Supplemental Material to: Rimple D. Almeida, Virginie Sottile, Matthew Loose, Paul A. De Sousa, Andrew D. Johnson and Alexey Ruzov. Semi-quantitative immunohistochemical detection of 5-hydroxymethylcytosine reveals conservation of its tissue distribution between amphibians and mammals. Epigenetics 2012; 7(2); DOI: 18949;

http://www.landesbioscience.com/journals/epigenetics/article/18949/

Materials and methods

Immunohistochemistry and imaging. Paraffin embedded formaldehyde fixed sections of axolotls, *Xenopus laevis*, wild type CD1 mouse embryonic and adult tissues were used for immunohistochemistry. Tissues were fixed in 4% formaldehyde for 12 hours. Tissue sections were de-waxed according to standard procedures and permeabilised for 15 min with PBS containing 0.5% Triton X-100. For 5-hmC and 5-mC staining, permeabilised tissue sections were incubated in 4N HCl for 1h at 37°C and then neutralised in 100 mM Tris-HCl (pH 8.5) for 10 min, followed by a standard immunostaining protocol. Anti-5-hmC (Active Motif, 1:5000; 1:50000 and 1:500 000 dilutions) and anti-5-mC (Eurogentec) primary antibodies were used. Peroxidase-conjugated anti-rabbit secondary antibody (Dako) and the tyramide signal enhancement system (Perkin Elmer) were employed for 5-hmC detection. 5-mC was visualised using 555-conjugated secondary antibody (Alexafluor). Control staining without primary antibody produced no detectable signal. Images were acquired using a Nikon ECLIPSE 90i immunofluorescence microscope and Volocity software.

Image quantification was performed using Fiji software. Slides with serial adjacent sections were processed under identical conditions with varying times of incubation with tyramide and were imaged at the same exposure settings. Mean intensities were measured for 10-20 random cell nuclei on each region of interest for each sample (examples of measured regions are presented in Figure 1D). Mean values of the mean intensities were plotted onto graphs. Experimental error is expressed as s.e.m. Reaction velocities were calculated as Δ Intensity/ Δ Time, with Δ as a difference between experimental points with minimal and maximal times of incubation with a fluorescent enhancer.

Dot blot assays were performed as reported previously[1] using anti-5-hmC (Active Motif, 1:5000 dilution) and anti-5-mC (Eurogentec, 1:1000 dilution) primary antibodies. Equal dilutions of axolotl tissues derived DNA were loaded onto membrane. The dilution rate between two neighbouring experimental points equalled 10X. Image quantification was performed using Fiji software. Mean intensities for different concentrations of genomic DNA were plotted onto graphs. Principally identical results were produced using anti-5-hmC antibody produced by Diagenode.

Bioinformatic analysis. The 14 Tet1/2/3 sequences from different species were aligned using RevTrans[1] and all positions containing gaps and missing data were removed. There were a total of 1518 positions in the final dataset. Evolutionary analyses were conducted in MEGA5[2]. NCBI Accession numbers for the sequences used are Homo sapiens Tet1:NM_030625, Tet2:NM_001127208, Tet3:NM_144993 Mus musculus Tet1:NM_027384, Tet2:NM_001040400, Tet3:NM_183138 Rattus norvegicus Tet2:XM_227694, Tet3:XM_001057850 Xenopus tropicalis Tet2:XM:002934777, Tet3:NM_001097187 Gallus galls Tet1:XM_421571. Danio rerio sequences were identified from Ensembl (version Zv9 of the Zebrafish genome) as Tet1:ENSDARG00000075230, Tet2:ENSDARG00000076928, Tet3:ENSDARG00000062646. The developmental stage and tissue distribution of Xenopus tropicalis Tet 2 and 3 transcripts is determined from NCBI UniGene Est profiles (Tet2:Str.52041 Tet3:Str.53063). Transcript counts are reported in TPM (transcripts per million).

Figure S1. (A) The distribution of 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) in embryonic skin of 17.5 dpc mouse embryo. 5-hmC, 5-mC staining and merge views are shown. Immunostaining was performed with 1:5000 dilution of a primary anti-5-hmC antibody. (B) 5-hmC immunostaining signal at indicated times of incubation with tyramide in serial adjacent sections of mouse embryonic skin. The primary anti-5-hmC antibody dilutions used (1:50000, and 1:500000) are indicated. Sections were stained in parallel under identical conditions with different times of incubation with tyramide. The exposures are identical for all the presented pictures. (C) The progress curves of peroxidase reactions produced by quantification of immunostaining data presented in (C) with indicated dilutions of an anti-5-hmC antibody.

