

Simultaneous deletion of Tet1 and Tet3 increases transcriptome variability in early embryogenesis

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TET enzymes produce oxidized methylcytosines, intermediates in DNA demethylation as well as new epigenetic marks. Here we show data suggesting that TET proteins maintain the consistency of gene transcription. Embryos lacking *Tet1* and *Tet3* (*Tet1/3 DKO*) displayed a strong loss of 5-hydroxymethylcytosine (5hmC) and a concurrent increase in 5-methylcytosine (5mC) at the 8-cell stage. Single cells from 8-cell embryos and individual E3.5 blastocysts showed unexpectedly variable gene expression compared to controls, and this variability correlated in blastocysts with variably increased 5mC/5hmC in gene bodies and repetitive elements. Despite the variability, genes encoding regulators of cholesterol biosynthesis were reproducibly downregulated in *Tet1/3 DKO* blastocysts, resulting in a characteristic phenotype of holoprosencephaly in the few embryos that survived to later stages. Thus TET enzymes and DNA cytosine modifications could directly or indirectly modulate transcriptional noise, resulting in the selective susceptibility of certain intracellular pathways to regulation by TET proteins.

Significance

Development of pre-implantation embryos entails global DNA demethylation on the zygotic genome. The original thought was that TET-deficient embryos would be unlikely to survive early embryogenesis since they would be unable to mediate genome-wide demethylation in the zygote and pre-implantation embryo. However, mice lacking the individual TET proteins *Tet1*, *Tet2* or *Tet3* have survived until birth and beyond, suggesting redundancy among TET proteins in the early embryogenesis. Here we report that pre-implantation embryos doubly disrupted for *Tet1* and *Tet3* show abnormal embryonic phenotypes, whose incomplete penetrance correlates with a high variability of transcriptional profiles and DNA methylation status. Our data suggest that in addition to facilitating DNA demethylation, TET proteins and oxidized methylcytosines may regulate the consistency of gene transcription during embryogenesis.

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The three mammalian TET proteins TET1, TET2 and TET3 alter the modification status of cytosines in DNA by successively oxidizing 5mC to 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (1-3). All three oxidised methylcytosines (oxi-mC) are intermediates in DNA demethylation, the complete conversion of 5mC to C (reviewed in (4, 5)). At least two mechanisms appear to be involved: inhibition of the maintenance methyltransferase activity of the DNMT1/UHRF1 complex (6), and excision of 5caC and 5fC by thymine DNA glycosylase (TDG) (3, 7, 8). Since DNA demethylation occurs in a genome-wide fashion during embryonic development, there has been considerable interest in the role of TET proteins in early embryogenesis.

Genome-wide DNA demethylation is observed at two stages of embryonic development, in the fertilized zygote and during the specification of primordial germ cells (PGCs) (9, 10). In PGCs, *Tet1* and *Tet2* contribute to DNA demethylation through a replication-dependent mechanism (9). In fertilized zygotes, *Tet3* was originally thought to oxidize 5mC preferentially in the paternally-inherited genome (11-13); more recently, however, reduced-representation bisulfite sequencing (RRBS) has been used to suggest that demethylation of the maternal genome is also catalyzed by *Tet3* (14). RRBS measures the sum of 5mC and 5hmC (versus C, 5fC and 5caC) at a fraction of cytosines in the genome, and the data show that loss of 5mC+5hmC in both maternal and paternal pronuclei occurs primarily through a passive, replication-dependent process (14, 15). Despite the high expression of *Tet3* in oocytes and zygotes (11), *Tet3*-deficient zygotes display only a marginal increase in 5mC+5hmC in either paternal or maternal pronuclei (14), suggesting the redundant involvement of other TET proteins.

Further attesting to the redundant functions of TET-family proteins, mice lacking individual TET proteins develop relatively normally until birth and beyond (reviewed in (16)). Specifically, adult *Tet1* and *Tet2* KO mice are viable and fertile, displaying relatively mild behavioral and hematopoietic phenotypes respectively (17-19), whereas *Tet3* KO mice die perinatally for unknown reasons (11). *Tet3*-deficient early embryos show no difference in gene expression compared to WT embryos (14), suggesting compensation from *Tet1*, *Tet2* or both. Even the double deletion of *Tet1* and *Tet2* has only minor consequences: a significant fraction of *Tet1/Tet2* doubly-deficient mice survive to adulthood, while the remainder succumb late in embryogenesis or shortly after birth (20). This relatively mild embryonic phenotype could reflect the potential involvement of *Tet3*, which is upregulated compared to control in embryonic stem (ES) cells, E13.5 embryos and adult brain and lung of *Tet1/2* DKO mice (20). *Tet3* is likely to be an important player, since triple *Tet1/2/3*-deficient ES cells show significant defects in differentiation (21) and triple TET-deficient mouse embryonic fibroblasts cannot be reprogramed to induced pluripotent stem cells (22). Thus a complete characterization of the roles of TET proteins and oxi-mC modifications in early embryogenesis will require significant interference with TET function, through analysis of embryos deficient for *Tet1/Tet3*, *Tet2/Tet3* or all of the three TET proteins.

In this study, we investigated the roles of *Tet1* and *Tet3* in early embryonic development. We show that single cells of *Tet1/3* DKO 8-cell embryos display a global loss of 5hmC and gain of 5mC, indicating that *Tet1* and *Tet3* are the primary contributors to oxi-mC production at this developmental stage. RNA sequencing of single blastomeres from these 8-cell embryos revealed an unexpected degree of transcriptome variability compared to controls, with a global effect on the majority of expressed genes irrespective of expression level. A similar global variability was observed at a slightly later stage of embryonic development, by RNA-sequencing of single blastocysts collected at E3.5. RRBS analysis of individual blastocysts showed that the variability of gene transcription correlated with variably increased 5mC/5hmC in gene bodies and repetitive elements, which in turn correlated with pronounced phenotypic variability at both early and late stages of embryonic development. However, a small number of genes were reproducibly downregulated in *Tet1/3* DKO blastocysts compared to

controls; many of these encoded enzymes and transcription factors involved in lipid and cholesterol biosynthesis, potentially explaining a phenotype resembling holoprosencephaly observed in the few embryos that survived to later developmental stages (E10.5).

