



An epigenetic switch repressing *Tet1* in gonadotropes activates the reproductive axis

Yahav Yosefzon^a, Cfir David^a, Anna Tsukerman^a, Lilach Pnueli^a, Sen Qiao^b, Ulrich Boehm^b, and Philippa Melamed^{a,1}

^aFaculty of Biology, Technion – Israel Institute of Technology, Haifa 32000, Israel; and ^bExperimental Pharmacology, Center for Molecular Signaling (PZMS), Saarland University School of Medicine, 66421 Homburg, Germany

Edited by William F. Crowley, Jr, Massachusetts General Hospital, Boston, MA, and accepted by Editorial Board Member David J. Mangelsdorf August 7, 2017 (received for review April 3, 2017)

The TET enzymes catalyze conversion of 5-methyl cytosine (5mC) to 5-hydroxymethyl cytosine (5hmC) and play important roles during development. TET1 has been particularly well-studied in pluripotent stem cells, but *Tet1*-KO mice are viable, and the most marked defect is abnormal ovarian follicle development, resulting in impaired fertility. We hypothesized that TET1 might play a role in the central control of reproduction by regulating expression of the gonadotropin hormones, which are responsible for follicle development and maturation and ovarian function. We find that all three TET enzymes are expressed in gonadotrope-precursor cells, but *Tet1* mRNA levels decrease markedly with completion of cell differentiation, corresponding with an increase in expression of the luteinizing hormone gene, *Lhb*. We demonstrate that poorly differentiated gonadotropes express a TET1 isoform lacking the N-terminal CXXC-domain, which represses *Lhb* gene expression directly and does not catalyze 5hmC at the gene promoter. We show that this isoform is also expressed in other differentiated tissues, and that it is regulated by an alternative promoter whose activity is repressed by the liganded estrogen and androgen receptors, and by the hypothalamic gonadotropin-releasing hormone through activation of PKA. Its expression is also regulated by DNA methylation, including at an upstream enhancer that is protected by TET2, to allow *Tet1* expression. The down-regulation of TET1 relieves its repression of the methylated *Lhb* gene promoter, which is then hydroxymethylated and activated by TET2 for full reproductive competence.

Tet1 | Tet2 | gonadotrope | enhancer | luteinizing hormone

TET enzymes catalyze the conversion of 5-methyl cytosine (5mC) to 5-hydroxymethyl cytosine (5hmC), which blocks some of the 5mC repressive effects while also catalyzing additional modifications of 5hmC to bases that are quickly removed, thus comprising a pathway to active demethylation (1–4). However, the TET proteins are found enriched at CpG-rich gene promoters and 5hmC is readily detected in many cell types, particularly at active or poised regulatory elements, suggesting a facilitating role in transcriptional activation (5–7).

Although the three TET proteins harbor the same catalytic activity, they are expressed in distinct developmental and tissue-specific patterns (8). TET1 is at its highest levels in ES cells (ESCs) and is down-regulated during differentiation. TET2 is also expressed in ESCs, and transcription of both genes is regulated by the pluripotency factors (9, 10). Despite this, both TET1 and TET2 are also expressed through largely unknown mechanisms in differentiated tissues, although mice lacking TET1 or TET2 are viable and, besides from the TET2 effects on hematopoiesis, their definitive roles are mostly not clear (11–13). In fact, the most overt phenotype of TET1-KO mice was a defect in ovarian development: their ovaries were small and had fewer mature follicles and reduced fertility, even though embryonic germ-cell development appeared normal (11, 12), pointing to a unique role for TET1 in the regulation of follicular development.

Ovarian growth and activity are regulated by the gonadotropins luteinizing hormone and follicle-stimulating hormone. These hormones are expressed in the pituitary gonadotropes during embryonic development, and are up-regulated during a neonatal period of

proliferation, but then become quiescent until puberty, when they are reactivated by the hypothalamic gonadotropin-releasing hormone (GnRH). We have shown previously that specific chromatin modifications are involved in determining the expression of these genes in a tissue-specific or hormonally induced context (14–19). However, the *Lhb* gene promoter is particularly rich in CpGs, prompting us to consider that it might be regulated by DNA methylation, and the effect of TET1 KO on ovarian development and function opened the possibility that this might be modified by TET1.

Our study revealed that an N-terminal truncated TET1 is expressed in poorly differentiated proliferating gonadotropes, which appears as the more common isoform in other differentiated tissues; it is regulated by an alternative proximal promoter and repressed by estradiol (E₂) and dihydrotestosterone (DHT), and also by GnRH via activation of PKA. Its expression can also be regulated by methylation, including at an upstream enhancer that is protected by TET2. TET1 down-regulation in the gonadotrope precursor cells relieves repression of the *Lhb* gene promoter, which is then hydroxymethylated by TET2 to allow *Lhb* expression and reproductive competence.

Results

Tet1 Negatively Correlates with Lhb Expression and Is Down-Regulated by GnRH and Gonadal Steroids. We detected all three *Tet* mRNAs in primary murine gonadotrope cells. However, the levels of *Tet1* were considerably higher in cells from immature 6-d-old mice in which the gonadotrope population is expanding than in the gonadotropes of adult (8–14 wk) sexually mature mice, whereas the levels of *Tet2* and *Tet3* mRNAs were similar in both groups (Fig. S1A). Thus, there is a negative correlation of *Tet1* with the mRNA levels of *Lhb*, which is expressed at a higher level in

Significance

We present an epigenetic switch in the central control of reproduction as a truncated TET1, expressed in proliferating gonadotrope-precursor cells, which inhibits *Lhb* expression and so must be repressed for reproductive development. Expression of this TET1 isoform is regulated by *cis*-elements mediating effects of gonadal steroids and PKA, and also a potentially methylated distal enhancer. As this isoform appears more common than the canonical TET1 in other differentiated tissues, our study has broader functional implications outside of the reproductive axis. Furthermore, our findings support the idea that distinct genomic regions are used at different developmental stages or in different tissues, and that a particular sequence can be part of the primary transcript in some tissues or an enhancer RNA in others.

Author contributions: Y.Y. and P.M. designed research; Y.Y., C.D., A.T., L.P., and S.Q. performed research; L.P., U.B., and P.M. contributed new reagents/analytic tools; Y.Y., C.D., A.T., S.Q., U.B., and P.M. analyzed data; and Y.Y., U.B., and P.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. W.F.C. is a guest editor invited by the Editorial Board.

