

1 ***H2A.X* mutants exhibit enhanced DNA demethylation in *Arabidopsis thaliana***

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32 **response, DNA glycosylase**

33

34 **Running title: hypomethylation in *h2a.x* mutant endosperm**

35

36 **Abstract**

37

38 *H2A.X* is an H2A variant histone in eukaryotes, unique for its ability to respond to DNA
39 damage, initiating the DNA repair pathway. *H2A.X* replacement within the histone octamer is
40 mediated by the FAcilitates Chromatin Transactions (FACT) complex, a key chromatin
41 remodeler. FACT is required for DEMETER (DME)-mediated DNA demethylation at certain
42 loci in *Arabidopsis thaliana* female gametophytes during reproduction, though it is not
43 known how FACT targets DME sites. Here, we investigated whether H2AX is involved in
44 DME- and FACT-mediated DNA demethylation during *Arabidopsis* reproduction. We show
45 that *h2a.x* mutants are more sensitive to genotoxic stress, consistent with previous reports.
46 *H2A.X* fused to the *Green Fluorescent Protein (GFP)* gene under the *H2A.X* promoter was
47 highly expressed in newly developing *Arabidopsis* tissues, including in male and female
48 gametophytes. We examined DNA methylation in *h2a.x* developing seeds using whole
49 genome bisulfite sequencing, and found that CG DNA methylation in the developing
50 endosperm, but not the embryo, is decreased genome-wide in *h2a.x* mutants, predominately
51 in transposons and intergenic DNA. Hypomethylated sites overlapped 62 % with canonical
52 DME loci. These data indicate that H2A.X is not required for DME function, but is important
53 for DNA methylation homeostasis in the unique chromatin environment of *Arabidopsis*
54 endosperm.

55

56 **Introduction**

57

58 DNA methylation regulates gene expression and silences transposable elements (TEs) in
59 plants and vertebrates (Law and Jacobsen, 2010), and epigenetic reprogramming by DNA
60 demethylation is vital for reproduction in mammals and flowering plants (Monk et al.,
61 1987;Feng et al., 2010;Wu and Zhang, 2017;Parrilla-Doblas et al., 2019). In *Arabidopsis*
62 *thaliana*, DNA demethylation during reproduction is catalyzed by the DNA glycosylase
63 DEMETER (DME) (Choi et al., 2002). DME is a dual function glycosylase/AP lyase, which
64 actively removes DNA methylation via the Base Excision Repair (BER) pathway (Gehring et
65 al., 2006).

66 DME-mediated DNA demethylation occurs genome-wide at discrete loci that fall into two
67 groups. The first consists of relatively euchromatic, AT-rich, small TEs that are nucleosome-
68 poor, and generally interspersed with genes in chromosome arms (Ibarra et al., 2012). The

69 second group of loci requires the Facilitates Chromatin Transactions (FACT) complex for
70 DME access, and are longer, heterochromatic TEs prevalent in pericentromeric, gene poor
71 regions, enriched with heterochromatic histone marks and H1 linker proteins (Frost et al.,
72 2018). During reproduction DME and DME-FACT mediated DNA demethylation occurs
73 specifically in male and female gamete companion cells, the vegetative and central cells,
74 respectively (Ibarra et al., 2012; Park et al., 2017), and is vital for *Arabidopsis* reproduction,
75 whereby loss of maternal DME or FACT results in development abnormalities, loss of
76 genomic imprinting and seed abortion (Choi et al., 2002; Gehring et al., 2006; Hsieh et al.,
77 2009; Ikeda et al., 2011; Ibarra et al., 2012).

78 FACT is required for several other vital cellular functions, including transcription
79 initiation and elongation, nucleosome assembly and disassembly, and for histone variant
80 exchange, specifically of H2A.X (Belotserkovskaya et al., 2003; Heo et al., 2008; Formosa,
81 2012; Piquet et al., 2018). In *Arabidopsis*, H2A.X is essential for the response to DNA
82 damage, whereby the phosphorylation of its SQEF motif by Ataxia Telangiectasia Mutated
83 (ATM) and ATR kinases, serves as a signal for recruitment of DNA repair and checkpoint
84 proteins (Du et al., 2006; Heo et al., 2008; Dantuma and van Attikum, 2016). It is not known
85 how FACT is recruited to DME target sites, and the apurinic/aprimidinic (AP) sites created
86 during base-excision repair (BER) can lead to the formation of double strand breaks
87 (Sczepanski et al., 2010). We therefore sought to explore whether recruitment of H2A.X to
88 sites of DME activity during BER may provide a functional link between H2A.X, FACT and
89 DME during *Arabidopsis* reproduction. In order to investigate this, we analyzed the
90 expression and activity of *H2A.X* during *Arabidopsis* reproduction, finding that *H2A.X* is
91 expressed throughout the plant, particularly in developing tissues and the male and female
92 gametophytes. The loss of H2A.X does not impair DME-mediated DNA demethylation,
93 instead leading to CG hypomethylation at DME sites, as well as other intergenic regions and
94 transposable elements, specifically in the endosperm.

95

96 **Materials and Methods**

97

98 **Plant materials and growth conditions**

99 *Arabidopsis* seeds were bleached and sown onto Murashige and Skoog plates, followed by
100 vernalisation in the dark at 4 degrees for 3 days, and two weeks growth in a light chamber,
101 before transplantation onto soil. Seedlings were grown in a greenhouse with a long-day

102 photoperiod (16 h light, 8 h dark). Seed stocks of T-DNA insertion mutants (SALK_012255 in
103 HTA3 and SAIL_382_B11/CS873648 in HTA5, Figure 1A) in the Columbia-0 (Col-0)
104 background were obtained from the ABRC stock center. Mutant alleles were, backcrossed five
105 times to wild-type, and finally crossed to obtain double *hta3/hta3; hta5/hta5* null plants,
106 designated *h2a.x*, as well as segregating wild-type siblings. Both T-DNA insertion alleles have
107 been studied and validated in recent reports (Lorkovic et al., 2017; Waterworth et al., 2019).

108

109 **Edu cell proliferation assay**

110

111 5-ethynyl-2'-deoxyuridine (EdU) staining using an Invitrogen Click-iT™ EdU Alexa Fluor™
112 488 HCS Assay (C10350) was performed to detect S phase cells. Seeds were grown in MS
113 media vertically for 3 days. Seedlings were collected in MS solution containing 1μM Edu
114 and incubated at 22°C for 30 minutes. And then, samples were fixed in 4% (w/v)
115 formaldehyde solution in phosphate-buffered saline (PBS) with 0.1% Triton X-100 for
116 30min, and washed three times with PBS each for 5 minutes. The samples were incubated in
117 Edu detection cocktail solution at room temperature for 30 minutes in the dark, and washed
118 with the Click-iT® rinse buffer and then three times with PBS. The photos were taken using
119 confocal microscopy (LSM700, Zeiss).

120

121 **Propidium Iodide (PI) staining**

122

123 Propidium Iodide (P-4170, sigma) staining was used to detect cell death and show the
124 anatomy of the roots. The samples were stained with working PI solution (5ml PI solution in
125 1ml of distilled water) at room temperature for 30s and washed with distilled water on slide
126 glass.

127

128 **Observation of root hair phenotypes**

129

130 Root hair phenotypes were observed under a stereomicroscope (M205 FA, Leica). Root hair
131 length was measured as previously described by (Lee and Cho, 2006) with slight
132 modifications. DAG3 seedling roots were photographed digitally using the stereomicroscope
133 at 40X magnification. The hair length of nine consecutive hairs which protruded
134 perpendicularly from each side of the root, for a total of 18 hairs from both sides of the root,

135 was calculated using ImageJ 1.50b software (National Institutes of Health).

136

137 ***H2A.X* expression localization**

138

139 HTA3 and HTA5 GFP fusion proteins were cloned alongside a hygromycin resistance cassette
140 using a Gibson assay (Invitrogen) and F1 seeds screened on MS containing hygromycin. F1
141 plants were screened manually using a fluorescence microscope and seeds collected from
142 plants expressing GFP. F2 seeds were grown on hygromycin and selected if we identified
143 segregation of the resistance allele, indicating the presence of a single copy transgene. F3
144 plants were then used for confocal microscopy.

145

146 **DNA damage assay**

147

148 *Arabidopsis* seeds were planted on MS containing 0.5ug/ml bleomycin sulphate and grown
149 vertically for 14 days under long day conditions, before measuring root length. MS without
150 bleomycin was used as a control. Values are from three independent experiments each
151 including 15 seedlings for each genotype. Truf leaf assay was performed as previously
152 described with 10-day-old seedlings (Min et al., 2019).

153

154 **Isolation of *Arabidopsis* endosperm and embryos**

155

156 WT Col-0 and *h2a.x* mutant *Arabidopsis* flower buds were emasculated at flower stage 12-13
157 using fine forceps and pollinated with *Ler* pollen 48 hours later. Eight to ten days after
158 pollination (DAP) developing F1 seeds (linear to bending cotyledon stage) were immersed in
159 dissection solution (filter-sterilized 0.3 M sorbitol and 5 mM pH 5.7 MES) on sticky tape and
160 dissected by hand under a stereo-microscope using fine forceps (Fine Science Tools, Inox
161 Dumont #5) and insect mounting pins. The seed coat was discarded, and debris removed by
162 washing collected embryos or endosperm five to six times with dissection solution under the
163 microscope.

