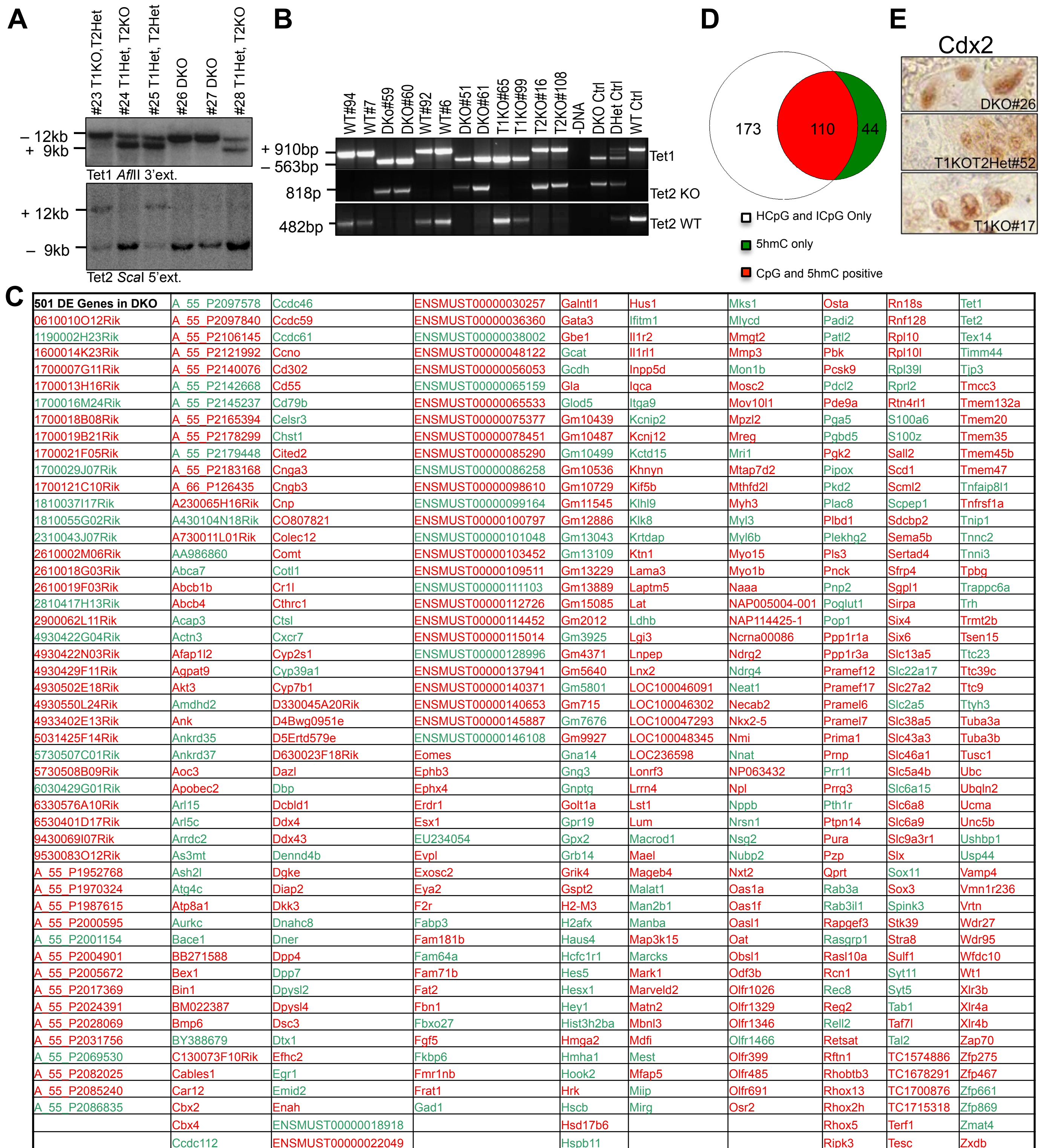
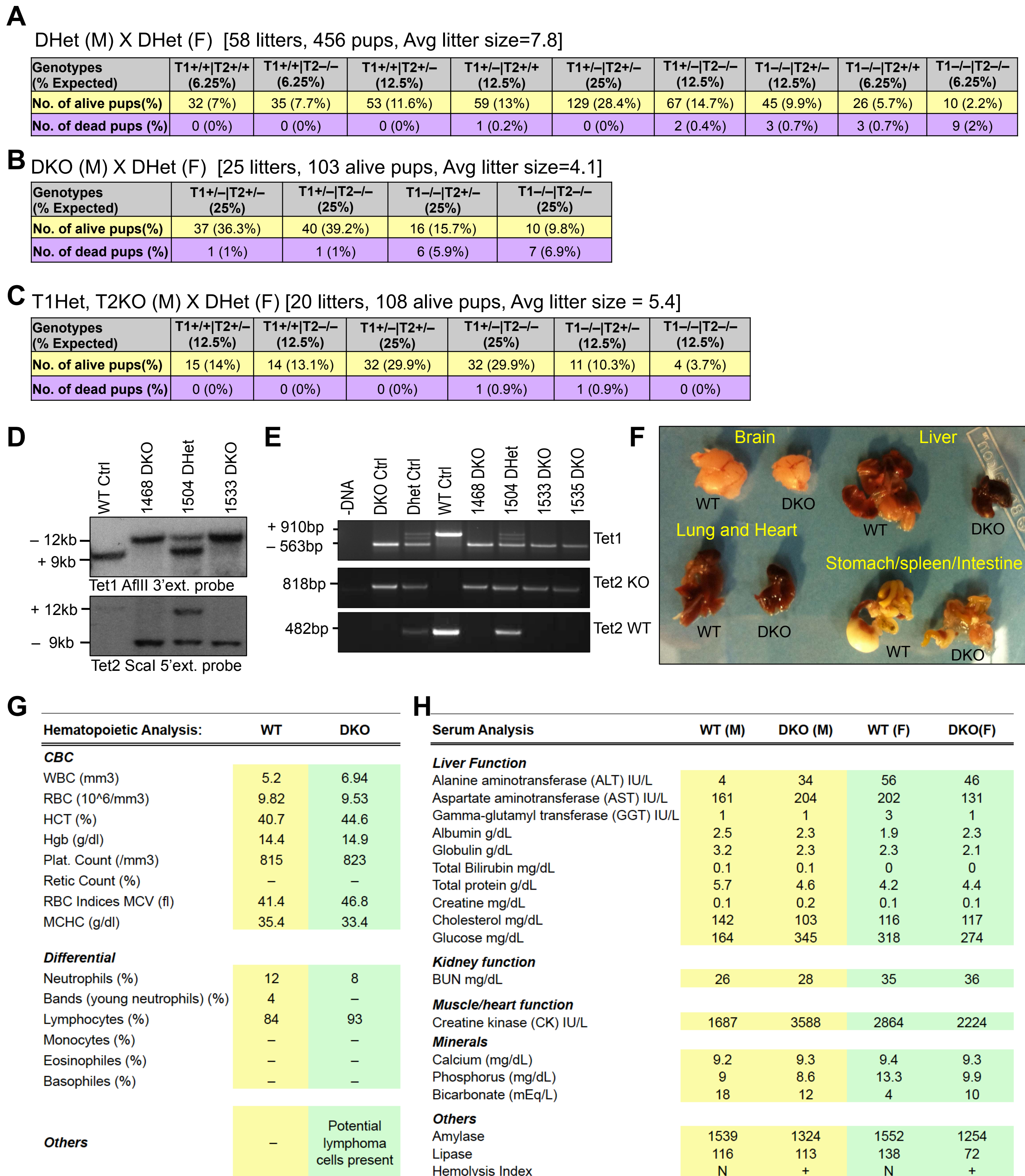