Results

Embryonic lethality of *Tet1/3* double-deficient mice

Consistent with previous reports (23, 24), we detected Tet1 protein at the 2-cell, 8-cell and blastocyst stages by immunocytochemistry, and Tet3 protein at the 2-cell but not the 8-cell stage (**Fig. 1A-1C**; for specificity of the Tet1 and Tet antisera see **SI Appendix, Figs. S1D and S1E**). Thus Tet3 and Tet1 likely act in succession during early embryonic development, leading us to examine mice conditionally disrupted for both the *Tet1* and the *Tet3* genes (**SI Appendix, Fig. S2A and S2B**).

Unlike *Tet1*-disrupted mice on a mixed genetic background (129Sv, C57BL/6) (17, 25), *Tet1*-disrupted C57BL/6 mice showed partial embryonic lethality – only 30% of the expected number of *Tet1* KO pups survived to birth (**SI Appendix, Fig. S3A, Table S1**). Like the mixed background mice, however, C57BL/6-background *Tet1*-disrupted mice were runted (**SI Appendix, Fig. S3B and S3C**); they showed hallmarks of hydrocephaly (**SI Appendix, Fig. S3B**), a phenomenon that may underlie some of the reported learning defects of the mixed background mice (17, 18); and the females were infertile with degenerated ovaries (**SI Appendix, Fig. S3D**), recalling the meiotic abnormality of *Tet1* gene-trap mice (26). *Tet3*-disrupted mice on the C57BL/6 background showed perinatal lethality, as observed for 129Sv-background *Tet3*^{-/-} mice (11). Intercrosses of *Tet1*^{+/-}*Tet3*^{+/-} double heterozygous parents yielded significantly smaller litters (3.4 pups/ litter) than those from *Tet1*^{+/-} or *Tet3*^{+/-} crosses (4.5 and 7.2 pups/ litter respectively), and *Tet1*^{-/-}*Tet3*^{-/-} pups did not survive to birth (**SI Appendix, Fig. S3E, Table S2**).

To generate *Tet1*^{-/-}*Tet3*^{-/-} progeny efficiently, we bred *Zp3*-Cre or *Stra8*-Cre mice to *Tet1*^{fl/fl}, *Tet3*^{fl/fl} mice (hereafter termed *Zp3*DKO and *Stra8*DKO mice). As control breeders, we used *Tet1*^{fl/fl}, *Tet3*^{fl/fl} littermates of the *Zp3*DKO females and *Stra8*DKO males. None of the resulting embryos (*Tet1*^{fl/fl}, *Tet3*^{fl/fl}, *Zp3*^{mat}, *Stra8*^{pat}, hereafter termed *Tet1/3* DKO) was viable beyond day 10.5: the majority of embryos (45/58 at day E10.5-11; 11/15 at E12-12.5) were resorbed and the remainder showed abnormal development (**SI Appendix, Fig. S3F**). Genotyping of *Tet1/3* DKO E10.5 embryos from two litters confirmed complete excision of the floxed *Tet1* and *Tet3* alleles (**SI Appendix, Fig. S3G**).

Early developmental abnormalities in *Tet1/3* DKO 8-cell embryos

Focusing on early embryonic stages, we found that *Tet1/3* DKO embryos showed variably delayed or aborted development relative to *Tet1*^{fl/fl}, *Tet3*^{fl/fl} (CTL) embryos: at embryonic day (E) 2.5, more than 95% of control embryos had reached the 8-cell stage, whereas ~25% of *Tet1/3* DKO embryos were 1- and 2-cell embryos displaying various abnormal phenotypes including mitotic defects, apoptosis and/or nuclear blebbing or fragmentation (**Fig. 1A and 1B**). The *Tet1/3* DKO embryos (~70%) that achieved the 8-cell stage by E2.5 were similar to control embryos, as judged by staining with antibodies to Nanog and Cdx2, lineage markers for primitive ectoderm and trophoectoderm respectively (**Fig. 1C**). In control 8-cell embryos, 5hmC and 5mC occupied distinct areas of the nucleus (**Fig. 1D and 1E**; the distinct staining patterns of 5mC and 5hmC confirm good antibody specificity), but in *Tet1/3* DKO 8-cell embryos, 5hmC was undetectable and instead there was uniform 5mC staining throughout the nucleus (**Fig. 1D and 1E**). Thus *Tet1* and *Tet3* are the major catalysts of 5mC oxidation at early embryonic stages; in their absence, 5mC is not converted to 5hmC.

Increased transcriptome variability in *Tet1/3* DKO 8-cell blastomeres compared to controls

To investigate how the global loss of 5hmC and increase of 5mC in *Tet1/3* DKO embryos affected gene expression profiles at the 8-cell stage, we performed RNA-seq on single cells (blastomeres) taken from 8-cell DKO and control embryos, a stage at which individual blastomeres are relatively, although not completely, homogeneous (27, 28). We collected six 8-cell embryos from control or *Tet1/3* DKO timed-matings, and dissociated them into two pools of 48 single blastomeres each; then randomly chose 10 control and 35 *Tet1/3* DKO single blastomeres and used them to generate single-cell RNA-seq libraries (**SI Appendix, Table S3**; the variability of the mutant phenotypes led us to collect more mutant than control blastomeres). When single-cell transcriptomes of all control and *Tet1/3* DKO blastomeres were analyzed with respect to spike-in controls (*red dots*), overall gene expression levels were similar between control and *Tet1/3* DKO blastomeres (**SI Appendix, Fig. S4A**), but the variability of gene expression was different (**Fig. 2**). Unsupervised clustering distinguished groups A-D (**Fig. 2A and SI Appendix, Fig. S4B**); principal component analysis showed considerably greater variability in the transcriptomes of single blastomeres from *Tet1/3* DKO 8-cell embryos compared to controls (**Fig. 2A**). The Group A cluster contained both control and DKO blastomeres, indicating that some *Tet1/3* DKO blastomeres in Group A possessed relatively normal transcriptomes (**Fig. 2A and SI Appendix, Fig. S4B**); however, even in Group A, the expression of a subset of genes was different between control and *Tet1/3* DKO single cells (**SI Appendix, Fig. S4C**). Gene ontology (GO) analysis of genes differentially expressed in Groups B, C and D compared to Group A identified distinct categories (**SI Appendix, Fig. S4D**), with the GO terms “cell cycle” in Group C and “programmed cell death” in Group D (**SI Appendix, Fig. S4D**) recalling the abnormal phenotypes observed in 2-cell embryos (**Fig. 1B**) and suggesting that the same failures occur in some blastomeres at the 8-cell stage.