164

165 **Bisulfite sequencing library construction**

166

167 As described previously, genomic DNA was isolated from endosperm and embryo (Hsieh et
168 al., 2009). Bisulfite sequencing libraries for Illumina sequencing were constructed as in

169 (Ibarra et al., 2012) with minor modifications. In brief, about 50 ng of genomic DNA was
170 fragmented by sonication, end repaired and ligated to custom-synthesized methylated
171 adapters (Eurofins MWG Operon) according to the manufacturer's instructions for gDNA
172 library construction (Illumina). Adaptor-ligated libraries were subjected to two successive
173 treatments of sodium bisulfite conversion using the EpiTect Bisulfite kit (Qiagen) as outlined
174 in the manufacturer's instructions. The bisulfite-converted library was split between two 50
175 μ l reactions and PCR amplified using the following conditions: 2.5 U of ExTaq DNA
176 polymerase (Takara Bio), 5 μ l of 10X Extaq reaction buffer, 25 μ M dNTPs, 1 μ l Primer 1.1
177 and 1 μ l multiplexed indexing primer. PCR reactions were carried out as follows: 95°C for 3
178 minutes, then 14-16 cycles of 95 °C 30 s, 65 °C 30 s and 72 °C 60 s. Enriched libraries were
179 purified twice with AMPure beads (Beckman Coulter) prior to quantification with the Qubit
180 fluorometer (Thermo Scientific) and quality assessment using the DNA Bioanalyzer high
181 sensitivity DNA assay (Agilent). Sequencing on either the Illumina HiSeq 2000/2500 or
182 HiSeq 4000 platforms was performed at the Vincent J. Coates Genomic Sequencing
183 Laboratory at UC Berkeley.

184

185 **Bisulfite data analysis**

186

187 Sequenced reads were sorted and mapped to the TAIR8 Col-0 and Ler genomes in cases of
188 seeds derived from Col x Ler crosses, or not sorted and mapped to TAIR8 Col-0. Gene and
189 TE ends analysis and kernel density plots were generated as previously described (Ibarra et
190 al., 2012), using only windows with at least 10 informative sequenced cytosines, and
191 fractional methylation of at least 0.7 (CG), 0.4 (CHG) or 0.08 (CHH) in at least one of the
192 samples being compared.

193

194 **Results**

195

196 ***Arabidopsis* seedlings lacking *H2A.X* have reduced DNA damage tolerance**

197

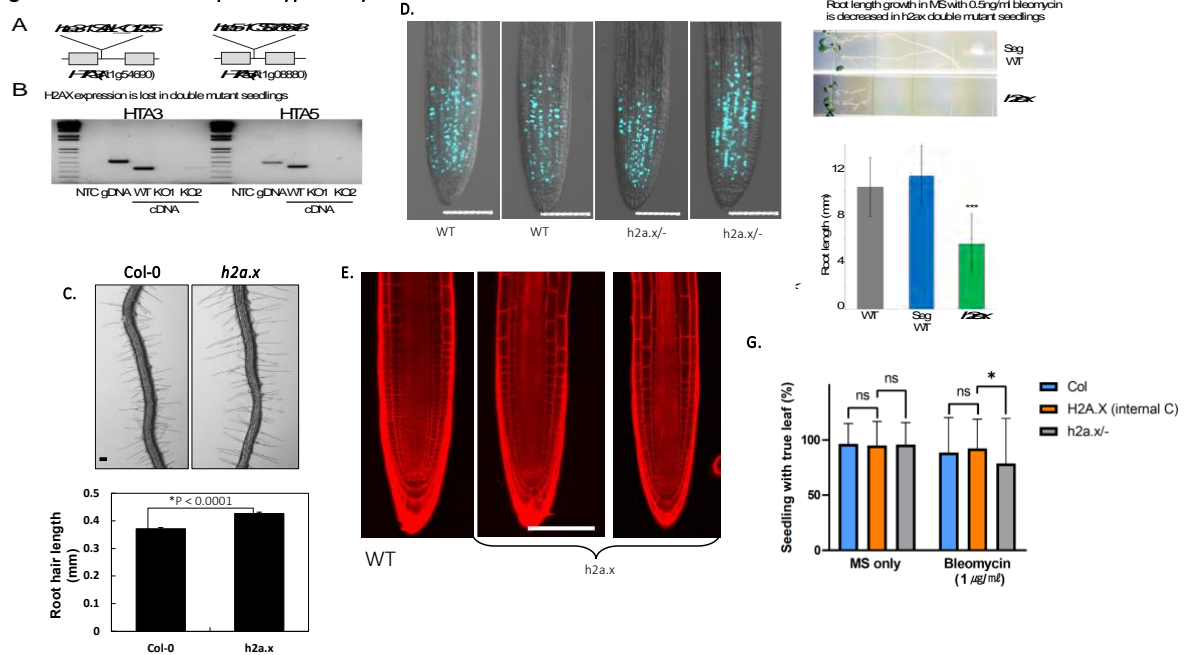
198 *H2A.X* is encoded by two genes in *Arabidopsis*, HTA3 (AT1G54690) and HTA5
199 (AT1G08880). To investigate the effect of *H2A.X* mutations, we generated double mutants
200 lacking both HTA3 and HTA5, verified the loss of transcripts using RT-PCR (Figure 1B) and
201 analyzed the sporophytic and gametophytic phenotypes of *h2a.x* plants. *h2a.x* mutant allele
202 segregation, plant morphology, growth rate and flowering time were all normal, except for a

203 significant increase in root hair length (Figure 1C, $p < 0.0001$). *h2a.x* root hairs were ~15 %
204 longer than WT three days after germination (DAG). We then tested whether cell
205 proliferation was normal in *h2a.x*, using the 5-ethynyl-2'-deoxyuridine (EdU), a thymine
206 analog, and click chemistry to measure incorporation in newly synthesized DNA (Kotogany
207 et al., 2010). We did not observe a difference in EdU-stained cells between wild-type and
208 *h2a.x* roots (Figure 1D), indicating that S-phase progression and cell proliferation are normal
209 in *h2a.x* mutants. We also measured whether there was increased DNA damage in mutant
210 roots using propidium iodide (PI) staining but did not observe any differences between WT
211 and *h2a.x* (Figure 1E). These observations are consistent with mutant phenotypes observed in
212 other DNA damage pathway genes, such as ATM or ATR kinases, which only exhibit a
213 phenotype under growth conditions that promote DNA damage (Culligan et al., 2006).

214 We therefore grew *h2a.x* and segregating WT seeds on MS plates containing
215 Bleomycin sulphate, which induces double strand breaks (DSB) in DNA. MS Bleomycin
216 concentrations of 0.5 ng/ml were used to test primary root formation and 1 ug/ml to test true
217 leaf formation, as root development was more sensitive to the drug. *h2a.x* mutant seedlings
218 had a significant reduction in root length compared to WT (Figure 1F). True leaf formation
219 rate is slightly reduced in *h2a.x* mutants (Figure 1G). These data are consistent with previous
220 findings, also showing aberrant true leaf and root growth in *h2a.x* double mutant seedlings
221 grown under genotoxic stress (Lorkovic et al., 2017). Thus, a lack of *H2A.X* resulted in
222 increased sensitivity of developing tissues to DNA damaging agents, showing that *H2A.X* is
223 required for the response to DNA damage in *Arabidopsis*.

224

Figure 1 – *h2a.x* mutant phenotype analysis



225

226 **Figure 1. *h2a.x* mutant phenotype analysis**

227 (A) HTA.3 and HTA.5 genomic loci, showing gene structure and location of T-DNA insertions. (B) qPCR
 228 analysis of each mutant, showing cDNA-specific PCR amplification and loss of gene product in mutant seedling
 229 tissue (C) Root hair phenotypes of wild-type (Col-0) and *h2a.x* mutant primary roots and length measurements
 230 in mm. Data represent mean \pm SEM (n = 1,419 root hairs for Col-0 and 1,159 root hairs for *h2a.x* from 35 ~ 40
 231 roots. The asterisk (*) indicates a significant difference (Student's t test). Scale bar, 100 μ m. (D) EdU staining
 232 of WT and *h2a.x* double mutant roots at 3 DAG. Scale bar, 100 μ m. (E) Propidium iodide (PI) staining of WT
 233 and *h2a.x* double mutant roots. Scale bar, 100 μ m. (F) Aberrant root growth of *h2a.x* mutant seedlings when
 234 grown in bleomycin MS. Root length measurements are in mm and the result of three replicate experiments,
 235 each with 15 seedlings. (G) The formation of true leaves was slightly reduced in *h2a.x* mutant seedlings when
 236 grown in bleomycin MS. Measurements are the result of duplicated experiments. Leaves counted are; 415 in MS
 237 only and 203 in bleomycin MS for Col-0, 395 in MS only and 209 in bleomycin MS for H2A.X internal
 238 segregated WT control, 406 in MS only and 202 in bleomycin MS for *h2a.x* mutant. The box height and whisker
 239 length indicate the mean and standard deviation of each sample, respectively. The significance of differences
 240 between samples was measured by the Kolmogorov-Smirnov test. ns, not significant; * p=0.0440

