

5-Hydroxymethyl-, 5-Formyl- and 5-Carboxydeoxycytidines as Oxidative Lesions and Epigenetic Marks

Florian Schelter^{+, [a]}, Angie Kirchner^{+, [a, b]}, Franziska R. Traube,^[a] Markus Müller,^[a] Wolfgang Steglich,^[a] and Thomas Carell^{*[a]}

In memory of François Diederich

Abstract: The four non-canonical nucleotides in the human genome 5-methyl-, 5-hydroxymethyl-, 5-formyl- and 5-carboxydeoxycytidine (mdC, hmdC, fdC and cadC) form a second layer of epigenetic information that contributes to the regulation of gene expression. Formation of the oxidized nucleotides hmdC, fdC and cadC requires oxidation of mdC by ten-eleven translocation (Tet) enzymes that require oxygen, Fe(II) and α -ketoglutarate as cosubstrates. Although these oxidized forms of mdC are widespread in mammalian genomes, experimental evidence for their presence in fungi and plants is ambiguous. This vagueness is caused by the fact that these oxidized mdC derivatives are also formed as oxidative lesions, resulting in unclear basal levels that are likely to have no epigenetic function. Here, we report the xdC levels in the fungus *Amanita muscaria* in comparison to murine embryonic stem cells (mESCs), HEK cells and induced pluripotent stem cells (iPSCs), to obtain information about the basal levels of hmdC, fdC and cadC as DNA lesions in the genome.

The genetic system consists of four deoxyribonucleotides (dA, dC, dG and T), which create the sequence information that encodes the construction of proteins from amino acids. In higher organisms, four additional nucleotides are present that are all derived from 5-methyl-deoxycytidine (mdC). These four epigenetic nucleotides are mdC itself and the three derivatives 5-hydroxymethyl-, 5-formyl-, and 5-carboxydeoxycytidine

(hmdC, fdC and cadC = xdC) formed by oxidation of mdC with the help of α -ketoglutarate and oxygen requiring Tet-enzymes.^[1–4] These epigenetic nucleotides control the transcriptional activity of the genes in which they are embedded in a poorly understood way. It is however known, that the nucleotides fdC and cadC are substrates for the DNA repair glycosylase Tdg.^[5–8] This enzyme is able to hydrolyse the glycosidic bond of fdC and cadC, thereby generating abasic (AP) sites. These AP sites may subsequently be processed to β -elimination sites (β E). Finally, AP and β E-sites are replaced by unmodified dC,^[9] which ultimately leads to the conversion of the xdCs back to dCs (Figure 1).^[10]

This whole process of dC methylation by dedicated DNA methyltransferases (Dnmt enzymes), Tet-induced oxidation to xdC and Tdg-initiated conversion of fdC and cadC to dC sites, creates a dynamic active methylation and demethylation circle depicted in Figure 1.^[11–14]

Although this circle is now well established in mammalian genomes, experimental data about the levels of xdC bases in other higher eukaryotes such as plants and fungi are ambiguous. This suggests that at least in some of these species the detected xdC levels might be caused by just oxidative stress

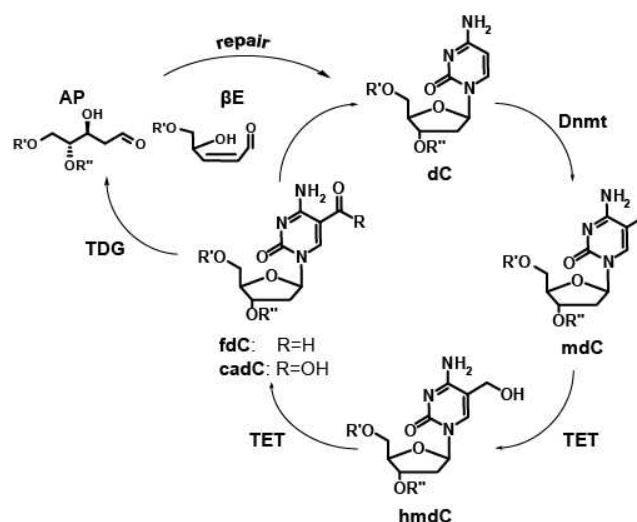


Figure 1. Depiction of the mdC oxidation cycle. Dnmt enzymes methylate dC to mdC, which is oxidized by Tet enzymes to give xdCs (hmdC + fdC + cadC). Tdg cleaves the glycosidic bond of fdC and cadC to create abasic sites (AP). Other bifunctional repair enzymes produce β E-sites. Both BER intermediates are further processed leading to an insertion of dC. (R' and R'' = DNA).

