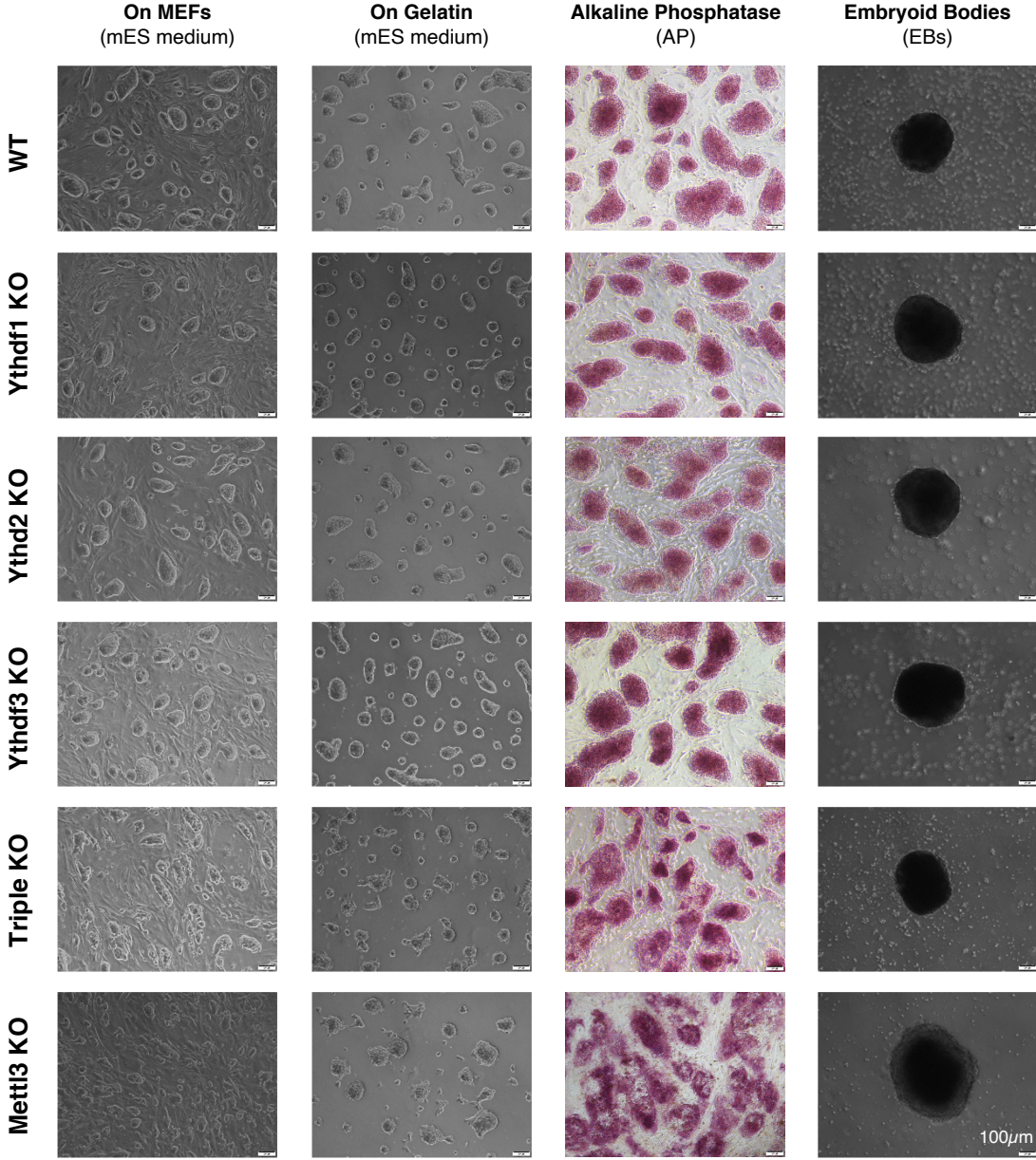


C

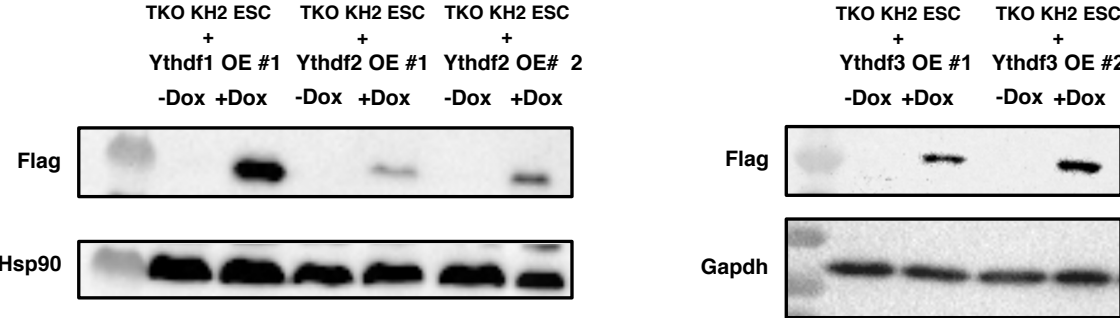


Supplemental Fig. S9

A

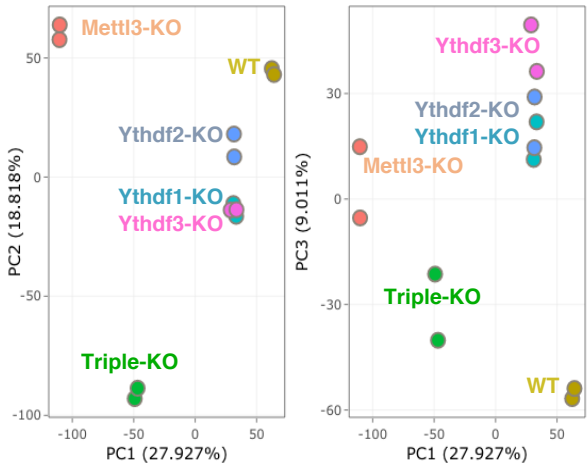


B

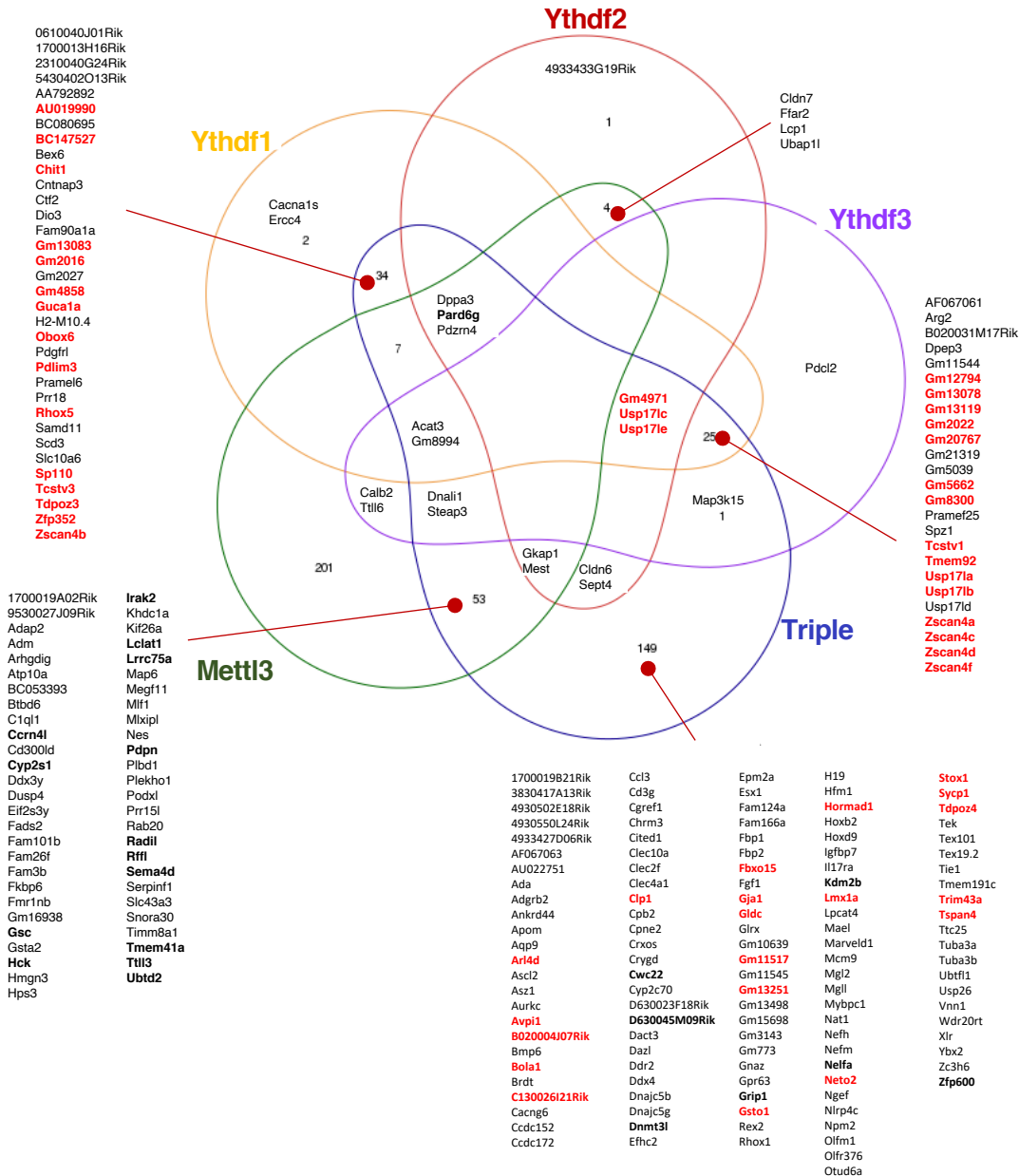


Supplemental Fig. S10

A

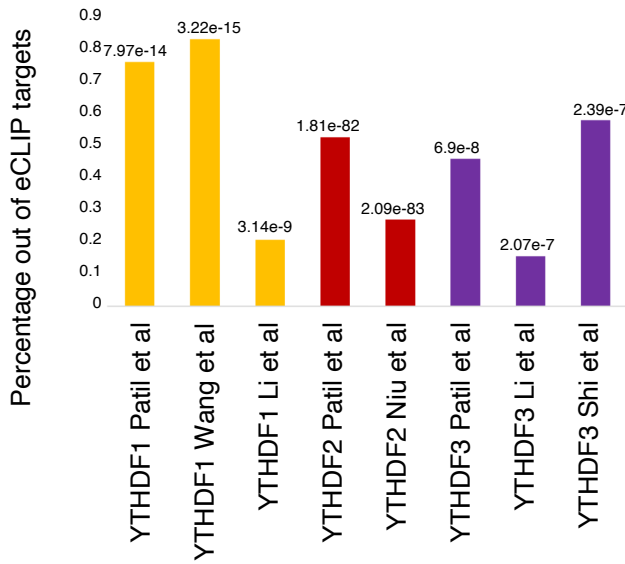


B

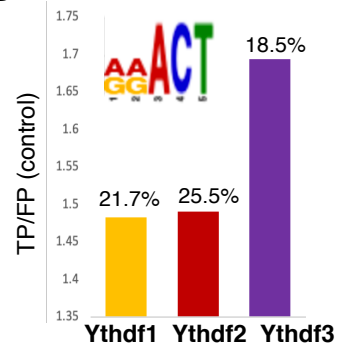


Supplemental Fig. S11

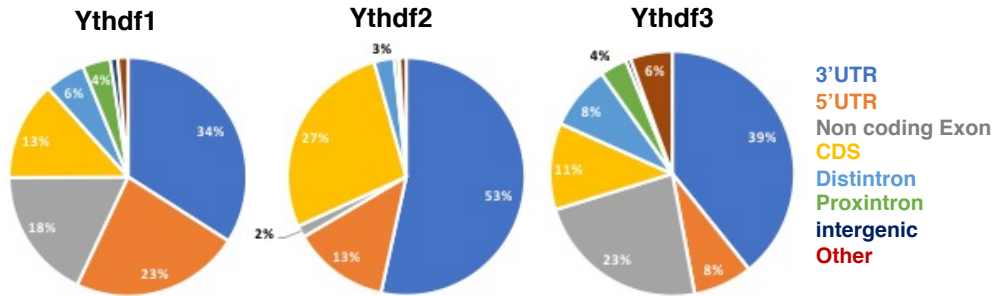
A