241

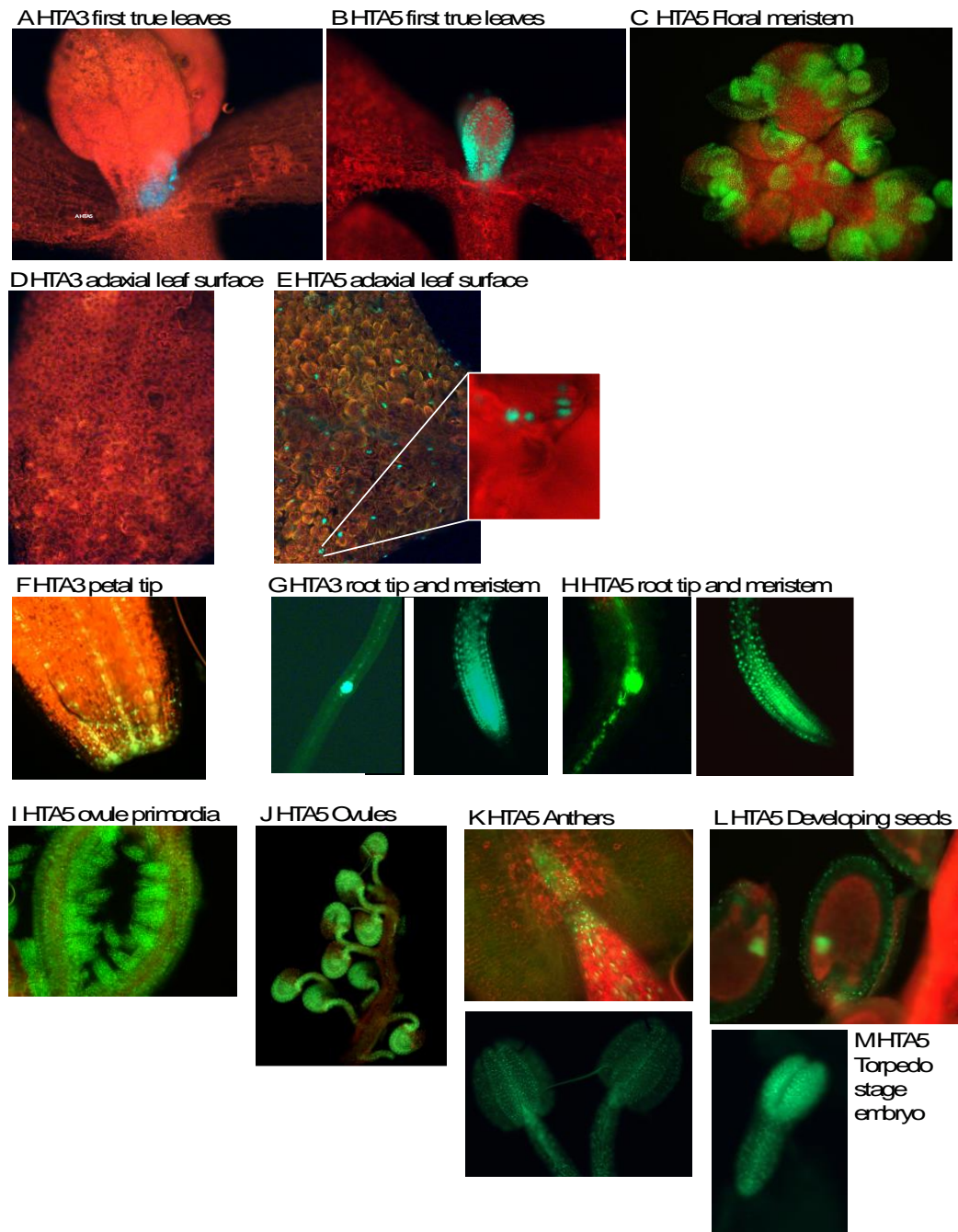
242 ***H2A.X* is widely expressed across *Arabidopsis* tissues, including in gamete companion**
 243 **cells**

244

245 To investigate the role of *H2A.X* in *Arabidopsis* development, we analyzed its expression
 246 pattern in sporophytic and reproductive tissues. We generated translational fusion constructs
 247 between the Green Fluorescent Protein (GFP) gene and the HTA3 and HTA5 genes,
 248 including their promoter sequences, and introduced them into WT Col-0 *Arabidopsis* plants

249 using *Agrobacterium* mediated transfer, deriving three and four independent lines for each
250 allele, respectively. GFP fluorescence was observed using confocal microscopy. Both HTA3
251 and HTA5 were expressed in dividing cells of the sporophyte: First true leaves (Figure 2A
252 and B), the floral meristem (Figure 2C), the adaxial leaf surface (Figure 2D and E), petal tips
253 (Figure 2F), in root meristem and root tips (Figure 2G and H). In reproductive structures
254 supporting gametophyte development, such as the ovule primordia (Figure 2I), ovules (Figure
255 2J), and anthers (Figure 2K) both isoforms were expressed. In the next generation seed, both
256 isoforms were present in the developing embryo, but not in the endosperm (Figure 2L and
257 M). In each case, HTA5 expression was more prevalent and more widely expressed than
258 HTA3. Conversely, in gametophytic development, both isoforms were again expressed but
259 HTA3 was the dominant isoform (Figure 3). In the male gametophyte, both HTA3 and HTA5
260 were present in the microspore prior to mitosis. After Pollen Mitosis 1 (PMI) HTA3 was
261 expressed in the generative and vegetative nucleus of bicellular pollen, and following Pollen
262 Mitosis II (PMII), in both sperm cells and the vegetative nucleus of mature, tricellular pollen
263 (Figure 3A). HTA5 expression was also present in both the generative and vegetative
264 nucleus following PMI, but was lost in the vegetative nucleus following PMII, in tricellular
265 pollen (Figure 3B). In the female gametophyte, egg cell expression was visible for both
266 HTA3 and 5, but was weak, conversely, HTA3 expression was very striking in the central
267 cell, where it persisted following fertilization in the first cell divisions of the developing
268 endosperm (Figure 3C and E). HTA5 expression was also observed in the central cell, but
269 expression in the surrounding ovule tissue was more prominent for this isoform (Figure 3D).

