Title: The DNMT1 inhibitor GSK-3484862 mediates global demethylation in murine embryonic stem cells

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Abstract

Background: DNA methylation plays an important role in regulating gene expression in mammals. The covalent DNMT1 inhibitors 5-azacytidine and decitabine are widely used in research to reduce DNA methylation levels, but they impart severe cytotoxicity which limits their demethylation capability and confounds interpretation of experiments. Recently, a non-covalent inhibitor of DNMT1 called GSK-3484862 was developed by GlaxoSmithKline. We sought to determine whether GSK-3484862 can induce demethylation more effectively than 5-azanucleosides. Murine embryonic stem cells (mESC) are an ideal cell type in which to conduct such experiments, as they have a high degree of DNA methylation but tolerate dramatic methylation loss.

Results: We determined the cytotoxicity and optimal concentration of GSK-3484862 by treating wild-type (WT) or *Dnmt1/3a/3b* triple knockout (TKO) mESC with different concentrations of the compound,

which was obtained from two commercial sources. Concentrations of 10 µM or below were readily tolerated for 14 days of culture. Known DNA methylation targets such as germline genes and GLN-family transposons were upregulated within two days of the start of GSK-3484862 treatment. By contrast, 5-azacytidine and decitabine induced weaker upregulation of methylated genes and extensive cell death. Whole genome bisulfite sequencing (WGBS) showed that treatment with GSK-3484862 induced dramatic DNA methylation loss, with global CpG methylation levels falling from near 70% in WT mESC to less than 18% after 6 days of treatment with GSK-3484862, similar to the methylation level observed in *Dnmt1* deficient mESCs.

Conclusions: GSK-3484862 mediates striking demethylation in mESCs with minimal non-specific toxicity.

Keywords: DNA methylation, DNMT1, GSK-3484862, 5-azacytidine, decitabine, demethylation, whole genome bisulfite sequencing

Introduction

DNA methylation is a key regulator of gene expression in mammals[1]. DNA methyltransferases (DNMTs) transfer a methyl group to the fifth carbon of the DNA base cytosine to form 5-methylcytosine. Typically, a high density of DNA methylation at a gene or transposon's transcriptional start site promotes transcriptional repression[2]. DNMT3A and DNMT3B methylate previously unmodified CpG sites, establishing the pattern of DNA methylation during early embryonic development[3]. DNMT1 in turn methylates the newly synthesized DNA strand after DNA replication. Because DNMT1 preferentially methylates CpGs complementary to, or in the vicinity of, existing CpG methylation, it can maintain CpG methylation patterns through many cycles of cell division[4, 5].

DNA methylation is critical for maintaining silencing of certain genes, particularly genes associated with germline development, and transposons[1, 6, 7]. Aberrant hypermethylation of the promoters of tumour suppressors and other genes occurs in a wide variety of cancers[8]. Consequently, there is high

45 interest in demethylating drugs for both research and therapeutic purposes[9]. Two such drugs are widely
46 used in research and as therapeutics: 5-azacytidine and decitabine (5-aza-2'-deoxycytidine). They were
47 synthesized in the 1960s as potential chemotherapeutics that might interfere with nucleic acid
48 metabolism in rapidly dividing cells [10, 11]. It was subsequently demonstrated that unlike other
49 chemotherapeutics, 5-azacytidine and related compounds induce differentiation of sarcoma cells to
50 myotubes, an activity attributable to apparent demethylating properties [12].

51 5-azacytidine and decitabine are cytidine analogs which contain nitrogen at the fifth position of
52 the pyrimidine ring [10, 11]. In cells, decitabine is converted to 5-aza-dCTP and incorporated into DNA
53 during replication[13]. The 5-azacytosine base forms a stable covalent bond with DNMT1, irreversibly
54 inhibiting the enzyme[14]. The resulting DNA-protein adduct is then repaired and DNMT1 is destroyed in
55 the proteosome[15]. Decitabine thus has two modes of activity: it causes cell death via the creation of
56 excessive DNA-protein adducts, and it reduces DNA methylation by the inhibition and destruction of
57 DNMT1[16]. 5-azacytidine is converted into a mixture of 5-aza-dCTP and 5-aza-CTP in the cell[17]. As such,
58 it is incorporated into both DNA and RNA, and may cause additional cytotoxicity by interfering with RNA
59 metabolism, methylation, and translation[18, 19].

60 The cytotoxicity of 5-azanucleosides limits their use as demethylating agents in research.
61 Excessive concentrations of 5-azanucleosides will cause cell death, and thus experiments must be
62 conducted in a narrow window of optimal concentration in which DNMT1 is inhibited but the cells do not
63 die[9]. At tolerated doses, DNMT1 inhibition may be incomplete, and 5-azanucleoside-treated cells show
64 only modest global reductions in DNA methylation when measured by highly quantitative whole genome
65 bisulfite sequencing (WGBS)[20, 21]. Even at lower concentrations, the genotoxic effects of 5-
66 azanucleoside make it unclear whether gene expression changes and cellular phenotypes are caused by
67 demethylation or non-specific toxicity. To identify genes regulated by DNA methylation, Hackett and
68 colleagues treated NIH3T3 cells with decitabine for 3 days and identified 344 upregulated genes[22]. Few

69 of these upregulated genes had heavily methylated promoters and many upregulated genes were
70 involved in immune and stress response, suggesting a response to toxicity rather than demethylation.
71 After 14 days of recovery, 49 genes showed persistent upregulation. These genes were disproportionately
72 enriched for heavily methylated promoters and likely to be direct targets of methylation-mediated
73 silencing. This strategy of treatment and recovery was successful at identifying genes regulated by DNA
74 methylation in NIH3T3s but carries obvious drawbacks. Any gene remethylated during the recovery period
75 would be missed. Furthermore, researchers studying the role of DNA methylation during a dynamic
76 process cannot necessarily incorporate a treatment and recovery period. A DNA methyltransferase
77 inhibitor lacking non-specific toxicity would be preferable from a research perspective.

