

1 **SETDB1 prevents TET2-dependent activation of IAP retroelements in naïve**
2 **embryonic stem cells**

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20 methylation

21 **Background:** Endogenous retroviruses (ERVs), which are responsible for 10% of
22 spontaneous mouse mutations, are kept under control via several epigenetic
23 mechanisms. The H3K9 histone methyltransferase SETDB1 is essential for ERV
24 repression in embryonic stem cells (ESCs), with DNA methylation also playing an
25 important role. It has been suggested that SETDB1 protects ERVs from TET-
26 dependent DNA demethylation, but the relevance of this mechanism for ERV
27 expression remains unclear. Moreover, previous studies have been performed in
28 primed ESCs, which are not epigenetically or transcriptionally representative of
29 preimplantation embryos.

30 **Results:** We used naïve ESCs to investigate the role of SETDB1 in ERV regulation
31 and, in particular, its relationship with TET-mediated DNA demethylation. Naïve
32 ESCs show an increased dependency on SETDB1 for ERV silencing when
33 compared to primed ESCs, including at the highly mutagenic intracisternal A particles
34 (IAPs). We found that, in the absence of SETDB1, TET2 activates IAP elements in a
35 catalytic-dependent manner. Surprisingly, however, TET2 does not drive changes in
36 DNA methylation levels at IAPs, suggesting that it regulates these transposons
37 indirectly. Instead, SETDB1 depletion leads to a TET2-dependent loss of H4R3me2s,
38 which is indispensable for IAP silencing during epigenetic reprogramming.

39 **Conclusions:** Our results demonstrate a novel and unexpected role for SETDB1 in
40 protecting IAPs from TET2-dependent histone arginine demethylation.

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45 **Background**

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47 Endogenous retroviruses (ERVs) are retroelements bearing long terminal repeats
48 (LTRs) and constitute approximately 10% of the mouse genome [1]. Whilst most
49 ERVs are inactive, a subset of these genetic parasites retain their transposition ability
50 and therefore pose a threat to genome integrity [2]. Indeed, around 10% of mouse
51 spontaneous mutations arise as a direct result of ERV insertions [3] and insertional
52 mutagenesis by ERVs is frequently associated with murine cancers [4,5]. Therefore,
53 numerous transcriptional and post-transcriptional host mechanisms have evolved to
54 suppress ERV activity. DNA methylation (or 5-methylcytosine; 5mC) plays an essential
55 role in ERV repression in postimplantation embryos and male germ cells [6,7].
56 However, during early preimplantation and primordial germ cell (PGC) development,
57 the genome undergoes genome-wide DNA demethylation [8–12] and additional
58 mechanisms are required to ensure ERV silencing. Indeed, ERV silencing in
59 embryonic stem cells (ESCs) is largely dependent on post-translational modification
60 of histones, in particular methylation at H3K9. Removal of the H3K9me3 histone
61 methyltransferase SETDB1 and its co-repressor, KRAB-associated protein (KAP1,
62 also known as TRIM28), leads to significant activation of ERVs in ESCs [13–15] and
63 PGCs [16]. Interestingly, 5mC and H3K9me3 regulate largely non-overlapping
64 subsets of ERVs in ESCs, with the notable exception of intracisternal A particles
65 (IAPs), whose silencing depends on the synergistic action of both epigenetic marks
66 [14,15].

67 IAP elements are relatively resistant to DNA demethylation during epigenetic
68 reprogramming [6,16–18], which is presumably a host defense mechanism against
69 these highly mutagenic ERVs. Maintenance of methylation at IAP and imprinting
70 control regions is driven by the G9a/GLP complex, which recruits *de novo* DNA
71 methyltransferases (DNMTs) independent of its H3K9 methyltransferase activity [19–
72 21]. On the other hand, H3K9me2-enriched regions are refractory to demethylation

73 during epigenetic reprogramming [22] via recruitment of the DNMT1 chaperone
74 NP95/UHRF1 [23,24].

75 A role for H3K9me3 in protecting ERVs from TET-mediated DNA demethylation has
76 also been proposed in ESCs [25]. TET enzymes oxidize 5mC into 5-
77 hydroxymethylcytosine (5hmC) and other oxidative derivatives as part of an active
78 DNA demethylation pathway [26–28]. We have previously shown that TET1 binds to
79 multiple retroelements in ESCs, and that both TET1 and TET2 help to maintain LINE-
80 1 elements in a hypomethylated state [29]. At LTR elements such as IAPs, however,
81 it has been shown that loss of SETDB1 enables TET1 binding, concomitant with an
82 accumulation of 5hmC at these sites [25]. However, this resulted in only very subtle
83 DNA methylation changes, and it remains unknown whether these alterations affect
84 the expression of IAP elements and other ERVs. Moreover, TET enzymes may act
85 on ERVs via non-catalytic pathways, similar to what we observed in LINE-1 elements
86 [29]. Finally, previous studies were performed using primed ESCs grown under
87 standard serum-containing conditions, which are highly methylated and express high
88 levels of the *de novo* methyltransferases DNMT3A and DNMT3B [17,18]. These
89 conditions may counteract and mask the catalytic activity of TET enzymes at IAPs
90 and other ERVs. Global DNA methylation can be induced *in vitro* by growing ESCs
91 under the so-called 2i conditions, which more closely resemble inner cell mass cells,
92 driving a naïve pluripotent state [17,30].

93 Here we investigated the role of SETDB1 in the regulation of ERVs in naïve ESCs
94 and its relationship with TET-mediated DNA demethylation. We find that SETDB1
95 has a markedly more prominent role in ERV silencing in naïve cells compared to
96 primed cells, including at IAP elements. The catalytic activity of TET2 contributes to
97 IAP activation upon SETDB1 depletion, but surprisingly this is not linked to DNA
98 methylation changes at IAPs. We show that instead TET2 drives a loss of the
99 repressive H4R3me2s mark at IAPs.

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101 **Results**

102 SETDB1 safeguards ERV silencing in naïve ESCs

103 To investigate the role of SETDB1 in ERV silencing in naïve ESCs, we switched
104 serum-grown (i.e., primed) E14 ESCs to 2i culture conditions. Using deep
105 sequencing of PCR amplicons from oxidative bisulfite (oxBS)-treated DNA [31,32],
106 we first confirmed that 5mC levels were substantially lower in naïve versus primed
107 ESCs at multiple ERVs, including IAP elements (Additional File 1: Fig. S1A).
108 RLTR4/MuLV elements were already hypomethylated in primed ESCs and showed
109 only a small decrease in 5mC levels in naïve ESCs. 5hmC levels were generally low
110 and similar between both culture conditions (Additional File 1: Fig. S1B). In line with
111 previous reports [33], transcript levels of these ERVs were not significantly higher in
112 naïve ESCs compared to primed ESCs, suggesting that other mechanisms
113 compensate the loss of DNA methylation to protect the genome against the activity of
114 ERVs (Additional File 1: Fig. S1C).