| 501 DE Genes in DKO | A_55_P2097578 | Ccdc46 | ENSMUST00000030257 | Galntl1 | Hus1 | Mks1 | Osta | Rn18s | Tet1 |
|---|---|---|---|---|---|---|---|---|---|
| 0610010O12Rik | A_55_P2097840 | Ccdc59 | ENSMUST00000036360 | Gata3 | Ifitm1 | Mlycd | Padi2 | Rnf128 | Tet2 |
| 1190002H23Rik | A_55_P2106145 | Ccdc61 | ENSMUST00000038002 | Gbe1 | Il1r2 | Mmgt2 | Patl2 | Rpl10 | Tex14 |
| 1600014K23Rik | A_55_P2121992 | Ccno | ENSMUST00000048122 | Gcat | Il1rl1 | Mmp3 | Pbk | Rpl10l | Timm44 |
| 1700007G11Rik | A_55_P2140076 | Cd302 | ENSMUST00000056053 | Gcdh | Inpp5d | Mon1b | Pcsk9 | Rpl39l | Tjp3 |
| 1700013H16Rik | A_55_P2142668 | Cd55 | ENSMUST00000065159 | Gla | Iqca | Mosc2 | Pdcl2 | Rprl2 | Tmcc3 |
| 1700016M24Rik | A_55_P2145237 | Cd79b | ENSMUST00000065533 | Glod5 | Itga9 | Mov10l1 | Pde9a | Rtn4rl1 | Tmem132a |
| 1700018B08Rik | A_55_P2165394 | Celsr3 | ENSMUST00000075377 | Gm10439 | Kcnip2 | Mpzl2 | Pga5 | S100a6 | Tmem20 |
| 1700019B21Rik | A_55_P2178299 | Chst1 | ENSMUST00000078451 | Gm10487 | Kcnj12 | Mreg | Pgbd5 | S100z | Tmem35 |
| 1700021F05Rik | A_55_P2179448 | Cited2 | ENSMUST00000085290 | Gm10499 | Kctd15 | Mri1 | Pgk2 | Sall2 | Tmem45b |
| 1700029J07Rik | A_55_P2183168 | Cnga3 | ENSMUST00000086258 | Gm10536 | Khnyn | Mtap7d2 | Pipox | Scd1 | Tmem47 |
| 1700121C10Rik | A_66_P126435 | Cngb3 | ENSMUST00000098610 | Gm10729 | Kif5b | Mthfd2l | Pkd2 | Scml2 | Tnfaip8l1 |
| 1810037I17Rik | A230065H16Rik | Cnp | ENSMUST00000099164 | Gm11545 | Klhl9 | Myh3 | Plac8 | Scpep1 | Tnfrsf1a |
| 1810055G02Rik | A430104N18Rik | CO807821 | ENSMUST00000100797 | Gm12886 | Klk8 | Myl3 | Plbd1 | Sdcbp2 | Tnip1 |
| 2310043J07Rik | A730011L01Rik | Colec12 | ENSMUST00000101048 | Gm13043 | Krtdap | Myl6b | Plekhg2 | Sema5b | Tnnc2 |
| 2610002M06Rik | AA986860 | Comt | ENSMUST00000103452 | Gm13109 | Ktn1 | Myo15 | Pls3 | Sertad4 | Tnni3 |
| 2610018G03Rik | Abca7 | Cotl1 | ENSMUST00000109511 | Gm13229 | Lama3 | Myo1b | Pnck | Sfrp4 | Tpbg |
| 2610019F03Rik | Abcb1b | Cr1l | ENSMUST00000111103 | Gm13889 | Laptm5 | Naaa | Pnp2 | Sgpl1 | Trappc6a |
| 2810417H13Rik | Abcb4 | Cthrc1 | ENSMUST00000112726 | Gm15085 | Lat | NAP005004-001 | Poglut1 | Sirpa | Trh |
| 2900062L11Rik | Acap3 | Ctsl | ENSMUST00000114452 | Gm2012 | Ldhb | NAP114425-1 | Pop1 | Six4 | Trmt2b |
| 4930422G04Rik | Actn3 | Cxcr7 | ENSMUST00000115014 | Gm3925 | Lgi3 | Ncrna00086 | Ppp1r1a | Six6 | Tsen15 |
| 4930422N03Rik | Afap1l2 | Cyp2s1 | ENSMUST00000128996 | Gm4371 | Lnpep | Ndrg2 | Ppp1r3a | Slc13a5 | Ttc23 |
| 4930429F11Rik | Agpat9 | Cyp39a1 | ENSMUST00000137941 | Gm5640 | Lnx2 | Ndrg4 | Pramef12 | Slc22a17 | Ttc39c |
| 4930502E18Rik | Akt3 | Cyp7b1 | ENSMUST00000140371 | Gm5801 | LOC100046091 | Neat1 | Pramef17 | Slc27a2 | Ttc9 |
| 4930550L24Rik | Amdhd2 | D330045A20Rik | ENSMUST00000140653 | Gm715 | LOC100046302 | Necab2 | Pramel6 | Slc2a5 | Ttyh3 |
| 4933402E13Rik | Ank | D4Bwg0951e | ENSMUST00000145887 | Gm7676 | LOC100047293 | Nkx2-5 | Pramel7 | Slc38a5 | Tuba3a |
| 5031425F14Rik | Ankrd35 | D5Ertd579e | ENSMUST00000146108 | Gm9927 | LOC100048345 | Nmi | Prima1 | Slc43a3 | Tuba3b |
| 5730507C01Rik | Ankrd37 | D630023F18Rik | Eomes | Gna14 | LOC236598 | Nnat | Prnp | Slc46a1 | Tusc1 |
| 5730508B09Rik | Aoc3 | Dazl | Ephb3 | Gng3 | Lonrf3 | NP063432 | Prr11 | Slc5a4b | Ubc |
| 6030429G01Rik | Apobec2 | Dbp | Ephx4 | Gnptg | Lrrn4 | Npl | Prrg3 | Slc6a15 | Ubqln2 |
| 6330576A10Rik | Arl15 | Dcbld1 | Erdr1 | Golt1a | Lst1 | Nppb | Pth1r | Slc6a8 | Ucma |
| 6530401D17Rik | Arl5c | Ddx4 | Esx1 | Gpr19 | Lum | Nrsn1 | Ptpn14 | Slc6a9 | Unc5b |
| 9430069I07Rik | Arrdc2 | Ddx43 | EU234054 | Gpx2 | Macrod1 | Nsg2 | Pura | Slc9a3r1 | Ushbp1 |
| 9530083O12Rik | As3mt | Dennd4b | Evpl | Grb14 | Mael | Nubp2 | Pzp | Slx | Usp44 |
| A_55_P1952768 | Ash2l | Dgke | Exosc2 | Grik4 | Mageb4 | Nxt2 | Qprt | Sox11 | Vamp4 |
| A_55_P1970324 | Atg4c | Diap2 | Eya2 | Gspt2 | Malat1 | Oas1a | Rab3a | Sox3 | Vmn1r236 |
| A_55_P1987615 | Atp8a1 | Dkk3 | F2r | H2-M3 | Man2b1 | Oas1f | Rab3il1 | Spink3 | Vrtn |
| A_55_P2000595 | Aurkc | Dnahc8 | Fabp3 | H2afx | Manba | Oasl1 | Rapgef3 | Stk39 | Wdr27 |
| A_55_P2001154 | Bace1 | Dner | Fam181b | Haus4 | Map3k15 | Oat | Rasgrp1 | Stra8 | Wdr95 |
| A_55_P2004901 | BB271588 | Dpp4 | Fam64a | Hcfc1r1 | Marcks | Obsl1 | Rasl10a | Sulf1 | Wfdc10 |
| A_55_P2005672 | Bex1 | Dpp7 | Fam71b | Hes5 | Mark1 | Odf3b | Rcn1 | Syt11 | Wt1 |
| A_55_P2017369 | Bin1 | Dpysl2 | Fat2 | Hesx1 | Marveld2 | Olfr1026 | Rec8 | Syt5 | Xlr3b |
| A_55_P2024391 | BM022387 | Dpysl4 | Fbn1 | Hey1 | Matn2 | Olfr1329 | Reg2 | Tab1 | Xlr4a |
| A_55_P2028069 | Bmp6 | Dsc3 | Fbxo27 | Hist3h2ba | Mbnl3 | Olfr1346 | Rell2 | Taf7l | Xlr4b |
| A_55_P2031756 | BY388679 | Dtx1 | Fgf5 | Hmga2 | Mdfi | Olfr1466 | Retsat | Tal2 | Zap70 |
| A_55_P2069530 | C130073F10Rik | Efhc2 | Fkbp6 | Hmha1 | Mest | Olfr399 | Rftn1 | TC1574886 | Zfp275 |
| A_55_P2082025 | Cables1 | Egr1 | Fmr1nb | Hook2 | Mfap5 | Olfr485 | Rhobtb3 | TC1678291 | Zfp467 |
| A_55_P2085240 | Car12 | Emid2 | Frat1 | Hrk | Miip | Olfr691 | Rhox13 | TC1700876 | Zfp661 |
| A_55_P2086835 | Cbx2 | Enah | Gad1 | Hscb | Mirg | Osr2 | Rhox2h | TC1715318 | Zfp869 |
|  | Cbx4 | ENSMUST00000018918 |  | Hsd17b6 |  |  | Rhox5 | Terf1 | Zmat4 |
|  | Ccdc112 | ENSMUST00000022049 |  | Hspb11 |  |  | Ripk3 | Tesc | Zxdb |