¹To whom correspondence should be addressed. Email: philippa@tx.technion.ac.il.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704393114/-DCSupplemental.

the adult mice (Fig. 1A). In gonadotrope cell lines, *Tet1* levels were much lower in the more fully differentiated L β T2 cells in which *Lhb* is expressed abundantly than in the poorly differentiated α T3-1 cells in which *Lhb* is barely expressed (Fig. 1B) (17), whereas levels of *Tet2* and *Tet3* differed less (Fig. S1B). This negative correlation of *Tet1* expression and gonadotrope maturation was confirmed in pituitary sections from adult mice, in which TET1 was notably lacking in the mature gonadotropes (Fig. 1C). Given that TET1 expression in the immature pituitary appears restricted to CGA-positive cells (Fig. S1C), and is found in the adult pituitary but not in the gonadotropes, these cells are very likely thyrotropes.

Given that the levels of *Tet1* gene expression shifted in accordance with a change in *Lhb* expression, we considered that regulatory hormones along the reproductive axis might play a role in determining its levels. Indeed, GnRH, the major activator of *Lhb* gene expression, reduced *Tet1* mRNA levels in both cell lines and primary cells. As we were able to mimic this effect by incubation of the cells with forskolin but not phorbol 12-myristate 13-acetate (PMA), we considered that the GnRH effect is via activation of PKA, which was confirmed by pretreatment with

H89 or transfection of a dominant-negative PKA, both of which completely abolished the GnRH repressive effect, and there was even a slight increase in *Tet1* expression (Fig. 1D).

We also exposed the gonadotrope cell lines and primary cells from immature mice of both sexes to E₂ or DHT (10 nM, 48 h). Both steroids repressed *Tet1* expression, although E₂ appeared more potent (Fig. 1E and F). To further understand the relationship between TET1 and the reproductive axis, we ovariectomized (OVX) or castrated adult mice to remove the gonadal steroid feedback to assess the effect on *Tet1* expression. As in the neonatal mice, the gonadotropes are proliferating in this state and include immature precursor cells (20). *Tet1* mRNA levels were significantly increased in these cells, especially in the males, although those of *Tet2* and *Tet3* were not affected (Fig. 1G and H and Fig. S1D and E). In males and females, average levels of *Lhb* were reduced in this precursor cell population compared with the fully differentiated population in intact mice (Fig. 1G and H), whereas the levels of *Fshb* were unaltered (Fig. S1D and E). In cells from the OVX mice, E₂ reduced the elevated *Tet1* expression, whereas no effect was apparent in cells from intact adult females (Fig. S1F).

The Gonadotrope Precursor TET1 Isoform is N-Terminally Truncated, Found in Other Differentiated Tissues, and Uses an Alternate Promoter That Binds the Liganded ESR1 and AR.

In PCR analyses of the *Tet1* mRNA in both cell lines and primary gonadotropes (from immature mice), we could barely detect the first coding exon 1 of the canonical *Tet1*, but an alternative exon, which we termed exon 1.5, was amplified (Fig. 2A and B). Analysis revealed this same pattern of exon expression in olfactory bulb and mammary glands, whereas, in the cerebellum, cerebral cortex, liver, and heart, both exons were detected but at differing ratios, and in the placenta, as in ESCs, exon 1 was expressed at a much higher level (Fig. S2A and B). RACE confirmed that the functional *Tet1* transcriptional start site (TSS) in the gonadotropes is indeed located 181 bp upstream of this exon 1.5 (Fig. S2C and D).

ChIP analysis for H3K4me3 and H3K27ac was carried out to determine whether this region of the *Tet1* gene carries histone marks characteristic of active promoters. Both modifications are clearly enriched at this region, and at much higher levels in α T3-1 than in L β T2 cells, but were not seen in either cell line around the canonical first exon (Fig. 2C and D). Cap analysis of gene expression (CAGE) data (FANTOM5) also indicates that this is the TSS for *Tet1* in other tissues, whereas the Encyclopedia of DNA Elements (ENCODE) data shows dual peaks of H3K4me3 and H3K27ac at this location, as well as H3K4me3 at a region upstream of the canonical exon 1 in all tissues except the ESCs and placenta, in which only the upstream peak is apparent (Fig. S3). This clearly indicates that TET1 can be expressed as a short or long isoform in distinct tissues, but, in many, including the immature gonadotropes, the dominant form is the short TET1 that lacks the CXXC domain.

The functional promoter of this TSS was demonstrated finally by ChIP, in which RNAPII S5p was seen to bind (Fig. 2E), as did the liganded ESR1 and AR; ESR1 was enriched at -550 bp upstream and also around -1.4 kbp, whereas AR appeared enriched throughout this region (Fig. 2F and G).

The Region Upstream of Tet1 TSS Can Be Methylated at Three Distinct CpGIs, but the Distal Part of the Most 5' CpGI Is Protected by TET2.

We postulated that the use of a different promoter by *Tet1* in the gonadotrope precursor cells might be the result of DNA methylation. Three CpG islands (CpGIs) identified by Methyl Primer Express Software (Applied Biosystems) using default parameters were found: CpGI 1 at a distal site 22.4 kbp upstream of the functional *Tet1* TSS, CpGI 2 just upstream of the canonical exon 1, and CpGI 3 immediately upstream of the functional TSS (Fig. 3A). Methylation at CpGI 1 and 3 was much higher in L β T2 than in α T3-1 cells, whereas CpGI 2 was highly methylated to a similar level in both cell types (Fig. 3B), indicating that *Tet1* expression might well be coupled to methylation of each of these regions.