115 To identify SETDB1 targets in naïve ESCs, we depleted SETDB1 by lentiviral
116 delivery of shRNAs (Fig. 1A) and performed RNA-seq on three independent
117 biological replicates. For comparison, we conducted the same experiment in primed
118 ESCs. Using an “inclusive mapping” strategy that harvests information from non-
119 uniquely aligned reads (see Material and methods), we identified classes of repetitive
120 elements that were differentially expressed by more than 2-fold ($p < 0.05$, DESeq2).
121 As expected, SETDB1 depletion in naïve ESCs led to the upregulation of many
122 repeat classes ($n=104$), the vast majority of which were ERVs (Fig. 1B). Notably,
123 about half of these repeat classes ($n=55$) were exclusively upregulated in naïve cells
124 and not in primed cells (Additional File 2). These naïve-specific repeats included
125 MERVL, LINE-1 and VL30 elements, amongst several others. In contrast, only 8
126 repeat classes were significantly upregulated exclusively in primed ESCs (Additional
127 File 2).

128 Intriguingly, although IAP elements were deregulated in both culture conditions, they
129 were substantially more activated in naïve ESCs when compared to primed ESCs
130 (Fig. 1B, Additional File 2). We validated these observations using quantitative
131 reverse transcription polymerase chain reaction (RT-qPCR), which confirmed that
132 IAPEz upregulation was more pronounced in naïve cells (Fig. 1C), even though
133 SETDB1 depletion led to a substantial loss of H3K9me3 at these elements in both
134 conditions (Fig. 1D). Similar results were obtained upon KAP1 depletion (Additional
135 File 1: Fig. S1D), as expected from the dependency of SETDB1 binding on KAP1
136 [13,15]. We then asked what fraction of IAP copies underwent this skewed IAP
137 activation in SETDB1-depleted naïve cells. We analysed data from uniquely mapped
138 RNA-seq reads and found that, out of 1,009 IAPs with detectable RNA-seq signal,
139 681 (67%) were found to be >10-fold upregulated upon SETDB1 depletion in naïve
140 ESCs, whereas in primed cells there were only 257 (25%). Notably, this pattern was
141 seen in both full-length and truncated elements (Fig. 1E), showing that SETDB1
142 removal leads to increased activation at the majority of mappable IAP elements in
143 naïve ESCs compared to primed ESCs.

144 All together, these data show that SETDB1 plays a more prominent role in ERV
145 silencing in naïve ESCs when compared to primed ESCs, including at IAP elements.

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147 IAP activation upon SETDB1 depletion depends on TET2 activity

148 In naïve ESCs the role of SETDB1 in ERVs suppression could be particularly critical
149 for genome integrity due to the hypomethylated state of these transposons.
150 Additionally, SETDB1 could protect ERVs from further DNA demethylation, by
151 preventing binding of TET enzymes [25]. However, it remains unclear to what extent
152 TET activity affects ERV methylation and expression. To address this question, we
153 first performed ChIP-qPCR on WT and TET-depleted ESCs, which revealed that both
154 TET1 and TET2 specifically bind IAP elements at the LTR and primer binding site
155 (PBS; where KAP1 is recruited) regions in both primed and naïve ESCs (Fig. 2A).

156 Notably, the enrichment of both TET enzymes at IAPs was similar to that seen at
157 LINE-1 elements, which we have previously shown to undergo TET-mediated DNA
158 demethylation [29]. To test whether TET enzymes were involved in the activation of
159 IAPs, we performed *Tet1* or *Tet2* knockdown (KD) in SETDB1-depleted naïve ESCs.
160 Our RT-qPCR analyses revealed that removal of TET2 markedly reduced IAPLTR1
161 activation (and IAPLTR2 to a lesser extent) in SETDB1-depleted cells, whereas this
162 effect was milder upon *Tet1* KD (Fig 2B). We also generated *Tet2* knockout (KO)
163 ESCs (Additional File 1: Fig. S2A,B) wherein, similarly to *Tet2* KD cells, upregulation
164 of IAPLTR1, as well as the coding *gag* region, was diminished when compared to
165 *Tet2* wild-type (WT) naïve ESCs (Fig. 2C). Experiments involving depletion of both
166 TET1 and TET2 showed that loss of TET2 alone was sufficient to maximally impair
167 IAP activation (Additional File 1: Fig. S2C).

168 We then asked whether the effect of TET2 is dependent on its 5mC-oxidising
169 catalytic activity. For this purpose, we used *Tet2* KO ESCs to establish stable cells
170 lines expressing either WT TET2 protein or a catalytically inactive mutant version of
171 the enzyme. Western blot analyses revealed that both wild-type and mutant proteins
172 were expressed at similar levels (Additional File 1: Fig. S2D). Upon SETDB1
173 depletion, naïve ESCs expressing the WT *Tet2* construct displayed similar activation
174 of IAPLTR1 and *gag* region to what was seen in *Tet2* WT cells, effectively rescuing
175 the loss of TET2 (Fig. 2C). In contrast, cells expressing the catalytic mutant TET2
176 failed to upregulate IAPs any further than what was seen in SETDB1-depleted *Tet2*
177 KO cells (Fig. 2C). These results show that the catalytic activity of TET2 contributes
178 to the activation of IAPs upon SETDB1 depletion. To test whether other ERVs were
179 targeted by the same mechanism, we performed RNA-seq on both TET2 rescue lines
180 in a SETDB1-depleted context. Strikingly, comparison of ERV expression between
181 WT and mutant TET2 rescue lines yielded only IAPEz elements as significantly
182 differentially expressed. However, as a group, SETDB1-regulated repeats displayed
183 higher expression levels in WT versus mutant TET2 rescue lines upon SETDB1

184 depletion, a tendency that was not seen at repeats that are not targeted by SETDB1
185 (Fig. 2D).

186 We also performed experiments in primed ESCs, wherein *Tet2* KO had no effect on
187 IAP upregulation upon SETDB1 depletion (Additional File 1: Fig. S2E). On the other
188 hand, both RT-qPCR and RNA-seq data showed that overexpression of wild-type
189 TET2 could also drive an increase in IAP activation in primed ESCs (Additional File
190 1: Fig. S2E).

