Processive DNA Demethylation via DNA Deaminase-Induced Lesion Resolution

[1] DNA Ed I . a d E e e c , IFOM-F da e I FIRC d O c a M ec a e, M a , I a. , [2] DNA Ed Lab, C a e Ha Lab a e , L d Re ea c I e, S M , U ed K d , [3] Lab a . De e e a Ge e c a d I , T e Bab a a I e, Ca b d e, U ed K d , [4] Ce e T b a Re ea c , U e . Ca b d e, Ca b d e, U ed K d

Ba e d ca c. e a e a a a ec c a b . , a e. ca d ec . e a e e e e, e , e DNA e a e e a e d ca e a e e e . . He e e c a ac e e c. e DNA dea a e AID ca a e DNA de e . a . In vitro, AID a ed a e ed DNA de e . a e . C G e c b a DNA e a c e e e, ac . Mec a ca . , ac e ed b. d c ba e a e a a ea e . -c. e, e e be e ed e e a e ba e b a e e c e ce e . e a e de e de e a . T e b c e ca d a e eca a ed a in vivo a e c a e a a. , a d de e

state, chromatin state, and location, the dU lesions will lead to

Treated DNA was purified via Qiagen Mini-prep (saved as input), isolated on streptavidin magnetic beads (Invitrogen-Dynal), collected in 100 μl TE, and 2 μl of this bead mixture subjected to real-time PCR. PCR reactions (20 μl) contained 10 μl of the LightCycler 480 SYBR Green I master mix (Roche Applied Sciences) and primers (1512 GGCCTAACTGGCCGGTAC Rev - 1518 GTCCACCTCGATATGTGC). The reaction was monitored in a LightCycler 480 Real-Time PCR System (Roche Applied Sciences); with the 'input' DNA analyzed in parallel as reference. C_t values for the biotinylated-DNA were correlated to the C_t values for the input DNA. Results were presented either as relative (fold-change) or absolute (% of input) quantification [22]. For fold-change, all samples were correlated to their input and then the FE alone sample (or another specified sample) was used as reference and set to one. Alternatively, in the % of input analysis the C_t qPCR values of input and output were converted to an

was added to the IVR, we observed a reduction without complete inhibition (Figure 1B). Although the use of Ugi could have minor side effects, the peptide has been extensively studied and characterized and shown remarkable specificity for inhibiting UNG2 (the predominant BER protein acting on dUs) [30,31] without affecting other uracil DNA glycosylases (e.g. TDG, MBD4, SMUG). This indicated that although UNG-dependent BER is important in AID-induced lesions resolution of methylated plasmids, other DNA repair pathways are also playing a role. During SP-BER the dU:dG (or dT:dG) lesion is repaired with the incorporation of a single dC, resulting in a dC:dG base pair. Processive polymerase-dependent repair pathways (e.g. LP-BER, MMR), not only resynthesize the lesion, but also incorporate nucleotides that are downstream of the initial dU. These pathways can be detected in the IVR by addition of biotinylated-dA (Figure 1B), where a significant amount of biotin incorporation can be seen after treatment with G-AID and FE. Importantly, by adding Ugi, we are able to discern between UNG-dependent LP-BER and other processive polymerase-dependent DNA repair pathways, such as non-classical MMR [21].

AID- d ced e a d ce e DNA de e , a

Aside from identifying the various DNA repair pathways acting on the AID-induced lesions of a methylated substrate, we also determined the extent of local DNA demethylation. Using bisulfite analysis of a region downstream of the GAL4 DNA-binding site, we identified both single site demethylation as well as consecutive (processive) demethylation events (Figure 2A), with AID activity leading to 43% cytosine demethylation (Figure 2B). One should note that although the DNA is CpG methylated, unmethylated dCs outside a 5mCpG context are still substrates for AID-induced deamination. When we treated the FE with Ugi, to inhibit UNG2 dependent BER, bisulfite analysis showed a significant decrease in the efficiency of the extract to induce DNA demethylation (Figure 2A & B). This strongly suggested that AID can induce DNA demethylation by acting on dCs, since dUs are the only substrate for UNG2. Furthermore, if dCs are deaminated to dUs and UNG2 lesion processing leads to 5mCpG demethylation, then LP-BER plays a role in local DNA demethylation.

L ca 5 C G c e, e c be DNA e a a a. c ce

uncut, or I and I digestion. The ratios of the differences were then used as a correction factor to determine AID-induced m6A demethylation. In Figure 3B and 3C, uncut G-AID treated samples were set to 100% and G-AID induced demethylation represented as % recovery to uncut. I reduced the efficacy of PCR amplification when compared to the uncut sample, indicating that the methylation of the four analyzed sites had been lost. This AID-dependent demethylation of sites was also observed when we restricted the plasmid with I and I. I restriction was not as complete as I (enhanced recovery), which also indicated a loss of A sites (Figure 3C).

