Supporting Information

Age-Dependent Levels of 5-Methyl-, 5-Hydroxymethyl-, and 5-Formylcytosine in Human and Mouse Brain Tissues**

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Supporting Information

1) Comparison of fC and hmC levels reported in this study to fC and hmC levels reported in literature

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Figure S1. Depiction of global fC levels in mouse and human tissues and cell types. Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because it amounts to the sum of cytosine (C) and its derivatives mC, hmC, and fC. Dark green bars represent fC levels reported in this study while light green bars represent levels of fC reported in literature. The corresponding hmC levels are depicted in Figure S2. Error bars represent the standard deviation between biological replicates (exceptions are the fC levels reported for cerebrum tissues of the human individual aged 85a, where error bars represent the standard deviation of technical replicates; see also Table S2). ESCs = serum-primed embryonic stem cells, p = postnatal day, pm = postnatal month, a = anno (year), av. = average.
Figure S2. Depiction of global hmC levels in mouse and human tissues and cell types. Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because it amounts to the sum of cytosine (C) and its derivatives mC, hmC, and fC. Dark blue bars represent hmC levels reported in this study while light blue bars represent levels of hmC reported in literature.[1] The corresponding fC levels are depicted in Figure S1. Error bars represent the standard deviation between biological replicates (exceptions are the hmC levels reported for cerebrum tissues of the human individuals aged 85a and 0.6a, where error bars represent the standard deviation of technical replicates; see also Table S2). ESCs = serum-primed embryonic stem cells, p = postnatal day, pm = postnatal month, a = anno (year), av. = average.

Table S1 (corresponding to Figures S1 and S2). List of global fC and corresponding global hmC levels in mouse and human tissues and cell types. Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because it amounts to the sum of cytosine (C) and its derivatives mC, hmC, and fC. Values reported for fC and hmC in this study are unmarked, while values taken from literature[1] are marked with "#". ESCs = serum-primed embryonic stem cells, p = postnatal day, pm = postnatal month, a = anno (year), biol. repl. = biological replicates, SD = standard deviation.

| Species  | Age  | Celltype / tissue | No. of biol. repl. | fC / G [%] | SD       |
|----------|------|-------------------|--------------------|------------|----------|
| Mouse    |      | ESCs: WT01 #      | 7                  | 5.19E-03   | 8.62E-04 |
|          |      | ESCs: J1 #        | 2                  | 1.03E-03   | 4.26E-06 |
|          |      | ESCs: R1 #        | 3                  | 8.19E-04   | 2.81E-04 |
| p1       | Cerebrum | 2              | 6.59E-04   | 7.11E-07   |
| p1       | Kidney   | 2               | 6.53E-04   | 2.22E-05   |
| p90      | Hippocampus # | 3          | 2.45E-04   | 6.58E-05   |
| p90      | Cerebrum  | 3               | 2.01E-04   | 4.84E-06   |
| p90      | Cerebellum # | 3          | 1.26E-04   | 2.86E-06   |
| p90      | Heart #   | 3               | 7.53E-05   | 9.21E-06   |
| p90      | Kidney    | 3               | 9.02E-05   | 1.49E-06   |
| p90      | Liver #   | 3               | 8.33E-05   | 2.92E-06   |
| pm18     | Cerebrum  | 3               | 1.65E-04   | 1.13E-05   |
| pm18     | Kidney    | 1               | 1.47E-04   |
| H. sapiens | 77-88a   | Neurons          | 2               | 7.08E-04   | 8.02E-06 |
|          | 77-88a   | Non-neur. cells  | 2               | 9.78E-04   | 1.95E-04 |
| 85a      | Cerebral grey matter | 2 (techn.) | 1.87E-04 | 1.48E-05 |
| 85a      | Cerebral white matter | 2 (techn.) | 2.68E-04 | 1.46E-06 |
| Species   | Age   | Celltype / tissue      | No. of biol. repl. | hmC / G [%] | SD   |
|-----------|-------|------------------------|--------------------|-------------|------|
| Mouse     | p1    | Cerebrum               | 2                  | 0.16        | 0.011|
|           | p1    | Kidney                 | 2                  | 0.05        | 0.001|
|           | p90   | Hippocampus            | 3                  | 0.76        | 0.034|
|           | p90   | Cerebrum               | 3                  | 0.57        | 0.054|
|           | p90   | Cerebellum             | 3                  | 0.32        | 0.010|
|           | p90   | Heart                  | 3                  | 0.20        | 0.014|
|           | p90   | Kidney                 | 3                  | 0.18        | 0.001|
|           | p90   | Liver                  | 3                  | 0.13        | 0.008|
|           | pm18  | Cerebrum               | 3                  | 0.81        | 0.002|
|           | pm18  | Kidney                 | 3                  | 0.24        | 0.013|
| H. sapiens| 77-88a| Neurons                | 4                  | 1.82        | 0.503|
|           | 77-88a| Non-neuronal cells     | 2                  | 0.82        | 0.042|
|           | 85a   | Cerebral grey matter   | 2 (techn.)         | 1.19        | 0.036|
|           | 85a   | Cerebral white matter  | 2 (techn.)         | 0.76        | 0.020|
|           | 0.6a  | Cerebral grey matter   | 1 (techn.)         | 0.42        | -    |
|           | 0.6a  | Cerebral white matter  | 1 (techn.)         | 0.59        | -    |
2) DNA isolation from human and mouse tissues and mass spectrometric quantification of the cytosine modifications fC, hmC, and mC