TET proteins have been reported to protect CpGIs from DNA methylation (21), and stable TET2 knockdown (KD) reduced *Tet1*

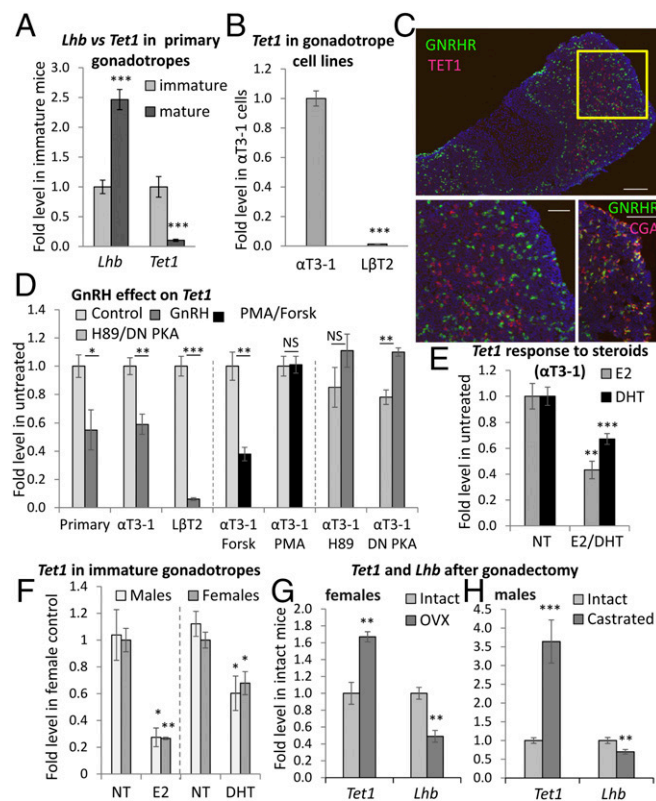


Fig. 1. *Tet1* negatively correlates with *Lhb* expression and is down-regulated by GnRH and gonadal steroids. (A and B) qPCR analyses in (A) primary gonadotropes from immature (6 d) or mature (8–14 wk) mice or (B) α T3-1 and L β T2 cells; mRNA levels are relative to immature mice or α T3-1 cells ($n = 3–9$). A t test was used to compare levels of the same gene between cells ($***P < 0.001$). (C) TET1 (red) in the adult pituitary of GRIC-GFP mice; GnRH⁺ cells appear green. (Scale bar: 100 μ m.) (Bottom Left) Enlargement of boxed region; (Bottom Right) CGA (red) in the same pituitary. (Scale bars: 50 μ m.) (D) *Tet1* levels in cell lines and primary cells from immature mice after exposure to GnRH in culture; α T3-1 cells were also exposed to forskolin or PMA; alternatively treated with H89 or transfected with dominant-negative (DN) PKA with and without GnRH exposure ($n = 2–6$; $*P < 0.05$ and $***P < 0.001$; NS indicates $P > 0.05$; all means shown relative to untreated controls). (E) α T3-1 cells or (F) primary gonadotropes from immature mice were exposed to E₂ or DHT; *Tet1* mRNA levels are presented as in Fig. 1D ($n = 4–6$). (G and H) *Tet1* and *Lhb* mRNA levels in gonadotropes 10 d after (G) ovariectomy ($n = 4$) or (H) castration ($n = 8$) shown relative to age-matched intact controls (Fig. S1).

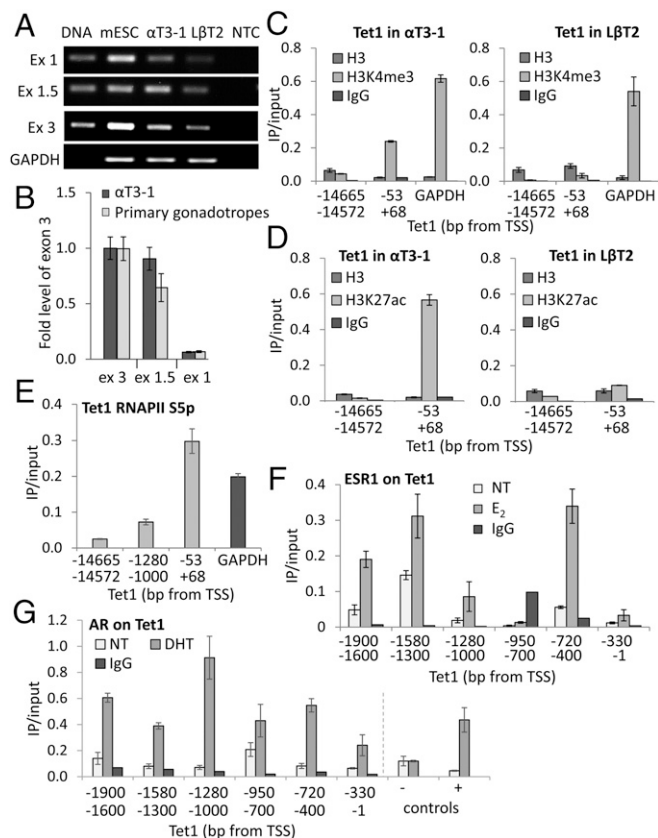


Fig. 2. The gonadotrope precursor TET1 isoform is N-terminally truncated, found in other differentiated tissues, and uses an alternate promoter that binds liganded ESR1 and AR. (A) RT-PCR analyses for the *Tet1* canonical exon 1, the alternative exon "1.5," and the canonical exon 3 in mouse ESCs, α T3-1 and β T2 cell lines, and gDNA, with *Gapdh* control. (B) qPCR of α T3-1 cells and primary gonadotropes from immature mice using the same primer sets and quantified by using a standard curve of gDNA, shown relative to levels of exon 3 ($n = 5$). (C–G) ChIP analysis for (C) H3K4me3 and (D) H3K27ac in α T3-1 (*Left*) and β T2 (*Right*) cells for the region upstream of the canonical exon 1 (–14,665 to –14,572 bp) and exon 1.5 (–53 to +68) or in (E) RNAPII S5p, (F) ESR1, and (G) AR in α T3-1 cells (exposed for 2 h to E_2 or DHT as noted). IP levels are relative to input ($n = 3$; Figs. S2 and S3).

expression by ~80% (Fig. 3C), suggesting its possible role in regulating *Tet1*. ChIP revealed that, in the immature α T3-1 cells, TET2 is indeed enriched at the two regions, corresponding to CpGIs 1 and 3 (Fig. 3D). Furthermore, methylated DNA immunoprecipitation (MeDIP) analysis showed that the *Tet1*CpGI 1 is nearly fourfold more methylated in *Tet2* KD than in control cells, although CpGI 3 was unaffected (Fig. 3E), clearly suggesting that TET2 plays a pivotal role at this distal CpGI 1.