Figure 2



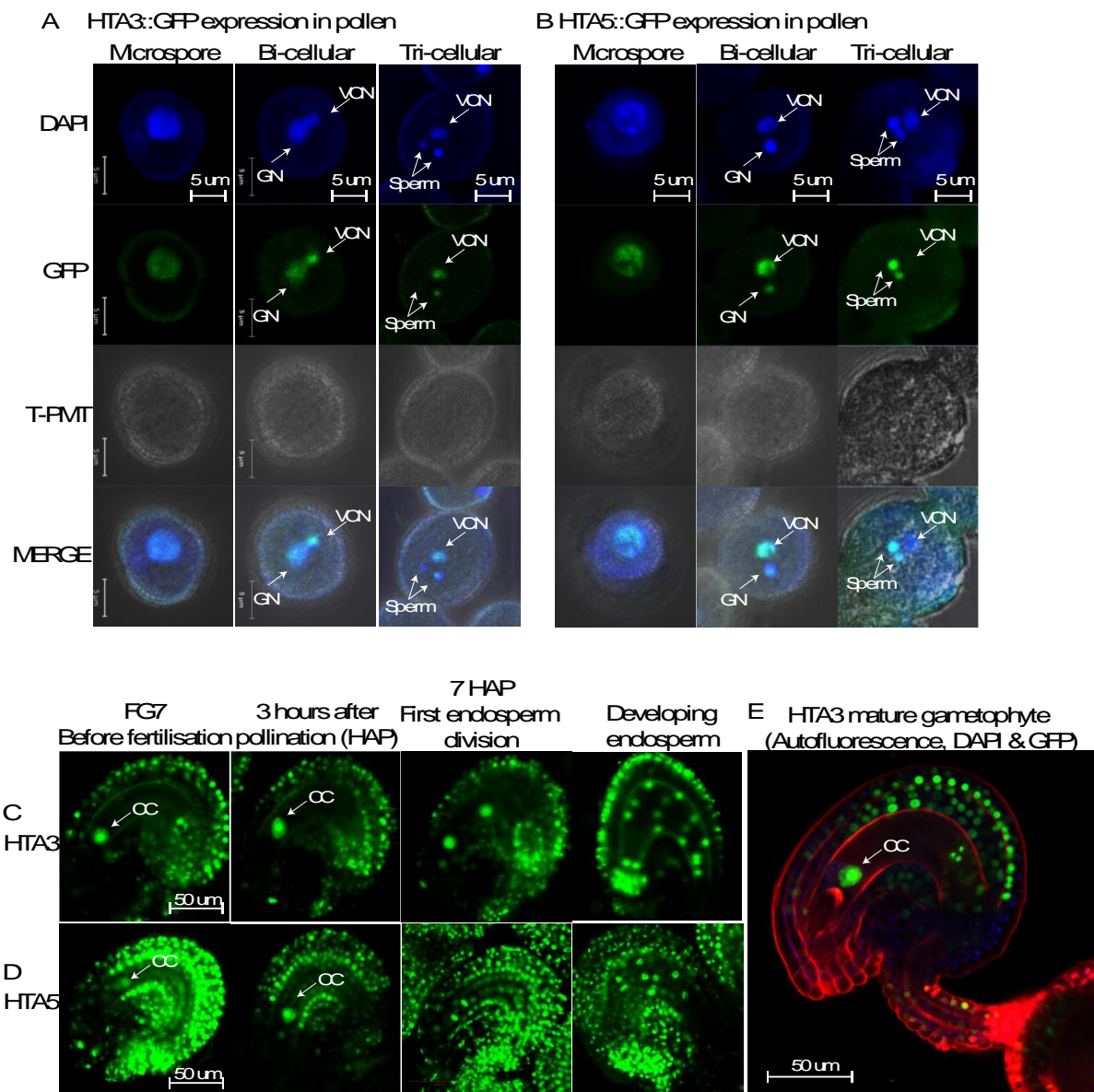
270

271 **Figure 2. HTA5 expression is more dominant in dividing cells of the sporophyte.**

272 First true leaves (A and B), the floral meristem (C), the adaxial leaf surface (D and E), petal tip
273 meristem and root tips (G and H). In reproductive structures supporting gametophyte development such as the
274 ovule primordia (I), ovules (J), and anthers (K), both isoforms were expressed. In the next generation seeds,
275 both isoforms were present in the developing embryos (L and M).

276

Figure 3



277

278 **Figure 3. *HTA3* expression is more dominant in gametophytic development.**

279 In the male gametophyte, both *HTA3* and *HTA5* are present in the microspore prior to mitosis. After Pollen

280 Mitosis I (PMI) *HTA3* was expressed in the generative and vegetative nucleus of bicellular pollen, and

281 following Pollen Mitosis II (PMII), in both sperm cells and the vegetative nucleus of mature, tricellular pollen

282 (A). *HTA5* expression was also present in both the generative and vegetative nucleus following PMI, but was

283 lost in the vegetative nucleus following PMII, in tricellular pollen (B). In the female gametophyte, egg cell

284 expression was visible for both *HTA3* and *HTA5*, but was weak. Conversely, *HTA3* expression was very striking

285 in the central cell, where it persisted following fertilization in the first cell divisions of the developing

286 endosperm (C and E). *HTA5* expression was also observed in the central cell, but expression in the surrounding

287 ovule tissue was more prominent for this isoform (D).

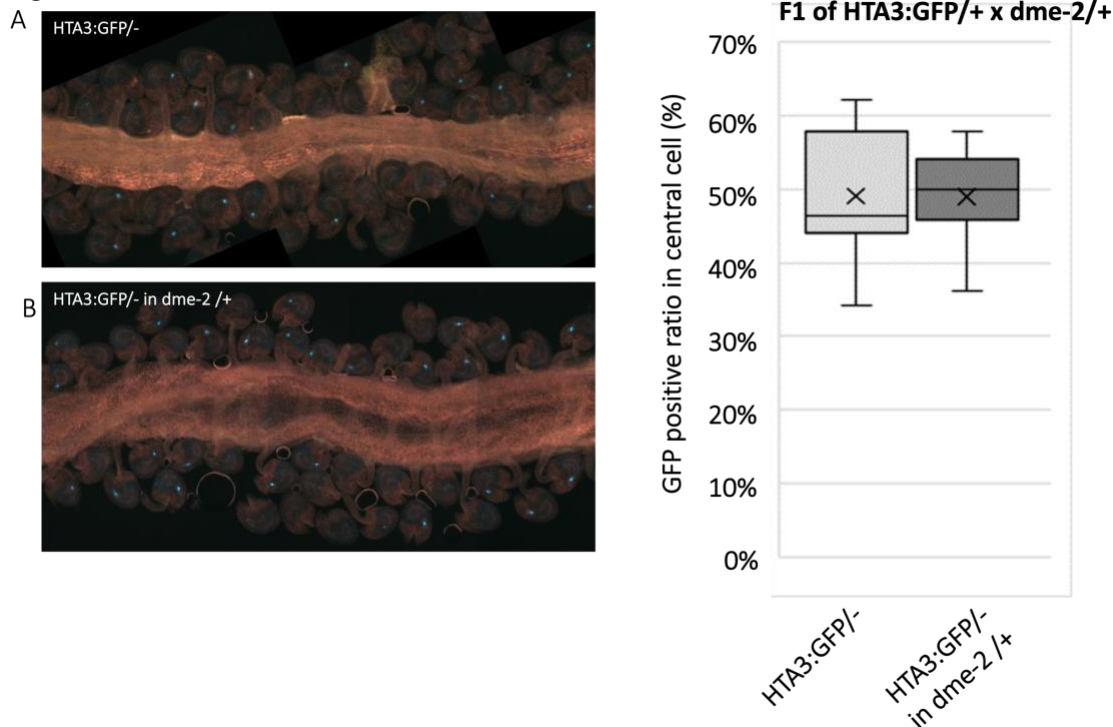
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289 **DME does not regulate *H2A.X* expression in the *Arabidopsis* gametophyte**

290

291 Since *H2A.X* expression was prominent in the central and vegetative cells, specifically where
292 DME-mediated demethylation and related BER activity takes place (Schoft et al., 2011; Park
293 et al., 2017), we reasoned that *H2A.X* expression may be regulated by promoter DNA
294 methylation, whereby DME might demethylate *HTA3* and *HTA5* promoter sequences in the
295 gametophyte, increasing expression of these transcripts. To test this hypothesis, we utilized
296 wild-type plants hemizygous for the *HTA3:GFP* transgene, for which strong *HTA3*
297 expression could be observed in the central cell in ~50 % of the developing ovules (Figure 4).
298 We crossed these plants with DME/*dme-2* heterozygotes to derive DME/*dme-2* plants that
299 were also hemizygous for the *HTA3:GFP* transgene. A maternally inherited *dme-2* mutation
300 generates embryo abortion and seed lethality, so analysis of seed development is generally
301 only possible in DME/*dme-2* heterozygotes. We then analyzed the incidence of *HTA3:GFP*
302 expression in DME/*dme-2* mutants and their segregating wild-type siblings in the F2
303 population. In both DME/DME *HTA3:GFP*⁻ (Figure 4A) and DME/*dme-2* *HTA3:GFP*⁻ F2
304 (Figure 4B) siblings we observed that ~50 % of the female gametophytes within ovules
305 produced a strong GFP signal, indicating that the loss of DME did not alter the expression of
306 *H2A.X* in the *Arabidopsis* female gametophyte. Consistent with this, when we compared
307 promoter DNA methylation for the *H2A.X* variants in *Arabidopsis* wild-type and *dme-2*
308 mutant central cells and endosperm (Hsieh et al., 2009; Park et al., 2016), we found that
309 *H2A.X* promoter methylation was low in both tissues, and unchanged in the *dme-2* mutant
310 (Supplementary Figure S1A and 1B). Other *H2A* variant gene loci were also unmethylated in
311 both wild-type and *dme-2* mutant central cell and endosperm, except for *H2A.Z.4*, which
312 exhibited promoter methylation in central cell and endosperm, that increased in *dme-2*
313 mutants, a hallmark of a DME-target promoter (Supplementary Figure S1C).

Figure 4



314

315 **Figure 4. *HTA.3:GFP* expression in WT and *dme-2* mutant central cells.**

316 (A) Confocal image of WT and *dme-2* mutant developing ovules expressing an *HTA.3:GFP* transgene.

317 Expression is confined to the central cell. (B) Box plot showing the distribution of GFP positive central cells

318 between WT and *dme-2* mutant ovules. There was no significant change in GFP positive central cells in

319 DME/*dme-2*. + mark in boxplot is mean of data. The line in a box is median (n = 554 mature ovules for

320 HTA3:GFP/- plants and 412 mature ovules for HTA3:GFP/- in DME/*dme-2* heterozygous plants. The values are

321 not significantly different by Student's *t* test.