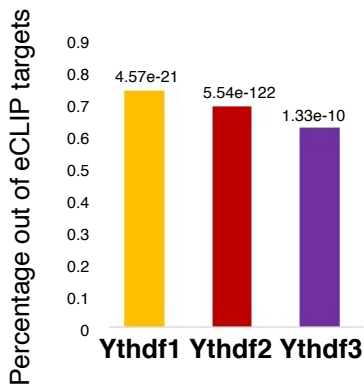
B



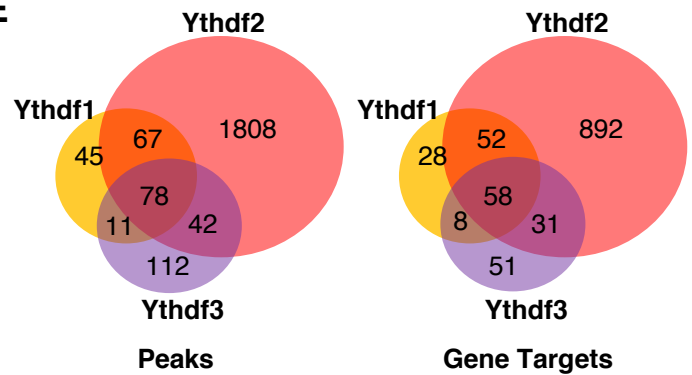
C



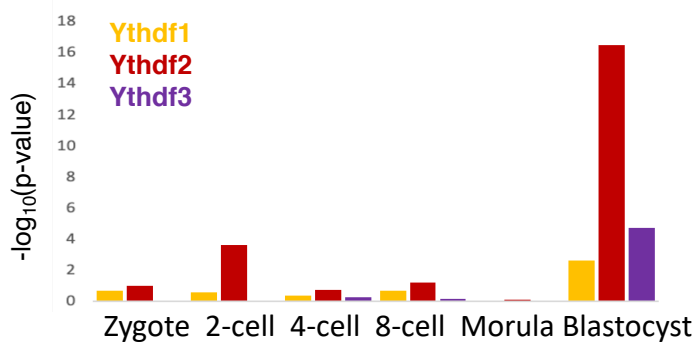
D



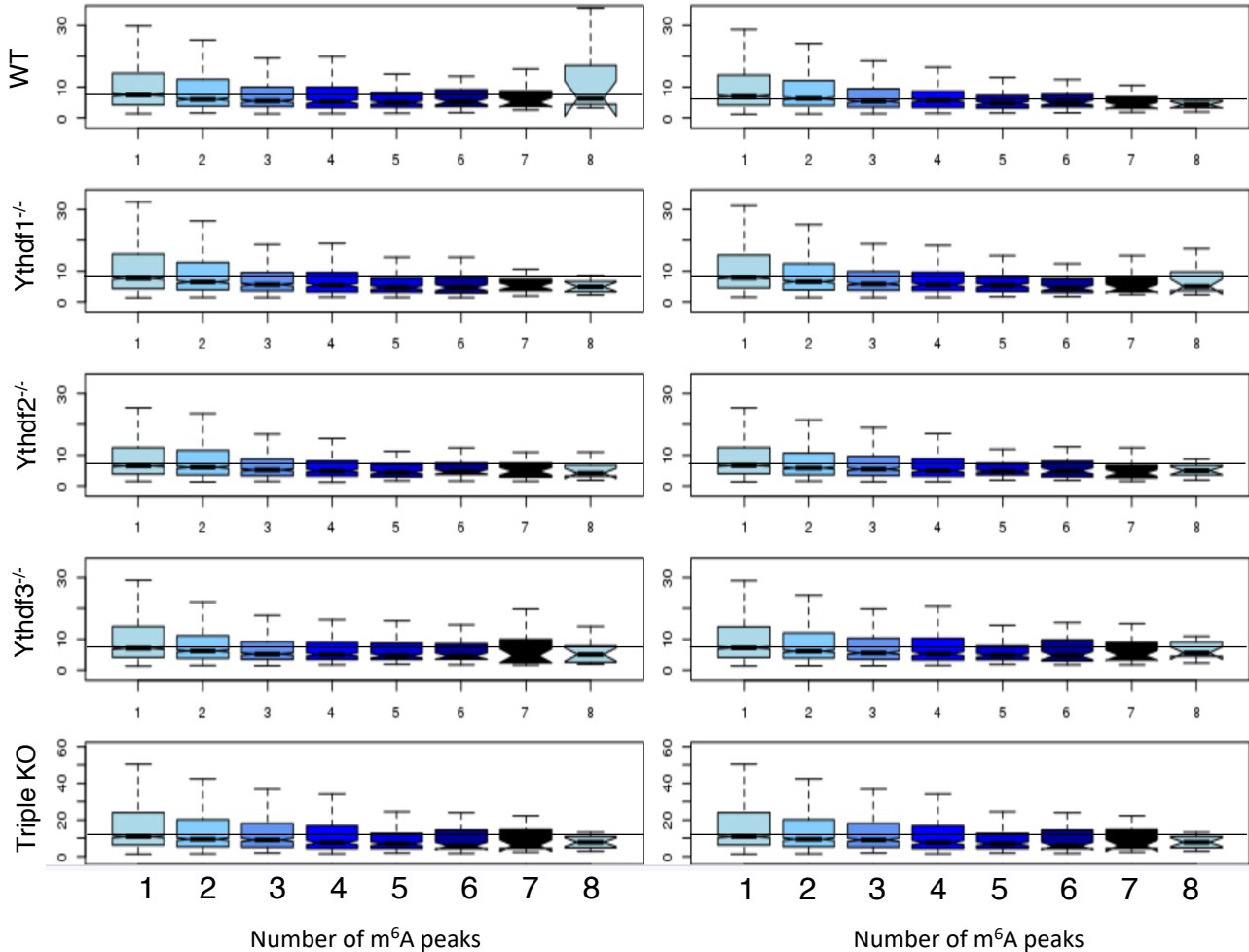
E



F



Supplemental Fig. S12



Supplemental Figure Legends

Supplemental Figure S1. Generating Ythdf1-KO, Ythdf2-KO and Ythdf3-KO mice.

- A) Multiple alignments of Ythdf1, Ythdf2 & Ythdf3 proteins, calculated using the Clustal