To confirm these findings and clarify which regions of CpGIs 1 and 3 are methylated, we analyzed the core of CpGI 1 (–22 to –21.3 kbp) and CpGI 3 (–296 to –42 bp) in bisulfite (BS)-converted DNA from control α T3-1 and β T2 cells, and also at the core of CpGI 1 in the DNA from TET2-KD cells. The methylation was much higher in β T2 cells than in the control α T3-1 cells at both CpGIs (Fig. 3F). In the TET2 KD cells, there was a clear increase in the methylation at CpGI 1 compared with the control cells, but only in the most distal part ($P < 0.001$, Fisher's exact test; Fig. 3F), suggesting that TET2 inhibits DNA methylation at this region, whereas the methylation at the proximal part of this CpGI, as well as the promoter (CpGI 3), is regulated via other mechanisms.

The Upstream CpGI Likely Comprises a Transcriptional Enhancer. These findings in which methylation of CpGI 1 was associated with very low levels of *Tet1* indicate that this distal region plays a role in

regulating *Tet1* expression, possibly acting as a transcriptional enhancer. We therefore performed ChIP analysis for H3K4me1, H3K4me3, and H3 on a single batch of α T3-1 cells. The distribution of the two H3K4 modifications clearly differed, with the core of the CpGI being enriched for H3K4me3 but not for H3K4me1 (Fig. 4A), whereas the region 5' to the CpGI had higher levels of H3K4me1 and much lower levels of H3K4me3 (~40-fold decrease). Thus, a distinct region at the 5' end of the CpGI contains a particularly high ratio of H3K4me1/H3K4me3, indicating that its likely function as an enhancer (22). However, this region was not enriched for H3K27ac (Fig. 4B), similar to other tissues expressing primarily the short *Tet1* isoform (Fig. S3B).

Active enhancers often produce noncoding RNAs [i.e., enhancer RNAs (eRNAs)], which may play crucial roles in their activity (e.g., refs. 18, 23). The region just upstream of the 5' end of the CpGI 1 was transcribed at a much higher level than the surrounding region (Fig. 4C and Fig. S4). Moreover, the level of this eRNA was higher in α T3-1 than in β T2 cells, consistent with the levels of the *Tet1* transcript (Fig. 4D), whereas, in TET2 KD cells, the eRNA levels, like those of the *Tet1* mRNA, were reduced (Fig. 4E). In both cases, the lower *Tet1* mRNA and eRNA levels corresponded with elevated levels of 5mC at the distal CpGI (Figs. 1B and 3B), suggesting that DNA methylation is involved in regulating

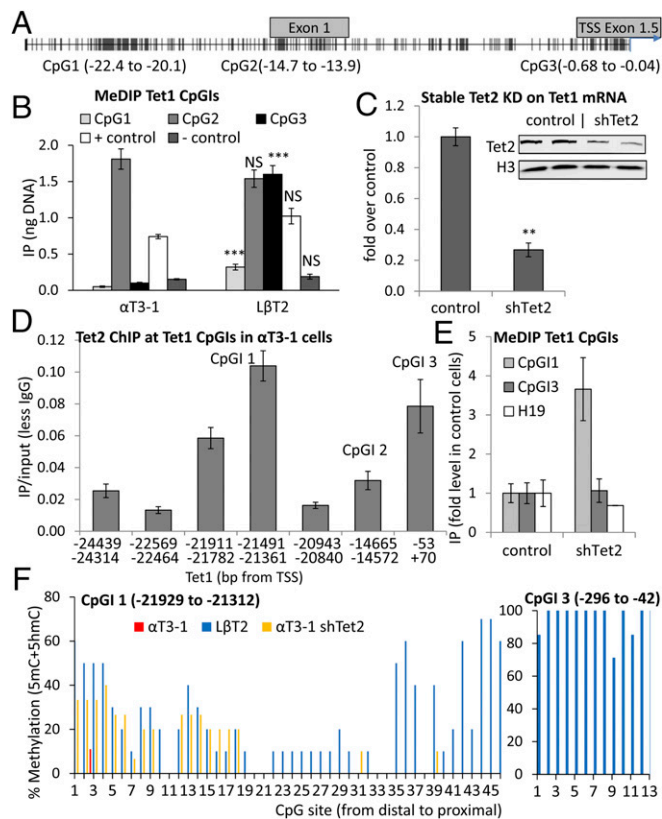


Fig. 3. The region upstream of this *Tet1* TSS can be methylated at three distinct CpGIs, but the distal part of the most 5' CpGI is protected by TET2. (A) CpGIs upstream of *Tet1* relative to the functional TSS. (B) Levels of 5mC DNA (relative to gDNA standard curve) at these CpGIs ($n = 4–5$). Statistical analysis (as in Fig. 1) compared each region between cell lines. (C) *Tet1* mRNA levels after TET2 KD (shTet2) in α T3-1 cells; data analyzed and presented as before ($n = 3–4$). Western blot analysis shows the TET2 KD ($n = 2$). (D) ChIP of TET2 at the CpGIs in α T3-1 cells, analyzed and presented as previously ($n = 4–7$). (E) MeDIP for the core of CpGI 1 (–21,491 to –21,361 bp) and CpGI 3 (–2 to –191 bp) from WT α T3-1 or shTet2 cells shown as fold in control cells, with *H19* as unaffected positive control ($n = 4–5$). (F) BS analysis of *Tet1*CpGIs 1 and 3 in the two cell lines and for CpGI 1 in shTet2 cells; percentage of each CpG was methylated in 6–15 independent clones from each cell line.