322

323 ***h2a.x* mutant endosperm is hypomethylated genome-wide**

324

325 To investigate whether changes in DNA methylation were present in *h2a.x*, we carried out

326 genome-wide bisulfite sequencing (BS-seq) of manually dissected endosperm and embryo

327 from homozygous *h2a.x* mutant and wild-type F1 seeds and their resulting seedlings,

328 following self-pollination of homozygous *h2a.x* mutants and wild-type sibling plants. We

329 observed that embryo DNA methylation in the *h2a.x* mutant was unchanged from wild-type,

330 with the peak of fractional methylation difference at zero (Figure 5A). However, DNA

331 methylation of *h2a.x* mutant endosperm was reduced compared to wild-type in the CG

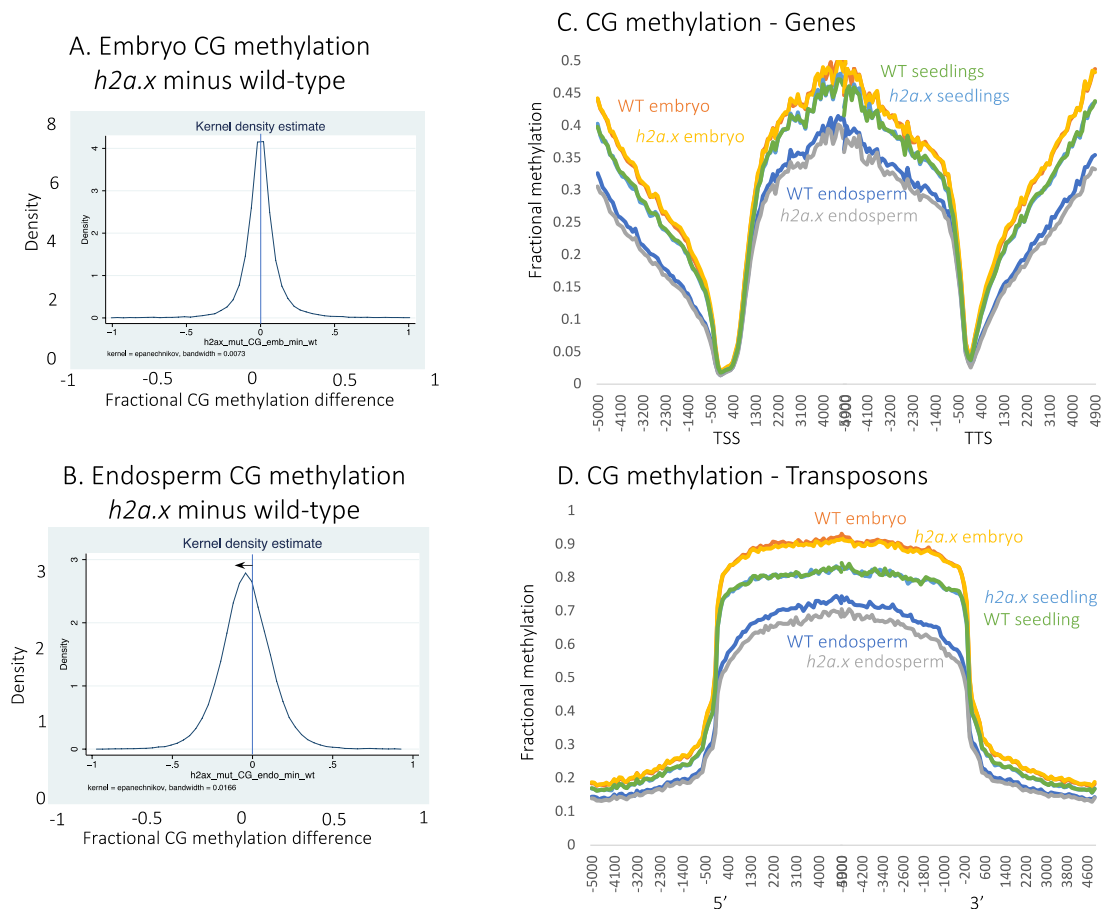
332 context, with the fractional methylation difference peak shifted to the left (Figure 5B).

333 To ascertain which endosperm loci were hypomethylated, we aligned our methylome data to

334 the 5' transcriptional start sites (TSS) and 3' transcriptional termination sites (TTS) of genes

335 and transposons, and also included methylome of *h2a.x* seedlings, which also showed that
 336 hypomethylation was present only in endosperm, and although present in gene bodies and
 337 intergenic regions, was most striking in transposon bodies, (Figure 5C and D). CHG and
 338 CHH methylation was also reduced in endosperm transposon bodies (Supplementary Figure
 339 S2A-D). In *h2a.x* embryos, CHH methylation in TEs was decreased (Figure S5D), although
 340 embryo CHH methylation increases steadily with time during embryo development
 341 (Papareddy et al., 2020) so it is possible the differences observed in embryos are a technical
 342 difference, whereby mutant seeds were dissected earlier than wild-type.

Figure 5 – Methylome analysis of homozygous H2AX mutant seeds (selfed) and seedlings



343
 344 **Figure 5. Genome-wide methylation analysis of selfed double *h2a.x* mutant developing embryo,**
 345 **endosperm and seedling.**
 346 (A) Fractional methylation difference between *h2a.x* double mutant and WT CG methylation from embryo
 347 (linear-bending cotyledon) is plotted, data in 50 bp windows with >10x sequence coverage. Data are from *h2a.x*
 348 Col selfed plants and segregating wild type siblings. (B) As for A, but with endosperm. (C) Ends analysis of
 349 *h2a.x* mutant genomic methylation in genes, with genes aligned according to their 5' and 3' ends. (D) Ends
 350 analysis of *h2a.x* mutant genomic methylation in transposons, with transposons aligned according to their 5' and
 351 3' ends.

352

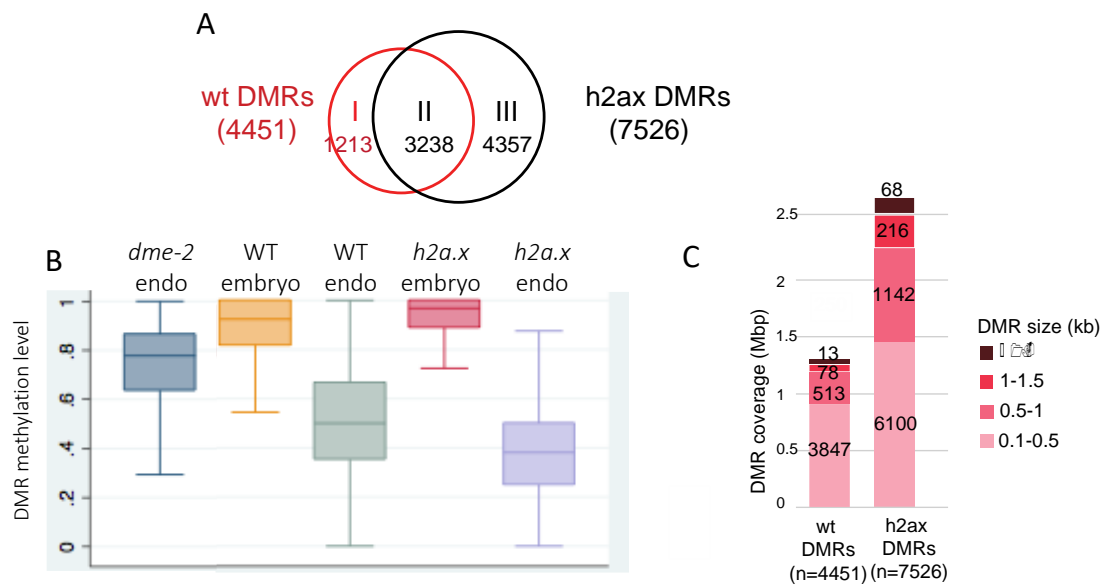
353 **H2A.X hypomethylation overlaps DME target loci**

354

355 Inheritance of a loss-of-function maternal *dme* allele or a loss-of-function maternal *ssrpl*
356 allele, which encodes one of the proteins in the FACT complex, results in a striking
357 phenotype of seed abortion and developmental delay. Seed viability in homozygous *h2a.x*
358 mutants, as well as in crosses from maternal *h2a.x* with wild-type Col-0 pollen, was normal,
359 suggesting that DME- and DME-FACT- mediated DNA demethylation occurred normally in
360 *h2a.x* mutant seeds, at least in the DME-regulated PRC2 genes critical for seed viability. In
361 wild type female gametophytes, DME and DME-FACT mediated DNA demethylation leads
362 to a hypomethylated endosperm compared to embryo (Hsieh et al., 2009;Ibarra et al.,
363 2012;Park et al., 2016;Frost et al., 2018). To assess whether *h2a.x* hypomethylation may still
364 be related to DME activity, we compared differential methylated regions (DMRs) between
365 endosperm and embryo in WT and *h2a.x* mutant seeds. There were 4,451 hypo-DMRs
366 between WT endosperm vs embryo, covering about 1.3 M bps, which reflect the activity of
367 DME in the central cell. In contrast, 7,526 hypo-DMRs were identified between *h2a.x*
368 endosperm vs embryo, covering 2.7 M bps in length, more than double the area of the wild-
369 type hypo-DMRs (Figure 6A). Of these, 4692 (62 %) overlapped with canonical DME DMR
370 loci (Ibarra et al., 2012). The hypo-DMRs consisted of both WT embryo-endosperm DMRs
371 (n=3238) and novel *h2a.x* specific DMRs (n = 4357, Figure 7A and B). There was also a
372 group of WT DMRs, which were only differentially methylated between WT embryo and
373 endosperm (n=1213). We delineated DMRs by size (0.1 kb->1.5 kb) and found that *h2a.x*
374 embryo-endosperm DMRs were represented across all size classes (Figure 6C).