191 Overall, these results reveal that the activation of IAPs seen upon SETDB1 loss
192 partly depends on the catalytic activity of TET2.

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194 SETDB1 does not protect IAPs from TET-mediated DNA demethylation

195 The contribution of TET2 catalytic activity to IAP activation in SETDB1-depleted
196 naïve cells suggests that SETDB1 protects IAPs from oxidation-driven DNA
197 demethylation. To directly address this hypothesis, we measured 5mC and 5hmC
198 levels at IAPs using oxBS. Surprisingly, we did not observe any significant changes
199 in 5mC levels in SETDB1-depleted naïve ESCs both at the LTR and PBS regions
200 (Fig. 3A). IAP 5hmC levels remained low after SETDB depletion and only the PBS
201 region displayed a small increase in 5hmC levels (Supplementary Fig.3A). In line with
202 these observations, we found that TET2 binding to IAPs was not enhanced by the
203 loss of SETDB1 in naïve ESCs (Fig. 3B).

204 Extending our analyses to other SETDB1-regulated ERVs, we found that ETn/MusD,
205 RLTR10C and VL30 elements also did not display a decrease in 5mC upon SETDB1
206 depletion in naïve ESCs (Fig. 3C). In contrast, primed ESCs displayed a small but
207 significant loss of 5mC at IAPLTR2, ETnII/MusD and RLTR10C ERVs upon SETDB1
208 depletion, which is consistent with previous findings [13,25] (Additional File 1: Fig.
209 S3B). However, these small reductions in 5mC levels were not associated with
210 changes in expression, as IAPLTR2 transcripts are not affected in primed *Tet2* KO
211 cells (Additional File 1: Fig. S2E). Furthermore, the lack of 5mC changes in naïve

212 cells suggests that TET2 does not affect ERV expression by driving their
213 demethylation.

214 We next considered the possibility that 5mC changes may be apparent in copies that
215 are more responsive to SETDB1 depletion, rather than in the pool of IAP copies that
216 are amplified by the consensus primers used above. Based on our RNA-seq data, we
217 designed specific primers for bisulfite sequencing that target three individual
218 IAPLTR1 and two individual IAPLTR2 elements that exhibited high activation upon
219 SETDB1 depletion. Notably, these individual elements show higher levels of TET2
220 binding compared to a pool of IAP copies, whereas H3K9me3 and TET1 levels are
221 similar (Additional File 1: 3C). Despite displaying higher TET2 levels, the tested IAP
222 copies did not show any significant alterations in DNA methylation in the absence of
223 SETDB1 in either naïve or primed ESCs (Fig. 3D, Additional File 1: Fig. S3D).

224 To confirm that TET2 did not modulate DNA methylation levels at IAPs, we
225 performed bisulphite sequencing in TET2-depleted naïve cells. As expected, removal
226 of TET2 did not affect methylation levels both for a pool of IAP copies as well as
227 individual copies (Additional File 1: Fig. S3E). Finally, we asked whether the effect of
228 TET2 on DNA methylation is evident only at its target IAP copies. To this end, we
229 analysed methylation levels of DNA that is immunoprecipitated by a TET2 antibody.
230 DNA methylation levels of TET2-bound IAPs were comparable to the whole pool of
231 IAPs (input) in all conditions, and no differences were seen between TET2-bound
232 elements before or after SETDB1 depletion (Fig. 3E).

233 Taken together, these observations reveal that SETDB1 does not safeguard IAPs
234 from TET-mediated DNA demethylation and that TET2 induces IAP activation by a
235 DNA methylation-independent mechanism.

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240 TET2 expression is associated with loss of H4R3me2 at IAPs

241 We hypothesized that the contribution of TET2 to ERV activation in SETDB1-
242 depleted naïve ESCs could be affecting key histone marks, possibly through an
243 indirect mechanism. Therefore, we first asked whether TET2 could contribute to the
244 loss of H3K9me3 seen upon SETDB1 depletion, but found that *Tet2* knockdown did
245 not affect the levels of H3K9me3 in naïve ESCs (Fig. 4A). It was previously shown
246 that TET enzymes can recruit O-GlcNac transferase (OGT) to chromatin in ESCs [34],
247 which in turn targets H3K4 methyltransferase SET1/COMPASS complex [35].
248 However, our ChIP-qPCR analyses demonstrated that TET2 depletion did not affect
249 H3K4me3 levels at IAPs, which remained very low in all conditions tested (Fig 4A).
250 We also found no differences in the levels of the H3K27me3 repressive mark
251 (Additional File 1: Fig. S4A). IAP elements were also shown to be highly enriched for
252 symmetrical arginine dimethylation at H4R3 (i.e., H4R3me2s) [36,37]. Importantly,
253 removal of the arginine methyltransferase PRMT5 leads to derepression of IAPs in
254 PGCs and blastocysts [37], suggesting that H4R3me2s is a key repressive mark of
255 IAPs during epigenetic reprogramming. Therefore, we next carried out ChIP-qPCR
256 for H4R3me2s and found reduced levels of this mark in SETDB1-depleted ESCs (Fig.
257 4B). Strikingly, this loss at IAPs was driven by the action of TET2, as the levels of
258 H4R3me2s remained stable

259 upon SETDB1 removal in *Tet2* knockdown cells (Fig. 4B). These results suggest
260 that TET2 contributes to IAP activation in SETDB1-depleted ESCs by modulating the
261 levels of the repressive H4R3me2s mark.

262 To test whether a similar mechanism could be responsible for the activation of other
263 ERVs, we mined publicly available ChIP-seq data for H4R3me2s in naïve ESCs [36].
264 Using uniquely mapped reads, we identified repeat classes that are enriched for
265 H4R3me2s peaks over a random control (Additional File 1: Fig. S4B). Interestingly,
266 H4R3me2s-enriched repeats were preferentially activated upon SETDB1 depletion
267 when compared to non-enriched repeats (Fig. 4C). Using ChIP-qPCR we validated

268 the enrichment of H4R3me2s on three selected SETDB1-regulated transposons
269 (RLTR4/MuLV, RLTR10C and L1Gf) and tested whether, similar to IAPs, SETDB1
270 depletion led to H4R3me2s loss at these sites. However, none of the tested elements
271 displayed a significant reduction in H4R3me2s levels upon SETDB1 removal (Fig.
272 4D). This is in line with the fact that, unlike IAPs, the expression of these transposons
273 was not mediated by the catalytic activity of TET2 (Additional File 1: Fig. S4C). These
274 data suggest that TET2-mediated loss of H4R3me2s is specific to IAPs and drives
275 their activation.