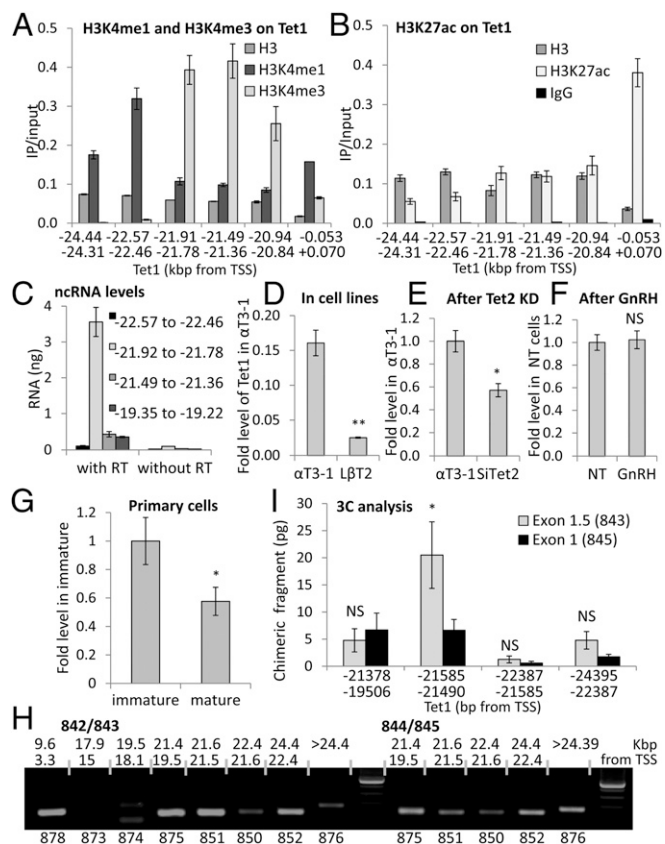


Fig. 4. The upstream CpGI likely comprises a transcriptional enhancer. (A and B) ChIP analysis of CpGI 1 for (A) H3K4me1, H3K4me3, and H3 or (B) H3K27ac, H3, and IgG in α T3-1 cells, as in Fig. 2 ($n = 2-4$; Fig. S3). (C) Total α T3-1 RNA was reverse transcribed (RT) for qPCR of the regions marked. Controls lacked reverse transcription (normalized to gDNA standards; $n = 3$; Fig. S4). (D-G) Levels of eRNA were measured similarly in (D) α T3-1 and $L\beta$ T2 cells; (E) WT and shTet2 α T3-1 cells; (F) $L\beta$ T2 cells with or without GnRH treatment; and (G) primary gonadotrope cells ($n = 3-6$). (H and I) A 3C assay was carried out in *Dpn2*-digested DNA from α T3-1 cells, and chimeric fragments were detected by using nested forward primers (nos. 842 and 843 or nos. 844 and 845) targeting the functional TSS or upstream of the canonical exon 1, with various primers targeting the upstream region, as detailed in Fig. S4. (H) Amplicons were resolved by electrophoresis and identity confirmed by sequencing or (I) measured by qPCR using standard curves of the cloned chimeric fragments ($n = 4-10$); a *t* test was used to compare interaction of the pairs of regions.

expression of the eRNA. Despite the dramatic decrease in *Tet1* expression following GnRH exposure in $L\beta$ T2 cells (Fig. 1D), the eRNA levels did not change (Fig. 4F). Also in primary cells, eRNA levels were lower in gonadotropes of mature than immature mice (Fig. 4G), in accordance with the levels of the *Tet1* transcript (Fig. 1A). These findings confirm that this region is likely an enhancer in which transcription of the eRNA is not determined directly by that of *Tet1*, but it may form part of a basal regulatory mechanism.

Finally, we performed chromatin conformation capture (3C) to assess whether this region interacts with the functional TSS. PCR was carried out on a *Dpn2*-digested and ligated 3C library using nested primers for semiquantitative analysis or individual primer sets for quantitative PCR (qPCR; Fig. S4). One set of primers targeted the functional TSS region (842 and 843; -221 bp and -191 bp) and the other targeted just upstream of exon 1 (844 and 845; $-14,991$ and $-15,011$ bp from the TSS). These were used with various upstream primers. Both methods showed interaction of the distal CpGI 1 region with the functional TSS, far more than with the region upstream of exon 1. This difference was further quantified by qPCR, normalized to the same cloned chimeric fragment, thus taking into account differences in the primer efficiency (Fig. 4H and I).

The Truncated TET1 Represses *Lhb* Gene Expression Regardless of DNA Methylation and Does Not Catalyze 5hmC. To determine whether this truncated TET1 isoform regulates *Lhb* gene expression, we first performed stable TET1 KD, which led to an increase in *Lhb* mRNA levels (Fig. 5A). Conversely, over-expression of the full-length or truncated isoform repressed *Lhb* similarly. This contrasted with the effect on the *Pgr* gene, whose expression was significantly, albeit marginally, increased by the truncated but not the full-length isoform (Fig. 5B), in accordance with reports that the catalytic domain (in both isoforms) but not the full-length TET1 decreases *Pgr* methylation (24). Binding of the TET1 CXXC domain was reported to be affected by DNA methylation (24), so we also treated the cells with 5-Aza-dC before over-expressing each isoform. The effect of the full-length TET1 on *Lhb* was lost after Aza treatment, whereas the truncated isoform was still inhibitory (Fig. 5B), indicating distinct mechanisms of recruitment.

We went on to evaluate by ChIP whether this repressive effect on *Lhb* is direct, and saw that the endogenous TET1 is associated with the *Lhb* promoter (Fig. 5C). Moreover, ChIP for H3K27 di- and trimethylation revealed that both modifications are enriched