DNA isolation from cells or tissue samples, DNA digestion to the nucleoside level, and quantification of absolute mC, hmC, and fC levels via HPLC-MS or UPLC-MS/MS were performed like previously reported by us.[1-2]

3) Preparation of human brain tissue samples and corresponding age-related quantification data for the bases mC, hmC, and fC in human brain tissues

3.1) Preparation of human brain tissue samples

Human tissue samples were provided by the BrainBank Munich, thereby all cases were clinically and neuropathologically characterized according to the BrainBank Munich standard protocols that were established by BrainNet Europe and BrainNet Germany. Tissue samples were snap-frozen and stored at -80°C. Written informed consent was obtained according to the guidelines of the local ethics committee.

3.2) Quantification data for the bases mC, hmC, and fC in human cerebrum and cerebellum

Table S2 (corresponding to Figures 2, 3, and 4). LC-MS quantification data regarding the DNA bases hmC, mC, and fC in human brain tissues. Compiled are mean values obtained from 2–4 independent technical replicates and their standard deviation (SD). Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because it amounts to the sum of cytosine (C) and its derivatives mC, hmC, and fC. Data regarding the two individuals aged 61a and the three individuals aged 84, 85 and 88a, respectively, are additionally compiled as biological replicates. In case of the 0.6 year old individual, due to limited sample size only one measurement was performed. Data for samples 61a (two individuals), 84a and 85a (here for cerebrum only) were taken from Kraus et al.[2a] *: Sampled at lobus occipitalis; ** and grey background: Sampled at lobus frontalis, grey + white matter; non-marked samples were sampled at lobus frontalis. WOP = week of pregnancy, a = anno (year), av. = average, techn. = technical replicates, biol. = biological replicates, G = guanine, SD = standard deviation.
### hmC

| Cerebrum | Grey matter | White matter |
|----------|-------------|--------------|
|          | No. of replicates | hmC / G [%] | SD | No. of replicates | hmC / G [%] | SD |
| 15. WOP  | 3 (techn.) | 0.09 | 0.02 | 3 (techn.) | 0.11 | 0.01 |
| 0.6 a    | 1 (techn.) | 0.42 | -    | 1 (techn.) | 0.59 | -   |
| 3.2 a**  | 4 (techn.) | 0.55 | 0.04 | -           | -     | -   |
| 22 a*    | 2 (techn.) | 1.07 | 0.02 | 3 (techn.) | 0.64 | 0.02 |
| 61 a*    | 3 (techn.) | 1.19 | 0.06 | 3 (techn.) | 0.71 | 0.04 |
| 61 a     | 2 (techn.) | 1.35 | 0.04 | 3 (techn.) | 0.76 | 0.04 |
| 84 a     | 3 (techn.) | 1.21 | 0.02 | 4 (techn.) | 0.77 | 0.02 |
| 85 a*    | 3 (techn.) | 1.17 | 0.08 | 4 (techn.) | 0.76 | 0.09 |
| 88 a*    | 2 (techn.) | 1.06 | 0.06 | 1 (techn.) | 0.85 | -   |
| av. 61 a | 2 (bion.)  | 1.27 | 0.11 | 2 (bion.)  | 0.74 | 0.04 |
| av. 84-88 a | 3 (bion.) | 1.14 | 0.08 | 3 (bion.)  | 0.79 | 0.05 |

| Cerebellum | Grey matter | White matter |
|------------|-------------|--------------|
|            | No. of replicates | hmC / G [%] | SD | No. of replicates | hmC / G [%] | SD |
| 22 a       | 2 (techn.) | 0.89 | 0.03 | 1 (techn.) | 0.61 | -   |
| 85 a       | 2 (techn.) | 1.06 | 0.11 | 2 (techn.) | 0.86 | 0.03 |