78 Despite their limitations, 5-azanucleosides have remained the best and most widely used drugs
79 for inducing DNA demethylation for the last 40 years. In 2019, GlaxoSmithKline announced the discovery
80 of a non-covalent DNMT1 inhibitor, GSK-3484862 [23]. As of this writing, there is no publication describing
81 its initial discovery in detail, but it has been used in two articles. Researchers at GlaxoSmithKline
82 demonstrate that GSK-3842364, a racemic mixture including GSK-3484862 and its enantiomer, selectively
83 inhibits DNMT1 with an IC_{50} of 0.4 μM[24]. Five days of treatment of GSK-3842364 induces a dramatic
84 global demethylation of erythroid progenitor cells as measured by mass spectrometry. In addition, GSK-
85 3842364 shows lower cytotoxicity than decitabine and induces transcription of the methylated fetal
86 hemoglobin genes, *HBG1* and *HBG2*, both in erythroid cells *in vitro* and in a murine model of sickle cell
87 anemia. In the second report, Haggarty and colleagues used GSK-3484862 to inhibit DNMT1 in murine
88 pre-implantation *Dnmt3a/3b* KO embryos[25]. Here, a concentration above 0.35 μM prevents blastocyst
89 formation. At this low concentration, only a 34% global drop in DNA methylation is observed relative to
90 untreated *Dnmt3a/3b* KO embryos; however, the treatment is sufficient to induce a marked increase in
91 IAP-Ez transposon expression, consistent with demethylating activity of the compound.

92 Considering the limited data concerning GSK-3484862 and the importance of a better DNA
93 methylation inhibitor for the scientific community, we tested the demethylating capacity of GSK-3484862
94 in murine embryonic stem cells (mESCs), which are ideal for testing methyltransferase inhibitors. When
95 cultured in classic serum+LIF conditions, mESCs have a high global level of DNA methylation[26]. However,
96 while most mammalian cells require some DNA methylation for survival[27], *Dnmt1/3a/3b* triple knockout
97 (TKO) embryonic stem cells self-renew and proliferate normally[28]. The set of genes regulated by DNA
98 methylation in mESCs is well characterized, as are individual and compound DNA methyltransferase
99 knockouts[29, 30]. In addition to wild-type cells, we used *Dnmt1/3a/3b* TKO mESCs because lacking DNA
100 methylation, these cells are suitable to assess non-specific cytotoxicity of GSK-3484862. Our results
101 indicate that GSK-3484862 mediates striking demethylation in mESCs, comparable to what is observed in
102 a complete DNMT1 knockout, with minimal non-specific toxicity.

103

104 **Results**

105 *mESCs tolerate high concentrations of GSK-3484862*

106 As of this writing, GSK-3484862 is not available for sale from GlaxoSmithKline, so we conducted
107 experiments using preparations purchased from ChemieTek and MedChemExpress. First, we sought to
108 determine the cytotoxicity and optimal concentration of GSK-3484862 (Fig. 1). Wild-type J1 line and
109 *Dnmt1/3a/3b* triple knockout (TKO) mESCs on a J1 background were seeded at low density on gelatin-
110 coated plates. WT mESCs showed classic mESC morphology, dome-shaped colonies with rounded edges,
111 while TKO ESCs showed jagged colony edges when cultured on gelatin. Cells were treated 24 hours after
112 plating with concentrations of GSK-3484862 ranging from 2 pM to 200 µM. Substantial precipitation of
113 the drug in media was observed at concentrations equal to or greater than 20 µM. After six days the
114 morphology of WT and TKO cells treated with GSK-3484862 was similar to that of corresponding control

115 cells that received only DMSO, with mortality only noticeable at 200 µM (Fig.1A). No differences were
116 observed in cell numbers after six days of treatment with GSK-3484862 at concentrations equal to or
117 below 20 µM in WT and TKO cells. (Fig. 1B).

118 We then evaluated the long-term cytotoxicity and demethylation efficacy of GSK-3484862. WT and TKO
119 cells were treated with 2 or 10 µM of GSK-3484862 for 14 days, 10 µM being the highest concentration at
120 which drug precipitation was not observed. WT drug-treated mESCs were indistinguishable from
121 corresponding vehicle-treated controls throughout the 14 days (Fig. 1C). A slight but reproducible
122 decrease in cell growth was observed in GSK-3484862-treated WT cells from day 6 onwards, whereas the
123 growth rate of TKO cells was constant throughout treatment (Fig. 1D). No difference was observed
124 between compound produced by the two companies (Fig. 1C, D). Altogether, the results indicate that
125 mESCs tolerate GSK-3484862 for up to two weeks at concentrations up to 10 µM.

126 *Genes repressed by DNA methylation show derepression in GSK-3484862*

127 We next assayed expression levels of seven germline genes (*Ctcfl, Magea4, Rhox1, Hormad1, Sohlh2, Dazl,*
128 *Tuba3b*) and one transposon class (*Gln*) reported to be repressed by DNA methylation in mESCs[29-31].
129 As expected, all tested transcripts were highly upregulated in TKO cells (Fig. 2A, Supplementary Table 1).
130 These transcripts were also upregulated after treatment with GSK-3484862, although often to a lesser
131 extent than in TKO cells, potentially reflecting continued expression of DNMT3A and DNMT3B in the WT
132 cells. GSK-3484862 produced by both companies showed similar levels of upregulation (Supplementary
133 Table 1). Upregulation of target transcripts was observed in WT cells treated with either 2 µM or 10 µM
134 GSK-3484862, starting as early as after two days of exposure (Figure 2), and all genes showed statistically
135 significant upregulation at both concentrations in a one-sided t-test (Supplementary Table 2).