[a] F. Schelter,⁺ Dr. A. Kirchner,⁺ Dr. F. R. Traube, Dr. M. Müller, Prof. Dr. W. Steglich, Prof. Dr. T. Carell
Ludwigs-Maximilian-Universität München
Butenandtstr. 5–13, 81377 Munich (Germany)
E-mail: thomas.carell@cup.lmu.de

[b] Dr. A. Kirchner⁺
Cancer Research UK Cambridge Institute, Li Ka Shing Centre
University of Cambridge
Cambridge CB2 0RE (UK)

[⁺] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/chem.202100551>

© 2021 The Authors. Published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

(ROS). In *Coprinopsis cinerea*,^[15] Tet homologues were identified, but data about the genomic xdC levels were not reported. It is a general problem that the xdC levels formed by oxidative stress are not known. In this context, it is important that repair enzymes such as the thymidine-DNA-glycosylase (Tdg) are able to remove fdC and cadC from the genome. The basal oxidative stress levels depend consequently not only on the amount of ROS, but also on the repair activity.^[16]

The availability of new ultra-high performance liquid chromatography (UHPLC)-MS² procedures in combination with synthetic isotope standards and reagents enable the quantification of AP and β E-sites,^[9,17] and the parallel determination of the xdC levels and BER repair events. This now allows us to gain information about the ROS induced xdC levels.^[18–19] We selected as a model organism the fungus *Amanita muscaria*,^[20] which was collected from its natural environment. The methylation levels in fungi of the basidiomycota are diverse between 2–5% per dC.^[21–22] Here, we measured for *Amanita muscaria* an mdC level of 3.3%, which is quite comparable to the levels in mammalian cell cultures^[23] and tissues.^[24–25] Furthermore, analysis of the annotated *Amanita* genome,^[20] regarding the content of Tet enzymes also in comparison with *Coprinopsis cinerea*, for which a Tet enzyme was recently reported,^[26] revealed no obvious Tet homologues making *Amanita muscaria* an ideal species to study the ROS induced levels of hmdC, fdC and cadC.

For the MS-based quantification of the non-canonical nucleotide levels in *Amanita muscaria*, we removed pieces from the stem and the cap and isolated the DNA material after bead milling of the cellular material using a reported procedure (Supporting Information).^[27] The DNA was enzymatically digested into single nucleosides and analysed according to a protocol that we reported recently in detail (Supporting Information).^[27–28] For exact quantification of the mdC and xdC levels, we added synthetic isotope standards of all bases as shown in Figure 2A. These function as internal references. Exact quantification was performed as recently described.^[27] In brief, the obtained nucleoside mixture was separated by UHPLC and characterised by coupling to a triple quadrupole mass spectrometer Figure 2B. We quantified the amounts of the xdC derivatives and for assessment of oxidative stress the oxidative lesion 8oxodG, again with the synthetically prepared standards depicted in Figure 2A. We then performed the same experiment with human iPSCs (neurogenin1/2-inducible iPSCs, so called iNGNs)^[29] and HEK 293T cells.

The obtained data depicted in Figure 2C show that the mdC levels measured in *Amanita muscaria* (cap and stem) are indeed comparable to those detected in HEK 293T and to data measured earlier in human and mouse tissues.^[25]

In contrast to the rather similar mdC levels, however, we detected as expected for an organism with putatively no Tet expression very little hmdC. The exact quantification revealed an extremely low level of hmdC with 2.5×10^{-3} per dG. This is ten-fold lower compared to HEK 293T cells, which have measurable but very low Tet expression levels.^[30] This strong reduction of the hmdC levels is interesting particularly because cell culture conditions tend to reduce mitochondrial content and oxidative phosphorylation. The hmdC value is furthermore