276 Notably, all of the transposons analysed above displayed similar levels of TET2
277 enrichment (Fig. 4E), showing that TET2 binding is not sufficient to impart a reduction
278 in H4R3me2s levels. This suggest that TET2 activates IAPs in an indirect manner,
279 possibly by regulating the expression of one or more chromatin modifiers that act on
280 a subset of SETDB1-regulated transposons.

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296 **Discussion**

297 Here we have used naïve mouse ESCs to show that SETDB1 protects IAPs from
298 TET2-dependent activation, but that instead of DNA demethylation this involves
299 modulation of H4R3me2s levels at these elements.

300 We first established that SETDB1 has a more prominent role in ERV silencing in
301 naïve ESCs than in primed ESCs. In contrast, in mouse embryonic fibroblasts ERV
302 suppression is largely independent of SETDB1 [13], suggesting that cell differentiation
303 is generally associated with a switch from an H3K9me3-dependent silencing
304 mechanism to a 5mC-dependent one. Our results suggest that such a reciprocal
305 relationship extends further back into naïve pluripotency, where there is a more
306 pronounced requirement for SETDB1-mediated deposition of H3K9me3 for
307 maintaining ERV silencing.

308 We show for the first time that the catalytic activity of TET2 contributes to IAP
309 activation in SETDB1-depleted naïve ESCs. Unexpectedly, TET2 does not drive DNA
310 demethylation at IAPs, including at individual IAP copies and at TET2-bound IAPs in
311 naïve ESCs (Fig. 4). In contrast, a previous report suggested that in primed ESCs
312 H3K9me3 deposition by SETDB1 protects IAPs from TET-mediated DNA
313 demethylation [25]. Our data in primed ESCs partly supports this (Additional File 1:
314 Fig. S5A), indicating that a potential direct relationship between SETDB1 and TET-
315 mediated DNA demethylation is exclusive to the primed state and seemingly lost in
316 naïve ESCs. Notably, even in primed ESCs, the extent of DNA demethylation is
317 relatively small and is not associated with expression changes. We find that, rather
318 than affecting IAP expression through changes in DNA methylation, TET2 drives a
319 decrease in H4R3me2s levels at IAPs in naïve ESCs (Fig. 4B). It has been shown
320 that in both PGCs and preimplantation embryos, deletion of *Prmt5* leads to loss of
321 H4R3me2s at IAPs, concomitant with their transcriptional activation [37]. Importantly,
322 *Prmt5*-null PGCs display no differences in IAP DNA methylation levels compared to
323 wild-type tissues [37]. Our data in naïve ESCs adds further support to a repressive

324 role of the H4R3me2s mark at IAPs, as other key histone modifications were
325 excluded as the mediators of TET2-dependent IAP activation (Fig. 4A; Additional File
326 1: Fig. S4A).

327 Whilst the catalytic activity of TET2 does not affect DNA methylation directly at IAPs,
328 it remains formally possible that TET2 oxidises methylated proteins or RNA
329 associated with IAP chromatin. However, our data suggest that the action of TET2 on
330 IAPs is likely to be indirect, involving the regulation of genes that in turn control IAP
331 expression. Our RNA-seq data show that neither the arginine methyltransferases
332 *Prmt5* and *Prmt7* nor the putative H4R3me2s demethylase *Jmjd6* are controlled by
333 TET2 activity (Additional File 1: Fig. S4D). It remains to be tested whether other
334 enzymes act *in vivo* to modify H4R3me2s, which could then mediate the activating
335 effect of TET2 on IAPs. Other more indirect scenarios are also possible, such as a
336 TET2-regulated gene that prevents recruitment of the enzymes involved in arginine
337 methylation.

338 If TET2-mediated demethylation indirectly affects IAP expression, then the same
339 could be true for other hypomethylation models, such as *Dnmt* KO ESCs. This raises
340 questions about reported roles of DNA methylation on IAP expression, namely its
341 synergistic action with H3K9me3 [14,38]. Similarly, *Tet1/Tet3* double KO
342 preimplantation embryos display a downregulation of IAPs [39], but direct evidence
343 for a causal relationship is missing. These considerations highlight the need for future
344 work to harvest the power of epigenetic editing tools to test for direct causal links
345 between ERV methylation and their activation.

346

347 **Conclusions**

348 We have demonstrated that SETDB1 has more prominent role in ERV silencing in
349 naïve ESCs than primed ESCs, with the removal of SETDB1 leading to increased
350 upregulation of ERVs. Our data show that activation of IAPs in SETDB1 depleted
351 naïve cells depends on the catalytic activity of TET2. However, surprisingly, TET2

352 does not play a role in DNA demethylation at IAPs, instead TET2-dependent
353 activation of IAPs is associated with the loss of H4R3me2s repressive mark upon
354 SETDB1 removal. In conclusion, our findings demonstrate a novel role of SETDB1 in
355 protecting IAPs from TET2-dependent loss of H4R3me2s in naïve ESCs.

356

357 **Methods**

358 Cell lines

359 E14 ESCs were used for all experiments unless otherwise stated. *Tet1* KO ESCs
360 were a kind gift from Guo-Liang Xu [40]. *Tet2* KO ESCs were generated by targeting
361 exon 14 of *Tet2* with a floxed neomycin resistance cassette (Additional File 1: Fig.
362 S4A). To rescue the expression of TET2, stable cell lines were derived from *Tet2* KO
363 ESCs using a PiggyBac transposon system. The *Tet2* catalytic mutant (H1304Y,
364 D1306A) construct was made from a wild-type clone (a kind gift from Kristian Helin)
365 by site-directed mutagenesis.