Figure S2. (A) 5-hmC immunostaining signal at indicated times of incubation with tyramide in serial adjacent sections of mouse adult brain and heart. The primary anti-5-hmC antibody was used at 1:50000 dilution. Sections were stained in parallel under identical conditions with different times of

incubation with tyramide. The exposures are identical for all the presented pictures. (B) The progress curves of peroxidase reactions produced by quantification of immunostaining data presented in (A) for mouse brain and heart (indicated). (C) The velocities of peroxidase reactions for mouse brain and heart immunostaining experiments.

Figure S3. The mosaic distribution of 5-hydroxymethylcytosine in axolotl skin and connective tissue. 5-hmC was visualised using green tyramide on a lateral section through the head region of young axolotl adult. 5-mC staining is shown in red. Merged view is presented. Cells strongly enriched with 5-hmC appear green or yellow on a merged view.

Figure S4. The mosaic distribution of 5-hydroxymethylcytosine in the skin of adult clawed frog *Xenopus laevis*. 5-hmC, 5-mC staining and merged views are shown.

Figure S5. (A) 5-hmC immunostaining signal at indicated times of incubation with tyramide in serial adjacent sections of axolotl neural tube and skeletal muscle. The primary anti-5-hmC antibody was used at 1:50000 dilution. Sections were stained in parallel in identical conditions with different times of incubation with tyramide. The exposures are identical for all the presented pictures. (B) The progress curves of peroxidase reactions produced by quantification of immunostaining data presented in (A) together with the progress curves obtained for mouse brain and heart staining experiments. (C) The velocities of peroxidase reactions for axolotl brain and skeletal muscle in comparison to those of mouse brain and heart.