**Figure S1: List of differentially expressed genes in Tet1/Tet2 double mutant ESCs (relates to Figure 1).**

(A&B) Southern blot and PCR to confirm the genotypes of derived ESCs. (C) 501 genes deregulated (1.5 folds or more). 175 down regulated genes are shown in green and 326 up regulated genes are shown in red. (D) Venn diagram showing the fraction of CpG rich and TSS 5hmC positive genes among all deregulated genes in DKO ESCs. (E) Trophoblast-like cells in teratoma sections stain positive for trophoblast marker Cdx2.



**A**

DHet (M) X DHet (F) [58 litters, 456 pups, Avg litter size=7.8]

| Genotypes (% Expected) | T1+/+\|T2+/+ (6.25%) | T1+/+\|T2–/– (6.25%) | T1+/+\|T2+/– (12.5%) | T1+/–\|T2+/+ (12.5%) | T1+/–\|T2+/– (25%) | T1+/–\|T2–/– (12.5%) | T1–/–\|T2+/– (12.5%) | T1–/–\|T2+/+ (6.25%) | T1–/–\|T2–/– (6.25%) |
|---|---|---|---|---|---|---|---|---|---|
| No. of alive pups(%) | 32 (7%) | 35 (7.7%) | 53 (11.6%) | 59 (13%) | 129 (28.4%) | 67 (14.7%) | 45 (9.9%) | 26 (5.7%) | 10 (2.2%) |
| No. of dead pups (%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (0.2%) | 0 (0%) | 2 (0.4%) | 3 (0.7%) | 3 (0.7%) | 9 (2%) |

**B** DKO (M) X DHet (F) [25 litters, 103 alive pups, Avg litter size=4.1]

| Genotypes (% Expected) | T1+/–\|T2+/– (25%) | T1+/–\|T2–/– (25%) | T1–/–\|T2+/– (25%) | T1–/–\|T2–/– (25%) |
|---|---|---|---|---|
| No. of alive pups(%) | 37 (36.3%) | 40 (39.2%) | 16 (15.7%) | 10 (9.8%) |
| No. of dead pups (%) | 1 (1%) | 1 (1%) | 6 (5.9%) | 7 (6.9%) |

**C** T1Het, T2KO (M) X DHet (F) [20 litters, 108 alive pups, Avg litter size = 5.4]

| Genotypes (% Expected) | T1+/+\|T2+/– (12.5%) | T1+/+\|T2–/– (12.5%) | T1+/–\|T2+/– (25%) | T1+/–\|T2–/– (25%) | T1–/–\|T2+/– (12.5%) | T1–/–\|T2–/– (12.5%) |
|---|---|---|---|---|---|---|
| No. of alive pups(%) | 15 (14%) | 14 (13.1%) | 32 (29.9%) | 32 (29.9%) | 11 (10.3%) | 4 (3.7%) |
| No. of dead pups (%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (0.9%) | 1 (0.9%) | 0 (0%) |