Omega tool. The area of YTH-domain is highlighted in red. Ythdf1-Ythdf3 protein sequence similarity is 70.11%, Ythdf1-Ythdf2 is 67.15%, and Ythdf2-Ythdf3 is 67.78%.
- B) CRISPR-Cas9 targeting strategy for knocking-out Ythdf readers *in vivo* in mouse zygotes.
- C) KO validation using PCR, showing successful primer integration in clones #12 (Ythdf1); #36, #46 (Ythdf2); #1, #3 & #4 (Ythdf3).

Supplemental Figure S2. Generating Mettl3 conditional knockout mouse model.

- A) Targeting strategy for generating Mettl3^{f/f} mice.
- B) Crossing strategy for generating different Mettl3^{f/f} Cre⁺ mice.

Supplemental Figure S3. Mettl3 is essential for female mice fertility.

- A) *In vitro* fertilization of Mettl3^{f/f} Zp3 Cre⁻ control oocytes with WT sperm, leads to creation of two-cell stage embryos, while the Mettl3^{f/f} Zp3 Cre⁺ oocytes fail to do so.
- B) PCA of transcriptional profile of Mettl3^{f/f} Zp3 Cre⁻ and Mettl3^{f/f} Zp3 Cre⁺ oocytes, showing a distinct expression pattern.
- C) RNA-seq landscape of selected differential genes, generated with IGV browser. Normalized coverage is presented.

Supplemental Figure S4. Mettl3 and Ythdf2 are essential for male mice fertility.