To quantify the variability of gene expression between single blastomeres from control and *Tet1/3* DKO 8-cell embryos we applied a variance-stabilizing transformation that normalizes variability to the level of gene expression (**Fig. 2B-2D**). The data revealed a global increase in the overall variance of gene expression in *Tet1/3* DKO blastomeres compared to controls (**Figs. 2B-D**). A correlation plot for the variance of gene expression among the 10 control and 35 DKO blastomeres showed that essentially all genes in DKO blastomeres were expressed more variably than in controls (**Fig. 2C**; each dot represents 1 gene, and the majority of dots fall above the diagonal), in a manner independent of the level of gene expression (**Fig. 2D**). To test if the greater number of *Tet1/3* DKO single-cells (n=35) of the blastomeres compared to the number of control single-cells (n=10), had an influence on the observed difference of variances, we randomly selected 10 DKO samples and calculated gene expression variances based on the variance-stabilized data in 20 iterations. We observed increased variation of gene expression in all groups of 10 randomly chosen *Tet1/3* DKO samples compared to the 10 control samples, demonstrating that the effect of increased variation is not dependent on the number of samples. *Zfp820* and *Zbed3* were the top variable genes in single blastomeres from control and *Tet1/3* DKO 8-cell embryos respectively, whereas *Invs* is an example of one of the few genes that was relatively stably expressed in both sets of blastomeres (**Fig. 2E, SI Appendix, Fig. S4E**).

We compared our RNA-seq data for single blastomeres from 8-cell embryos with data from two previous reports: one study profiled gene expression by RNA-seq in zygotes and single cells from 2-cell and 4-cell embryos (28), whereas the second examined the expression of 48 selected genes associated with embryonic development in single cells from the 1-cell (zygote) until the 64-cell stage (27). Principal component analysis of the combined data from our study and that of Biase et al. (2014) showed that the transcriptional profiles of single cells from control 8-cell embryos clustered tightly into a single group distinct from 4-cell embryos, whereas single cells from *Tet1/3* DKO 8-cell embryos showed a more variable distribution spread between the

4-cell and 8 cell stages (**Fig. 2F**). Comparison with the data of Guo et al. (2010) yielded a similar result (**SI Appendix, Fig. S4F**).

Together, these results emphasize the striking increase in the variability but not the level of gene expression in single blastomeres from 8-cell embryos lacking Tet1 and Tet3. This variability was maintained at later embryonic stages, as discussed for E3.5 blastocysts below.

Developmental abnormalities in *Tet1/3* DKO pre-implantation and peri-implantation embryos

We examined slightly later stages of embryonic development by immunocytochemistry (**Fig. 3, SI Appendix, Fig. S5**). All control (CTL) E3.5 pre-implantation blastocysts showed normal specification of the inner cell mass and trophectoderm, as judged by distinct expression of Nanog and Cdx2 (**Fig. 3A, upper panel; Fig. 3B, left panels**). In contrast, more than 60% (10/16) of E3.5 *Tet1/3* DKO embryos displayed either a severe loss of Nanog expression or aberrant co-expression of Nanog and Cdx2 (*arrows*); in many cases, the Nanog-expressing cells failed to form a well-defined inner cell mass (**Fig. 3A, lower panels, Fig. 3B, right panels; SI Appendix, Fig. S5A**). A fraction of *Tet1/3* DKO embryos (6/16) had a relatively normal appearance (e.g. **SI Appendix, Fig. S5B, left**), but several of these showed variable coexpression of Nanog and Cdx2 (**Fig. 5B, right**).

A similar failure of early lineage specification was observed at the peri-implantation blastocyst stage (E4.5). In control embryos, we observed well-developed layers of primitive ectoderm expressing Nanog, primitive endoderm expressing Gata6, and trophectoderm expressing Cdx2 (**Fig. 3C, SI Appendix, Fig. S5C, top panels**), whereas *Tet1/3* DKO embryos showed strong dysregulation of these three lineage markers, with aberrant co-expression of the markers, poor separation of germ cell layers, and poor or failed development of primitive ectoderm and primitive endoderm as judged by Nanog and Gata6 staining (**Fig. 3C, SI Appendix, Fig. S5C, bottom panels**). Again, some *Tet1/3* DKO embryos (5/15) had a relatively normal appearance at E4.5, with high Nanog and Gata6 expression (**SI Appendix, Fig. S5D**), but most embryos showed variably delayed development as judged by size (**Fig. 3C, SI Appendix, Fig. S5C and S5D; compare lengths of the 50 mm scale bars in each panel**). Overall, there was marked phenotypic variability in *Tet1/3* DKO embryos at each of the three stages profiled (E2.5, 3.5 and 4.5), and a steadily increasing fraction of abnormal embryos at later times.

Increased transcriptome variability in *Tet1/3* DKO blastocysts compared to controls

To investigate the variable phenotypes of *Tet1/3* DKO blastocysts at a molecular level, we performed RNA-sequencing (RNA-seq) on single blastocysts (**SI Appendix, Table S4**). At day 3.5 after timed-matings, we randomly chose and sequenced 3 control blastocysts from a litter of 7, and 5 *Tet1/3* DKO blastocysts from a litter of 8 (again, the highly variable phenotypes of mutant embryos led us to sequence more mutant than control embryos). Despite low levels of *Tet3* transcripts in control blastocysts, RNA-sequencing confirmed excision of the floxed *Tet1* and *Tet3* exons in *Tet1/3* DKO blastocysts (**SI Appendix, Fig. S6A, Dataset S1**); *Tet2* mRNA was expressed at considerably higher levels and showed no compensatory upregulation in *Tet1/3* DKO embryos relative to controls (**SI Appendix, Fig. S6B**). *IDAX* transcripts were not detected by RNA-seq (**SI Appendix, Dataset S1**), suggesting that Tet2 protein expression was unlikely to be controlled by IDAX (29) at this developmental stage. Expression of *Eif2s3y*, *Zfy1*, *Ddx3y*, *Usp9y* and *Uty* on the Y chromosome and *Xist* on the X chromosome showed that the three control embryos were female whereas the *Tet1/3* DKO group had 3 male and 2 female embryos (**SI Appendix, Fig. S6C**). The expression of imprinted genes in the *Tet1/3* DKO blastocysts was normal compared to controls.