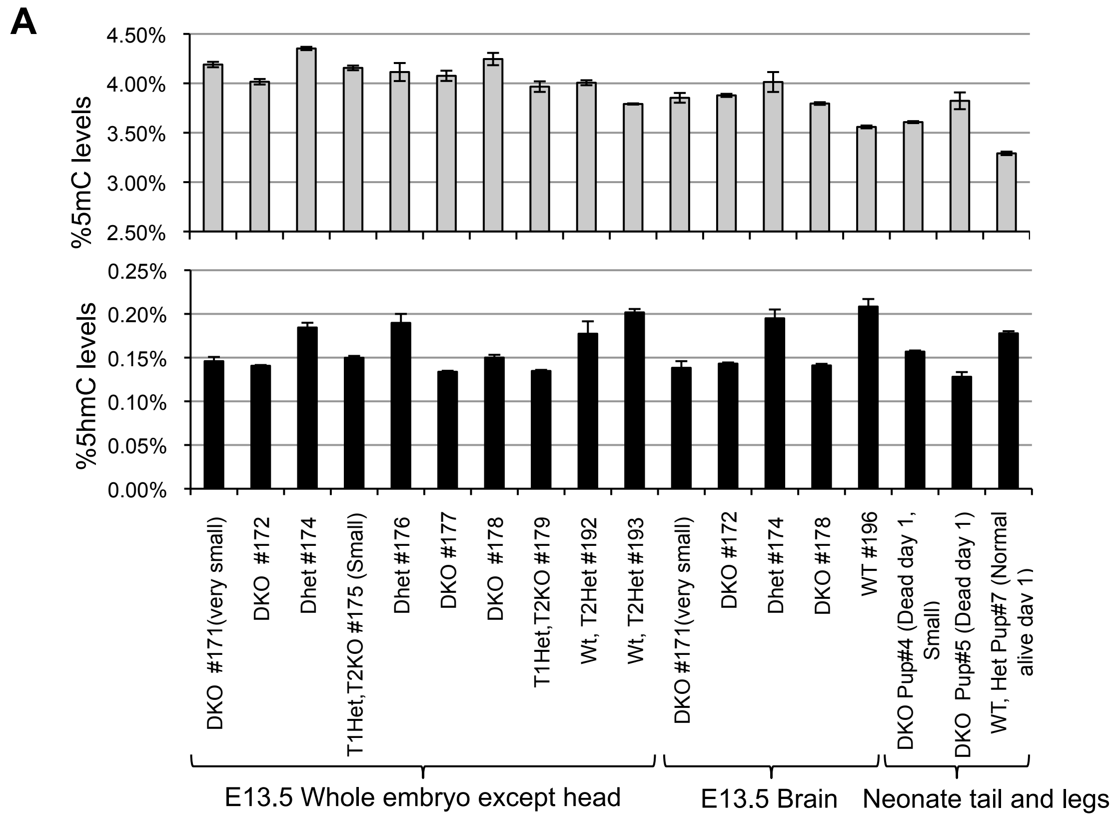
**G**

| Hematopoietic Analysis: | WT | DKO |
|---|---|---|
| ***CBC*** | | |
| WBC (mm3) | 5.2 | 6.94 |
| RBC (10^6/mm3) | 9.82 | 9.53 |
| HCT (%) | 40.7 | 44.6 |
| Hgb (g/dl) | 14.4 | 14.9 |
| Plat. Count (/mm3) | 815 | 823 |
| Retic Count (%) | – | – |
| RBC Indices MCV (fl) | 41.4 | 46.8 |
| MCHC (g/dl) | 35.4 | 33.4 |
| ***Differential*** | | |
| Neutrophils (%) | 12 | 8 |
| Bands (young neutrophils) (%) | 4 | – |
| Lymphocytes (%) | 84 | 93 |
| Monocytes (%) | – | – |
| Eosinophiles (%) | – | – |
| Basophiles (%) | – | – |
| ***Others*** | – | Potential lymphoma cells present |

**H**

| Serum Analysis | WT (M) | DKO (M) | WT (F) | DKO(F) |
|---|---|---|---|---|
| ***Liver Function*** | | | | |
| Alanine aminotransferase (ALT) IU/L | 4 | 34 | 56 | 46 |
| Aspartate aminotransferase (AST) IU/L | 161 | 204 | 202 | 131 |
| Gamma-glutamyl transferase (GGT) IU/L | 1 | 1 | 3 | 1 |
| Albumin g/dL | 2.5 | 2.3 | 1.9 | 2.3 |
| Globulin g/dL | 3.2 | 2.3 | 2.3 | 2.1 |
| Total Bilirubin mg/dL | 0.1 | 0.1 | 0 | 0 |
| Total protein g/dL | 5.7 | 4.6 | 4.2 | 4.4 |
| Creatine mg/dL | 0.1 | 0.2 | 0.1 | 0.1 |
| Cholesterol mg/dL | 142 | 103 | 116 | 117 |
| Glucose mg/dL | 164 | 345 | 318 | 274 |
| ***Kidney function*** | | | | |
| BUN mg/dL | 26 | 28 | 35 | 36 |
| ***Muscle/heart function*** | | | | |
| Creatine kinase (CK) IU/L | 1687 | 3588 | 2864 | 2224 |
| ***Minerals*** | | | | |
| Calcium (mg/dL) | 9.2 | 9.3 | 9.4 | 9.3 |
| Phosphorus (mg/dL) | 9 | 8.6 | 13.3 | 9.9 |
| Bicarbonate (mEq/L) | 18 | 12 | 4 | 10 |
| ***Others*** | | | | |
| Amylase | 1539 | 1324 | 1552 | 1254 |
| Lipase | 116 | 113 | 138 | 72 |
| Hemolysis Index | N | + | N | + |

**Figure S2: Breeding summary and blood analysis of Tet1/Tet2 double knockout mice. (relates to Figure 3)** (A-C) Summary of breeding for generating DKO mice from indicated crosses. (D &E) Genotype confirmation of mice by Southern blot and PCR. (F) Gross images of various organs of formalin fixed control and DKO neonates. (G&H) Blood analysis of adult 2-month-old DKO and wild type mice M=Male, F=Female, N=Normal.



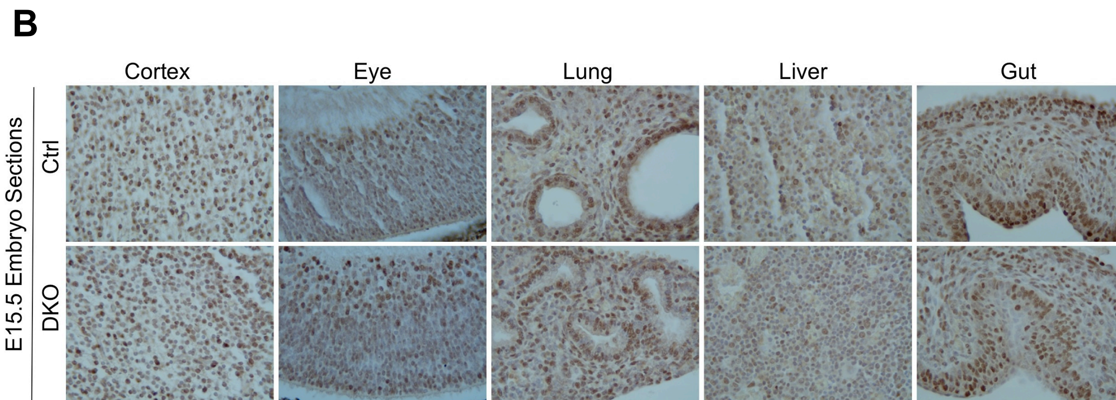
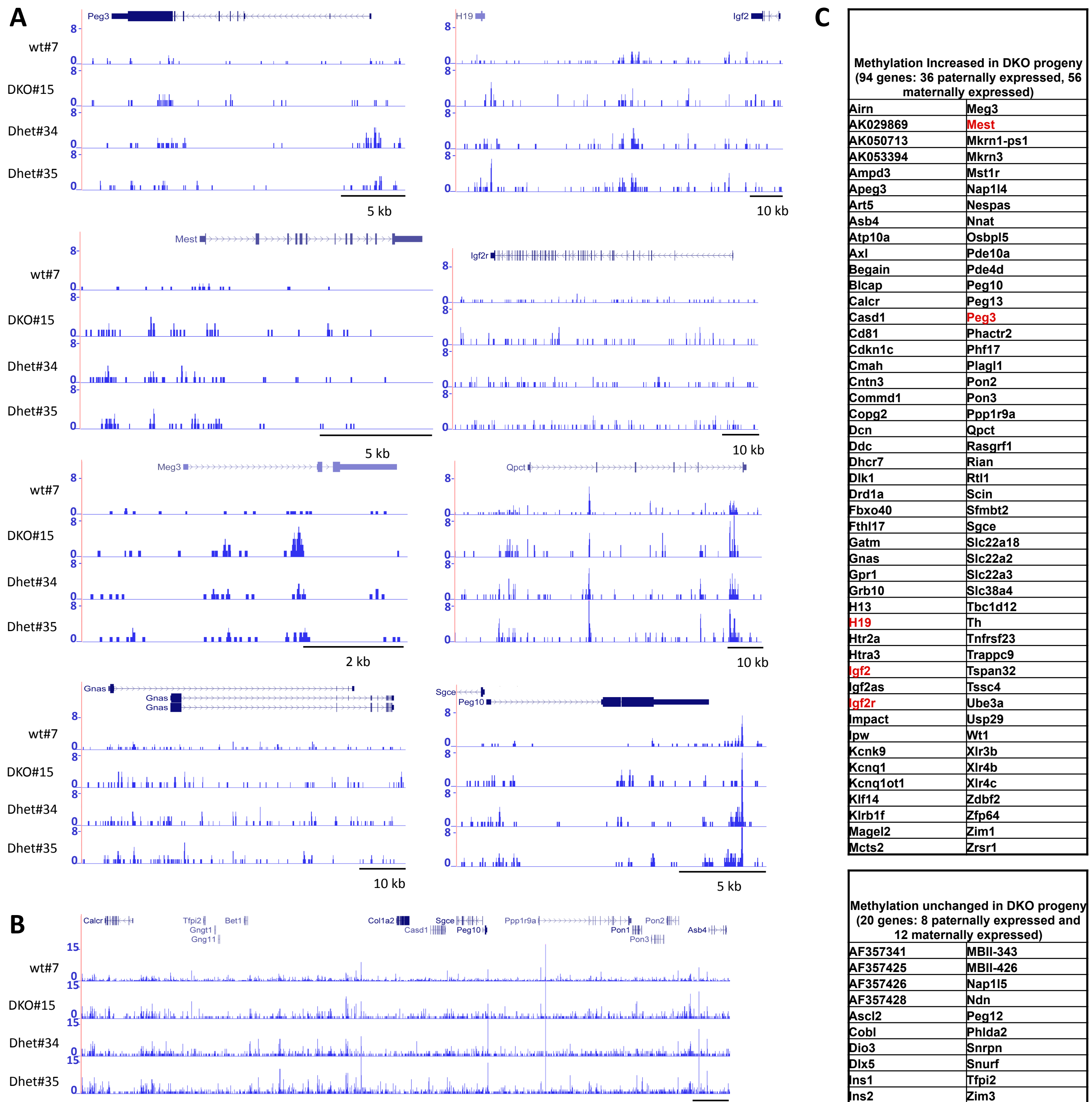