136 *Severe toxicity from 5-azacytidine or decitabine treatment.*

137 WT and TKO cells were exposed to 5-azacytidine or decitabine for 48 hours. Even 0.1 µM concentrations
138 of these compounds inflicted widespread cell death in WT cells (Fig 3A,B). TKO cells, which lack DNMT1
139 and thus will not form DNMT1-DNA adducts, appeared completely unaffected. After 1 – 2 days of recovery
140 time, some WT ESC colonies of typical morphology were observed from the 0.1 µM and 0.3 µM 5-
141 azacytidine and the 0.1 µM decitabine samples (Fig. 3A-D), and these were used for subsequent analysis.

142 WT cells treated with 0.3 µM 5-azacytidine showed upregulation of the genes *Tuba3b*, *Ctcfl*, *Magea4* and
143 *Rhox1*, while there was no change in the expression of the other transcripts tested (Fig. 3E). Minimal or
144 no upregulation of methylation-repressed transcripts was observed in WT cells treated with decitabine
145 (Fig. 3F.)

146 Comparable decitabine-mediated toxicity was observed for WT mESCs from the genetic hybrid V6.5 line,
147 confirming that this was not a cell line specific effect (Fig. 3G).

148 *Whole genome bisulfite sequencing showed dramatic demethylation in mESCs after GSK-3484862*
149 *treatment*

150 Whole genome bisulfite sequencing (WGBS), even at low sequencing depth, provides an accurate
151 quantitative estimate of total CpG methylation[32]. WT mESCs showed near 70% CpG methylation,
152 dropping below 18% after six or 14 days of treatment with GSK-3484862, for both companies and at both
153 concentrations (Fig. 4A). Strikingly, the methylation level of treated mESCs was almost identical to the
154 published DNA methylation level of *Dnmt1* KO mESCs[29], consistent with total and specific inhibition of
155 DNMT1. Mapping statistics and conversion efficiencies are shown in Supplementary Table 3.

156 Cells treated with 5-azacytidine showed far more modest reductions in 5-methylcytosine (Fig. 4B), but
157 they may have regained some DNA methylation during the 46-hour recovery period after treatment.

158

159 **Discussion**

160 From a research perspective, GSK-3484862 shows a great deal of promise. 5-azanucleosides have
161 substantial non-specific toxicity and work within a narrow concentration band. We observed
162 reactivation of some methylated genes after treatment with 0.3 µM 5-azacytidine but very few cells
163 survived at this concentration, while 0.1 µM 5-azacytidine was inadequate to reactivate methylated
164 genes. Furthermore, we had to allow two days for colonies of surviving cells to emerge in culture. By
165 contrast, 2 µM or 10 µM GSK-3484862 reactivated methylated gene expression and produced only
166 modest reduction in growth, most noticeable between days 6 and 10 of treatment. This reduced growth
167 may reflect specific activity of the compound. When mESCs undergo demethylation mediated by
168 treatment with MEK inhibitor, GSK3 inhibitor and high concentration of ascorbic acid, they have a burst
169 of transposon expression and undergo reconfiguration of heterochromatin state approximately during
170 this interval[31].

171 DNA methylation of the GSK-3484862-treated mESCs never fell below 17% regardless of the
172 dosage used or time of treatment, and methylated genes were not reactivated to the extent observed in
173 the TKO cells. This likely reflects the high level of DNMT3A and DNMT3B activity in mESCs, as evidenced
174 by the similar level of DNA methylation in published *Dnmt1* KO mESCs[29]. The *Dnmt1* deficient or
175 inhibited cells potentially reach an equilibrium in which methylation is constantly added by DNMT3A
176 and DNMT3B and lost through replication and Tet-protein activity. Other cell types may respond to
177 *Dnmt1* inhibition differently. Most somatic and cancer cells do not express such high levels of the *de*
178 *novo* DNA methyltransferases and may not be able to maintain such high levels of DNA methylation in
179 the absence of *Dnmt1* activity. At the same time, somatic or cancer cells may not survive dramatic DNA
180 methylation loss[27, 33]. *Dnmt1* deficient and *Dnmt1/3a/3b* TKO mESCs are not viable upon
181 differentiation, and DNMT1 becomes essential after implantation[6, 28]. This shift may reflect the fact
182 that mESCs depend heavily on Trim28 to silence transposons, but upon differentiation of mESCs or

183 uterine implantation of embryos, DNA methylation gains importance for transposon repression [7, 30,
184 34, 35]. Thus, researchers working with other cell types may well observe specific toxicity at lower
185 doses. We also cannot rule out that non-specific toxicity may occur in some cell types, a result suggested
186 by the GSK-3484862's ability to halt mouse development at relatively low concentrations[25].
187 Nonetheless, this novel DNA methyltransferase inhibitor appears to be a substantial improvement over
188 5-azanucleosides and a promising research tool.

189

190 **Conclusions**

191 GSK-3484862 mediates dramatic demethylation in murine embryonic stem cells. With regard to both
192 activation of methylated genes and non-specific toxicity, GSK-3484862 performs far better than 5-
193 azanucleosides.

194

195 **Methods**

196 *Cell Culture*

197 Mouse embryonic stem cells (mESC) lines used in this study included *Dnmt1/3a/3b* triple knockout [28]
198 on a J1 background (129S4/SvJae), as well as wildtype J1 and V6.5 lines (C57BL/6 x 129S4/SvJae). Cells
199 were cultured in Knockout-DMEM (Gibco) with 15% ES qualified FBS (Gibco), ESGRO mouse LIF 1000U/mL
200 (Millipore), 1X PenStrep (Invitrogen), 1X Glutamax (Invitrogen), 55µM b-mercaptoethanol (Invitrogen), 1X
201 non-essential amino acids (Invitrogen) and 1X Primocin (Invivogen). Cells were maintained at 37 °C and
202 5% CO_2 on dishes coated with 0.1% gelatin (Millipore) and a layer of mitomycin C-treated mouse
203 embryonic fibroblasts (MEFs) (Thermofisher Scientific).