RNA sequencing revealed that *Tet1/3* DKO blastocysts displayed considerably more transcriptome variability than the corresponding controls. Unsupervised clustering analysis based on similarities of the transcriptomes showed that control embryos clustered together whereas *Tet1/3* DKO embryos formed two clusters (**SI Appendix, Fig. S6D**). The clustering was not influenced by the sex of the embryos: DKO 1 and 2 (male) clustered with the three control embryos (female), whereas the other three embryos (DKO 3, male; DKO 4 and 5, female) formed a distinct group (**SI Appendix, Fig. S6D**). Principal component analysis based on the expression profiles (with sex chromosome data removed) confirmed that control embryos clustered together whereas *Tet1/3* DKO embryos showed a high degree of variance (**Fig. 4A**). Emphasizing this variability, the number of genes differentially-expressed in each of the five *Tet1/3* DKO blastocysts compared to the average of the 3 control blastocysts ranged from 172 differentially expressed genes in DKO2 to 1153 in DKO4 (adjusted p-value<0.05), with only 23 differentially expressed genes common to all 5 DKO blastocysts relative to controls (**Fig. 4B**).

Even in the averaged transcriptomes of E3.5 blastocysts, the vast majority of expressed genes showed a much greater variability of expression in *Tet1/3* DKO blastocysts relative to controls (**Fig. 4C-4E**; each gene is represented by a dot). As in 8-cell blastomeres, a correlation plot for the variance of gene expression showed that essentially all genes in the 5 DKO blastocysts were expressed more variably than in the 3 controls (**Fig. 4D**), again in a manner independent of the level of gene expression (**Fig. 4E**). Consistent with our immunocytochemistry data, *Nanog* was one of the most variably expressed genes in DKO blastocysts compared to controls (**Fig. 4C, 4F and 4G; SI Appendix, Dataset S1**). Other genes, such as *Cdx2* and *Oct4*, showed less variability: *Cdx2* mRNA expression was similar between the two groups (**Fig. 4F, middle**) whereas *Oct4* mRNA expression showed a slight decrease in DKO embryos relative to control (**Fig. 4F, bottom**). A heatmap comparing the expression of the top 1% of variable genes (n=178; indicated by red dots in **Fig. 4C**) in DKO embryos relative to controls showed that the most common pattern for the highly variable genes was expression comparable to controls in DKO embryos 1 and 2, versus poor expression relative to controls in DKO embryos 3, 4 and 5 (**Fig. 4G**).

Together, these results show that in E3.5 blastocysts as well as in single blastomeres from 8-cell embryos, deletion of *Tet1* and *Tet3* results in a profound and global increase in the variability of gene expression. We asked whether this variability was reflected in a corresponding global increase in the variability of DNA cytosine modification at different annotated regions of the genome (see section below).

A few genes are reproducibly downregulated in *Tet1/3* DKO blastocysts

Comparing the group of five *Tet1/3* DKO blastocysts to the group of three control blastocysts, and applying an adjusted p-value of <0.01, we identified only 40 genes as consistently differentially expressed in DKO blastocysts compared to controls (**SI Appendix, Dataset S1**). Among these 40 genes, the gene ranked at third place by p-value was *Lifr*, the receptor for the cytokine LIF (**Fig. 4H, left; SI Appendix, Dataset S1**). The active LIF receptor complex contains LIFR and its obligate binding partner, Gp130 (30), and *Gp130* expression was also reproducibly low in *Tet1/3* DKO blastocysts (**Fig. 4H, right**). Mice deficient in LIFR subunits show variable phenotypes ranging from impaired implantation to perinatal lethality (31), potentially partly explaining the variable onset of embryonic lethality in *Tet1/3* DKO embryos both before and after gastrulation. Embryonic stem cells (ESC) cannot be derived from *Lifr*-deficient blastocysts (31), and indeed we consistently failed to derive ESCs from *Tet1/3* DKO blastocysts (18 embryos from 3 litters), despite successful establishment of 5 different ESC lines from 11 control embryos. We also observed consistently reduced expression of several pluripotency-associated genes in *Tet1/3* DKO embryos, including *Tcl1* (T-cell leukemia-1, a gene involved in translocations to the TCR locus in T cell leukemias and also a target of *Tet1* in ES cells (32)) (**Fig. 4I, SI**

Appendix, Fig. S6E; Dataset S1). *Sca1/2* expression was diminished in *Tet1/3* DKO embryos (**SI Appendix, Fig. S6F**), potentially explaining the hematopoietic defects observed in some embryos at later developmental stages (**SI Appendix, Fig. S3F**). Genes involved in lipid and cholesterol biosynthesis were also reproducibly downregulated, as discussed below.

Increased levels and variability of DNA modification in *Tet1/3* DKO blastocysts compared to controls

To investigate whether transcriptional variability reflected a corresponding variability in DNA modification status, we used targeted bisulfite sequencing to assess DNA cytosine modification at the *Nanog* locus, for which variable expression was strongly apparent both by immunocytochemistry and by RNA-seq. Because 5mC is not distinguished from 5hmC, nor unmodified C from 5fC and 5caC, in bisulfite sequencing (33, 34), and because Tet2 expressed in *Tet1/3* DKO embryos can generate oxi-mC, we use the term “DNA modification” in preference to “DNA methylation”, and specify the modified bases actually examined in each case.

We first examined *Nanog* regulatory regions using bisulfite treatment followed by targeted PCR. At the 8-cell stage, reduced representation bisulfite sequencing (RRBS) still shows evidence of widespread 5mC/5hmC (35). However we did not observe any substantial difference in the extent or variability of DNA modification at the *Nanog* promoter /transcription start site (TSS) or a distal (-5 kb) regulatory region (36) at either the E3.5 blastocyst or the E2.5 8-cell stage (**SI Appendix, Fig. S7A and S7B**).