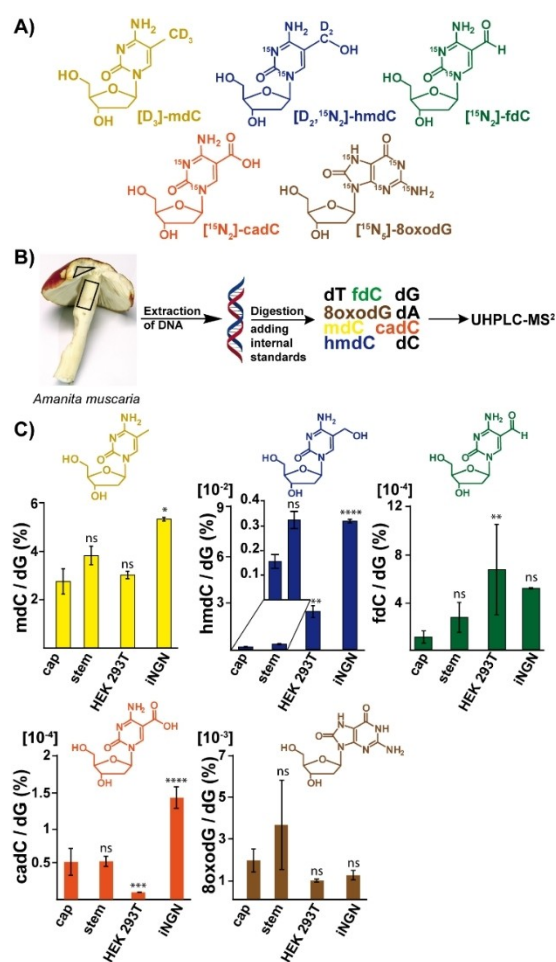


Figure 2. A) Depiction of the synthetic isotopically labelled nucleosides used as internal standards. B) Experimental procedure. C) Data about the absolute levels of mdC, xdC bases and the main oxidative lesion 8oxodG from six biological replicates. Bars show mean, error bars show standard deviation (s.d.). (Ordinary one way ANOVA with Tukey's multiple comparisons test: ns: $p \geq 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

40-times lower compared to induced pluripotent stem cells (iNGN), also kept in culture, where TET-mediated epigenetic processes play a vital regulatory role.^[31–32] We concluded that the low hmdC level in *Amanita muscaria* represents a level that we would consider to be a basal level of hmdC that is formed by oxidative stress under natural (wild life) conditions. In order to gain support for this idea, we next measured the 8oxodG levels. 8oxodG is an oxidative lesion derived from dG. Indeed, we found that the 8oxodG levels are similar to those of hmdC. Interestingly, we detected slightly more 8oxodG in the *Amanita muscaria* stem, where the hmdC was higher as well, showing a potential correlation between the 8oxodG, the hmdC levels and hence oxidative stress.

The comparison of the fdC and cadC levels in the fungus *Amanita muscaria*, in HEK 293T- and iNGN cells resulted in a wide range of values. The fdC levels are lowest in *Amanita muscaria*, again in agreement with lacking epigenetic relevance. Regarding cadC, we detected the highest values in iNGNs, again supported by epigenetic activity. In general, however, the

differences are surprisingly small given that we compare data from an organism collected from nature with cells kept under controlled cell culture conditions, with likely reduced oxidative phosphorylation.

We next turned our attention to mESCs, where epigenetic oxidation of mdC is a well-studied process.^[33] Here, we investigated three different cell lines (Figure 3). The first is a wildtype cell line containing biallelic copies of Tdg (Tdg^{+/+}). The second cell line is deficient in Tdg (Tdg^{-/-}) and the third mESC line contains a point mutation in the catalytic centre that encodes for a catalytically mutant Tdg (Tdg^{CM}) with impaired glycosylase activity.^[8,34] This is a very important cell line, because a complete Tdg knockout could also affect protein complexes that contain Tdg as a component,^[8,35–36] which could potentially influence the experimental outcome. These cell lines were generated by CRISPR-Cas9-mediated genome editing. A single-stranded repair template containing either mutations in exon 2 (Tdg^{-/-}) or exon 4 (Tdg^{CM}), respectively, was transiently transfected together with a plasmid containing the endonuclease Cas9 and the respective guide RNA. Earlier work could demonstrate that a D151A point mutation in the catalytic centre of Tdg preserves fdC binding by the glycosylase, but does not lead to base excision.^[37] For the knockout, mutations causing a shift in the reading frame were predicted. The genomic sequence of the repair template was altered accordingly, and a restriction site was introduced or removed to identify targeted clonal populations (Supporting Information). Tdg knockout and catalytic mutant candidates were validated by Sanger sequencing and Western blotting (Supporting Information).