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Figure 6 – Endosperm-Embryo hypomethylated DMR analysis in WT and *h2ax* mutants



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377 **Figure 6.** Analysis of *h2ax* methylomes, comparing DMRs between endosperm and embryo. (A) Venn diagram
 378 illustrating that WT embryo and endosperm harbour 4451 DMRs, the majority of which (3238) are shared with
 379 *h2ax* embryo and endosperm. *h2ax* embryo and endosperm have an additional 4357 DMRs. (B) Box plots
 380 showing the relative methylation level of DMRs in embryo and endosperm, in wild type, *h2ax* and *dme-2*
 381 mutants (C) Characterization of *h2ax*-specific embryo-endosperm DMRs; wild-type and *h2ax* Endosperm-
 382 Embryo DMRs grouped by size, with the cumulative total length they covered shown, whereby they are
 383 represented across all DMR sizes, and represent an overall increase in size distribution.

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385 ***h2ax* mutant endosperm hypomethylation is not allele-specific**

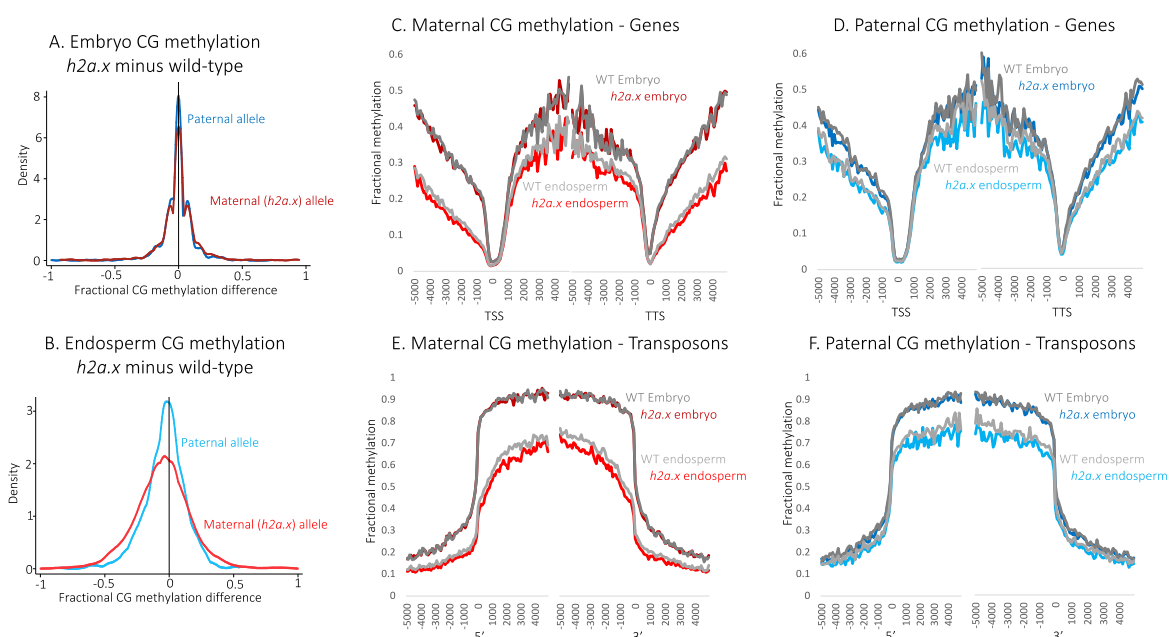
386

387 Seeds are formed following fertilization of both egg and central cell with sperm, resulting in
 388 the diploid zygote and triploid endosperm, respectively. Since *h2ax* hypomethylation is
 389 confined to the endosperm, we deduced that this effect may result from a loss of *h2ax* in
 390 the central cell. Consistent with this idea, both *H2A.X* isoforms are strongly expressed in the
 391 wild-type central cell (Figure 3C and D). To identify changes in DNA methylation in the
 392 *h2ax* central cell, we pollinated maternal *h2ax* mutant plants in the Columbia ecotype with
 393 wild-type *Ler* pollen. We manually-dissected embryo and endosperm from mutant and
 394 segregating wild-type seeds and following BS-Seq, sorted the reads according to their
 395 parental ecotype. In this way, the maternal endosperm genome can be used as a proxy for the
 396 central cell genome. In *h2ax* embryos, both maternal and paternal allele CG methylation is
 397 identical to WT (peak aligns on zero. Figure 7A), consistent with our observations in self-
 398 pollinated *h2ax* mutants (Figure 5A). However, in endosperm, a slight shift is visible
 399 towards the left, indicating mutant hypomethylation, present for both maternal and paternal

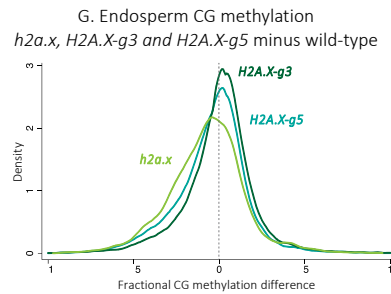
400 endosperm (Figure 7B). This indicates that whilst hypomethylation may be inherited from the
 401 central cell, resulting in hypomethylated maternal alleles, hypomethylation of the paternal
 402 allele must manifest post-fertilization, perhaps due to a reduction in CG methylation
 403 efficiency or maintenance. To ascertain which loci were hypomethylated, we again aligned
 404 our methylomes to the TSS/5' and TTS/3' ends of genes and transposons (Figure 7C-F and
 405 Supplementary Figure S3A-D). As in the previous analysis, CG methylation in embryo is not
 406 different from wild-type in *h2a.x* mutant gene and transposon bodies, but both maternal and
 407 paternal *h2a.x* endosperm alleles are hypomethylated in genes, intergenic regions and TEs,
 408 with hypomethylation in TE bodies being most striking. CHG methylation is the same in
 409 wild-type and *h2a.x* mutant embryo and endosperm (Supplementary Figure S3E-H) whereas
 410 CHH methylation is decreased on both parental end alleles, in both *h2a.x* embryo and endosperm
 411 (Supplementary Figure S3I-L).

412 *H2A.X* is encoded by two almost identical isoforms, HTA3 and HTA5. To determine
 413 whether one isoform may have an effect independent of the other, we dissected developing
 414 seeds from both *hta3/hta3 hta5/+* (*H2A.X-g3*) and *hta3/+ hta5/hta5* mutants (*H2A.X-g5*),
 415 crossed to Ler, so that the sporophyte had one remaining copy of one of the isoforms, but
 416 both *H2A.X* isoforms are lost in 1/2 of the gametophytes. Following BS-seq, we determined
 417 that both isoforms act redundantly, whereby endosperm methylation was not substantially
 418 affected in either *hta3/hta3 hta5/+* or *hta5/hta5 hta3/+* seeds (Figure 7G, *H2A.X-g3* and
 419 *H2A.X-g5*, kdensity peaks on zero) compared to hypomethylated *h2a.x* double mutant
 420 endosperm (Figure 7G, *h2a.x* peak shifted to the left).

Figure 7 – Methylome analysis of seeds from *h2a.x* maternal and WT paternal plant crosses



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Figure 7: Genome-wide methylation analysis of *h2a.x* mutant developing embryo and endosperm, comparing maternal and paternal alleles in wild-type (WT) and *h2a.x* mutant crosses whereby the maternal allele is either WT Columbia or *h2a.x* homozygous mutant Columbia, and the paternal allele is always wild-type Ler; ‘*h2a.x* paternal’ denotes a wild-type paternal allele now resident in a heterozygous *h2a.x* mutant seed. (A) Fractional methylation difference between *h2a.x* double mutant gametophyte crossed with Ler WT pollen in CG methylation from embryo (linear-bending cotyledon) is plotted, data in 50 bp windows with >10x sequence coverage (B) As for A, but with endosperm. A slight shift towards the left can be seen for the maternal endosperm allele (inherited from *h2a.x* mutant central cell). (C) Ends analysis of maternal (*h2a.x* mutant) genomic methylation in genes, with genes aligned according to their 5’ and 3’ ends. (D) Ends analysis of paternal genomic methylation in genes, with genes aligned according to their 5’ and 3’ ends. (E) Ends analysis of maternal (*h2a.x* mutant) genomic methylation in transposons, with transposons aligned according to their 5’ and 3’ ends. (F) Ends analysis of paternal genomic methylation in transposons, with transposons aligned according to their 5’ and 3’ ends. (G) Fractional CG DNA methylation difference between mutant and WT maternal endosperm in developing seeds from both *hta3/hta3 hta5/+* (*H2A.X-g3*) and *hta5/hta5 hta3/+* mutants (*H2A.X-g5*) were crossed to Ler, so that the sporophyte had one remaining copy of one of the isoforms, but both *H2A.X* isoforms are lost in ½ of the gametophytes. *H2A.X-g5* and *H2A.X-g3* are plotted alongside *h2a.x*. For both *H2A.X-g3* and *H2A.X-g5*, the curve peaks are close to zero, whereas the full *h2a.x* mutant is skewed to the left, indicating redundancy between HTA5 and HTA3 isoforms in the context of mutant endosperm DNA hypomethylation.