To examine DNA modification more globally, we prepared and sequenced RRBS libraries from 3 control blastocysts (from a litter of 6), and 5 *Tet1/3* DKO blastocysts (from a litter of 6, **SI Appendix, Table S5**). As expected, control blastocysts were substantially demethylated on all chromosomes (7-10% 5mC/5hmC; **Fig 5A**). In contrast, the overall levels of 5mC/5hmC in the five different *Tet1/3* DKO embryos were highly variable, with two blastocysts (DKOa, DKOb) resembling controls, and the three others showing variably increased levels of 5mC/5hmC (**Fig 5A**). Detailed analysis of DNA modification levels at specific genomic regions showed a substantial but variable increase in 5mC/5hmC at all genomic regions in *Tet1/3* DKO embryos compared to controls (**Fig. 5B**), as shown for selected promoters and gene bodies in **Figure 5C and SI Appendix, S7C**. We evaluated the variability of DNA modification (5mC/5hmC) at each CpG, eliminating CpG's covered by < 8 reads as well as CpGs with < 10% 5mC+5hmC (**Fig. 5D**). *Tet1/3* DKO blastocysts displayed a much higher variability of DNA modification relative to controls, regardless of the actual level of modification present at each CpG.

Together, our data indicate that loss of TET function results both in increased DNA modification as well as in increased variability of modification, consistent with the increased variability of gene expression observed in **Figure 4**. Because RNA-seq and RRBS DNA methylation analysis cannot currently be performed on the same blastocysts, a direct correlation of gene expression with DNA modification status is technically out of reach.

Increased levels and variability of DNA modifications at repetitive elements in *Tet1/3* DKO embryos correlates with altered and variable expression

We examined 5mC/5hmC levels at repetitive elements in control and *Tet1/3* DKO blastocysts. All elements – non-LTR (LINE, SINE) and LTR (LTR, IAP) retrotransposon groups, DNA transposons and satellite sequences – showed a variable increase in 5mC+5hmC in *Tet1/3* DKO blastocysts compared to controls (**Fig. 6A, top**). To illustrate this point in more detail, the greater variability of DNA modification in LINE and LTR elements of *Tet1/3* DKO blastocysts is shown at the level of individual CpGs in **Figures 6B and 6C**. In parallel, we assessed the expression levels of these repetitive elements using our single-embryo RNA-seq data (obtained

from a different set of blastocysts for the reason outlined above). The expression of LTR and IAP elements was significantly downregulated in *Tet1/3* DKO compared to control blastocysts (**Fig. 6A, bottom**); expression of LINEs and SINEs showed a decrease in mean expression levels as well as an increase in the variability of expression.

Since single cells are technically not yet amenable to RRBS, we were unable to measure 5mC/5hmC in single blastomeres from control and *Tet1/3* DKO 8-cell embryos. Instead, we assessed the expression levels of retrotransposons, DNA transposons and satellite sequences at this stage (**Fig. 6D**). Confirming the results in blastocysts, expression of IAP elements was significantly downregulated in *Tet1/3* DKO compared to control 8-cell blastomeres whereas expression of SINES and LTRs was increased (**Fig. 6D**).

Thus *Tet1/3* DKO embryos show variably increased 5mC+5hmC as well as an increase in the variability of expression of transposable elements compared to control embryos, and may positively control IAP expression at the 8-cell and blastocyst stages of embryonic development.

Deficiency of cholesterol synthesis in *Tet1/3* DKO blastocysts results in failure of the sonic-hedgehog pathway in late embryogenesis

Gene ontology (GO) analysis for the 40 genes consistently differentially regulated in *Tet1/3* DKO embryos (adjusted p-value <0.01) identified a number of genes whose products regulate cellular lipid metabolism and cholesterol biosynthesis that were downregulated in *Tet1/3* DKO embryos relative to controls (**Fig. 7, SI Appendix, Fig. S8A and S8B; Dataset S1**). The transcription factor Srebp2, encoded by the *Srebf2* gene, regulates the expression of a large number of these enzymes; it is normally resident in the endoplasmic reticulum but is escorted to the Golgi by a chaperone, Scap, and then cleaved to release its DNA-binding domain when cells become depleted of cholesterol (37). Four of the five *Tet1/3* DKO embryos (DKO2-5) showed a strong reduction of *Srebf2* mRNA expression; the fifth (DKO1) showed normal *Srebf2* expression but strongly diminished expression of *Scap* mRNA (**SI Appendix, Fig. S8C**). Using antibodies to N-terminal and C-terminal regions of Srebp2, which detect total (ER and nuclei) and ER-localised forms of Srebp2 respectively (37), we confirmed that Srebp2 protein expression was also significantly downregulated in *Tet1/3* DKO embryos (**SI Appendix, Fig. S8D and S8E**). However, there was no change in the unmethylated (C/5fC/5caC) status of the *Srebp2* TSS and promoter at early embryonic stages in *Tet1/3* DKO embryos compared to controls (**SI Appendix, Fig. S8F**).

Since sonic hedgehog (*Shh*) requires covalent modification by cholesterol for its activity (38), we speculated that defective cholesterol synthesis during the development of *Tet1/3* DKO embryos might result in decreased functioning of the hedgehog-signaling pathway and contribute to developmental abnormalities. Indeed, the phenotypes of late stage (E10.5) *Tet1/3* DKO embryos – poor forebrain formation and abnormal facial structures (**SI Appendix, Fig. S8G**) – resembled the phenotypically heterogeneous holoprosencephaly with consistent forebrain defects and facial abnormalities observed in *Shh* deficiency as well as in mice with defects in cholesterol synthesis (39, 40). Indeed mRNAs encoding signaling components (Patched1 (*Ptch1*), hedgehog interacting protein (*Hhip*)), transcription factors (*Gli1/2*, *Pax1/9*), and other targets (Cyclin D2 (*Ccnd2*)) of the Hedgehog signaling pathway were significantly decreased in E10.5 *Tet1/3* DKO embryos relative to controls (**SI Appendix, Fig. S8H**); these transcripts were not detectably expressed at either the 8-cell or the blastocyst stage. Consistent with neural defects, mRNAs encoding neural (*Nestin*, *Sox1*, *Pax6*) but not cardiac (*Fgf10*, *Nkx2-5*, *TnnT2*, *Myh7*) markers were also significantly reduced (**SI Appendix, Fig. S8I and S8J**).