204 *Determining GSK-3484862 toxicity*

205 An assay to determine the optimal concentration and toxicity of GSK-3484862 (ChemieTek) was
206 performed using J1-line WT and DNMT TKO mESCs. 30,000 cells were seeded in 24-well plates pre-coated
207 with 0.1% gelatin. The next day, medium was changed to fresh mESC medium or medium containing
208 DMSO (0.1% or 1%) for the following concentrations of GSK-3484862: 2 pM, 20 pM, 200 pM, 2 nM, 20
209 nM, 200 nM, 2 µM, 20 µM (in 0.1% DMSO) and 200 µM (in 1% DMSO). The medium was refreshed every
210 day for the next six days, after which cell morphology was assessed, followed by cell dissociation with
211 0.05% Trypsin-EDTA (Gibco) for cell counting.

212 Next, the demethylation efficacy and long-term cytotoxicity of GSK-3484862 from two companies,
213 ChemieTek and MedChemExpress, was evaluated in duplicate experiments. To improve solubility, after
214 resuspension in DMSO, GSK-3484862 was subjected to ultrasonication. GSK-3484862 from both
215 companies were sonicated for six minutes at 42 kHz in an ultrasonic water bath (Sper Scientific). Still, drug
216 precipitation was observed for concentrations at or above 20 µM in media, and therefore an upper
217 concentration of 10 µM was chosen. WT and DNMT TKO cells were seeded in 12-well plates pre-coated
218 with 0.1% gelatin and had 0.1% DMSO, 2 µM or 10 µM GSK3484862 added in medium + LIF from day zero.
219 The medium was refreshed every day and cells were counted using the Countess II FL instrument and
220 passaged every 2-3 days for the next 14 days.

221 *5-azacytidine assay*

222 We found that WT mESCs died if treated with 5-azacytidine immediately after plating (data not shown).
223 Therefore, we plated cells at low density (1.4 x 10^4 cells/ cm^2) and added 0.1% DMSO, 0.1 or 0.3 µM of
224 the drug 24 hours later. WT and DNMT TKO cells were exposed to 5-azacytidine for 48h, with a media
225 change at the 24-hour timepoint to replenish 5-azacytidine. The cultures were then maintained in media
226 without the drug for 46 hours. Cells were then dissociated with 0.05% Trypsin-EDTA, counted, and

227 collected for subsequent analysis. Because of their higher survival and consequent higher density, DNMT
228 TKO cells were harvested after only 24 hours recovery time.

229 *Decitabine assay*

230 Concentrations of decitabine as low as 0.1 µM proved lethal to WT mESCs plated on gelatin (data not
231 shown), so mESCs were seeded at 1.4 x 10^4 cells/ cm^2 on a monolayer of MEF feeder cells to enhance
232 survival. 24 hours later, 0.1% DMSO or decitabine at concentrations of 0.1 µM, 0.3 µM or 1 µM was added
233 and cells were treated for 48 hours, with a media change at the 24-hour timepoint. Cells were allowed to
234 recover for another 24 hours and then harvested. Because of the low viability of the WT J1 line mESCs,
235 the experiment was repeated with V6.5 mESCs, a robust line on a hybrid genetic background (C57BL/6 x
236 129S4/SvJae).

237 *Cell imaging and counting*

238 All cells in this paper were imaged with an EVOS M5000 microscope (Invitrogen) and counted using
239 Countess II FL Automated Cell Counter (Invitrogen). Images were processed using Adobe Photoshop and
240 ImageJ, and graphs were created using GraphPad Prism software.

241 *DNA and RNA extraction*

242 Cell pellets were snap frozen and kept at –80 °C until extraction. Samples from the GSK-3484862 and 5-
243 azacytidine treated assays had RNA and DNA isolated simultaneously using the AllPrep DNA/RNA kit
244 (Qiagen), whereas only RNA was isolated from decitabine samples by using the RNAzol total RNA protocol
245 (Sigma). DNA and RNA concentrations were measured with the Qubit dsDNA and RNA HS Assay Kits
246 respectively (ThermoFisher).

247 *Quantitative RT-PCR*

248 500 ng of RNA was used for cDNA synthesis using Froggabio SensiFAST cDNA synthesis kit and following
249 the manufacturer's instructions. The qPCR reaction was performed using PowerUp SYBR green mix
250 (Invitrogen) containing cDNA generated from the equivalent of 5ng RNA and 0.5 µM of primer mix
251 (forward and reverse) in a final reaction of 6 µL per duplicate. Quantification and analysis were performed
252 with the QuantStudio5 instrument (Applied Biosystems), and the cycling conditions were: (50°C 2 min,
253 95°C 20 s, 55x (95°C 3 s, 60°C 30 s), 95°C 1 s). The following primers were used:

254 *Actin*: F:5’- ACTGGGACGACATGGAGAAG -3’ R:5’- GGGGTGTTGAAGGTCTCAAA -3’

255 *Gapdh*: F:5’- CATCAAGAAGGTGGTGAAGC -3’ R:5’- GGGAGTTGCTGTTGTAAGTCG -3’

256 *Tuba3b*: F:5’- AGGAAGATGCAGCCAACAATTA -3’ R:5’- TGCACAGATCGGCCAGTTT -3’

257 *Ctcfl*: F:5’- GCCTTCAGCATTGCGTGAC -3’ R:5’- AGCAGGTGAAAATGTATCCGC -3’