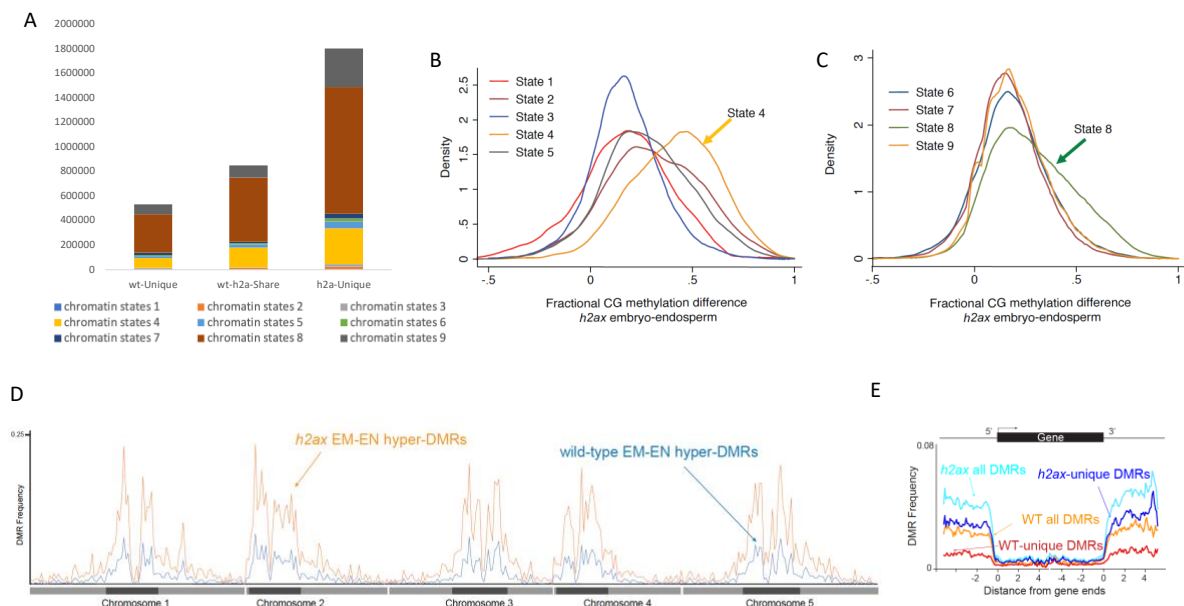
***h2a.x* hypomethylation is widespread in intergenic DNA**

To assess if *h2a.x* endosperm hypomethylated loci are associated with particular chromatin states, we used the published histone marks and genomic characteristics that topologically group the *Arabidopsis* genome into 9 distinct chromatin states (Sequeira-Mendes et al., 2014) and used them to compare methylation differences between *h2a.x* vs wildtype endosperm. For the hypo-DMRs specific to *h2a.x* endosperm vs embryo, the majority reside in non-coding, intergenic sequences, including distal promoters (chromatin state 4, Figure 8A) and AT-rich heterochromatic regions (chromatin states 8 and 9, Figure 8A), consistent with what we observed in TE metaplots (Figure 5C and D, Figure 6E and F). In addition, when we used fractional methylation differences to analyse the chromatin states

454 of hypomethylated loci unique to the *h2a.x* mutant, i.e. not including those present in
 455 between wild-type embryo and endosperm, chromatin states 4 and 8 exhibit the largest shift
 456 (Figure 8B and C). These data indicate that the novel, *h2a.x*-specific DMRs lie primarily in
 457 chromatin states 4, 8 and 9.

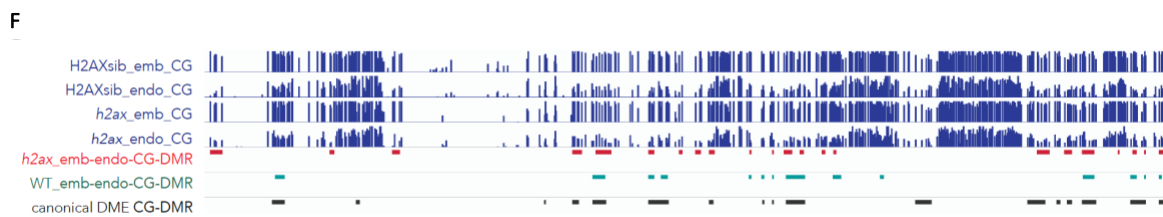
458 In order to characterize the location of *h2a.x* embryo-endosperm DMRs, we plotted their
 459 co-ordinates across the *Arabidopsis* genome in 300 kb bins (see Materials and Methods;
 460 Figure 8D). This analysis showed that *h2a.x* DMRs, in general, mirror the distribution of
 461 wild-type (DME-mediated) embryo-endosperm DMRs, which are enriched in pericentric
 462 heterochromatin (Figure 8D). To gain further resolution on DMR location, we aligned wild-
 463 type and *h2a.x* DMR coordinates according to 5' and 3' ends of genes, which revealed that
 464 *h2a.x* hypomethylation is enriched in intergenic regions, consistent with its enrichment in
 465 chromatin states 4 and 8 (Figure 8E). To determine whether the *h2a.x* endosperm
 466 hypomethylation represented novel sites of demethylation, or resulted from increased
 467 demethylation at already demethylated sites (e.g., resulting in longer DMRs), we took a
 468 locus-specific approach, using the IGV genome browser to view aligned methylation data and
 469 DMRs (Figure 8F). The majority of *h2a.x*-specific hypomethylation represented stand-alone,
 470 novel DMRs (red outline). *h2a.x* DMRs overlapped DME-mediated wild-type
 471 endosperm/embryo DMRs (green outline), but did not make them longer.

Figure 8 – Chromatin states of *h2a.x* DMRs



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475 **Figure 8:** (A) Comparison of the chromatin states comprising each group of DMRs from Figure 6A. Chromatin
476 state distribution, and the total length they covered, within wt-unique, wt-*h2a.x* shared, and *h2a.x*-unique
477 Endosperm-Embryo DMRs. States 1 to 7 correspond to euchromatin, and states 8 and 9 correspond to AT- and
478 GC-rich heterochromatin, respectively. The chromatin states most increased (as a fraction of their total) in *h2a.x*
479 embryo-endosperm DMRs are 4, 8 and 9. (B and C) Kernel density plots showing the fractional methylation
480 difference for *h2a.x* mutant embryo minus endosperm, plotted according to chromatin state, demonstrating that
481 the largest shift to endosperm hypomethylation (i.e. to the right) lies in State 4 (yellow in B) and State 8 (green
482 in C). (D) Arabidopsis chromosome view of genome-wide methylation levels for *h2a.x* mutant DMRs between
483 embryo and endosperm, and WT DMRs between embryo and endosperm, represented by the distribution
484 frequency of DMRs along the 5 chromosomes. Dark blocks represent centromere and peri-centromeric regions
485 of each chromosome. (E) Ends analysis plot showing distribution frequency of DMRs with respect to coding
486 genes. Genes were aligned at the 5'- or the 3'-end, and the proportion of genes with DMRs in each 100-bp
487 interval is plotted. DMR distribution is shown with respect to all wt-DMRs (orange trace), wt-unique DMRs
488 (red trace), all *h2a.x*-DMRs (cyan trace), and *h2a.x*-unique DMRs (blue trace).
489 (F) IGV browser view of methylome data and DMR calls for H2AX segregating wild-type embryo and
490 endosperm (Green), *h2a.x* mutant embryo and endosperm (Red), as well as DMRs identified between *dme-2*/wt
491 endosperm (Black) (Ibarra et al., 2012).

492

493 Discussion

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495 *H2A.X* is one of the H2A variants in higher eukaryotes and differs from canonical H2A by its
496 rapid phosphorylation to γ -*H2A.X* in response to DNA double-strand breaks. Unmodified
497 *H2A.X* is ubiquitously expressed and distributed throughout the genome as a component of
498 nucleosome core structure, estimated to represent approximately 10 % of H2A variants
499 present in chromatin at any given time (Rogakou and Sekeri-Pataryas, 1999; Celeste et al.,
500 2003a; Celeste et al., 2003b; Seo et al., 2012). We show that H2AX is widely expressed in
501 *Arabidopsis* newly developing tissues and reproductive cells including the companion cells of
502 the male and female gametophytes. Loss of H2A.X results in endosperm hypomethylation at
503 intergenic regions, and heterochromatic TEs that include DME target sites, revealing a
504 potential link between H2AX deposition and DME-mediated DNA demethylation.