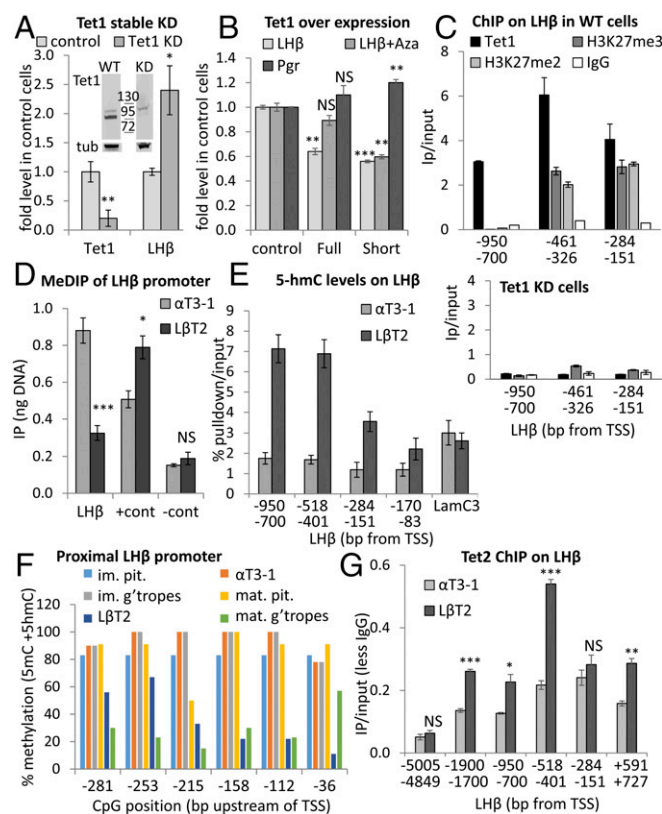


Fig. 5. The truncated TET1 represses *Lhb* gene expression, regardless of DNA methylation, and does not catalyze 5hmC. (A) *Tet1* and *Lhb* mRNA in α T3-1 cells after TET1 KD; data were analyzed and presented as before ($n = 3-4$). Western blot shows TET1 KD (lanes are from one blot, localized exactly as shown). (B) The canonical (full) or the truncated *Tet1* isoform (short) were over-expressed in α T3-1 cells, some of which were treated with Aza for 48 h, and levels of *Lhb* or *Pgr* mRNA were measured, and are shown relative to control cells and analyzed as before ($n = 2-3$). (C) ChIP for TET1, H3K27me2, and me3 at the *Lhb* promoter in WT α T3-1 cells (Top) or for TET1 and H3K27me3 in TET1 KD cells (Bottom) as in Fig. 2. (D) MeDIP analysis at the *Lhb* gene promoter, calculated and presented as in Fig. 3B ($n = 6$). (E) 5hmC DNA at the *Lhb* promoter, with *LamC3* as positive control, shown relative to input ($n = 4-6$). (F) BS analysis of the *Lhb* promoter in gonadotropes from immature and mature mice, nongonadotrope pituitary cells (pit), and the gonadotrope cell lines. Data presented as in Fig. 3F; $n = 6-20$ clones from individual or pooled mice pituitaries and $n = 9-10$ clones for each cell line. (G) ChIP for TET2 upstream of *Lhb*, presented as in Fig. 3C ($n = 3$). Statistical analysis compared levels at each region between the cell lines.

in the proximal region of the promoter, but not in the upstream region; this was absent in the TET1 KD cells (we tested only K27me3; Fig. 5C). Thus, the endogenous truncated TET1 appears to repress expression of the *Lhb* gene directly, possibly involving recruitment of histone H3K27 methyltransferases.

We next determined the methylation status of the *Lhb* promoter and saw a much higher level of 5mC in α T3-1 cells than in the L β T2 cells (Fig. 5D). We also analyzed levels of 5hmC, which, conversely, were higher in L β T2 cells than in α T3-1 cells, especially at the more distal region (Fig. 5E). Thus, the binding of TET1 in immature gonadotrope precursor cells concurs with a region that is 5mC- but not 5hmC-methylated, whereas the 5hmC in mature cells is clearly not the result of TET1. The methylation status of the proximal region was further confirmed in primary cells from immature and mature mice by BS analysis. Despite the inherently heterogeneous gonadotrope populations, the *Lhb* proximal promoter was clearly the least methylated in the differentiated gonadotrope cells of the mature mice, as in the L β T2 cells, whereas the nongonadotrope pituitary cells, immature primary gonadotropes, and cell line were all methylated to a similarly high level (Fig. 5F).

Given that the truncated TET1 was clearly not responsible for 5hmC upstream of the *Lhb* gene promoter in the L β T2 cells, we next examined whether TET2 might be responsible for this modification. ChIP revealed that TET2 is indeed bound at significantly higher levels in the L β T2 cells than the α T3-1 cells, over the entire upstream region and particularly upstream of -401 bp from the TSS (Fig. 5G), correlating with the most enriched region of the 5hmC. Thus, in cells in which the *Lhb* gene is expressed, its promoter is 5hmC-modified, apparently catalyzed by TET2, whereas, in partially differentiated cells, TET1 has a predominant and repressive role on this gene to which it recruits other repressors, and does not catalyze 5hmC (Fig. S5). Expression of *Lhb* is thus dependent on down-regulation of *Tet1* in these cells.

Discussion

We have shown here that a truncated TET 1 isoform directly represses expression of the *Lhb* gene, necessitating its down-regulation for final gonadotrope differentiation, and have also revealed the means of its regulation. Inhibition of this TET1 isoform by GnRH during the prepubertal period facilitates *Lhb* gene expression, but might also comprise an additional mechanism to curb proliferation of the gonadotrope precursor cell population (25). Certainly, the elevated *Tet1* expression in proliferating immature gonadotropes suggests that it plays a role in establishing this population of cells during development, which would at least partially explain the effect of its KO on fertility (11, 12). Subsequent to its repression by increasing GnRH levels, there is further transcriptional inhibition of this *Tet1* isoform by the gonadal steroids, in keeping with previous reports on the repressive effects of a synthetic estrogen on *Tet1* in the uterus (26); both would affect *Tet1* in gonadotropes but not thyrotropes. Notably, the *Lhb* gene appeared more sensitive to the increase in TET1 in females than in males, possibly relating to the differing phenotypes in the *Tet1* KO mice, although this is likely also the result of the complex hormonal interplay in the gonadotrope during the estrous cycle. Our findings thus not only extend a recent report that this isoform is expressed in various adult somatic tissues (27), but also place it in a physiological context, describing its role and regulation in the gonadotrope, through an alternative promoter as well as a distal enhancer.

The TET1-mediated repression of *Lhb* is direct, with TET1 binding the CpG-rich region on the more distal part of the promoter, to which it appears to recruit histone-modifying enzymes responsible for repressive modifications. Such effects have been reported in ESCs, and TET1-bound promoters were seen to be occupied by the polycomb repression complex 2 (PRC2), leading to the suggestion that TET1 facilitates PRC2 binding, associates with the Sin3A complex, and/or may help recruit the MBD3/NuRD complex (21, 28, 29). Although this seems a likely mechanism of TET1-mediated repression of the *Lhb* gene, the lack of CXXC domain in this isoform suggests some differences in binding of the TET1 to the DNA, and possibly also its function. Indeed, the

region of the *Lhb* gene promoter bound by the truncated TET1 is clearly 5mC- but not 5hmC-methylated, and hypomethylation by Aza had no effect on its repressive activity. This contrasted with the full-length TET1, whose repressive activity on *Lhb* was lost in the hypomethylated cells, presumably because of competition from the increased number of unmethylated regions (24).