258 *Magea4*: F:5’- GTCTCTGGCATTGGCATGATAG -3’ R:5’- GCTTACTCTGAACATCAGTCAGC -3’

259 *Rhox1*: F:5’- CCGGTTTTCTGGAGTATGAGAGA -3’ R:5’- CCAGCCGTTTTCTGTCTTGTG -3’

260 *Hormad*-1: F:5’- TGAAAACTCTGGAGCTTCTGAAA -3’ R:5’- ACTGACTAACTGTTCAACCTGACA -3’

261 *Sohlh*-2: F:5’- CCATCGAGCTGTTCCTTCCA -3’ R:5’- GGAATACACGTTCAGGCCCC -3’

262 *Dazl*: F:5’- GTGGCTTCTGCTCCACCTTCG -3’ R:5’- CCTTGACTTGTGGTTGCTGA -3’

263 *Gln*: F:5’- CGTAAGGACCCTAGTGGCTG -3’ R:5’- GCACTCACTCTTCTTCACTCTG -3’

264 The *Dazl* and *Gln* primers were taken from published sources[30].

265 *Analysis of qRT-PCR data*

266 To calculate which genes showed statistically significant upregulation relative to control cells,
267 expression of each gene in the DNMT TKO or GSK-3484862 cells was normalized to the control or control

268 lines in the same experimental replicate and time-course. Data from cells treated with GSK-3484862 from
269 the two manufacturers (ChemieTek and MedChemExpress) was combined. A paired, one tailed t-test was
270 conducted, pairing control cells with treated cells for each time point and calculating one p-value for each
271 GSK-3484862 concentration across all timepoints.

272 *Whole-genome bisulfite library (WGBS) preparation*

273 Genomic DNA (500 ng) with spiked-in lambda DNA (1.25 ng) (NEB) was sheared using an M220 ultrasound
274 sonicator (Covaris) to an average size of 350 bp. The size of the DNA fragments was confirmed by
275 electrophoresis on an 1.5% agarose gel. 200 ng of sheared DNA was subjected to bisulfite conversion using
276 EZ DNA Methylation-Gold Kit (Zymo Research) as per manufacturer's instructions. Libraries were prepared
277 using 50-100 ng of bisulfite-converted DNA and the Accel-NGS Methyl-Seq DNA Library prep kit (Swift
278 Biosciences) according to manufacturer's instructions, with 8-11 cycles of PCR-amplification. The integrity
279 of the libraries was assessed using agarose gel electrophoresis.

280 Libraries were sequenced on an Illumina HiSeq 4000 instrument at the Michael Smith Genome
281 Sciences Centre at BC Cancer.

282 *WGBS analysis*

283 Raw reads were quality checked with FastQC and adapters at either end were trimmed with the Trim
284 Galore! (v0.6.7) software. Reads were then aligned to the mm10 reference genome using Bismark
285 (v0.21.0). In cases where spike-in DNA was used to quality check bisulfite conversion rates, reads were
286 aligned either to pUC19 or lambda DNA, as specified (Supplementary Table 3). PCR duplicates were
287 removed using the deduplicate command in Bismark. DNA methylation was subsequently calculated using
288 the methylation extractor command in Bismark. In all cases, default parameters were used.

289

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392

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394 WP wrote the manuscript. CM and WP supervised research.

395

396 **Figure Captions**

397 **Figure 1. GSK-3484862 inhibitor treatment in mouse embryonic stem cells (mESC). A** Brightfield images
398 showing morphology of WT and TKO cells after 6 days of treatment with 0.1% DMSO or GSK-3484862 (2
399 µM, 20 µM and 200 µM) obtained from ChemiTek. **B** Cell numbers after 30,000 WT or TKO mESC were
400 treated with indicated culture conditions for six days. **C** Brightfield images of WT cells after 14 daysof
401 treatment with 0.1% DMSO, 2 µM or 10 µM of GSK-3484862 from ChemieTek or MedChemExpress. **D**
402 Graphs from two independent experiments show the cell growth of WT or TKO cells counted every 2-3
403 days.

404 **Figure 2. Expression of methylation-regulated transcripts in GSK-3484862 treated cells.** RT-qPCR,
405 normalised to *Actb*, of known methylation-regulated germline genes/transposons in cells treated with

406 GSK-3484862 over the number of days indicated. N=2 – 6 biological replicates per sample. Mean and
407 standard error are indicated.