**Figure S3: Presence of 5hmC in Tet1/Tet2 double knockout embryos and neonates (relates to Figure 6)** (A) Quantification of 5hmC and 5mC by mass spectrometry in embryos and dead pups. (B) Immunohistochemistry for 5hmC in sections of Ctrl (Dhet) and DKO E15.5 embryo. Nuclei are counter stained with Hematoxylin.




| Methylation Increased in DKO progeny (94 genes: 36 paternally expressed, 56 maternally expressed) | |
|---|---|
| Airn | Meg3 |
| AK029869 | Mest |
| AK050713 | Mkrn1-ps1 |
| AK053394 | Mkrn3 |
| Ampd3 | Mst1r |
| Apeg3 | Nap1l4 |
| Art5 | Nespas |
| Asb4 | Nnat |
| Atp10a | Osbpl5 |
| Axl | Pde10a |
| Begain | Pde4d |
| Blcap | Peg10 |
| Calcr | Peg13 |
| Casd1 | Peg3 |
| Cd81 | Phactr2 |
| Cdkn1c | Phf17 |
| Cmah | Plagl1 |
| Cntn3 | Pon2 |
| Commd1 | Pon3 |
| Copg2 | Ppp1r9a |
| Dcn | Qpct |
| Ddc | Rasgrf1 |
| Dhcr7 | Rian |
| Dlk1 | Rtl1 |
| Drd1a | Scin |
| Fbxo40 | Sfmbt2 |
| Fthl17 | Sgce |
| Gatm | Slc22a18 |
| Gnas | Slc22a2 |
| Gpr1 | Slc22a3 |
| Grb10 | Slc38a4 |
| H13 | Tbc1d12 |
| H19 | Th |
| Htr2a | Tnfrsf23 |
| Htra3 | Trappc9 |
| Igf2 | Tspan32 |
| Igf2as | Tssc4 |
| Igf2r | Ube3a |
| Impact | Usp29 |
| Ipw | Wt1 |
| Kcnk9 | Xlr3b |
| Kcnq1 | Xlr4b |
| Kcnq1ot1 | Xlr4c |
| Klf14 | Zdbf2 |
| Klrb1f | Zfp64 |
| Magel2 | Zim1 |
| Mcts2 | Zrsr1 |

| Methylation unchanged in DKO progeny (20 genes: 8 paternally expressed and 12 maternally expressed) | |
|---|---|
| AF357341 | MBII-343 |
| AF357425 | MBII-426 |
| AF357426 | Nap1l5 |
| AF357428 | Ndn |
| Ascl2 | Peg12 |
| Cobl | Phlda2 |
| Dio3 | Snrpn |
| Dlx5 | Snurf |
| Ins1 | Tfpi2 |
| Ins2 | Zim3 |

**Figure S4. DKO tissues show increased 5mC levels at imprinting loci (relates to Figure 6)**
(A) MeDIP-seq profile of the indicated mouse imprinted loci in newborn wt pup#7 (progeny of WT male x Dhet female), DKO pup#15 (progeny of DKO male x Dhet female), Dhet#34 (progeny of DKO male x WT female) and Dhet#35 (progeny of WT male x DKO female). Enrichments are indicated as normalized read counts per CpG. UCSC transcription units are indicated on top in dark blue. Genomic features are viewed as custom tracks in the UCSC genome browser (Dreszer et al., 2012). (B) MeDIP-seq profile of the distal part of the prox6 imprinting region of mouse chromosome 6 in the same samples as described in A. Enrichments are indicated as normalized read counts per CpG. Genomic features are viewed as custom tracks in the UCSC genome browser (Dreszer et al., 2012). (C) List of analyzed imprinted gene loci in mouse genome for increased methylation. The methylation status of genes shown in red are confirmed by sodium bisulfite sequencing in this study as shown in table S1.