The exact quantification data of all mESC cell lines are depicted in Figure 3. The first observation is that the hmdC

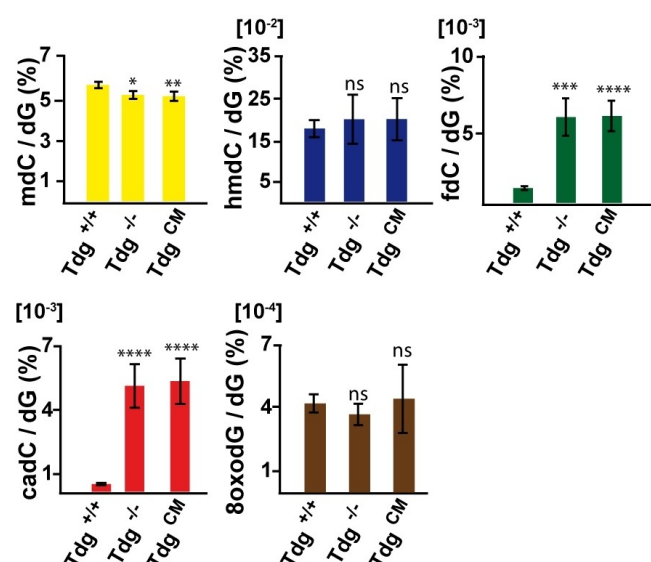


Figure 3. Depiction of the modified xdc levels in Tdg-proficient (Tdg^{+/+}), knockout (Tdg^{-/-}) and catalytic mutant (Tdg^{CM}, N151A) mESCs. Graphs show data from four biological replicates, bars represent mean, error bars represent s.d. (Ordinary one-way ANOVA with Dunnett's multiple comparison test: ns: $p \geq 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

levels are similar in all three cell lines, arguing that all three cell lines have a comparable Tet activity, as expected. The second observation is that the hmdC levels are two orders of magnitude higher compared to *Amanita muscaria*. These high Tet-generated hmdC values support the epigenetic relevance of this non-canonical base in mESCs. In agreement with the idea that the glycosylase Tdg removes fdC and cadC, we see interesting differences in deficient and mutant Tdg mESCs. In fully repair competent Tdg^{+/+} cells we see low fdC and cadC levels, but the fdC value is still ten times higher compared to *Amanita* showing that the repair process is unable to remove all fdC formed in mESCs. This finding is in full agreement with previous reports showing that fdC is a permanent or semi-permanent base that has a long life-time in specific genome regions.^[5,38–39] Interestingly, we see that the Tdg^{-/-} and Tdg^{CM} cells behave similar, arguing that disruption of Tdg-containing protein complexes has only a minor impact on the xdc levels. Both cell lines (Tdg^{-/-} and Tdg^{CM}) have the expected elevated fdC and cadC levels due to lack of repair. The levels of oxidative damage, as measured by the 8oxodG levels, were in the mESC cultures lower than *Amanita muscaria* but comparable to the HEK 293T and iNGN data. This can be explained with the fact that *Amanita muscaria* is an organism that was collected from nature, while in culture, cells are kept under controlled laboratory conditions. The low 8oxodG levels but more strikingly the 10-times higher cadC levels in the Tdg^{+/+} mESCs, compared to *Amanita muscaria*, let us conclude that the vast majority of the xdc levels in mESCs are epigenetically formed.

Our data led us to assume that the xdc levels detected in *Amanita muscaria* and potentially also those seen in HEK 293T cells represent the levels that are derived exclusively from oxidative stress (hmdC = 5×10^{-6} , fdC = 5×10^{-7} and cadC = 1×10^{-7}). This leads to the conclusion, that in mESCs, the levels of hmdC are 100-times above the ROS damage level. The fdC value is about 10-times above the ROS damage level in mESCs. Interestingly, in mESCs (Tdg^{+/+}) the cadC level is reduced to the ROS level. The elevated levels of fdC and cadC in BER-deficient mESC cells (Tdg^{-/-} and Tdg^{CM}) support the idea of a quick fdC and cadC repair by Tdg.