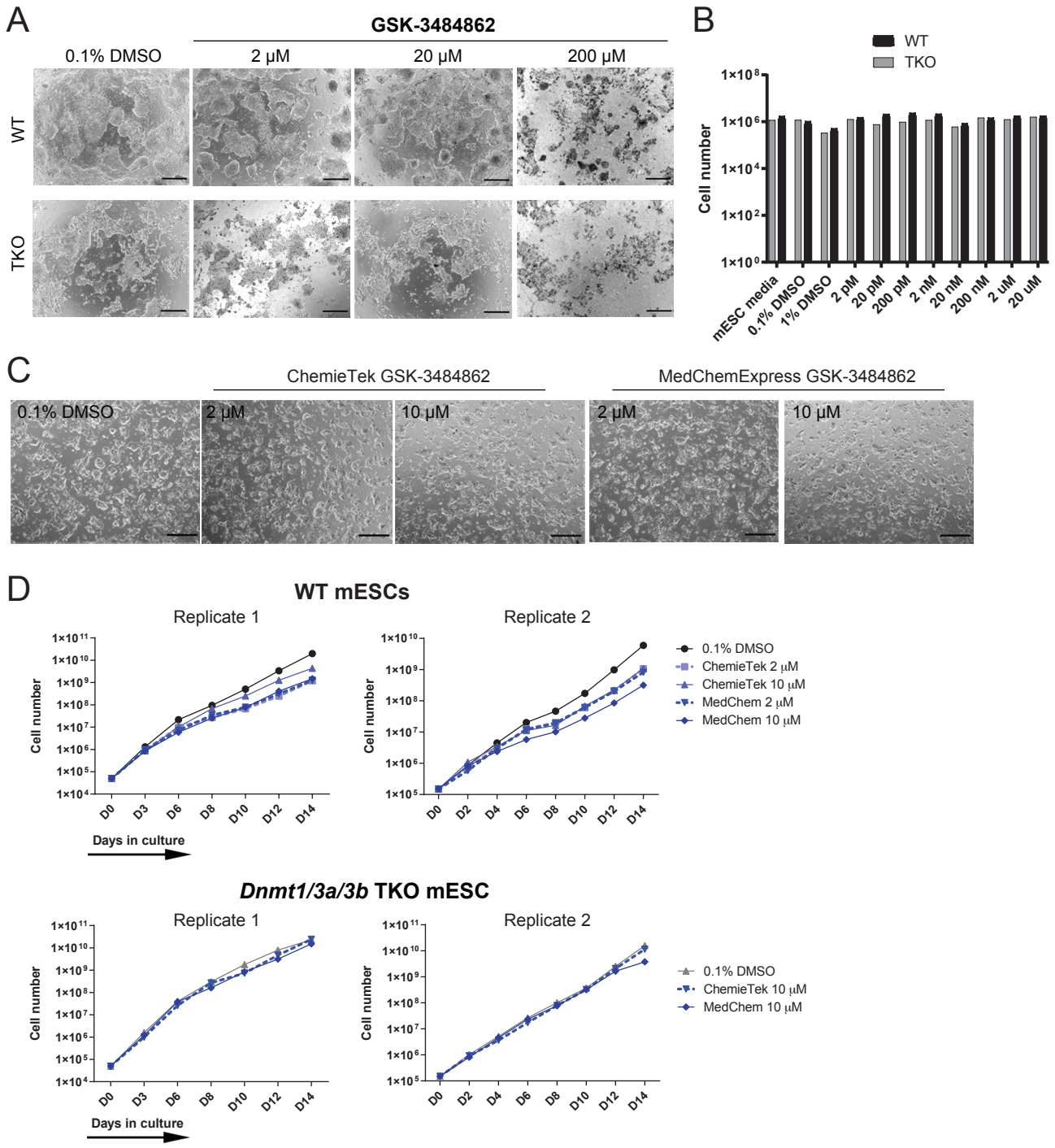
408 **Figure 3. Treatment of mouse embryonic stem cells with 5-azanucleosides. A** Brightfield images of WT
409 and *Dnmt1/3a/3b* TKO cells after 48 hours of exposure to 0.1% DMSO, 0.1 or 0.3 µM 5-azacytidine (left
410 columns) and WT cells 46 hours after the end of treatment with 5-azacytidine (right column). **B**
411 Brightfield images taken of WT and TKO cells after 48 hours of exposure to 0.1% DMSO, 0.1 or 0.3 µM
412 decitabine. To enhance survival, these cells were cultured on murine embryonic feeders (MEFs) rather
413 than gelatin, hence the distinct morphology. **C, D** Cell numbers after treatment and recovery for 5-
414 azacytidine (**C**) and decitabine (**D**). **E, F** Expression of methylated genes upon 5-azacytidine (**E**) or
415 decitabine (**F**) treatment and recovery, normalized to *Actb* and *Gapdh* respectively. Mean and standard
416 error of two technical replicates is indicated. **G** V6.5 mESCs after exposure to decitabine. These cells
417 showed similar lethality to J1 WT mESCs.

418 **Figure 4. CpG methylation reduction after treatment with GSK-3484862 or 5-azacytidine. A** Global CpG
419 methylation levels of WT cells treated with 0.1% DMSO, *Dnmt1/3a/3b* TKO cells treated with 0.1%
420 DMSO, and WT cells after 6 or 14 days of treatment with 2 µM or 10 µM GSK-3484862. Methylation
421 levels of published *Dnmt1* knockout cells are shown in comparison[29]. **B** Methylation levels in WT
422 mESCs after treatment with 0.1% DMSO, 0.1 or 0.3 µM 5-azacytidine. WGBS = Whole-genome bisulfite
423 sequencing.

424 **Supplementary Table 1. qRT-PCR data for all samples.**

425 **Supplementary Table 2. P-values for upregulation of indicated genes.**

426 **Supplementary Table 3. Mapping and conversion statistics for Whole Genome Bisulfite Sequencing**
427 **data used in this publication.**