### mC

| Cerebrum | Grey matter | White matter |
|----------|-------------|--------------|
|          | No. of replicates | mC / G [%] | SD | No. of replicates | mC / G [%] | SD |
| 15. WOP  | 3 (techn.) | 3.71 | 0.07 | 3 (techn.) | 3.99 | 0.04 |
| 0.6 a    | 1 (techn.) | 4.44 | -    | 1 (techn.) | 4.49 | -   |
| 3.2 a**  | 4 (techn.) | 5.13 | 0.26 | -           | -     | -   |
| 22 a*    | 3 (techn.) | 5.85 | 0.23 | 3 (techn.) | 4.50 | 0.35 |
| 61 a*    | 4 (techn.) | 5.41 | 0.15 | 3 (techn.) | 4.40 | 0.37 |
| 61 a     | 2 (techn.) | 5.35 | 0.18 | 3 (techn.) | 4.14 | 0.18 |
| 84 a     | 3 (techn.) | 5.08 | 0.75 | 4 (techn.) | 3.93 | 0.17 |
| 85 a*    | 3 (techn.) | 5.16 | 0.07 | 4 (techn.) | 4.24 | 0.42 |
| 88 a     | 2 (techn.) | 3.81 | 0.06 | 1 (techn.) | 3.75 | -   |
| av. 61 a | 3 (bion.)  | 5.30 | 0.01 | 2 (bion.)  | 4.27 | 0.19 |
| av. 84-88 a | 3 (bion.) | 4.68 | 0.76 | 3 (bion.)  | 3.97 | 0.25 |

| Cerebellum | Grey matter | White matter |
|------------|-------------|--------------|
|            | No. of replicates | mC / G [%] | SD | No. of replicates | mC / G [%] | SD |
| 22 a       | 2 (techn.) | 4.59 | 0.15 | 2 (techn.) | 5.15 | 0.36 |
| 85 a       | 4 (techn.) | 4.13 | 0.29 | 2 (techn.) | 4.82 | 0.00 |
4) Preparation of sorted nuclei from human cerebrum tissue and corresponding quantification data for the bases mC, hmC, and fC in neurons and non-neuronal cell populations

4.1) Preparation of sorted nuclei from human cerebral occipital cortex tissue

For analysis of cytosine modification levels in human brain cell populations, cerebral occipital cortex tissue (Brodmann area 17–19) was sampled from five individuals aged 77a (3 individuals), 83a, and 88a. Tissue samples were sorted into neuronal and non-neuronal nuclei using a NeuN selective antibody. Nuclei of both brain cell populations were then analyzed separately.

4.2) Quantification data for the bases mC, hmC, and fC in human neuronal and non-neuronal cell populations

Table S3 (corresponding to Figures 2, 3, and 4). UPLC-MS/MS-based quantification data regarding the levels of mC, hmC, and fC in neuronal and non-neuronal cell populations of cerebral occipital cortex tissue of five individuals aged 77–88a. Depicted are mean values of the biological replicates and their standard deviation (SD). Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because it amounts to the sum of cytosine (C) and its derivatives mC, hmC, and fC. biol. = biological replicates.

5) Preparation of mouse tissues and corresponding age-related quantification data for the bases mC, hmC, and fC in mouse cerebral cortex and kidney tissue

5.1.) Preparation of mouse tissues

All mice used were on the wild-type C57-BL6 genetic background. All procedures concerning animals were performed with permission of the local authority (Regierung von Oberbayern). Cerebral cortex was isolated using fine forceps and spatula under a Stemi 2000 stereo microscope (Zeiss,
Oberbochen, Germany) from freshly dissected brain of mice aged 1 day (p1, newborn), 14 days (p14), 90 days (p90), 12 months (pm12), and 18 months (p18). Kidneys from the same animals were also isolated. Tissues were immediately placed in 2 mL Eppendorf-tubes, snap frozen in liquid nitrogen and stored at -80 °C until use.