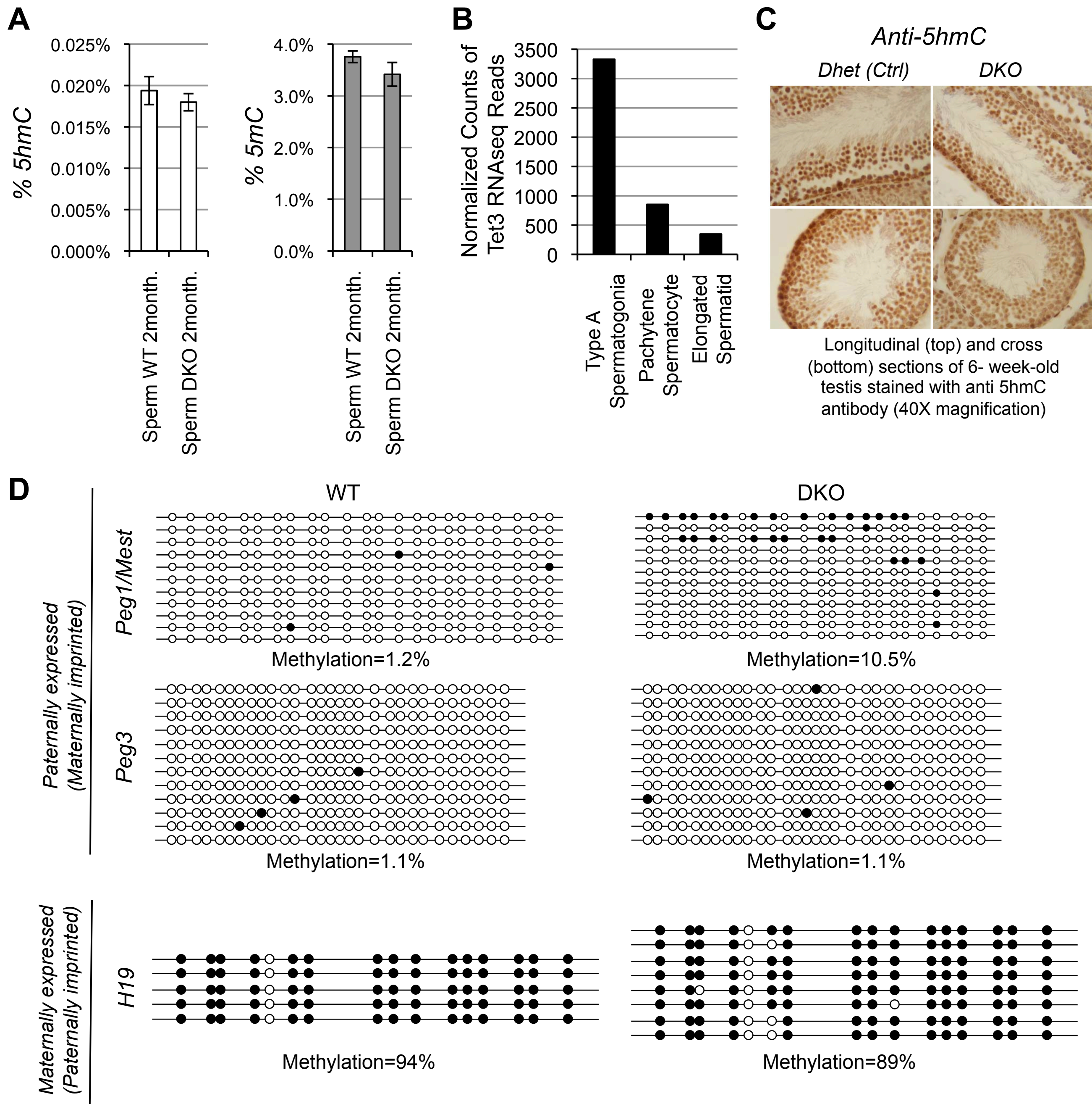



**Figure S5: 5hmC quantification and bisulfite sequencing of selected imprinted genes in WT and DKO sperm DNA (relates to Figure 5)** (A) Global 5hmC and 5mC levels in sperm DNA quantified by mass spectrometry. (B) Normalized counts of RNAseq reads from different stages of spermatogenesis in GEO data set GSE35005 (published March 2012) that maps to Tet3 transcript. (C) Sections of testes stained for 5hmC. Nuclei are counter stained with hematoxylin. Note the presence of 5hmC in various cell types inside the tubules of DKO testis. Specificity of this antibody for this staining protocol has been confirmed in liver sections in figure S3. (D) Bisulfite sequencing of paternally expressed gene Peg3 and paternally imprinted gene H19 in WT and DKO sperm DNA.

## Table S1: Methylation analysis of selected imprinted genes by sodium bisulfite sequencing, relates to figure 7 and table 1.

### *Table S1A: Analysis of paternally expressed genes Mest and Peg3*

| Sample | Parents | Genotype | Phenotype | % Mest Methylation | % Peg3 Methylation | *Maternally expressed* % H19 Methylation |
|---|---|---|---|---|---|---|
| Embryo#174 | DKO male X Dhet female | Dhet | Normal | 97 | 9 | 65 |
| Embryo#172 | DKO male X Dhet female | DKO | Normal | 50 | 63 | 37 |
| Embryo#171 | DKO male X Dhet female | DKO | Very small | 58 | 7 | – |
| Embryo#178 | DKO male X Dhet female | DKO | Slightly large | 49 | 1.3 | – |
| Embryo#192 | WT male X WT female | WT | Normal Viable | 36 | 1.3 | 36 |
| Pup#28 | DKO male X Dhet female | T1KO,T2Het | Dead P1 | 76 | 19 | 73 |
| Pup#8 | DKO male X Dhet female | T1KO,T2Het | Viable and Small | 70 | 12 | 48 |
| Pup#34 | DKO male X WT female | Dhet | Viable | 44 | 55 | – |
| Pup#15 | DKO male X Dhet female | DKO | Dead P3 | 56 | 13 | – |
| Pup#29 | DKO male X Dhet female | T1KO,T2Het | Dead P1 | 56 | 10 | – |
| Pup#25 | DKO male X Dhet female | DKO | Dead P1 | 54 | 21 | – |
| Pup#16 | DKO male X Dhet female | DKO | Dead P1 | 52 | 8 | – |
| Pup#4 | DKO male X Dhet female | DKO | Dead P1 | 25 | 18 | – |
| Pup#2182 | WT male X Dhet female | T1WT,T2Het | Viable | 57 | 17 | 44 |
| Adult#2286 | DKO male X WT female | Dhet | Viable | 57 | 14 | – |
| Adult#2287 | DKO male X WT female | Dhet | Viable | 56 | 11 | – |
| Adult#2285 | DKO male X WT female | Dhet | Viable | 42 | 10 | – |
| Adult#2284 | DKO male X WT female | Dhet | Viable | 29 | 12 | – |
| Adult#2288 | WT male X WT female | WT | Viable | 43 | 8 | – |
| Sperm | 2month old Male (from DhetXDhet) | DKO | Viable | 10.5 | 1.1 | 89 |
| Sperm | 2month old Male (from DhetXDhet) | WT | Viable | 1.2 | 1.1 | 94 |

### *Table S1B: Analysis of maternally expressed genes H19 and Igf2r*

| Sample | Parents | Genotype | Phenotype | % H19 Methylation | % Igf2r Methylation | *Paternally expressed* % Mest Methylation |
|---|---|---|---|---|---|---|
| Pup#17 | WT male X DKO female | Dhet | Dead P1 or P2. | 85 | – | 75 |
| Pup#41 | WT male X DKO female | Dhet | Dead P1 or P2. | 82 | 58 | 60 |
| Pup#42 | WT male X DKO female | Dhet | Dead P1 or P2. | 77 | 81 | 83 |
| Pup#46 | WT male X DKO female | Dhet | Dead P1 or P2. | 75 | 50 | – |
| Pup#35 | WT male X DKO female | Dhet | Dead P1 or P2. | 72 | – | – |
| Pup#44 | WT male X DKO female | Dhet | Dead P1 or P2. | 70 | 48 | 57 |
| Pup#40 | WT male X DKO female | Dhet | Dead P1 or P2. | 68 | 65 | – |
| Pup#43 | WT male X DKO female | Dhet | Dead P1 or P2. | 68 | 78 | 78 |
| Pup#39 | WT male X DKO female | Dhet | Dead P1 or P2. | 60 | 47 | – |
| Pup#45 | WT male X DKO female | Dhet | Dead P1 or P2. | 54 | 44 | – |
| Adult#2310 | WT male X DKO female | Dhet | Viable | 70 | 38 | – |
| Adult#2309 | WT male X DKO female | Dhet | Viable | 66 | 39 | – |
| Adult#2311 | WT male X DKO female | Dhet | Viable | 52 | 40 | – |
| Adult#2288 | WT male X WT female | WT | Viable | 27 | 39 | 45 |
| Pup#34 | DKO male X WT female | Dhet | Viable | 55 | – | – |
| Sperm | 2month old Male (from DhetXDhet) | DKO | Viable | 89 | – | 10.5 |
| Sperm | 2month old Male (from DhetXDhet) | WT | Viable | 94 | – | 1.2 |