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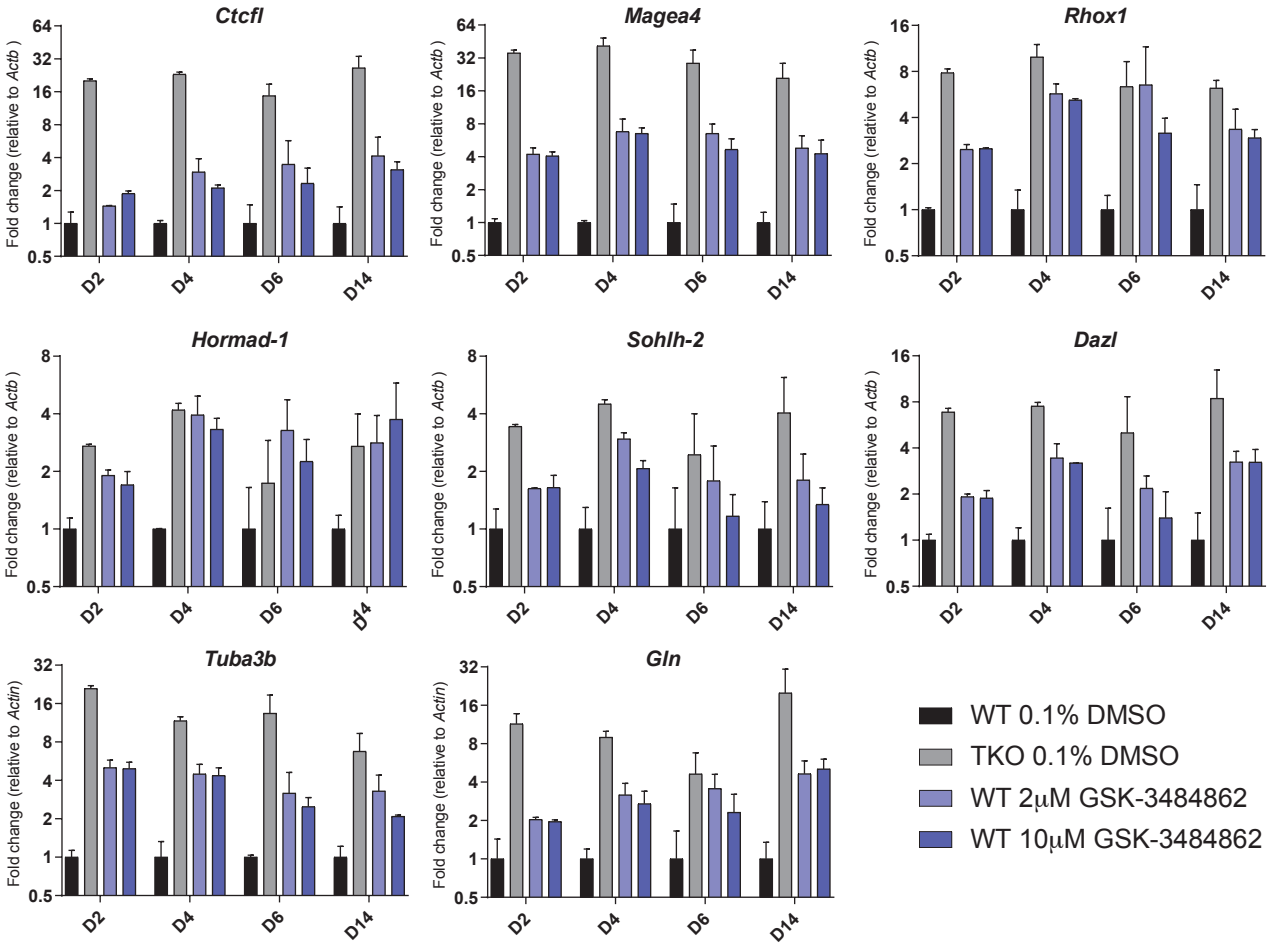

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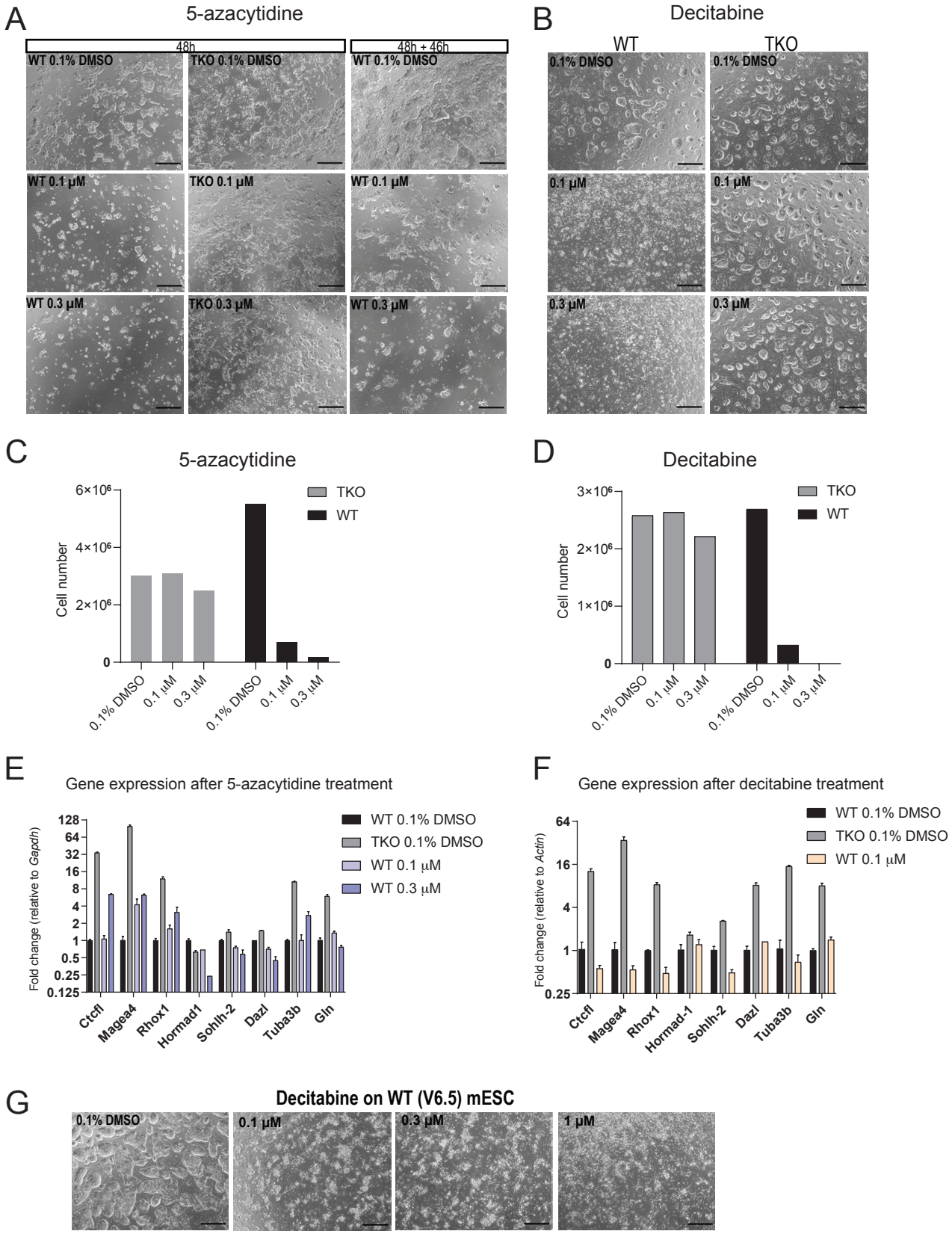
A
5-azacytidine
48h
48h + 46h
WT 0.1% DMSO
TKO 0.1% DMSO
WT 0.1% DMSO
WT 0.1 μM
TKO 0.1 μM
WT 0.1 μM
WT 0.3 μM
TKO 0.3 μM
WT 0.3 μM
B
Decitabine
WT
TKO
0.1% DMSO
0.1% DMSO
0.1 μM
0.1 μM
0.3 μM
0.3 μM
C
5-azacytidine
Cell number
6×10⁶
4×10⁶
2×10⁶
0
TKO
WT
0.1% DMSO
0.1 μM
0.3 μM
0.1% DMSO
0.1 μM
0.3 μM
D
Decitabine
Cell number
3×10⁶
2×10⁶
1×10⁶
0
TKO
WT
0.1% DMSO
0.1 μM
0.3 μM
0.1% DMSO
0.1 μM
0.3 μM
E
Gene expression after 5-azacytidine treatment
Fold change (relative to Gapdh)
128
64
32
16
8
4
2
1
0.5
0.25
0.125
WT 0.1% DMSO
TKO 0.1% DMSO
WT 0.1 μM
WT 0.3 μM
Ctcfl
Magea4
Rhox1
Hormad1
Sohlh-2
Dazl
Tuba3b
Gln
F
Gene expression after decitabine treatment
Fold change (relative to Actin)
64
16
4
1
0.25
WT 0.1% DMSO
TKO 0.1% DMSO
WT 0.1 μM
Ctcfl
Magea4
Rhox1
Hormad-1
Sohlh-2
Dazl
Tuba3b
Gln
G
Decitabine on WT (V6.5) mESC
0.1% DMSO
0.1 μM
0.3 μM
1 μM