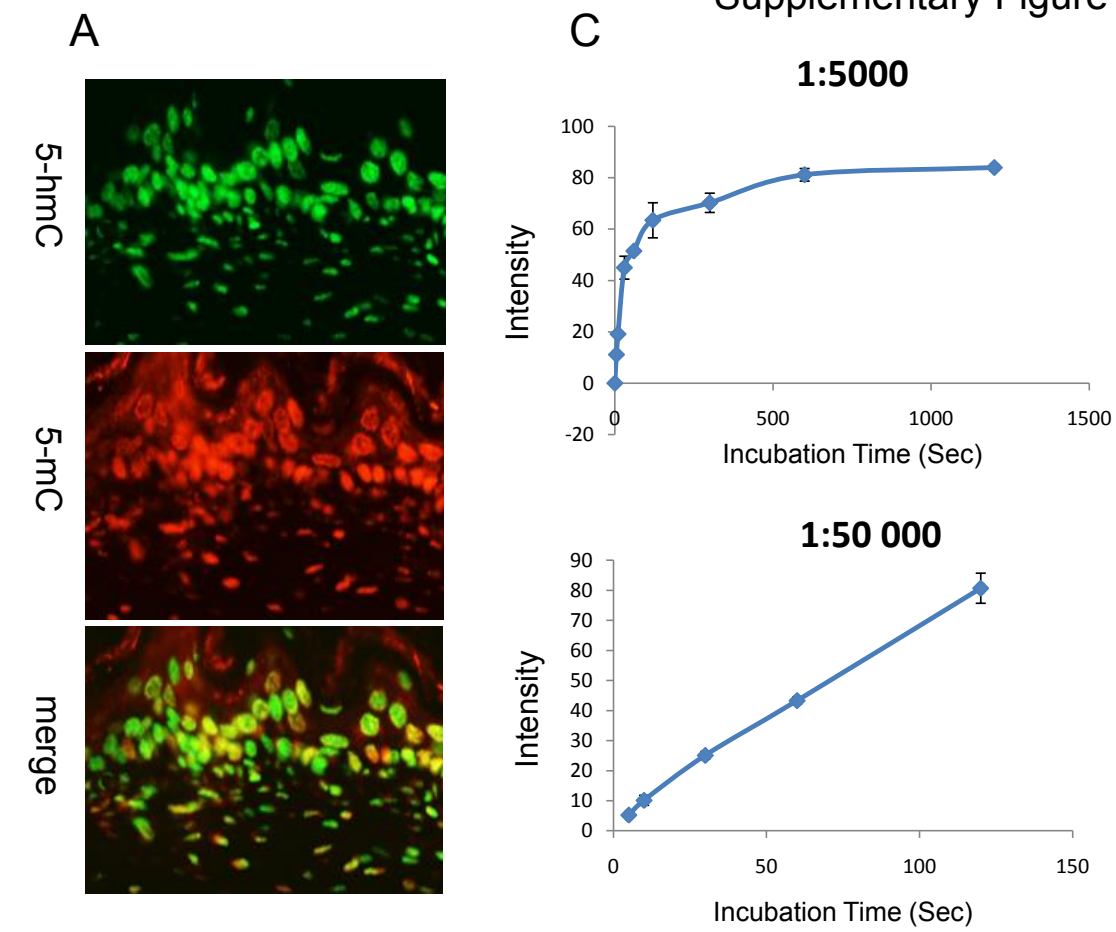
References

1. Nestor C, Ruzov A, Meehan R, Dunican D. Enzymatic approaches and bisulphite sequencing cannot distinguish between 5-methylcytosine and 5-hydroxymethylcytosine in DNA. Biotechniques 2010; 48**:** 317-319.

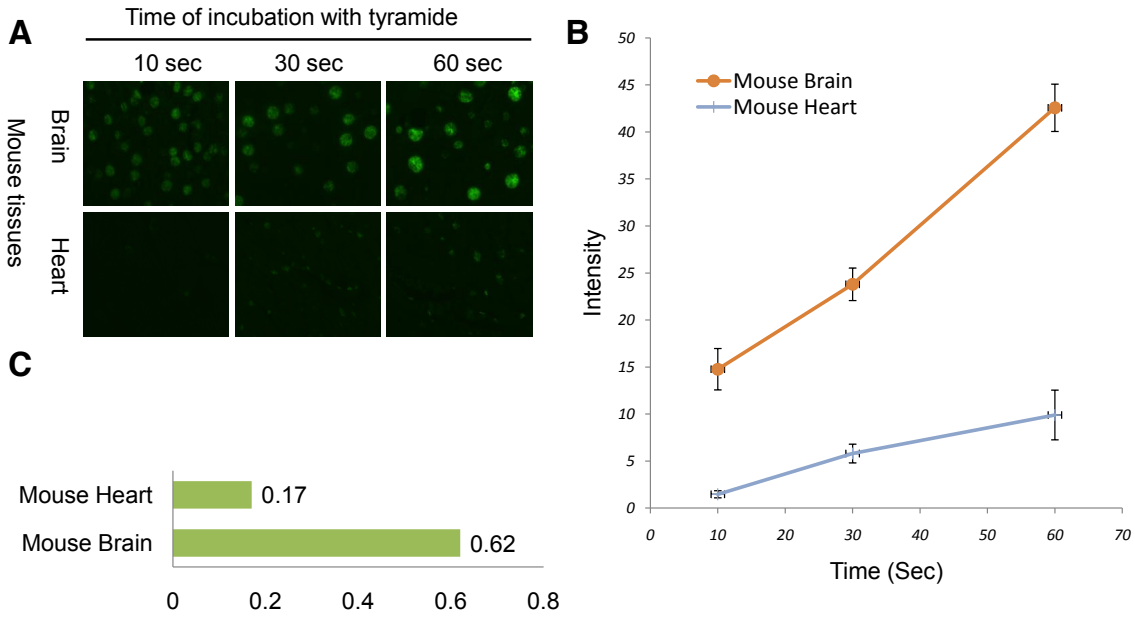
2. Wernersson R, Pedersen AG. RevTrans - Constructing alignments of coding DNA from aligned amino acid sequences. Nucl. Acids Res. 2003; 31:3537-3539.