- A) Gross morphology of testis and epididymis of Mettl3^{f/f} Vasa Cre⁺ and Mettl3^{f/f} Vasa Cre⁻ males. Cre⁺ males show a massive decrease in testis and epididymis size compared to Cre⁻ control.
- B) H&E staining showing severe degenerative changes in the seminiferous tubules of Mettl3^{f/f}Vasa Cre⁺ and lack of sperm in the cauda epididymis.
- C) Number of pups per plug produced by Mettl3^{f/f} Vasa Cre⁺ males, compared to Mettl3^{f/f} Vasa Cre⁻ control males. The mothers in both cases were WT. In this case there is a significant hypo fertility of the KO ($p < 0.0001$, Mann-Whitney test).
- D) Gross morphology of testis and epididymis of Mettl3^{f/f} Stra8 Cre⁺ and Mettl3^{f/f} Stra8 Cre⁻ males. Cre⁺ males show a reduced-size testis and epididymis compared to Cre⁻ control.
- E) H&E staining showing mild degenerative changes in the seminiferous tubules of Mettl3^{f/f}Stra8 Cre⁺ and ~75% reduction in sperm quantity in the cauda epididymis, compared to Mettl3^{f/+}Stra8 Cre⁺ sibling control.
- F) Same as in (C), for Stra8 Cre, showing a significant hypo fertility of the KO ($p < 0.0001$, Mann-Whitney test).
- G) Vasa, Stra8 and Prm1 are expressed during spermatogenesis, in different stages, as indicated.

- H) Same as in (C), for Prm1 Cre, showing no significant difference between Cre⁺ and Cre⁻ male fertility.
- I) H&E staining of seminiferous tubules showing a normal morphology in Mettl3^{f/f}Prm1 Cre⁺ males.

Supplemental Figure S5. Ythdf1 knockout and Ythdf3 knockout mice are viable and fertile.

- A) Number of pups per plug produced by mating Ythdf1-KO males, compared to Ythdf1-HET males. The mothers in both cases are WT. Here there is no significant difference between KO and HET male fertility (Mann-Whitney test).
- B) Number of pups per plug produced by mating Ythdf1-KO females, compared to Ythdf1-HET females. The fathers in both cases are WT. Here there is no significant difference between KO and HET female fertility (Mann-Whitney test).
- C) Number of pups per plug produced by mating Ythdf3-KO males, compared to Ythdf3-HET males. The mothers in both cases are WT. Here there is no significant difference between KO and HET male fertility (Mann-Whitney test).
- D) Number of pups per plug produced by mating Ythdf3-KO females, compared to Ythdf3-HET females. The fathers in both cases are WT. Here there is no significant difference between KO and HET female fertility (Mann-Whitney test).
- E) H&E staining of seminiferous tubules showing a normal morphology in Ythdf1-KO and Ythdf3-KO males.
- F) The morphology of Ythdf1-KO and Ythdf3-KO flushed oocytes appears to be normal, similar to the Ythdf1-heterozygous (HET) and Ythdf3-HET flushed oocytes.
- G) The morphology of Ythdf2-KO flushed oocytes appears to be normal, similar to the WT flushed oocytes.
- H) Number of pups per plug produced by mating Ythdf2^{-/-} females, compared to Ythdf2^{+/-} control females. The fathers in both cases are WT. A significant difference between the fertility of KO and heterozygous females is observed ($p < 0.0001$, Mann-Whitney test).
- I) PCA of transcriptional profile of Ythdf2-KO and WT oocytes, showing a distinct expression pattern.
- J) Transcriptional profile of genes that are differentially expressed between Ythdf2-KO and WT oocytes, along with selected enriched categories; 311 downregulated in KO, and 339 upregulated in KO.
- K) Transcriptional profile of genes that are differentially expressed between Ythdf2-KO and WT round spermatids, along with selected enriched categories. m⁶A-methylated genes appear in bold; 145 downregulated in KO, and 156 upregulated in KO.

Supplemental Figure S6. Oocytes staining for Ythdf1, Ythdf2 and Ythdf3 proteins.

- A) Immunostaining of Ythdf1, Ythdf2 and Ythdf3 in ICR WT oocytes after hormone priming (PMS & hCG). More expanded presentation of images shown in Figure 1B.
- B) Immunostaining of Ythdf1, Ythdf2 and Ythdf3 in ICR WT oocytes after PMS & HCG - negative control (NC), without primary antibody.
- C) Immunostaining of Ythdf1, Ythdf2 and Ythdf3 in ICR WT oocytes without hormone priming.

Supplemental Figure S7. Generation and validation of Ythdf1/2/3 knockout mESC lines.