Dysregulation of imprinted gene expression at E10.5

Even at this late stage, 5hmC levels were considerably lower in *Tet1/3* DKO relative to control E10.5 embryos suggesting incomplete compensation from Tet2 (SI Appendix, Fig. S9A, left); however overall 5mC levels were not detectably affected in most embryos (SI Appendix, Fig. S9A, right), suggesting that any changes in DNA modification occurred at a locus-specific level. We tested the DNA modification status of imprinted genes in genomic DNA of 4 control and 4 *Tet1/3* DKO E10.5 embryos by BstUI digestion and qPCR quantification of methylated genomic DNA (41), after generating standard curves for amplification of short control and long differentially methylated regions (DMR)-containing amplicons (SI Appendix, Fig. S9B). Four different DMRs from 2 paternally and 2 maternally imprinted genes were analyzed for their level of DNA modification (5mC+5hmC) and for expression of the corresponding genes. Both maternally (*Peg3* and *Snrpn*) and paternally (*H19* and *Igf2r*) imprinted genes showed increased DNA modification at their DMRs and concomitantly decreased expression in E10.5 embryos (SI Appendix, Fig. S9C), suggesting that *Tet1* and *Tet3* regulate the expression and modification status of these imprinted genes during later embryonic development.

Discussion

Here we report the effects of TET loss-of-function in early embryogenesis. With one exception (20), most previous work in this area has focused on loss of function of individual TET proteins (17, 19, 25, 42). Because TET proteins clearly function redundantly (20, 25, 43), we preferred to examine mice with a very substantial loss of TET function during early embryonic development. We chose to delete both *Tet1* and *Tet3*: *Tet3* is present in zygotes and at the 2-cell stage whereas *Tet1* is expressed from the 2-cell stage to the blastocyst. We find (as expected) that the effects of double deletion are considerably more striking than the effects of individual deletion of either *Tet1* or *Tet3*. More importantly, however, analysis of *Tet1/3* DKO embryos revealed an unexpected phenotype of a global increase in transcriptional variability.

Even in a population of genetically identical cells, individual cells exhibit a remarkable degree of diversity, most easily visualized through flow cytometric profiles of protein or reporter expression, which often span 1-2 orders of magnitude. Variations in gene expression patterns within a single cell can be assessed by comparing the levels of two different reporter genes driven off the same promoter (intrinsic noise), whereas those that occur between cells (extrinsic noise) can be examined in many ways including single-cell RNA sequencing, the technique we have employed here (44-46). Cell-to-cell variability in gene expression reflects stochastic variation, differences in cell state or environmental input, and gene regulatory processes including the frequency and robustness of transcriptional initiation (46), the relative abundance of transcriptional regulators (47, 48), the size and frequency of transcriptional bursts (49), the spatial organization of the genome (50), and the relative efficiency of transcription versus translation (51) (reviewed in (45, 52)).

Studies in yeast indicate that several epigenetic regulators can impose transcriptional consistency and suppress transcriptional noise. In many cases, this involves modulation of transcriptional elongation by RNA polymerase II (Pol II), a process linked to cotranscriptional histone modifications that regulate nucleosome disassembly and reassembly. Eviction of acetylated nucleosomes ahead of Pol II permits efficient elongation, while reassembly of deacetylated nucleosomes behind the elongating polymerase prevents initiation at spurious sites. Consistent with this scenario, mutations in histone chaperones that promote nucleosome assembly/ disassembly, or in enzyme complexes that catalyze or erase histone modifications associated with gene bodies (e.g. H3K9/14 and H4 acetylation, H2B ubiquitylation, H3K36 di- and tri-methylation), affect transcriptional consistency in yeast (49, 53-55).

Our data suggest that in mammals, TET-mediated DNA modifications may play a similar role. In support of this hypothesis, 5hmC is present at the highest levels in gene bodies of the most highly expressed genes (43, 56-60), and TET proteins have been linked to many processes and histone modifications associated with transcriptional variability. *(i)* In yeast, the Set2 methyltransferase complex travels with RNA pol II and methylates H3K36 in gene bodies; the H3K36me3 modification is recognized by the Sin3a/Rpd3 histone deacetylase (HDAC) complex, which permits nucleosome reassembly by deacetylating lysines on histones 3 and 4, thus preventing spurious initiation at intragenic sites (53). In mammalian cells, TET functions are linked to both types of histone modifications: high levels of 5hmC are present in the gene bodies of expressed genes; 5hmC distribution in gene bodies is strongly correlated with that of H3K36me3 (43, 56-60); and TET1 co-immunoprecipitates with SIN3A and HDAC1/2 in mouse ES cells (61). *(ii)* Also in yeast, H2B ubiquitylation has been implicated in nucleosome dynamics (54, 55), and a screen for chromatin regulators that altered the variability of reporter expression in yeast showed that H2B ubiquitylation recruited the Set3C HDAC complex, thereby limiting the high rate of Pol II elongation associated with transcriptional bursts (49). Similarly in mammalian cells, TET2 and TET3 have been linked to H2B-ubiquitination through their association with the enzyme O-GlcNAc transferase (OGT) (62-64); OGT attaches O-linked N-acetylglucosamine to H2B (63), a modification required for subsequent H2B ubiquitylation (65). *(iii)* Finally, the linear extent (“breadth”) of H3K4me3 enrichment around gene promoters has been correlated with transcriptional elongation, Pol II pausing/ processivity, increased DNase I accessibility and increased transcriptional consistency in mammalian cells (66); the TET proteins TET2 and TET3 are implicated in this process because of their association with the SET/COMPASS complex, which contains the SETD1A lysine methyltransferase that deposits H3K4me3 (64).

In a recent elegant study, interference with one of two redundant pathways of gene regulation was shown to result in variable penetrance of an organismal phenotype, ultimately stemming from increased transcriptional noise (48). In *C. elegans*, *end-1* and *end-3* both control the expression of *elt-2*, a transcriptional regulator required for specification of the “E” cell, a precursor cell required for intestinal development. Mutations in upstream regulators (*skn-1*, *med-1/2*) that caused decreased expression of *end-3* transcripts were also associated with an increased variability of *end-1* expression, resulting in the emergence of a threshold effect that decreased *elt2* expression and led to incomplete penetrance of the final phenotype, the total number of intestinal cells and formation of a normal intestine. Although TET enzymes are likely to be ubiquitous global regulators that affect the expression of many genes in mammalian cells, the parallels are clear – double deficiency of Tet1 and Tet3, through strong interference with TET function and pronounced alterations in DNA cytosine modification, may not so much interfere with gene expression per se, as impose a global phenotype of increased transcriptional variability.