366

367 Cell culture and gene knockdown

368 ESCs were grown in feeder-free conditions using either DMEM-based medium with
369 15% FBS and 1,000 U/ml ESGRO LIF (Millipore) or in 2i culturing conditions using
370 DMEM F-12 (Gibco 21331) and neurobasal media (Gibco 21102) supplemented with
371 N2 (Life tech. 17502048), B27 (Gibco 17504-044), 1,000 U/ml ESGRO LIF (Millipore),
372 Mek inhibitor (PD0325901) and GSK3b inhibitor (CHIR99021). For shRNA-mediated
373 gene knockdown, ESCs were infected with viral particles carrying pLKO.1 constructs
374 harbouring gene-specific shRNAs from The RNAi Consortium, (shSETDB1:
375 CCCGAGGCTTTGCTCTTAAAT, TRCN0000092975; shTET1:
376 TTTCAACTCCGACGTAAATAT, TRCN0000341848; shTET2:
377 TTCGGAGGAGAAGGGTCATAA, TRCN0000250894) or a non-targeting sequence
378 (shScr: CCTAAGGTTAAGTCGCCCTCGCTC;). After 48 hours, cells were selected
379 with 1µg/ml puromycin or 50µg/ml hygromycin for 3 days. For siRNA-mediated gene

380 knockdown, cells were transfected twice (at day one and three) with Kdm5d-specific
381 siRNAs (Dharmacon siGENOME siRNA Cat. D-054675-02-0002 and ThermoFisher
382 Silencer Select siRNA Cat. s74014) or non-targeting siRNA (Dharmacon siGENOME
383 Non-Targeting siRNA #2 Cat.D-001210-02-20) at 125nM using Lipofectamin 3000
384 (Thermo Scientific, Cat. L3000008). The cells were collected four days after the first
385 transfection and the knockdown was confirmed by RT-qPCR.

386

387 RNA Isolation and RT-qPCR

388 RNA was extracted using AllPrep DNA/RNA mini kit (Qiagen 80204) and DNase
389 treated with the TURBO DNA-free™ Kit (Ambion, AM1907). RNA (1 µg) was
390 retrotranscribed using Revertaid Reverse Transcriptase (Thermo Scientific EP0441)
391 and the cDNA was diluted 1/50 for qPCRs using MESA BLUE MasterMix
392 (Eurogentec, 10-SY2X-03+NRWOUB) on a LightCycler® 480 Instrument II
393 (Roche). A list of primers used can be found in Additional File 3.

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395 RNA-seq Library Preparation

396 For analysing the effects of SETDB1 depletion, ribosomal RNA-depleted RNA-seq
397 libraries were prepared from 200-500 ng of total RNA using the low input ScriptSeq
398 Complete Gold Kit (Epicentre). For the TET2 rescue samples, mRNA libraries were
399 prepared using the Dynabeads mRNA purification kit (Life Technologies) and the
400 NEBnext Ultra RNA library prep kit (NEB). Libraries were sequenced on an Illumina
401 NextSeq 500 with single-end 75 bp reads.

402

403 Chromatin Immunoprecipitation

404 ChIP was performed as described in Latos et al [41], with modifications. For the
405 detection of TET1, TET2 and ATRX, cells were fixed with an initial cross-linking step
406 of 45 minutes with 2 mM Di(N-succinimidyl) glutarate (Sigma-Aldrich Cat. 80424) in
407 PBS at room temperature, followed by a PBS wash and a second fixation step of 12

408 minutes with 1% formaldehyde in PBS. For histone ChIPs (H4K20me3, H3K9me3,
409 H3, H3K4me3, H4R3me2s) the cells were fixed with % formaldehyde for 12 minutes
410 in PBS. After quenching with glycine, washes and lysis, chromatin was sonicated
411 using a Bioruptor Pico from Diagenode, to an average size of 200-700 bp.
412 Immunoprecipitation was performed using 100 µg of chromatin and 7.5 µg of
413 antibody (TET1, TET2, ATRX) or 20 µg of chromatin and 2.5 µg of antibody
414 (histones). Final DNA purification was performed using the GeneJET PCR
415 Purification Kit (Thermo Scientific. Cat. K0701) and elution in 80 µL of elution buffer.
416 This was diluted 1/10 and analysed by qPCR, using the KAPA SYBR® FAST Roche
417 LightCycler® 480 2X qPCR Master Mix (Kapa Biosystems, Cat. KK4611). A list of
418 primers and antibodies used can be found in Additional Files 3 and 4, respectively.

419

420 Oxidative bisulphite sequencing

421 Deep sequencing of PCR products from BS- and oxBS-converted DNA was
422 performed as previously described [31]. Briefly, precipitated DNA (without glycogen)
423 was resuspended in water and further purified using Micro Bio-Spin columns (Bio-
424 Rad), after which half of the DNA was oxidised with 15 mM K₂Cr₂O₇ (Alpha Aesar) in
425 0.5 M NaOH for 1 hour. Following bisulphite conversion of both DNA fractions with
426 the EpiTect Bisulfite kit (QIAGEN), a two-step PCR amplification was used: a first
427 PCR amplifies the region of interest and adds part of the sequencing adaptors; a
428 second PCR on pooled amplicons then completes the adaptors and adds sample
429 barcodes, allowing for multiplexing (see primers in Additional File 3). Paired-end
430 sequencing of pooled samples was done using an Illumina MiSeq.

431

432 High-throughput sequencing data processing

433 Reads were trimmed using Trim_galore! v0.3.3 with default parameters. External
434 ChIP-seq data for H4R3me2s (GEO accession GSE37604) [36] were aligned to the
435 mm9 genome assembly using Bowtie2 v2.1.0 [42] and uniquely aligned reads were

436 extracted for peak detection using MACS2. To identify repeats enriched for
437 H4R3me2s, the number of ChIP-seq peaks overlapping each repeat class were
438 compared with a random control where peaks were shuffled (using bedtools) over
439 mappable regions of the genome. RNA-seq data were aligned to mm9 using Tophat
440 v2.0.9 [43] with -g 1 option, which assigns reads with multiple hits of equal quality to
441 one of those locations at random (i.e., “inclusive mapping”). Raw read counts for
442 each gene or Repeatmasker class were used in DESeq2 for differential expression
443 analysis and for generating normalised gene and repeat expression values. For
444 expression analysis of individual IAP copies, only uniquely mapped reads were used,
445 together with a custom annotation of IAPs which merged same-strand IAP fragments
446 within 100bp into a single element; elements longer than 5 kb were classified as full-
447 length. Only elements with >0.25 RPM in any of the analysed samples were used.
448 OxBS data were aligned with Bismark [44] to a custom genome containing the
449 amplicon sequences; only CpGs covered by at least 100 reads were used to
450 calculate 5mC/5hmC levels.

451

452 **Declarations**

453 Availability of data and material

454 The datasets generated during the current study (RNA-seq) are available in the GEO
455 repository under the accession number GSE100864. ChIP-seq data for H4R3me2s
456 (GEO accession GSE37604) [36] were downloaded from the NCBI Gene Expression
457 Omnibus.