3. Tamura K, Dudley J, Nei M, and Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 2007; 24:1596-1599.

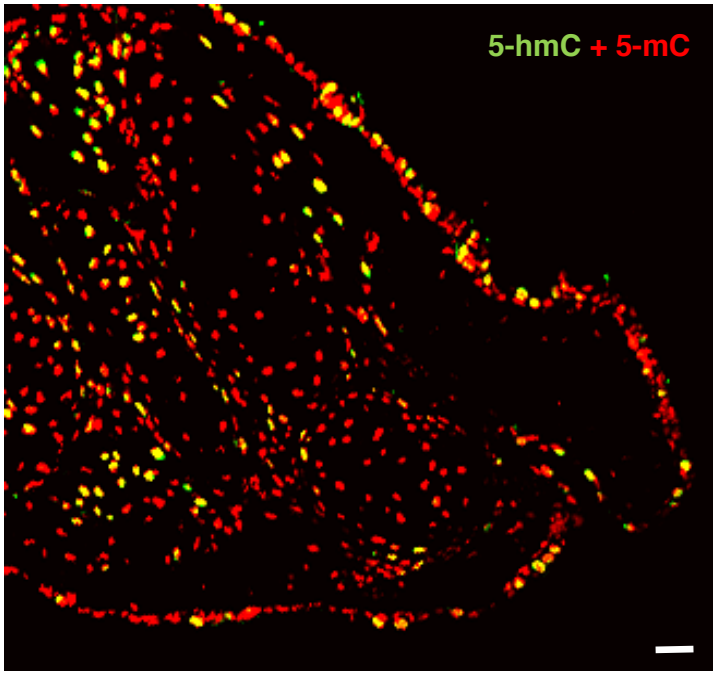
Supplementary Figure S1



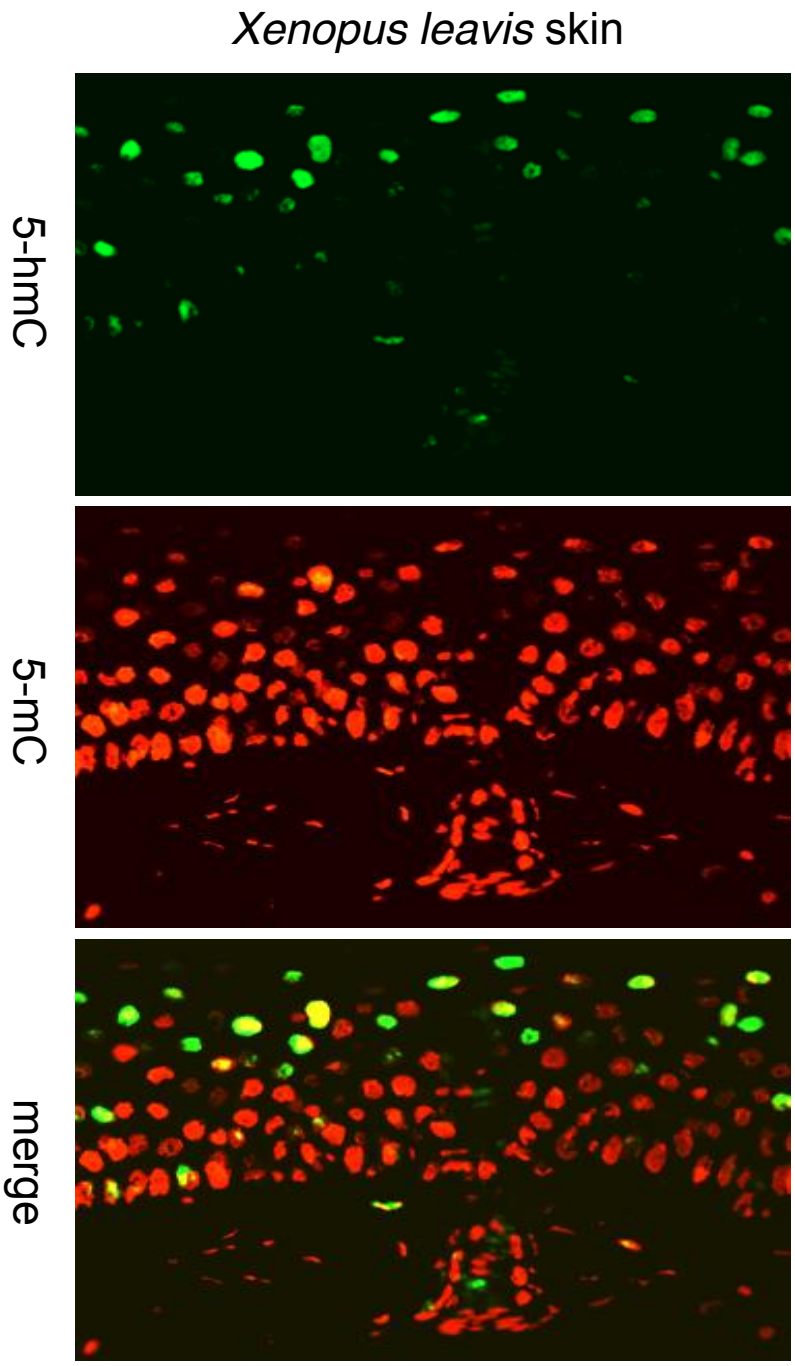
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5