Apart from this difference in sensitivity to the methylation status of the DNA, the truncated TET1 appears to be catalytically inactive, certainly in the current context of the *Lhb* gene promoter, but also apparently in other differentiated cells in which this truncated isoform is the dominant form, according to our PCR analysis and also ENCODE and CAGE data for histone modifications and TSSs in a large number of tissues. Unlike in ESCs, in which TET1, PRC2, and H3K27me3 colocalize with 5hmC, in these differentiated tissues, colocalization with 5hmC is not apparent (30). This was clarified in a recent study (27) reporting that, aside from the CXXC domain, the N terminus of the full-length TET1 also contains a domain that promotes the demethylation activity, which is thus reduced in the short isoform. Therefore, the truncated isoform clearly harbors characteristics distinct from those of the canonical full-length isoform, not only in the way it is recruited, but also in its function. In fact, the *Lhb* gene is 5hmC-methylated only when *Tet1* expression is reduced, and TET2 then binds the same region to catalyze the modification, facilitating *Lhb* expression.

Although we have shown that this truncated *Tet1* isoform is regulated through an alternative proximal promoter regulated by gonadal steroids and PKA, we also describe an upstream enhancer. Methylation of this enhancer plays a crucial role in determining *Tet1* expression, but it can be protected by TET2. This distal region is enriched at its 5' end with a high ratio of HK4me1 to HK4me3, whereas the CpGI core is enriched with HK4me3, characteristic of unmethylated CpGIs (31). Moreover, the region adjacent to that enriched with H3K4me1 is transcribed, and the transcript levels were found to vary in accordance with basal, but not GnRH-regulated, expression of *Tet1* mRNA. The 3C assay showed that this region interacts physically with the functional *Tet1* TSS, all of which suggest that this distal region acts as a transcriptional enhancer for the truncated *Tet1* isoform.

Another group recently reported that, in stem cells, this distal CpGI comprises one of two alternative TSSs for a longer *Tet1* transcript that includes exon 1, and these are used differently at distinct stages of development (32). However, we were unable to amplify any fragment by using various forward primers targeting the CpGI 1 and reverse primers targeting exon 1 [termed exon 2 in their paper (32)], confirming that this gene is indeed regulated very differently through cell differentiation and development. In support of these distinct regulatory mechanisms, the aforementioned study (32) showed enrichment of H3K27 acetylation at this upstream region (i.e., 1a and vicinity), which was completely lacking in the gonadotropes, and does not appear in other differentiated tissues, in accordance with the lack of exon 1 expression. This distinct regulation of *Tet1* in stem cells is perhaps not surprising given that its expression is activated by pluripotent factors in these cells (9, 10), and the absence of such factors in differentiated cells would necessitate alternative regulatory mechanisms and the use of additional *cis*-elements.

The present study supports the idea that distinct genomic regions can be used differently at various stages of development or in different tissues (23) and extends the possibilities of a particular sequence being transcribed as part of the primary transcript in some tissues, or as an eRNA from a functional enhancer in others. We have shown that this truncated *Tet1* isoform, which is more common in differentiated tissues than the canonical ESC form, is regulated by methylation at two genomic regions, as well as a common kinase signaling pathway and gonadal steroids, opening the way for studies of its regulation in diverse contexts. These findings therefore have broad implications in understanding the regulation of *Tet1* expression in other tissues, especially hormonally regulated cancers. Regardless of its precise role in proliferating gonadotrope precursor cells, we have shown that down-regulation of this *Tet1* isoform is essential for *Lhb* expression and

therefore also in gonadotrope cells completing differentiation and subsequently acquiring reproductive competence.

Materials and Methods

Mice, Cells, and Culture. α T3-1 and L β T2 murine gonadotrope-derived cells were cultured and treated (17, 18) as detailed in *SI Materials and Methods*. Pituitaries were extracted from GRIC-GFP or GRIC-tdTomato mice; gonadotropes were enriched by FACS (as in refs. 33,34), and some were cultured for 24 h before treatment (as in ref. 25 and *SI Materials and Methods*). Ovariectomy and orchietomy (www.iacuc.ucsf.edu/Policies/Gonadectomy.doc) and other animal experiments were performed after protocol approved by the Institutional Animal Care and Use Committee of Technion – Israel Institute of Technology and the State of Saarland review board, and according to institutional animal care and use committee guidelines.

RNA Extraction, Real-Time PCR, and 5'RACE. RNA was isolated with TRIzol, DNase I digested, and cDNA synthesized (High Capacity cDNA reverse transcription kit; Applied Biosystems). qPCR was performed with Perfecta Taq SYBR Green FastMix (Quanta) with primers listed in Table S1. Calculation of amplicon levels and their analysis are detailed in *SI Materials and Methods*. RACE reactions were as previously described (18).

Methylation Analysis. Sonicated denatured genomic DNA was precipitated with mouse anti-5mC (Diagenode) and sheep anti-mouse IgG Dynabeads (Invitrogen). After washing and proteinase K digestion, the DNA was extracted and precipitated before resuspension in water for qPCR. BS analysis used the EZ-DNA Methylation-Direct Kit (Zymo) and Red Load Taq Master (Larova). After purification (PCR purification kit; Qiagen) and cloning into pGEM-T-Easy (Promega), inserts from at least six random clones were sequenced and analyzed by Quantification Tool for Methylation Analysis software (quma.cdb.riken.jp/). Hydroxymethyl Collector Kit (Active Motif) was used to label the 5mC, and the eluted DNA was analyzed by qPCR as detailed earlier (primers in Table S1).

ChIP. ChIP was carried out after cross-linking (as in ref. 18) with the following antibodies: TET1 and ESR1 (ab-191698 and ab-32063; Abcam); TET2 and AR (sc-136926 and sc-816 X; Santa Cruz Biotechnology); histones and RNAPII as in ref. 18; or IgG as control. The DNA was purified and regions amplified by qPCR from input and immunoprecipitated (IP) samples as detailed earlier (primers in Table S1).

Protein Extraction. Western analysis was carried out as described previously (35) with TET1 (09–872; Millipore), TET2 (ab94580; Abcam), H3 (ab1791; Abcam), and secondary (sc-2004; Santa Cruz Biotechnology) antibodies.