It seems self-evident that in a given cell type, transcriptional variability would affect certain gene networks more than others. Indeed, *Tet1/3* DKO blastocysts showed reproducible downregulation of a core set of genes involved in cholesterol biosynthesis and in Hedgehog signaling. Similarly, Tet1-deficient ES cells show clear skewing towards the trophectoderm lineage, both in cell culture and in teratomas (67), and Tet2-deficient haematopoietic stem/precursor cells show skewed differentiation towards the myeloid lineage (19, 68). If TET proteins maintain the consistency of gene transcription during cell lineage specification as suggested here, their loss might be expected to disturb preferentially the expression of gene products – including critical transcription factors and proteins in key signalling pathways – involved in determining cell fate.

Whether TET loss-of-function acts through increased DNA methylation, loss of oxi-mC production or both remains unclear. The genome-wide DNA demethylation observed in early embryos appears to be a default pathway independent of TET function or oxi-mC modification: both the 5hmC-modified paternal genome and

the unmodified maternal genome are passively demethylated at similar rates during the robust cell divisions that occur in the early embryo (69). Even in zygotes, demethylation of both maternal and paternal pronuclei requires DNA replication, a process only partially dependent on Tet3 (14, 15). For each observed outcome, the relative contributions of oxi-mC production and DNA demethylation may be distinguished by comparing cells deficient in DNA methyltransferases, which would be depleted for both 5mC and oxi-mC, with cells deficient in TET proteins, which lack only oxi-mC. Specific downstream mechanisms include altered recruitment of key transcription factors to their binding sites in DNA, either through direct modification of the binding sequences, modulation of chromatin accessibility, or both; retrotransposon modification and expression, since insertion of these elements is known to create alternative promoters for host genes during early embryogenesis (70, 71); changes in local histone modifications, as suggested by an early study in which DNA methylation in the gene body was shown to regulate Pol II elongation efficiency through H3K4-di/tri methylation and H3K9/14-acetylation (72). Future studies will clarify these points.

Materials and Methods

Generation of *Tet1*- and *Tet3*- deficient mice

The endogenous *Tet1* locus was targeted for conditional *Tet1* and *Tet3* alleles were generated by excision of exons 8, 9, and 10 (**Fig. S1A, top**) and exon 2 respectively (**Fig. S1B**).

Single- embryo and cell RNA sequencing

CTL and *Tet1/3* DKO 8-cell embryos and blastocysts were collected at E2.5 and E3.5 respectively. 8-cell embryos were dissociated, lysed and converted into double stranded cDNA using SMARTer ultra low RNA kit for Illumina sequencing (Clontech). 1 ng of cDNA for each sample was used for preparing libraries using Nextera XT DNA sample preparation kit (Illumina) and sequenced using an Illumina HISEQ 2500 instrument. RNA-seq data was mapped against mm9 using Tophat. Sequencing read counts per gene were calculated using htseq-count.

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Author Contributions

JK designed and did experiments; LC, ML and AC performed computational and statistical analyses; WAP, ECT and SBK generated gene-targeted animals; AT set up breedings and examined mouse phenotypes early

in the study; MN and RSL provided technical help for the single-cell sequencing experiments; and SK trained JK in mouse embryology techniques. WAP, SBK, MAS and SK critically evaluated the manuscript. AR directed the study; AR, JK and LC analyzed data and wrote the manuscript.

Figure Legends

Figure 1. Analyses of control and *Tet1/3* DKO 2-cell and 8-cell embryos. **(A)** Bar graph showing the percentage of 1-cell, 2-cell, 4-cell and 8-cell embryos in control (CTL) and *Tet1/3* DKO embryos harvested at E2.5. Almost all CTL embryos have reached the 8-cell stage whereas a substantial fraction of *Tet1/3* DKO embryos show aborted or delayed development. **(B)** Apoptosis (*left*), nuclear fragmentation (*middle*) and mitotic defects (*right*) in representative non-viable 2-cell embryos from *Tet1/3* DKO mice revealed by DAPI staining. **(C)** Immunocytochemistry for the lineage markers Nanog and Cdx2 does not reveal any pronounced differences between CTL and *Tet1/3* DKO 8-cell embryos. Cdx2 was expressed in all blastomeres whereas Nanog expression was typically not detected in 8-cell embryos (or occasionally detected in one or a very few blastomeres). **(D)** Immunocytochemistry using antibodies to 5mC and 5hmC shows global loss of 5hmC and concomitant increase of 5mC in nuclei of *Tet1/3* DKO 8-cell embryos compared to CTL. Scale bar 50 μ m. **(E)** *Top*, Merged and enlarged images of a second control embryo; *bottom*, *Tet1/3* DKO embryo from **Figure 1d** *middle*, stained with antibodies to 5mC and 5hmC.

Figure 2. Transcriptional variability associated with *Tet1/3* deficiency revealed by single-cell RNA sequencing of individual blastomeres from 8-cell embryos. **(A)** Principal component analysis of the transcriptomes of 10 CTL and 35 *Tet1/3* DKO with the sex chromosome information removed, showing that the 35 *Tet1/3* DKO blastomeres are more widely separated than the 10 CTL blastomeres for both principal component (PC) 1 (absolute gap, 37 vs. 8) and PC2 (17 vs. 13). Groups A-D identified by the clustering analysis of **Figure 2A** are indicated. **(B)** The box-and-whisker plot depicts the distribution of stabilized variances (gene expression variances across samples independent of their expression strength (see *Methods*)) in 8-cell embryos. Median variances are shown at the bottom. Upper and lower whiskers represent >75% and <25%, respectively. **(C)** Correlation plot of expression variances of all genes expressed in single cells of CTL and *Tet1/3* DKO 8-cell embryos. Each gene is represented by a dot. Most genes fall above the diagonal, indicating greater variability of expression in *Tet1/3* DKO blastomeres compared to controls. **(D)** The ratios of gene expression variance between CTL and *Tet1/3* DKO blastomeres are plotted against the average expression level for each gene. The increased gene expression variance observed in blastomeres from *Tet1/3* DKO 8-cell embryos is independent of the level of gene expression. **(E)** The genes expressed with highest variance in CTL and *Tet1/3* DKO blastomeres, *Zfp820* (*left panel*) and *Zbed3* (*right panel*) respectively, and a gene that shows low variance in both samples, *Invs* (*bottom panel*), are indicated (also see **Fig S4E**). **(F)** Principal component analysis of the combined RNA-seq data from our study and the single-cell analysis of zygotes, 2-cell and 4-cell embryos performed by Biase *et al.* (2014).