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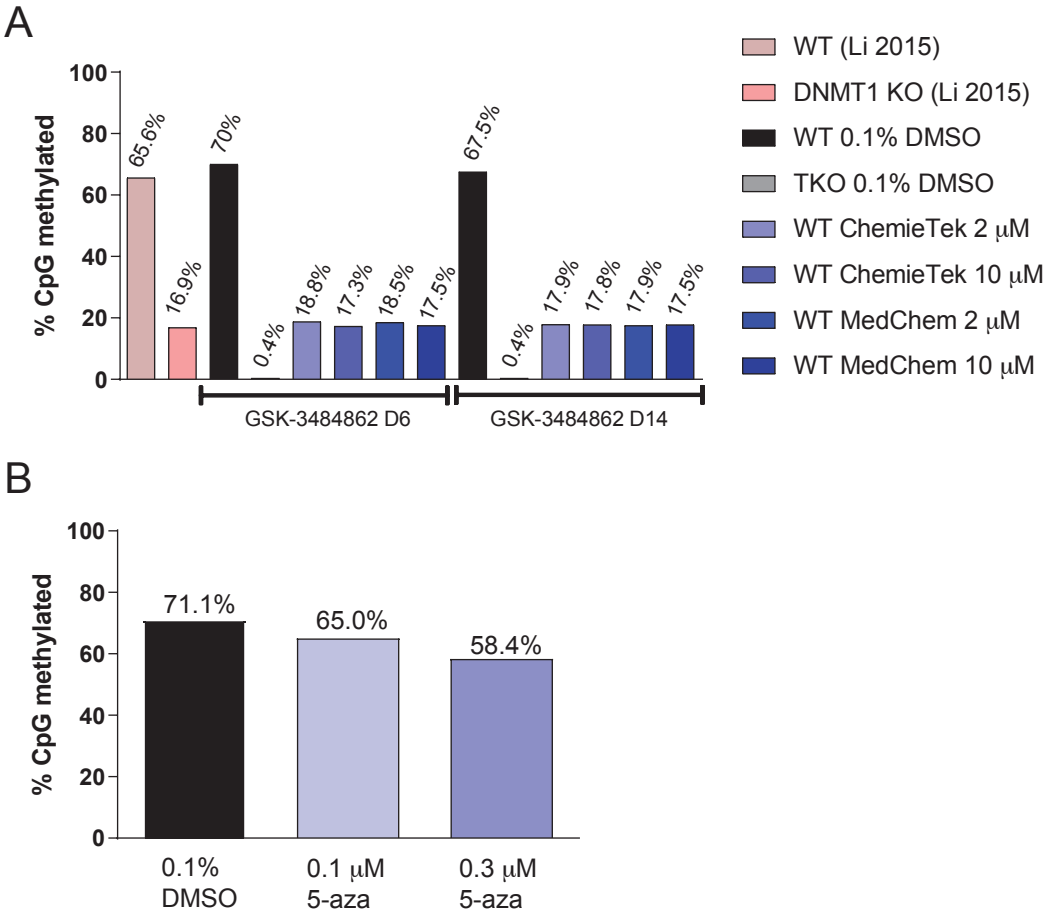

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