Chromatin Conformation Capture Assay. Using a *Dpn2*-digested library of ligated fragments from α T3-1 cells (as in ref. 18), PCR used nested primers at the exon 1.5 or exon 1 TSS and various upstream primers, as in Fig. S3 and Table S1. Amplicons were resolved by electrophoresis and sequenced or measured by qPCR relative to standards comprising the same cloned chimeric fragment.

Microscopy. Formaldehyde-fixed frozen pituitaries were sectioned at 14 μ m and processed (as in ref. 32) before incubation with primary antibody [TET1 (ab191698; Abcam) at 1:500 or CGA (NIDDK) at 1:1,500] overnight, and then secondary antibody (1:500; 706-166-148; Jackson Labs) for 2 h before application of Hoechst (1:10,000) for 7 min, rinsing, and covering.

Statistical Analysis. Data are from at least three independent experiments, combined or shown as a representative, as mean \pm SEM. The *n* values represent biological repeats transfected or treated and measured independently. Statistical analysis was performed by Student's *t* test (two-tailed), with significant differences at $P \leq 0.05$, or Fisher's exact test for the BS analysis.

ACKNOWLEDGMENTS. We thank Pamela Mellon for the gonadotrope cell lines and Stan McKnight for the dominant-negative PKA. This research was supported by German Research Foundation (Deutsche Forschungsgemeinschaft) Grants BO 1743/7-1 (to U.B. and P.M.) and SFB894 (to U.B.), and Israel Science Foundation Grant 840/12 (to P.M.).

- Zhang H, et al. (2010) TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. *Cell Res* 20:1390–1393.
- Hashimoto H, et al. (2012) Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. *Nucleic Acids Res* 40:4841–4849.
- Ito S, et al. (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333:1300–1303.
- He Y-F, et al. (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 333:1303–1307.
- Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324:929–930.
- Song C-X, et al. (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nat Biotechnol* 29:68–72.
- Xu Y, et al. (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. *Mol Cell* 42:451–464.
- Tan L, Shi YG (2012) Tet family proteins and 5-hydroxymethylcytosine in development and disease. *Development* 139:1895–1902.
- Koh KP, et al. (2011) Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell* 8:200–213.
- Neri F, et al. (2015) TET1 is controlled by pluripotency-associated factors in ESCs and downmodulated by PRC2 in differentiated cells and tissues. *Nucleic Acids Res* 43:6814–6826.
- Dawlaty MM, et al. (2011) Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. *Cell Stem Cell* 9:166–175.
- Dawlaty MM, et al. (2013) Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. *Dev Cell* 24:310–323.
- Moran-Crusio K, et al. (2011) Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell* 20:11–24.
- Melamed P, Kadir MN, Wijeweera A, Seah S (2006) Transcription of gonadotropin beta subunit genes involves cross-talk between the transcription factors and co-regulators that mediate actions of the regulatory hormones. *Mol Cell Endocrinol* 252:167–183.
- Lim S, et al. (2007) Distinct mechanisms involving diverse histone deacetylases repress expression of the two gonadotropin beta-subunit genes in immature gonadotropes, and their actions are overcome by gonadotropin-releasing hormone. *Mol Cell Biol* 27:4105–4120.
- Melamed P (2008) Histone deacetylases and repression of the gonadotropin genes. *Trends Endocrinol Metab* 19:25–31.
- Wijeweera A, et al. (2015) Gonadotropin gene transcription is activated by menin-mediated effects on the chromatin. *Biochim Biophys Acta* 1849:328–341.
- Pnueli L, Rudnizky S, Yosefzon Y, Melamed P (2015) RNA transcribed from a distal enhancer is required for activating the chromatin at the promoter of the gonadotropin α -subunit gene. *Proc Natl Acad Sci USA* 112:4369–4374.
- Rudnizky S, et al. (2016) H2A.Z controls the stability and mobility of nucleosomes to regulate expression of the LH genes. *Nat Commun* 7:12958.
- Smith PF, Keefer DA (1982) Immunocytochemical and ultrastructural identification of mitotic cells in the pituitary gland of ovariectomized rats. *J Reprod Fertil* 66:383–388.
- Williams K, et al. (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 473:343–348.
- Heintzman ND, et al. (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet* 39:311–318.
- Melamed P, Yosefzon Y, Rudnizky S, Pnueli L (2016) Transcriptional enhancers: Transcription, function and flexibility. *Transcription* 7:26–31.
- Jin C, et al. (2014) TET1 is a maintenance DNA demethylase that prevents methylation spreading in differentiated cells. *Nucleic Acids Res* 42:6956–6971.
- Savulescu D, et al. (2013) Gonadotropin-releasing hormone-regulated prohibitin mediates apoptosis of the gonadotrope cells. *Mol Endocrinol* 27:1856–1870.
- Jefferson WN, et al. (2013) Persistently altered epigenetic marks in the mouse uterus after neonatal estrogen exposure. *Mol Endocrinol* 27:1666–1677.
- Zhang W, et al. (2016) Isoform switch of TET1 regulates DNA demethylation and mouse development. *Mol Cell* 64:1062–1073.
- Wu H, et al. (2011) Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. *Nature* 473:389–393.
- Yildirim O, et al. (2011) Mbd3/NURD complex regulates expression of 5-hydroxymethylcytosine marked genes in embryonic stem cells. *Cell* 147:1498–1510.
- Neri F, et al. (2013) Genome-wide analysis identifies a functional association of Tet1 and Polycomb repressive complex 2 in mouse embryonic stem cells. *Genome Biol* 14:R91.
- Ooi SKT, et al. (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* 448:714–717.
- Sohni A, et al. (2015) Dynamic switching of active promoter and enhancer domains regulates Tet1 and Tet2 expression during cell state transitions between pluripotency and differentiation. *Mol Cell Biol* 35:1026–1042.
- Wen S, et al. (2008) Functional characterization of genetically labeled gonadotropes. *Endocrinology* 149:2701–2711.
- Hoivik EA, et al. (2011) DNA methylation of intronic enhancers directs tissue-specific expression of steroidogenic factor 1/adrenal 4 binding protein (SF-1/Ad4BP). *Endocrinology* 152:2100–2112.
- Feng J, Lawson MA, Melamed P (2008) A proteomic comparison of immature and mature mouse gonadotrophs reveals novel differentially expressed nuclear proteins that regulate gonadotropin gene transcription and RNA splicing. *Biol Reprod* 79:546–561.