Interesting is the observation that in mESCs BER reduces cadC to the ROS level arguing that, if at all, only very few cadCs might have a permanent or at semi-permanent character as it was seen for fdC.^[5,39]

In order to finally investigate if *Amanita muscaria* has a normal base excision repair process, we next quantified the AP and β E-site levels. To this end the isolated DNA was treated with the recently introduced AP- and β E-site detection reagent.^[9] The hydroxylamine reagent reacts efficiently with the aldehyde functional group to give stable adducts, which after full digestion of the DNA, generate AP- and β E-adducts that can be quantified by UHPLC-MS².

For exact quantification we synthesized isotopically modified versions of the AP- and β E-site adducts (shown in Figure 4A), which were again used as internal standards. The reagent allows extremely sensitive detection, because it eliminates N₂ in the gas phase, which leads to a defined MS transition, which can be easily detected in MS/MS experiments

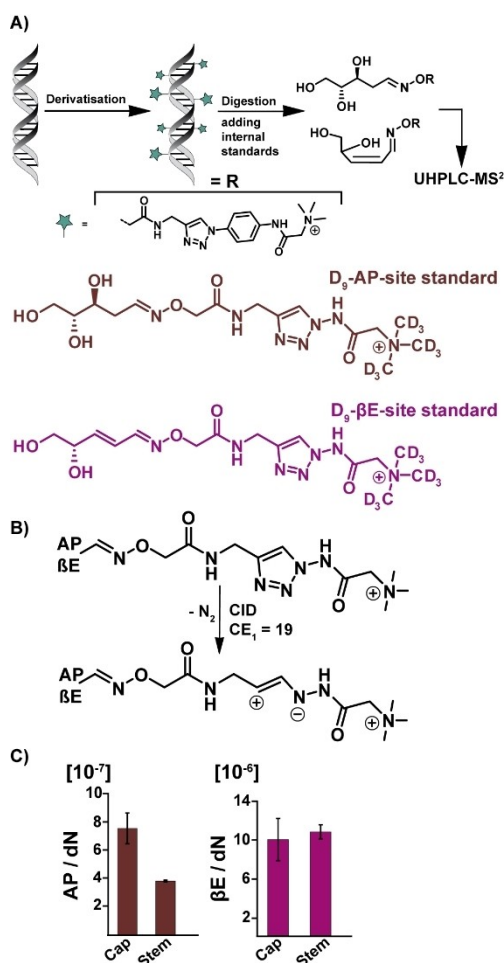


Figure 4. A) Workflow of the experiment used to quantify the AP- and β E-sites as BER intermediates. B) Depiction of fragmentation pattern, which gives a defined daughter ion after loss of N_2 . C) Depiction of absolute values of AP- and β E-sites in stem and cap of *Amanita muscaria*. Three biological replicates each measured as technical duplicate.

(Figure 4B).^[9] The experimental workflow and the data are shown in Figure 4. With this method we quantified the AP-sites to be $4\text{--}8 \times 10^{-7}$ and β E-sites to be around 1×10^{-5} per dN. These values are very comparable to previously published data from HEK 293T cells (8.8×10^{-7} AP- and 2.0×10^{-6} β E sites per dN),^[9] supporting functional BER in *Amanita muscaria*. The measured basal ROS levels of the xDCs are consequently generated in a repair competent system.^[9]

In summary, we used ultrasensitive UHPLC-MS² methods with synthetic internal isotope standards to quantify the levels of mdC, hmdC, fdC, cadC, 8oxodG, AP- and β E-sites in two systems that have no or low Tet activity (*Amanita muscaria* and HEK 293T cells) and compared the levels with data from cells that are epigenetically active at low (iNGN) and high (mESC) levels. We could deduce that the levels of hmdC = 5×10^{-6} , fdC = 5×10^{-7} and cadC = 1×10^{-7} are levels that can be considered to be the basal, steady state levels generated by oxidative stress and base excision repair. Epigenetic activity, created by Tet induced oxidation, increases the hmdC levels by

a factor of 100 and the fdC value by a factor of 10. If repair is switched off as in Tdg^{-/-} or Tdg^{CM} cells, the levels of fdC and cadC increase as expected by a factor of 5–10. Our data suggest that, while some of the fdCs have a permanent or semi-permanent character, because in mESCs even with full BER (Tdg^{+/+}) the levels never drop to the ROS level, this is questionable for cadCs. Removing fdC at these (semi) permanent sites then requires Tdg-independent pathways that were recently investigated in detail.^[19]