5.2) Age-related quantification data for the bases mC, hmC, and fC in mouse cerebral cortex and kidney tissue

Table S4 (corresponding to Figure 5). Age-related quantification data for the bases fC, hmC, and mC in mouse cerebral cortex and kidney tissue. Compiled are mean values obtained from 2–3 independent biological replicates and their standard deviation (SD). Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because the overall G content is equal to the sum of cytosine and its derivatives mC, hmC, and fC. p = postnatal day, pm = postnatal month, biol. repl. = biological replicates.

### fC

| Age  | No. of biol. repl. | fC / G [%] | SD  | No. of biol. repl. | fC / G [%] | SD  |
|------|--------------------|------------|-----|--------------------|------------|-----|
| p1   | 2                  | 6.59E-04   | 7.11E-07 | 2                  | 6.53E-04 | 2.22E-05 |
| p14  | 3                  | 5.99E-04   | 3.63E-05 | 3                  | 2.27E-04 | 2.66E-05 |
| p90  | 3                  | 2.01E-04   | 4.84E-06 | 3                  | 9.02E-05 | 1.49E-06 |
| pm12 | 3                  | 1.60E-04   | 4.47E-05 | 3                  | 9.45E-05 | 2.32E-05 |
| pm18 | 3                  | 1.85E-04   | 1.13E-05 | 1                  | 1.47E-04 | -   |

### hmC

| Age  | No. of biol. repl. | hmC / G [%] | SD  | No. of biol. repl. | hmC / G [%] | SD  |
|------|--------------------|-------------|-----|--------------------|-------------|-----|
| p1   | 2                  | 0.16        | 0.011 | 2                  | 0.05       | 0.001 |
| p14  | 3                  | 0.24        | 0.018 | 3                  | 0.08       | 0.003 |
| p90  | 3                  | 0.57        | 0.054 | 3                  | 0.18       | 0.001 |
| pm12 | 3                  | 0.74        | 0.027 | 3                  | 0.28       | 0.022 |
| pm18 | 3                  | 0.81        | 0.002 | 3                  | 0.24       | 0.013 |

### mC

| Age  | No. of biol. repl. | mC / G [%] | SD  | No. of biol. repl. | mC / G [%] | SD  |
|------|--------------------|------------|-----|--------------------|------------|-----|
| p1   | 2                  | 2.89       | 0.09 | 2                  | 2.08       | 0.07 |
| p14  | 3                  | 2.51       | 0.08 | 3                  | 2.43       | 0.04 |
| p90  | 3                  | 4.38       | 0.05 | 3                  | 3.98       | 0.08 |
| pm12 | 3                  | 4.19       | 0.16 | 3                  | 3.72       | 0.43 |
| pm18 | 3                  | 4.83       | 0.18 | 3                  | 3.84       | 0.21 |
6) Supplementary Literature

[1] T. Pfaffeneder, F. Spada, M. Wagner, C. Brandmayr, S. K. Laube, D. Eisen, M. Truss, J. Steinbacher, B. Hackner, O. Kotljarova, D. Schürmann, S. Michalakis, O. Kosmatchev, S. Schiesser, B. Steigenberger, N. Raddaoui, G. Kashiwazaki, U. Müller, C. G. Spruijt, M. Vermeulen, H. Leonhardt, P. Schär, M. Müller, T. Carell, Nat. Chem. Biol. 2014, 10, 574-581.

[2] a) T. F. J. Kraus, D. Globisch, M. Wagner, S. Eigenbrod, D. Widmann, M. Münzel, M. Müller, T. Pfaffeneder, B. Hackner, W. Feiden, U. Schüller, T. Carell, H. A. Kretzschmar, Int. J. Cancer 2012, 131, 1577-1590; b) S. Schiesser, T. Pfaffeneder, K. Sadeghian, B. Hackner, B. Steigenberger, A. S. Schroder, J. Steinbacher, G. Kashiwazaki, G. Hofner, K. T. Wanner, C. Ochsenfeld, T. Carell, J. Am. Chem. Soc. 2013, 135, 14593-14599; c) A. Perera, D. Eisen, M. Wagner, S. K. Laube, A. F. Kunzel, S. Koch, J. Steinbacher, E. Schulze, V. Splith, N. Mittermeier, M. Muller, M. Biel, T. Carell, S. Michalakis, Cell rep. 2015, 11, 283-294.