*16-24 clones were analyzed for each sample.*
*Controls for each category of samples are shown in blue.*
*– indicates not tested.*
*Embryos used in this analysis were harvested at E13.5*
*Aberrantly methylated samples (>65%) are show in red.*

**Table S2: Classification of deregulated genes in DKO mouse ESCs (relates to Figure 1).**

Deregulated genes in DKO ESCs are classified into CpG containing genes, 5hmC containing genes and bivalent gene categories.

## Supplemental Experimental Procedures

### Super-ovulation of mice and Derivation of ESCs

*Tet1*$^{+/-}$|*Tet2*$^{+/-}$ females were primed with 5 IU of pregnant mare serum gonadotrophin (PMSG) (IP injection) and 45hr later administered 5 IU of human chorionic gonadotropin (HCG) (IP injection) and mated with DKO or double heterozygote males. Next day, plugged females were sacrificed and fertilized eggs were harvested from oviduct and cultured in KSOM for three days. Developed E3.5 blastocysts were used to derive ES cells as explained before (Markoulaki et al., 2008). Briefly, blastocysts were treated with acid Tyrode's solution (Sigma Cat# T-1788) to remove zona and immediately washed and cultured in ES cell derivation medium containing MEK inhibitor on gamma-irradiated mouse feeders. Six days later the cultures were passaged and expanded on feeders using regular ESCs media containing LIF. Genotyping was performed by Southern blot as described before (Dawlaty et al., 2011; Li et al., 2011) and PCR using primers listed below:

| | |
|---|---|
| Tet1 For | 5'- AACTGATTCCCTTCGTGCAG -3' |
| Tet1 Rev | 5'- TTAAAGCATGGGTGGGAGTC -3' |
| Tet2WT For | 5'- CCATGCAGGGAAGACAAGAGTAGC -3' |
| Tet2WT Rev | 5'- ATCTTGTTTGGATGGAGCCCAGAG -3' |
| Tet2KO For | 5'- CCCATTGTTCCTTTGCTCCATGCA-3' |
| Tet2KO Rev | 5'- CGTCGCCGTCCAGCTCGACCAG -3' |
| SRY For | 5'- GGAATGAATGTGTTCCATGTCG -3' |
| SRY Rev | 5'- CTCATGTAGACCAAGATGACC -3' |

### Mouse colony generation and blood analysis

Additional breeding schemes to those described in the main text involved crossing male DKOs or *Tet1*$^{-/-}$|*Tet2*$^{+/-}$ or *Tet1*$^{+/-}$|*Tet2*$^{-/-}$ animals with female double heterozygote mice to obtain DKO mice. Pregnant females were monitored daily for delivery of newborns. Neonates were monitored daily for signs of poor health. Dead or weak/sick/dehydrated pups that were removed from nest were collected

for genotyping. Mice were weaned and weighed at 3-4 weeks of age. Genotyping was performed using tail DNA and the same primer used for genotyping ESCs. For blood analyses, 400 µl of blood was collected retro-orbitally from 2-month old mice and analyzed at the Massachusetts Institute of Technology Department of Comparative Medicine Diagnostic and Comparative Pathology lab.

**Teratoma assay**

ESCs ($1x10^6$) were injected subcutaneously into SCID mice (Taconic). Mice were euthanized 3 weeks after injection and tumors were collected and fixed in formalin for two days followed by imbedding in paraffin, sectioning and staining with hematoxylin and eosin for histological analysis following standard procedures.

**DNA extraction and quantification of 5mC and 5hmC**

Following a published procedure (Le et al., 2011), pelleted ESCs and mouse tissues were incubated overnight in 100mM Tris-HCl containing 5mM EDTA, 0.2% SDS, 200 mM NaCl and 400 µg/ml proteinase K, and DNA was then isolated using a standard protocol with phenol:chloroform:isoamyl alcohol and Phase Lock Gel™ separation tubes (5-PRIME). The DNA content of the final samples was then determined by the optical density at 260 nm using a NanoDrop spectrophotometer. Two µg of DNA (except for sperm DNA when 200ng was used) was digested (37 ºC, >1 hr) with a cocktail of nuclease enzymes (DNA Degradase Plus™, Zymo Research; 2.5 µl 10X DNA Degradase Reaction buffer, 1 µl DNA Degradase Plus and water, total volume of 25 µl). Aqueous formic acid was then added (25 µl, 0.1% v/v; final concentration 40 ng digested DNA/µl), and aliquots of the mixture were injected onto a reverse phase UPLC column (Eclipse C18 2.1 x 50 mm, 1.8 µ particle size, Agilent) equilibrated with buffer A (0.1% aqueous formic acid) and eluted (200 µL/min) with an increasing

concentration of buffer B (methanol: min/%B; 0/0, 2/0, 4/5, 6/5, 8/0, 10/0). The injection volume for each sample was adjusted such that the dC peak area was at least 1 million area counts (Agilent MassHunter Quantitative Analysis, version B.04.00), which is equivalent to injection of approximately 100 ng of digested DNA. The effluent from the column was directed to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole mass spectrometer (Agilent 6460 QQQ) operating in the positive ion multiple reaction monitoring mode using previously optimized conditions, and the intensity of specific $MH^+$→fragment ion transitions were recorded (5mdC m/z 242.1→126.1, 5hmdC 258.1→142.1 and dC m/z 228.1→112.1). Peak areas for dC, 5mdC and 5hmdC were measured using instrument manufacturer-supplied software (Agilent Mass Hunter Quantitative Analysis version B.04.00/build 4.0.225.19). Three 897bp DNA standards, each homogenous for either unmodified 2'-deoxycytidine (dC), 5-methyl-2'deoxycytidine (5mdC), or 5-hydroxymethyl-2'-deoxycytidine (5hmdC), were purchased (Zymo, Irvine, CA), and used to generate calibration curves. The standards had been prepared by PCR using the appropriate nucleotides and were spin-column purified by the manufacturer to obtain 50 ng/uL aqueous Tris buffered solutions. By MRM criteria these standards were all more than 99.6% pure. With each batch of experimental samples a series of standard samples was simultaneously prepared using the DNA standards. The standard samples contained increasing amounts of 5mdC and 5hmdC in the presence of the same amount of dC (0, 0.1, 1, 5 and 10% for 5mdC and 0, 0.1, 0.5, 1, and 2% for 5hmdC). Calibration curves were constructed for 5mdC and 5hmdC from the data obtained from the standard samples (measured 5mdC or 5hmdC peak area/measured dC + 5mdC + 5hmdC peak areas) plotted against actual percentage of either 5mdC or 5hmdC in the samples. The measured percentage of 5mdC and 5hmdC in each experimental sample was then converted to actual percentage 5mdC and 5hmdC by interpolation from the calibration curves. This provided a correction for any differences that might

exist in the molar MRM responses of the various nucleosides. With this assay a sample with 0.1% 5hmdC can be reliably detected following injection of the hydrolysis mixture from 50 ng of digested DNA. The DKO samples reported here did not have a detectable peak for 5hmdC, even following injection of the hydrolysis mixture from 400 ng of digested DNA.