Acknowledgements

We thank Kerstin Kurz and Claudia Scherübl for experimental help. Further we would like to thank the Deutsche Forschungsgemeinschaft (DFG) for financial support via GRK2338 (Project ID 321812289), via CRC1309 (Project ID 325871075) and CRC1361 (Project ID 893547839). This project has received additional funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme under grant agreement No 741912 (EpiR) and Marie Skłodowska-Curie grant agreement No 765266 (LightDyNAmics). We are grateful for funding from the Volkswagen Foundation. Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: DNA repair · DNA modification · epigenetics · mass spectrometry · oxidative lesion

- [1] M. Münzel, D. Globisch, T. Carell, *Angew. Chem. Int. Ed.* **2011**, *50*, 6460–6468; *Angew. Chem.* **2011**, *123*, 6588–6596.
- [2] S. Ito, L. Shen, Q. Dai, S. C. Wu, L. B. Collins, J. A. Swenberg, C. He, Y. Zhang, *Science* **2011**, *333*, 1300–1303.
- [3] C. G. Spruijt, F. Gnerlich, A. H. Smits, T. Pfaffeneder, P. W. Jansen, C. Bauer, M. Münzel, M. Wagner, M. Müller, F. Khan, H. C. Eberl, A. Mensinga, A. B. Brinkman, K. Lephikov, U. Müller, J. Walter, R. Boelens, H. van Ingen, H. Leonhardt, T. Carell, M. Vermeulen, *Cell* **2013**, *152*, 1146–1159.
- [4] M. Tahiliani, K. P. Koh, Y. Shen, W. A. Pastor, H. Bandukwala, Y. Brudno, S. Agarwal, L. M. Iyer, D. R. Liu, L. Aravind, A. Rao, *Science* **2009**, *324*, 930–935.
- [5] M. Su, A. Kirchner, S. Stazzoni, M. Müller, M. Wagner, A. Schröder, T. Carell, *Angew. Chem. Int. Ed.* **2016**, *55*, 11797–11800; *Angew. Chem.* **2016**, *128*, 11974–11978.
- [6] T. Fu, L. Liu, Q. L. Yang, Y. Wang, P. Xu, L. Zhang, S. Liu, Q. Dai, Q. Ji, G. L. Xu, C. He, C. Luo, L. Zhang, *Chem. Sci.* **2019**, *10*, 7407–7417.
- [7] A. Maiti, A. C. Drohat, *J. Biol. Chem.* **2011**, *286*, 35334–35338.
- [8] S. Cortellino, J. Xu, M. Sannai, R. Moore, E. Caretti, A. Cigliano, M. Le Coz, K. Devarajan, A. Wessels, D. Soprano, L. K. Abramowitz, M. S. Bartolomei, F. Rambow, M. R. Bassi, T. Bruno, M. Fanciulli, C. Renner, A. J. Klein-Szanto, Y. Matsumoto, D. Kobi, I. Davidson, C. Alberti, L. Larue, A. Bellacosa, *Cell* **2011**, *146*, 67–79.
- [9] R. Rahimoff, O. Kosmatchev, A. Kirchner, T. Pfaffeneder, F. Spada, V. Brantl, M. Müller, T. Carell, *J. Am. Chem. Soc.* **2017**, *139*, 10359–10364.
- [10] F. Neri, D. Incarnato, A. Krepelova, S. Rapelli, F. Anselmi, C. Parlato, C. Medana, F. Dal Bello, S. Oliviero, *Cell Rep.* **2015**, *10*, 674–683.