Figure 3. Expression of the lineage markers Nanog, Cdx2 and Gata6 in control and *Tet1/3* DKO E3.5 and E4.5 embryos. **(A)** Immunocytochemistry for Nanog and Cdx2 expression shows dysregulation of the first step of lineage commitment in *Tet1/3* DKO E3.5 embryos compared to control (CTL). Arrows indicate aberrant co-expression of Nanog and Cdx2. **(B)** Two more control blastocysts (*left panel*) and three *Tet1/3* DKO blastocysts with poor or failed Nanog expression (*right panel*). **(C)** Immunocytochemistry for Nanog, Cdx2 and Gata6 expression shows dysregulation of the second step of lineage commitment in *Tet1/3* DKO E4.5 embryos. Scale bar 50 μ m. Listed in red are the numbers of embryos displaying the indicated normal or abnormal phenotype, out of the total number of embryos examined. For more examples see **Figure S5**.

Figure 4. Transcriptional variability associated with *Tet1/3* deficiency revealed by RNA sequencing of single E3.5 blastocysts. **(A)** Principal component analysis (PCA) of three CTL and five *Tet1/3* DKO transcriptomes with the sex chromosome information removed, showing that the three control blastocysts cluster together whereas the five *Tet1/3* DKO blastocysts are widely separated. **(B)** Numbers of genes differentially expressed

in each of the *Tet1/3* DKO blastocysts compared to the average of the CTL blastocysts. Only 23 genes are differentially expressed in all 5 *Tet1/3* DKO blastocysts relative to control blastocysts. (C) Overall increase in the variance of gene expression in *Tet1/3* DKO compared to CTL blastocysts. Each gene is represented by a dot, with *Nanog* indicated in green. Genes shown in red are those with highest (top 1%, n=178) vsd variance in *Tet1/3* DKO blastocysts. (D) Correlation plot of expression variances of all genes from CTL and *Tet1/3* DKO embryos. Most genes fall above the diagonal, indicating greater variability of expression in *Tet1/3* DKO blastocysts compared to controls. (E) The ratios of gene expression variance between CTL and *Tet1/3* DKO blastocysts are plotted against the average expression level for each gene. The increased gene expression variance observed in *Tet1/3* DKO blastocysts is independent of the level of gene expression. (F) *Nanog*, *Cdx2* and *Oct4* mRNA expression in control (CTL) and *Tet1/3* DKO E3.5 blastocysts quantified by RNA-seq. (G) Heatmap showing expression of the top 1% of variable genes in *Tet1/3* DKO blastocysts compared to controls. *Nanog* is indicated by an arrow. (H) *Lifr*, *Gp130* and (I) *Tcl1* expression levels in control (CTL) and *Tet1/3* DKO blastocysts assessed by RNA-seq (**adjusted p-value<0.01).

Figure 5. The variable levels of DNA modification (5mC+5hmC) in *Tet1/3* DKO E3.5 blastocysts parallel the transcriptional variability observed above. (A) Modification levels on each chromosome were plotted for 3 CTL and 5 *Tet1/3* DKO E3.5 embryos based on RRBS analysis. Note that the DKOa blastocyst is the only one with a Y chromosome and that cytosines in control unmodified lambda DNA show more than 98% conversion to T in all embryos. (B) The levels of DNA modification at CpG islands (CGI), transcription factor binding sites (TFBS), enhancers, DNaseI accessible regions, promoter (± 2 kb relative to the TSS), gene bodies (subdivided into 5'UTR, exon, intron, 3'UTR) and intergenic regions. Each circle represents one blastocyst, with the embryo ID shown within each circle: *blue circles*, control blastocysts a-c; *red circles*, *Tet1/3* DKO blastocysts a-e. (C) Modification levels of part of the gene body of *Sfi1* and the entire gene body of *Prdm16* from individual control and *Tet1/3* DKO blastocysts. Each bar represents one CpG, and the full length of each bar represents 100% modification (*red*, 5mC+5hmC; *blue*, C+5fC+5CaC). (D) The correlation plot depicts variances in the DNA modification level of individual CpGs from CTL and *Tet1/3* DKO embryos. Each dot represents one CpG (>8 reads and >10% 5mC+5hmC). The level of DNA modification at each CpG is graded from blue to red. Most dots fall above the diagonal regardless of modification level, indicating that *Tet1/3* DKO blastocysts show greater variability of DNA modification compared to controls.

Figure 6. Variability of DNA modification and expression of repetitive elements in *Tet1/3* DKO early embryos. (A) *Top*, DNA modification (5mC+5hmC) levels associated with LINE, SINE, LTR, IAP, DNA transposon and satellite sequences taken from RRBS data. *Blue circles*, control blastocysts; *red circles*, *Tet1/3* DKO blastocysts. *Bottom*, expression levels of the corresponding elements taken from RNA-seq data using a separate set of blastocysts. *Blue circles*, control blastocysts; *red circles*, *Tet1/3* DKO blastocysts (see **Fig. 3**). (B, C) The variance ratios of DNA modification for individual CpGs within LINEs (B) and LTRs (C) of CTL and *Tet1/3* DKO embryos are plotted against average DNA modification level (*top*) and RRBS coverage (*bottom*). FC, fold change. (D) Expression of LINE, SINE, LTR, IAP, DNA transposon and satellite sequences taken from RNA-seq data on 10 individual CTL and 35 individual *Tet1/3* DKO blastomeres from 8-cell embryos. Note the strongly diminished expression of IAP elements, also observed for E3.5 blastocysts in **Figure 6A**.

Figure 7. The reproducible downregulation of genes in the cholesterol biosynthetic pathway in *Tet1/3* DKO E3.5 embryos compared to controls is associated with a phenotype that resembles that of sonic hedgehog deficiency. Shown is the sequence of enzymes in the cholesterol biosynthesis pathway, indicating the degree of impaired expression based on RNA-seq data. The averaged normalized counts of three control and five *Tet1/3* DKO embryos are plotted. Adjusted p-values are shown at right.

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