458

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469

470 Author's Contributions

471 Ö.D. and M.B. designed the study and experiments. Ö.D. performed cell culture,
472 shRNA knockdowns, RT-qPCR, Western blotting, oxBS and ChIP experiments. L.R.
473 performed ChIP experiments. K.C. performed qPCR analyses. D.S. generated the
474 *Tet2* KO ESCs. M.B. performed oxBS, RNA-seq and bioinformatic analyses. Ö.D.
475 and M.B. wrote the manuscript with all other authors.

476

477 Competing interests

478 The authors declare that they have no competing interests.

479

480 Ethics

481 Not applicable.

482

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627 **Figure Legends**

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630

631 **Figure 1 – SETDB1 is pivotal for ERV silencing in naïve ESCs.** **a.** Western blot
632 (representative data from n=2) and RT-qPCR analyses (n=7) show SETDB1
633 depletion by lentiviral delivery of shRNAs (ANOVA with Tukey's multiple comparison
634 test, *** p<0.001, **** p<0.0001). **b.** RNA-seq data using inclusive mapping from
635 primed and naïve cells was overlaid with the RepeatMasker annotation to
636 determine repeat classes that are differentially expressed upon SETDB1 removal
637 (highlighted in red, n=3). **c.** Analysis of IAP expression by RT-qPCR in SETDB1 KD
638 primed and naïve ESCs. The error bars show s.d. (n=6-12; ANOVA with Tukey's
639 multiple comparison test, * p<0.05, **p<0.01, **** p<0.0001). **d.** H3K9me3 ChIP-
640 qPCR at IAPs upon SETDB1 loss (ANOVA with Tukey's multiple comparison test, *
641 p<0.05, **p<0.001). Data are shown as mean ± s.d. of seven independent
642 experiments. **e.** Expression data from uniquely mapped RNA-seq reads at truncated
643 and full-length (>5kb) IAP elements.

644

645 **Figure 2 – TET2 drives IAP expression in the absence of SETDB1.** **a.** ChIP-qPCR
646 data showing TET1 and TET2 enrichment at IAPs and L1 elements in primed (in the
647 presence and absence of TETs) and naïve ESCs. Data points from two independent
648 experiments are shown. **b.** Expression of IAPLTR1 and IAPLTR2 in SETDB1 and
649 TET1 or TET2-depleted cells in naïve ESCs (n=2; one-way ANOVA, Dunnet's
650 multiple comparison test, * p<0.05, **p < 0.01). Each bar represents the mean ± s.d.
651 **c.** RT-qPCR analysis of *Tet2* WT, KO and rescue cell lines at IAPs in naïve ESCs
652 (n=3-4; ANOVA with Tukey's multiple comparison test, * p<0.05, **** p<0.0001). The
653 results are presented as mean ± s.d. **d.** Fold-change in expression between TET2
654 wildtype and mutant rescue lines for different repeats classes, depending on whether
655 they are repressed by SETDB1 or not (n=3, t-test) .

656

657 **Figure 3 – TET2 does not affect DNA methylation levels at IAPs in naïve ESCs.**

658 **a.** 5mC levels using oxBS at IAPs upon SETDB1 knockdown in naïve ESCs; each
659 data point represents the average value from two biological replicates at a given CpG
660 within the amplicon. **b.** Normalised enrichment of TET2 using a spike-in control at
661 IAPLTR1 and the PBS region in naïve ESCs (representative replicate from n=3) **c.**
662 5mC levels at additional ERVs upon SETDB1 knockdown in naïve ESCs (ANOVA
663 with Tukey's multiple comparison test, **** p<0.0001). **d.** 5mC+5hmC levels using
664 BS at individual IAP copies (sclAPs) upon SETDB1 depletion, in primed and naïve
665 ESCs. **e.** 5mC levels at TET2-bound IAPLTR1 elements; each data point represents
666 the value at a given CpG within the amplicon (ANOVA with Tukey's multiple
667 comparison test, * p<0.05).

668

669 **Figure 4 – SETDB1 protects IAPs from TET2-dependent loss of H4R3me2s. a,b.**

670 ChIP-qPCR data for H3K4me3 and H3K9me3 (a) or H4R3me2s (b) at IAPs upon
671 SETDB1 loss in the presence and absence of TET2 (n=2-3; ANOVA with Sidak's
672 multiple comparison test, **p<0.01, *** p<0.001). **c.** Fold-change in expression upon
673 SETDB1 knockdown for different repeats classes, depending on whether they are
674 enriched for H4R3me2s peaks or not (n=3, t-test). **d.** ChIP-qPCR data for H4R3me2s
675 at additional ERVs upon SETDB1 loss in the presence and absence of TET2 (n=2,
676 each bar represents the mean ± sd). **e.** TET2 enrichment levels at ERVs. Data are
677 shown as mean ± s.d. of two independent experiments.