505 There are some precedents for H2A variant interactions with DME-like proteins in
506 *Arabidopsis*: H2A.Z is present at the transcriptional start sites of genes, where it promotes
507 transcription by preventing DNA methylation (Zilberman et al., 2008). ROS1/DML2/DML3
508 act to remove DNA methylation in vegetative tissues and are recruited to a subset of their
509 targets by the IDM1 complex, although they do not directly interact, instead H2A.Z recruits
510 the DME homologue ROS1 to its targets in vegetative tissues (Nie et al., 2019). This is
511 reminiscent of the activity of DME in the central cell, where FACT is required for DME
512 access to certain targets. Evidence exists to suggest that DME and FACT interact directly,
513 and so may not require intermediary proteins (Frost et al., 2018). Interestingly, our analysis
514 of promoter DNA methylation at H2A variants showed that the H2A.Z.4 locus may be
515 regulated by DME in the developing seed (Supplementary Figure S1) (Ibarra et al., 2012;Park
516 et al., 2016).

517 It was previously shown that the *HTA3* and *HTA5* gene promoters exhibited differences in
518 activity, with *HTA5* observed to be less active in the floral bud (Yi et al., 2006). Consistent
519 with this, we show that whilst HTA5 is the predominant protein isoform expressed in the
520 sporophyte, HTA3 predominates in the developing gametophytes, though both are highly
521 expressed in pollen. One explanation for H2A3/5 high expression in the vegetative and
522 central cells is that DME activity creates AP sites during BER, that may lead to the formation
523 of double strand breaks, thereby requiring high levels of H2A.X (Sczepanski et al., 2010).
524 Intriguingly, however, in heterochromatin, the mechanism of DNA repair is different; an
525 H2A.W variant, H2A.W.7 is phosphorylated by ATM to initiate the response in constitutive
526 heterochromatin to DNA damage (Lorkovic et al., 2017).

527 We observed a significant increase in root hair length in *h2a.x* mutants compared to wild-
528 type in the absence of any DNA damaging conditions. Intriguingly, reduction in H2A.Z
529 incorporation into chromatin also results in an increase in root hair length, since the altered
530 chromatin state mimics phosphate deficiency - activating phosphate deficiency response gene
531 locus (Deal et al., 2005;Smith et al., 2010). In a similar context, *h2a.x* mutations may
532 indirectly affect the expression of root hair-growth genes (Won et al., 2009;Hwang et al.,
533 2017;Mangano et al., 2017). Defective *H2A.X* expression may also cause nutrient-stress,
534 resulting in the modulation of genes involved in root hair growth.

535 The mechanism of DNA methylation loss in *h2a.x* endosperm remains unclear. Since we
536 identified hypomethylation on both male and female endosperm alleles, but not in embryo,
537 hypomethylation must occur post-fertilization in endosperm, at least on the paternal allele.
538 On the maternal allele, both pre- and post-fertilization DNA methylation dysregulation may

539 be present. Endosperm is a highly unique tissue; it is triploid, and the site of parental
540 competition for generational resources, in part reflected in the activities of DME and FACT
541 in the central cell, which confer deep hypomethylation. Endosperm exhibits highly distinct
542 higher-order chromatin structure compared to other tissues, being less condensed and
543 subsequently featuring increased trans-interactions, increased expression of TEs, and
544 encroachment of heterochromatin into euchromatic regions (Baroux et al., 2007; Yadav et al.,
545 2021). As such, the role of H2A.X in endosperm chromatin may well be distinct from that in
546 other tissues.

547 Our embryo minus endosperm DMR analyses revealed the following characteristics of
548 *h2a.x*-specific hypomethylated DMRs: enriched in chromatin states 4, 8 and 9 (Sequeira-
549 Mendes et al., 2014) and located in TEs, pericentric heterochromatin and intergenic regions.
550 Chromatin states 4 and 8 are strikingly enriched in intergenic DNA (66.2 and 58.2 %,
551 respectively, (Sequeira-Mendes et al., 2014). Chromatin state 4 is also characterized by the
552 presence of histone variants H3.3 and H2A.Z, and high levels of H3K27me3, but is not
553 highly associated with active transcription. It is also noted to likely contain distal promoters
554 and regulatory elements. Chromatin states 8 and 9 are highly enriched in TEs, and feature
555 H3.1, H3K9me2, and H3K27me1, and although state 8 is a transitional, more decondensed
556 state, they both represent Arabidopsis heterochromatin (Sequeira-Mendes et al., 2014). The
557 enrichment of DNA hypomethylation of these regions in *h2a.x* mutants is intriguing. Whilst
558 *h2a.x* hypomethylated DMRs exhibit overlap with DME target sites, they also represent
559 additional regions not normally demethylated, and their presence in intergenic chromatin and
560 in heterochromatic TEs may be indicative of normal requirement of H2A.X for DME
561 exclusion from these regions; that is, to prevent inappropriate remodeling of regulatory DNA
562 and heterochromatic TEs.

563 Nucleosome cores are crucial for nuclear DNA organization and function, and a complete
564 loss of *H2A.X* is likely quickly replaced by other H2A variants, such as H2A.Z, H2A.W, or
565 by canonical H2A. Intriguingly, in human cells, H2A.X phosphorylation increases the
566 accessibility of chromatin to DNA methylases, (Heo et al., 2008). We therefore speculate
567 that substitution for other H2A variants in *h2a.x* mutant endosperm contributes to endosperm
568 hypomethylation, perhaps by allowing DME to access loci normally not permitted.

569 In conclusion, we demonstrate that *H2A.X* is expressed widely in developing *Arabidopsis*
570 tissues and gamete companion cells, and show that the DNA damage response is impaired in
571 *h2a.x* mutant roots and seedlings. We show that *h2a.x* mutant endosperm exhibits DNA
572 hypomethylation at intergenic regions and heterochromatic TEs, creating a large number of

573 embryo-endosperm DMRs, not present in wild-type. We hypothesize that the presence of
574 H2A.X in endosperm contributes to a chromatin structure that is refractive to DME and
575 DME-FACT activity, preventing inappropriate DNA demethylation in intergenic and
576 heterochromatic DNA.

577

578 **Data Availability Statement**

579 The datasets for this study can be found in GEO accession (TBA)

580

581 **Conflict of Interest**

582

583 The authors declare that the research was conducted in the absence of any commercial or
584 financial relationships that could be construed as a potential conflict of interest.

585

586 **Author Contributions**

587

588 RLF, YC and JMF conceived the project; JMF, JL, PHH, SJHL, YM, MB, AMR, HTC
589 performed the experiments; JMF, YC, TFH, and RLF analyzed the data; JMF, YC, and RLF
590 wrote the article with contributions of all the authors. All authors contributed to the article
591 and approved the submitted version.

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595

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601

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603

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760 **Supplementary Materials**

761

762 **Supplementary Figure S1. Analysis of CG DNA methylation at H2A variant genomic** 763 **loci in WT (Col-0) and *dme-2* mutant Arabidopsis.**

764 (A) *HTA3* (H2A.X). (B) *HTA5* (H2A.X). (C) *HTA4* (H2A.Z). CC = Central cells (Park et
765 al., 2016), Endo = Endosperm (Hsieh et al., 2009). Transcription start site and promoter
766 regions are highlighted with orange box. All cytosine methylations are included without read
767 cutoff. All bars of histogram indicate the methylation % level of single cytosine. Bismark
768 was used for bisulphite sequencing data read alignment and Seqmonk was used for
769 visualization.

770

771 **Supplementary Figure S2. Genome-wide non-CG methylation analysis of *h2a.x* mutant**
772 **developing embryo, endosperm and seedling.**

773 Ends analysis of selfed *h2a.x* mutant and segregating WT genomic CHG methylation in
774 genes (A) and TEs (B), as well as CHH methylation in genes (C) and TEs (D), with those
775 aligned according to their 5' and 3' ends. Data for seedling, endosperm and embryo (linear-
776 bending cotyledon) are shown. Endosperm and embryo are hypomethylated at CHH at gene
777 edges and in TEs. Since embryo CHH methylation levels are incredibly sensitive to
778 gestational age, they may be indicative of slightly later dissection of WT for this sample.

779

780 **Supplementary Figure S3. Allele-specific genome-wide CG methylation analysis of**
781 ***h2a.x* mutant developing embryo and endosperm.**

782 Female WT Col-0 or *h2a.x* homozygous mutants were crossed with WT Ler pollen and the
783 methylation levels in F1 seeds were analyzed. '*h2a.x* paternal' denotes a WT paternal allele
784 now resident in a heterozygous *h2a.x* mutant seed. Ends analysis of embryo and endosperm
785 CG genomic methylation in genes (A and B, respectively) as well as those in TEs (C and D,
786 respectively) are shown, with genes and TEs aligned according to their 5' and 3' ends. The
787 maternal *h2a.x* endosperm allele is hypomethylated compared to WT. Ends analysis of
788 embryo and endosperm CHG genomic methylation in genes (E and F, respectively) as well as
789 those in TEs (G and H, respectively) are shown, with genes and TEs aligned according to
790 their 5' and 3' ends. Ends analysis of embryo and endosperm CHH genomic methylation in
791 genes (I and J, respectively) as well as those in TEs (K and L, respectively) are shown, with
792 genes and TEs aligned according to their 5' and 3' ends. CHH methylation in genes at
793 intergenic regions is decreased on both *h2a.x* endosperm alleles compared to WT. CHH
794 methylation in TE bodies is decreased on both *h2a.x* embryo and endosperm alleles
795 compared to WT.