**Immunohistochemistry**

For immunohistochemistry of embryos sections with DAB staining, E15.5 embryos were fixed in formalin for 24 hrs, paraffin embedded, sectioned and stained with anti 5hmC (Active Motif 1:2000) overnight at 4C as reported before (Yang et al., 2012). For immunohistochemistry of gonads with fluorescence staining, E13.5 gonads were fixed in 4% paraformaldehyde overnight and paraffin embedded. 5 micron longitudinal sections were prepared, denatured with HCl and stained with anti-5hmC, anti-5mC and anti-Mvh antibodies as described before (Gill et al., 2011). Indirect immunofluorescence for Oct4 and Nanog in ESCs were performed as explained before (Dawlaty et al., 2011). Description of antibodies used are listed below:

| | | |
|---|---|---|
| Anti 5hmC (rabbit) | Active Motif (#39769) | 1:2500 for IHC |
| Anti 5mC (mouse) | Active Motif (#39649) | 1:1000 for IHC |
| Anti Mvh (goat) | R&D Systems | 1:250 for IHC |
| Anti Cdx2 (mouse) | BioGenex | Ready-to-use mix |
| Anti Oct4 (mouse) | Santa Cruz (Clone C10) | 1:100 for IHC |
| Anti Nanog (rabbit) | Bethyl Laboratories | 1:250 for IHC |

**RNA extraction, cDNA synthesis and RTqPCRs**

RNA was extracted from pre-plated ESCs or snap-frozen and powdered mouse tissue using Qiagen RNAeasy kit. About 1.5µg of RNA was used to synthesize cDNA (Invitrogen Superscript III kit).

Real time quantitative PCR was performed in an ABI 7900 cycler (Applied Biosystems) using primers listed below. Gene expression was normalized to Gapdh.

| | |
|---|---|
| Tet1 For | 5’GCTGGATTGAAGGAACAGGA3’ |
| Tet1 Rev | 5’GTCTCCATGAGCTCCCTGAC3’ |
| Tet2 For | 5'GTCAACAGGACATGATCCAGGAG3' |
| Tet2 Rev | 5'CCTGTTCCATCAGGCTTGCT3' |
| Tet3 For | 5'CCGTGACTGTGCTCTCAACT3' |
| Tet3 Rev | 5'TTCTATCCGGGAACTCATGG3' |

Tet1 primers are from (Dawlaty et al., 2011) and Tet2 primers are from (Li et al., 2011). In addition to the primers listed above, RTqPCR primers for Nanog, Oct4 and Gapdh (Dawlaty et al., 2011), Esrrb (Buganim et al., 2012), Peg3 and Mest (Ciccone et al., 2009) were used in this study.

**Southern Blotting**

10 µg of DNA isolated from ESCS or mouse tail was digested overnight and ran on 0.8% agarose gel. Transfer, hybridization of blots and probe sequences were performed as described (Dawlaty et al., 2011; Li et al., 2011).

**Isolation and Sodium Bisulfite treatment of sperm DNA**

For the analysis of imprinted genes in sperm, sperm was collected from epididymis of 2-month-old mice and DNA was extracted as described (Tash and Bracho, 1998; Walsh and Bestor, 1999). 500ng of DNA was treated with sodium bisulfite using EpiTect kit (Qiagen) following manufacturers protocol. Bisulfite sequencing primers used were as explained before: Mest, Peg3 and H19 (Lucifero et al., 2002) and Igf2r (Hiura et al., 2006).

**Mapping of sequencing data**

Processing and mapping of the sequencing data were carried out using the short read analysis pipeline SHORE (Ossowski et al., 2008) and can be found in supplemental experimental procedures. Reads were trimmed by removing stretches of bases having a quality score <30 at the ends and to a maximal length of 60 bp. The trimmed reads were mapped to the mouse genome assembly mm9 using the mapping program BOWTIE (Langmead et al., 2009), allowing up to 2 mismatches per read. After the mapping duplicate reads were removed using PICARD (http://picard.sourceforge.net) and the position-wise coverage of the genome by sequencing reads was determined and visualized as custom tracks in the UCSC genome browser (Dreszer et al., 2011).

**Gene expression profile analysis**

RNA was extracted from mouse embryonic stem cells using Qiagen RNAeasy Kit, labeled and hybridized to Agilent Whole Mouse Genome 4 × 44K v2 microarrays (two-channel). Two independent male WT cell lines were hybridized on the same arrays as two independent male DKO cell lines. One of these comparisons was performed in replicate. Similarly two independent female WT cell lines were compared to two independent female DKO cell lines, with one comparison done in replicate. Two male T1 KO cell lines were compared to two male T2 KO cell lines in replicate. One of the T2 KO cell lines was left out of the analysis of differentially expressed genes because we discovered inconsistencies in sample preparation and quality. Data were normalized within array by loess normalization and between arrays by quantile normalization of average intensities (“Aquantile”) using Bioconductor. Probes for the same gene were summarized by mean and technical replicas were combined by average. Differential expression was assayed by moderated t-test as implemented by the

limma package in Bioconductor. The cutoffs used for differential expression were |logFC| > 0.7 and p-value < 0.01.

**Enrichment analysis of deregulated genes in ESCs**

For enrichment analysis of CpG islands and bivalent histone marks, the differentially expressed genes in DKO ESCs were compared to lists of high CpG, intermediate CpG and low CpG enriched genes or H3K4me3, H3K4me27, H3K4me3+H3K27me27 enriched genes in mouse embryonic stem cells (Bernstein et al., 2006). For enrichment analysis of 5hmC marked genes, the differentially expressed genes in DKO ESCs were compared to a list of TSS-5hmC enriched genes in mouse embryonic stem cells (Pastor et al., 2011). The significance of enrichment of different classes of genes within the deregulated gene set compared to all expressed genes was assessed by hypergeometric test using R. Gene ontology analysis was performed using GeneGo with default parameters.

**Tet3 RNAseq read count analysis**

The following samples for public dataset GSE35005 (published March 2012) were downloaded from GEO: GSM860182 (SG-A_RNAseq), GSM860185 (pacSC_RNAseq), GSM860187 (eST_RNAseq). The two runs for each sample were combined. Reads that did not have at least a quality of 20 in 90% of the read were removed with fastq_quality_filter, from the FASTX-Toolkit. Reads were mapped to the mouse genome (mm9) using tophat v2.0.4, without looking for novel junctions and using a gtf file with mm9 refseq annotations downloaded from UCSC. All other tophat options were the default. We used htseq-count (http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html) on the intersection-strict mode to count how many reads mapped to each gene. Gene counts were normalized using DEseq (Trapnell et al., 2009; Anders and Huber, 2010)

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