- A) CRISPR-Cas9 targeting strategy for knocking out Ythdf readers in mESC cell lines.
- B) Sequencing validation of the single-KO lines selected for further validation and analysis.
- C) IGV browser view showing the missing fragments in the KO of Ythdf1, Ythdf2 & Ythdf3.
- D) Western blot analysis for Ythdf2 and Ythdf3 in WT, single-KO and triple-KO mES cell lines.
- E) Left: Normalized expression levels, Right: Normalized ribosomal footprint (ribo-seq) of Ythdf1,2&3 and Mettl3 proteins in the indicated mES cell lines. Translation of KO proteins is typically significantly lower compared to WT control (* t-test <0.05).

Supplemental Figure S8. Immunostaining of Ythdf1/2/3 KO mESC lines for pluripotency markers.

- A) Immunostaining of Ythdf1 (red), Nanog (green), Oct4 (purple) and DAPI (blue) in WT, Ythdf1-KO, Triple-KO and Mettl3-KO cells.
- B) Immunostaining of Ythdf2 (red), Nanog (green), Oct4 (purple) and DAPI (blue) in WT, Ythdf2-KO, Triple-KO and Mettl3-KO cells.
- C) Immunostaining of Ythdf3 (red), Nanog (green), Oct4 (purple) and DAPI (blue) in WT, Ythdf3-KO, Triple-KO and Mettl3-KO cells.

Supplemental Figure S9. Morphology of Ythdf knockout mESC lines.

- A) Phase and alkaline phosphatase (AP) staining of WT, single-KOs and Triple-KO mESCs, and phases of their EBs.
- B) Triple-KO ESCs were rendered transgenic for either one of the Ythdf proteins under DOX induced promoter. Western blot of correctly targeted rescued lines showing the over expression (OE) of Ythdf1/2/3 proteins that can be induced by Dox, on the background of triple-KO cell line.

Supplemental Figure S10. Overlap of ESC signatures.

- A) PCA clustering of KO and WT mESCs samples, showing that in PC1, single reader KO samples are closer to WT, compared to triple-KO and Mettl3-KO.
- B) Overlap between upregulated gene signatures, measured in Ythdf single-KO and triple-KO, and in Mettl3-KO. Genes that are m⁶A-methylated are bold. Genes that are two-cell markers are highlighted in red.

Supplemental Figure S11. CLIP data evaluation of Ythdf proteins.

- A) Targets of Ythdf1, Ythdf2 and Ythdf3 highly overlap targets that were published before in human cancer cell lines.
- B) Enrichment of RRACT (R=G/A) motif among binding peaks of Ythdf readers. Bars indicate enrichment ratio (True positive/False positive), numbers indicate the percentage of peaks which contain the motif.
- C) Distribution of Ythdf peaks in various genomic entities, showing that the three readers have a tendency to bind 3' UTR, particularly Ythdf2.
- D) Significant overlap of Ythdf targets, with m⁶a-methylated genes.
- E) Significant overlap between Ythdf1, Ythdf2 and Ythdf3 targets

- F) Enrichment of Ythdf targets that were identified in mESCs, to early embryo genes (Gao et al. 2017), showing significant overlap with blastocyte genes.

Supplemental Figure S12. mRNA half-life as a function of number of m⁶A peaks

The half-life of m⁶A genes is plotted as a function of m⁶A peak number in the transcript, showing a slight yet significant decrease in half-life (shorter), as the number of m⁶A peaks increase.

Supplemental Table Legends

Supplemental Table S1. Differentially expressed genes in gametogenesis KO experiments: Mettl3-KO oocytes, and Ythdf2-KO oocytes and spermatoids, compared to matched controls.

Supplemental Table S2. Differentially expressed genes in mESCs, that carry a single-KO (Ythdf1, Ythdf2, Ythdf3 or Mettl3) or triple-KO (Ythdf1/2/3), compared to WT controls.

Supplemental Table S3. eCLIP binding targets of Ythdf1, Ythdf2 and Ythdf3, measured in mESCs.

Supplemental Table S4. Normalized expressed along with transcript half-life, calculated for each gene in the single-KO (Ythdf1, Ythdf2, Ythdf3), triple-KO Ythdf1/2/3, and WT control.