678

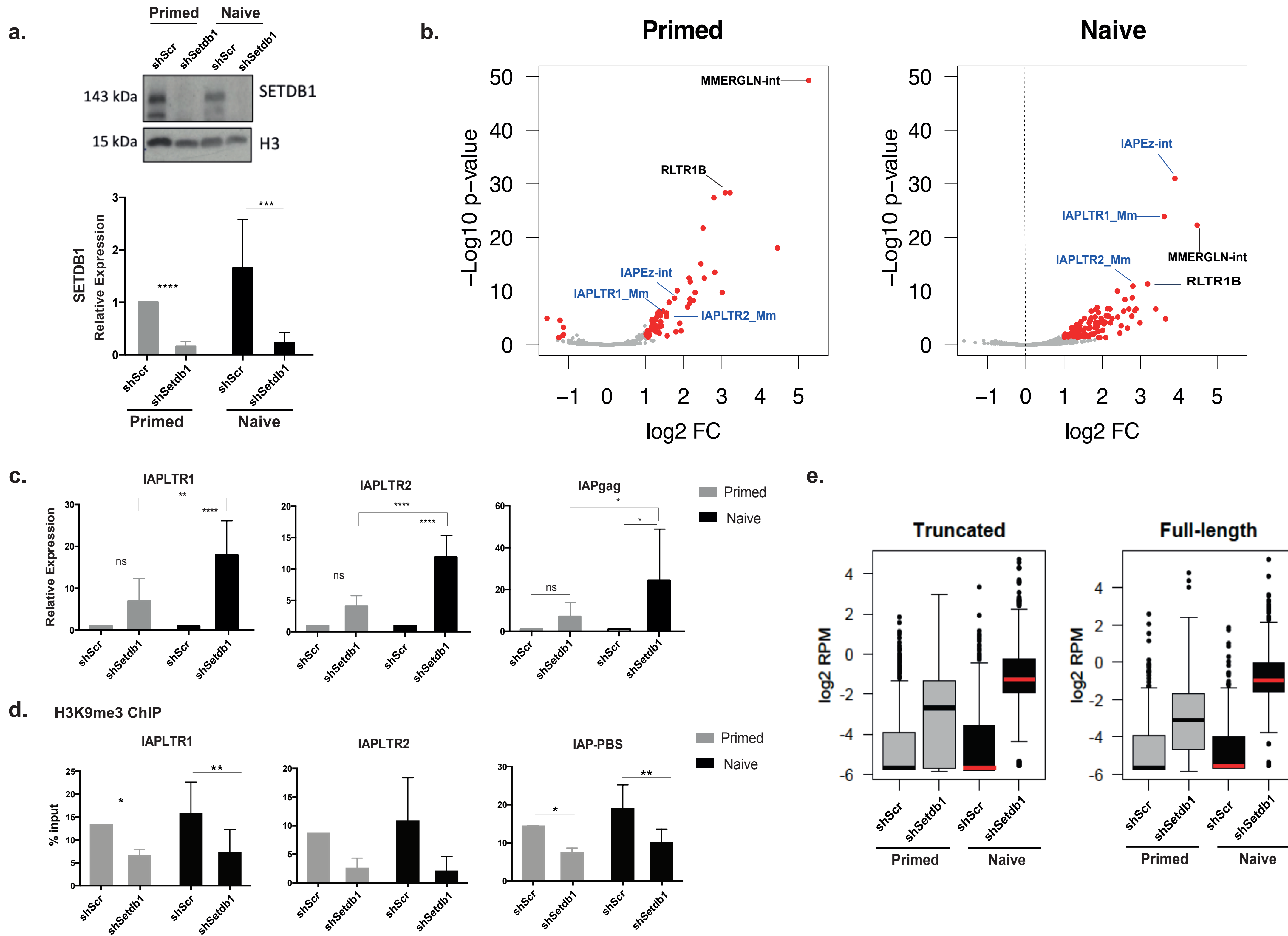


Figure 1

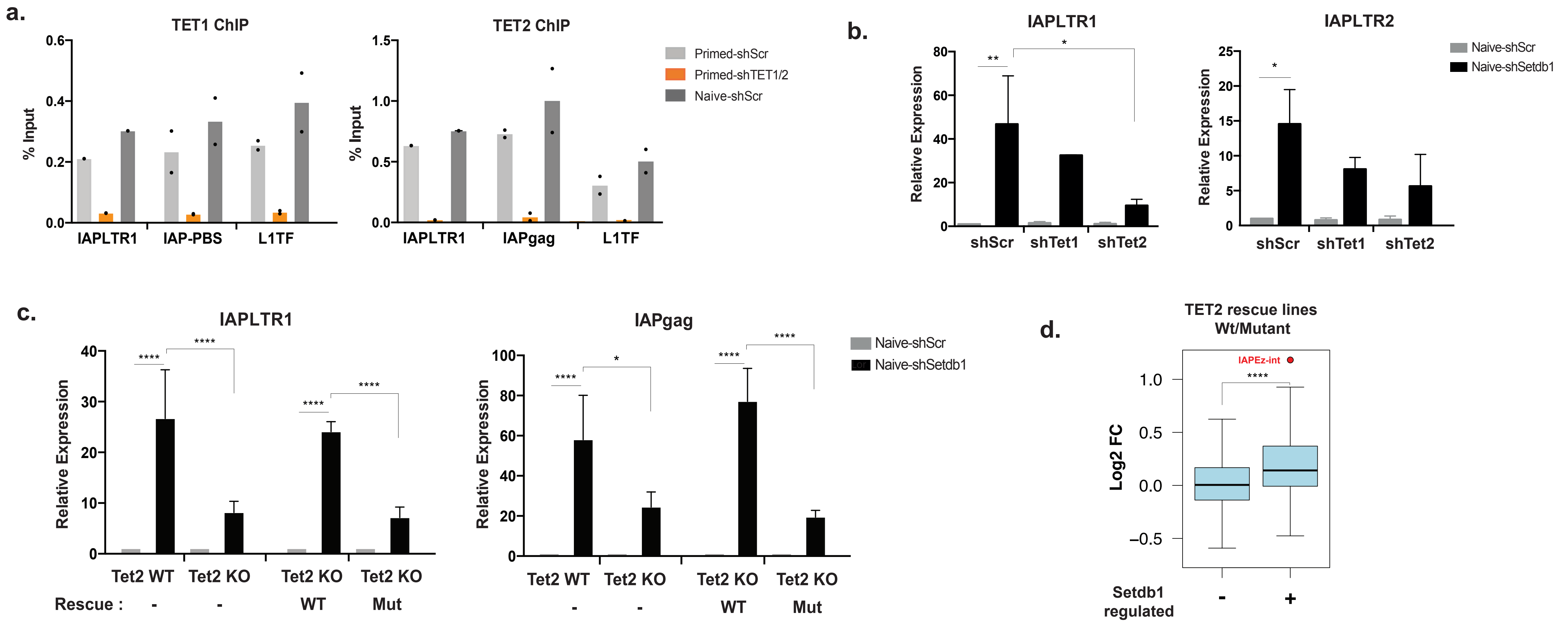


Figure 2