These results clearly demonstrate that AID-induced lesions (dU) can be repaired with a processive polymerase-dependent repair system, leading to substitution of methylated adenosine by unmethylated adenosine. Therefore, DNA repair from AID-induced lesions is sufficient to induce demethylation without

3. . ( ) T e ab e a e e e e a e
ade e (m6A) GATC e e ce c b. e e c e e MboI, Sau3AI DpnI. O e e e e e e ce c de e d
e a e ade e- e a . I e dd e e ab e PCR ec e a e d e d ca ed. T e c e e PCR ec e
d e ed DNA a e AID- d ced de e a m6A d ca ed e . ( ) ( ) AID d ce de e a e -ade e. S e c ed
a d (m6A(+) a a ed a da d *E. coli*

4. 4- ( ) GAL4-AID cDNA e e e ed a CMV e c a

postnatal tissues, as tested by RT-PCR (Figure 4B) and by immunofluorescence in zygotes (Figure 4C).

We bred males harboring the H19 DMR-UAS locus with females carrying the GAL4-AID, GAL4-ΔAID mutants, or the previously described CMV GAL4-Myc [33] expressing transgenes, and determined the extent of DNA methylation in F1 offspring. Due to technical limitation of obtaining enough material from fertilized oocytes we could not perform bisulfite analysis on homogenous tissues right after fertilization (zygote). Hence, we choose to analyze tissue samples for methylation analysis of various regions surrounding the UAS (Bi-2, -3, -4) from neonatal liver (Figure 5). Since adult liver did not express the transgene itself (Figure 4B), it was likely that any observed demethylation had to occur in earlier stages of development. The H19 locus is an imprinted locus, with the paternal allele being methylated and the maternal allele unmethylated. Due to the genetic manipulations of the system regions Bi-2 & 3 can be amplified from the paternal allele, while region Bi-4 can only be amplified from the maternal allele (Figure 5B). As shown in Figure 5C, Bi-2 was significantly more demethylated in GAL4-AID than in GAL4-Myc mice. More importantly, the demethylation required AID catalytic activity, as transgenic mouse 7 (TG 7 - harboring a catalytic inactive GAL4-AID) did not show extensive DNA demethylation in this region. Bisulfite analysis of the Bi-3 region confirmed the results for the demethylation capacity of a catalytic active AID (Figure 5D and summarized in Figure S5A), where TG 4 and TG 5 induced over 95% demethylation. Loss of the methylation on the paternal allele may induce methylation on the maternal allele – possibly via dosage compensation [38]. Yet targeting of AID to the paternal allele did not influence the DNA methylation status on the maternal allele, since bisulfite analysis of Bi-4 showed no change in any of the mice analyzed (Figure 5E and Figure S5B). The paternal DMR DNA methylation status (Bi-2 and Bi-3) was also analyzed from embryos and placenta (Figure 6 and Figure S5C),

and analogous to the results from the liver tissue, catalytic AID induced local DNA demethylation.

AID- d ced de e , a de a e

We estimated the extent of AID-induced DNA demethylation of at least 1,000 bases at the H19 locus, as the paternal specific Bi-2 and Bi-3 are each about 500 bases long and were substantially demethylated. The upstream border of DNA demethylation could be situated near the 5′ part of the Bi-2 (further upstream are no polymorphisms to distinguish between maternal and paternal alleles); the downstream border is likely the G-repeats, inhibiting the DNA demethylation.

AID favors the hot spot motif WRC (A/T,A/G,C) and strongly disfavors SYC (cold spot - G/C, C/T, C) sequences for deamination [27], which is even more pronounced on a 5mC containing substrate [6]. Sequence context analysis of the Bi-2

bly via LP-BER) to occur in the second phase of active DNA demethylation [43], mimicking our [illegible] and [illegible] findings and supporting our insights into the mechanisms of active DNA demethylation. Future work will determine if the findings form our

outcome [50,51]. Future work using the IVR and AID will also allow for the uncovering of the precise molecular mechanisms of how AID-induced demethylation can proceed during primordial germ cell formation [13], during pluripotency reprogramming

- 5mC) regions. GAL4-AID (circle-triangle) is bound at the UAS with arrows representing individual DNA deaminations, leading to complete demethylation (open circle). Number of total dCs (582) and 5mC (88) in this region is indicated, followed by a set of assumptions for the calculation. After n deaminations, the probability for a single target of being never hit is (581/582)^n and its probability of being hit at least once is 1-(581/582)^n. Deriving at a formula representing the number of deaminations that have to occur in order to have 'hit' 88 5mC in 582 dC with 99% confidence.
(PDF)

Table S1 Primers used for bisulfite analysis. List of primers used in the bisulfite analysis.
(DOC)

References S1
(DOC)

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Conceived and designed the experiments: DMF HM WR SKPM. Performed the experiments: DMF CFC HM EI GR WD FS SKPM. Analyzed the data: DMF CFC WD WR SKPM. Wrote the paper: DMF WR SKPM.

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