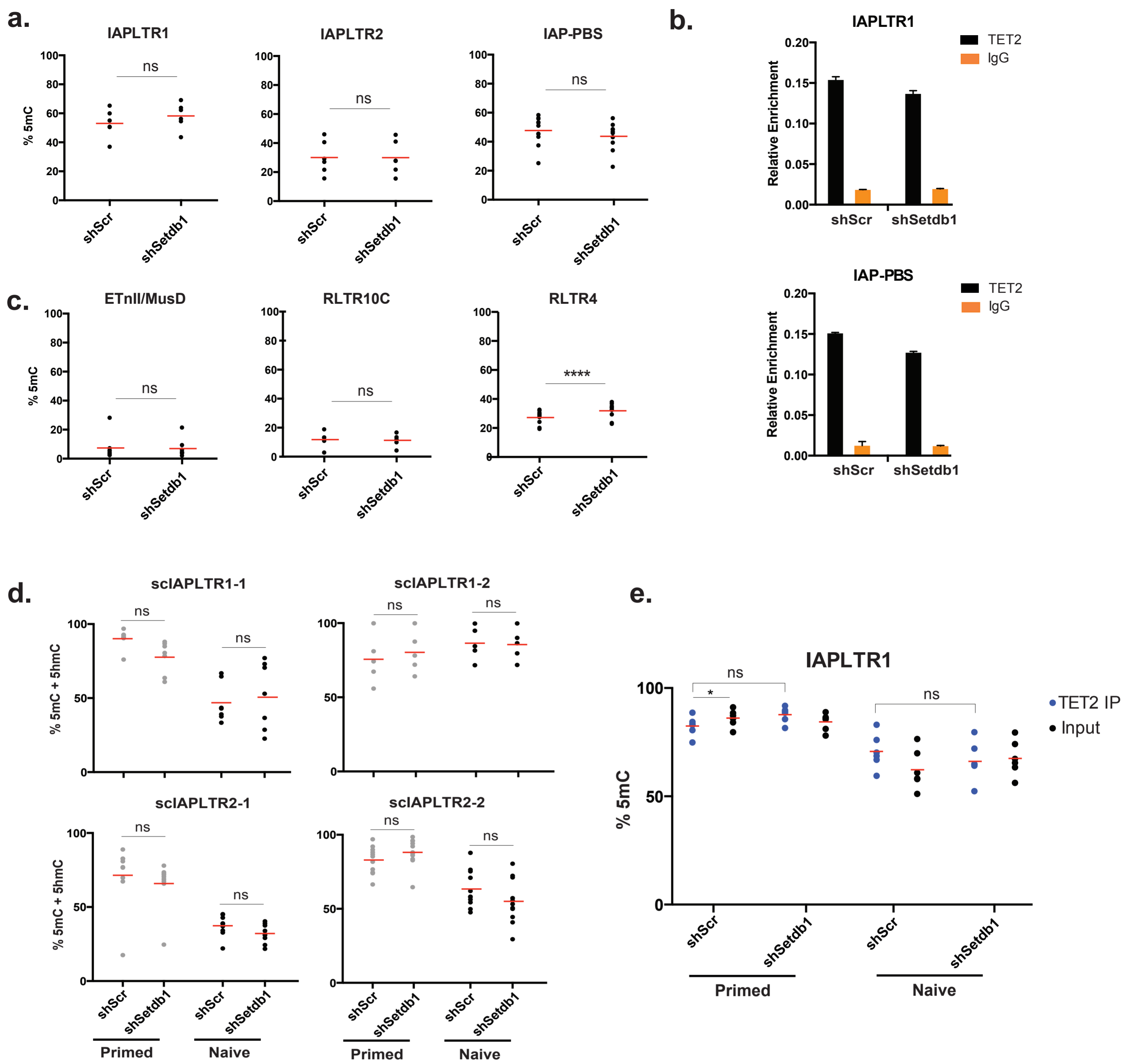


Figure 3

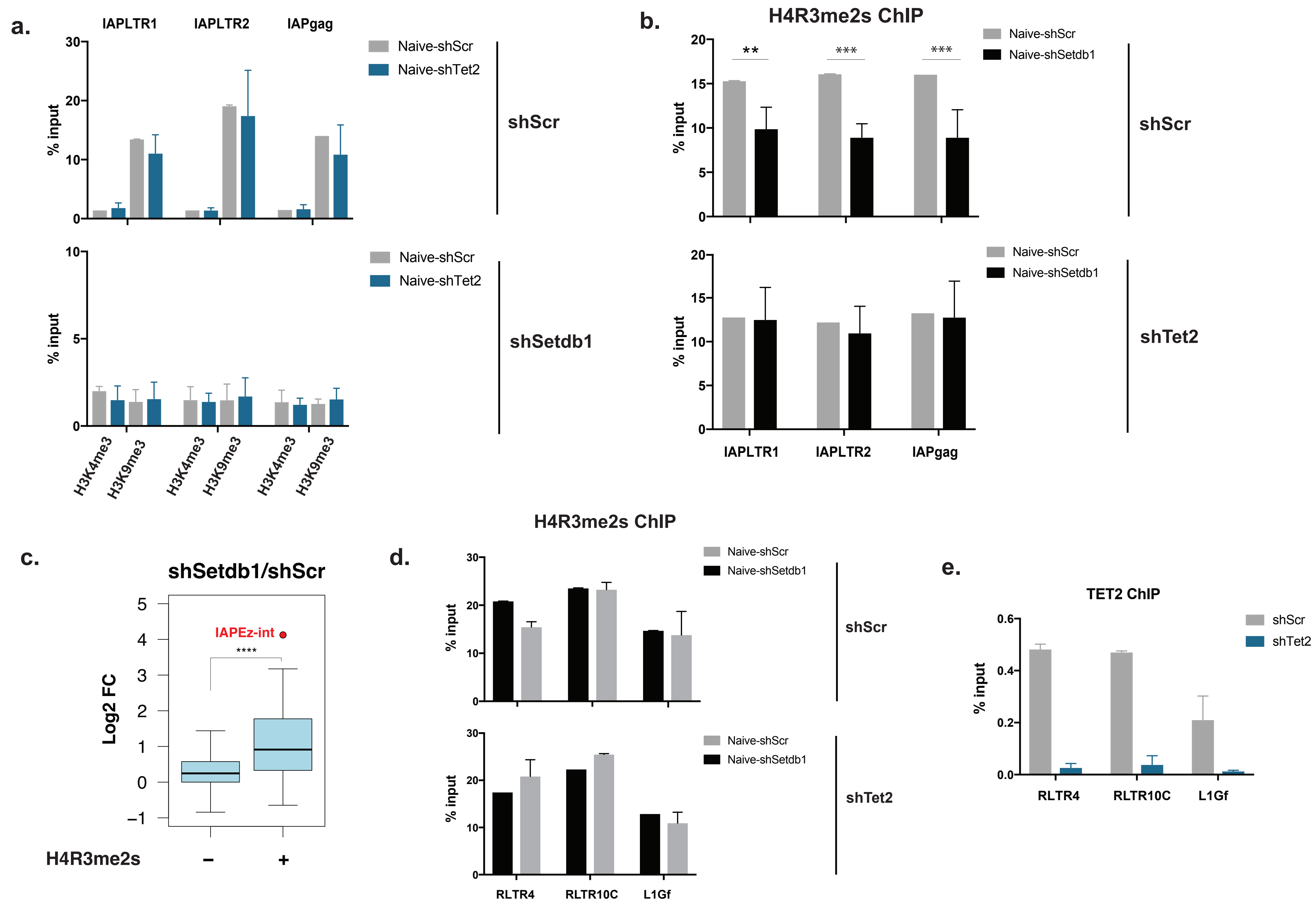


Figure 4