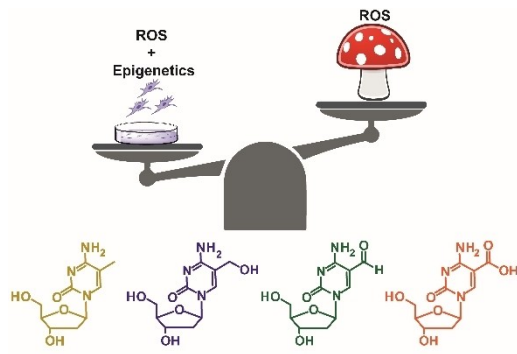
- [11] A. Schön, E. Kaminska, F. Schelter, E. Ponnkone, E. Korytiaková, S. Schiffers, T. Carell, *Angew. Chem. Int. Ed.* **2020**, *59*, 5591–5594; *Angew. Chem.* **2020**, *132*, 5639–5643.
- [12] T. Pfaffeneder, F. Spada, M. Wagner, C. Brandmayr, S. K. Laube, D. Eisen, M. Truss, J. Steinbacher, B. Hackner, O. Kotljarova, D. Schuermann, S. Michalakakis, O. Kosmathech, 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.
- [13] A. Jeltsch, *ChemBioChem* **2002**, *3*, 382–382.
- [14] Q. Du, Z. Wang, V. L. Schramm, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 2916–2921.
- [15] L. M. Iyer, D. Zhang, R. F. de Souza, P. J. Pukkila, A. Rao, L. Aravind, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 1676–1683.
- [16] S. Ito, I. Kuraoka, *DNA Repair* **2015**, *32*, 52–57.
- [17] A. C. Drohat, C. T. Coey, *Chem. Rev.* **2016**, *116*, 12711–12729.
- [18] T. Carell, M. Q. Kurz, M. Müller, M. Rossa, F. Spada, *Angew. Chem. Int. Ed.* **2018**, *57*, 4296–4312; *Angew. Chem.* **2018**, *130*, 4377–4394.
- [19] F. Spada, S. Schiffers, A. Kirchner, Y. Zhang, G. Arista, O. Kosmathech, E. Korytiaková, R. Rahimoff, C. Ebert, T. Carell, *Nat. Chem. Biol.* **2020**, *16*, 1411–1419.
- [20] A. Kohler, A. Kuo, L. G. Nagy, E. Morin, K. W. Barry, F. Buscot, B. Canbäck, C. Choi, N. Cichocki, A. Clum, J. Colpaert, A. Copeland, M. D. Costa, J. Doré, D. Floudas, G. Gay, M. Girlanda, B. Henrissat, S. Herrmann, J. Hess, N. Högberg, T. Johansson, H.-R. Khouja, K. LaButti, U. Lahrmann, A. Levasseur, E. A. Lindquist, A. Lipzen, R. Marmeisse, E. Martino, C. Murat, C. Y. Ngan, U. Nehls, J. M. Plett, A. Pringle, R. A. Ohm, S. Perotto, M. Peter, R. Riley, F. Rineau, J. Ruytinx, A. Salamov, F. Shah, H. Sun, M. Tarkka, A. Tritt, C. Veneault-Fourrey, A. Zuccaro, A. Tunlid, I. V. Grigoriev, D. S. Hibbett, F. Martin, C. Mycorrhizal Genomics Initiative, *Nat. Genet.* **2015**, *47*, 410–415.
- [21] T. Binz, N. D’Mello, P. A. Horgen, *Mycologia* **1998**, *90*, 785–790.
- [22] A. Zemach, I. E. McDaniel, P. Silva, D. Zilberman, *Science* **2010**, *328*, 916.
- [23] H. G. Leitch, K. R. McEwen, A. Turp, V. Encheva, T. Carroll, N. Grabole, W. Mansfield, B. Nashun, J. G. Knezovich, A. Smith, M. A. Surani, P. Hajkova, *Nat. Struct. Mol. Biol.* **2013**, *20*, 311–316.
- [24] D. Globisch, M. Münzel, M. Müller, S. Michalakakis, M. Wagner, S. Koch, T. Brückl, M. Biel, T. Carell, *PLoS One* **2010**, *5*, e15367.
- [25] M. Wagner, J. Steinbacher, T. F. J. Kraus, S. Michalakakis, B. Hackner, T. Pfaffeneder, A. Perera, M. Müller, A. Giese, H. A. Kretzschmar, T. Carell, *Angew. Chem. Int. Ed.* **2015**, *54*, 12511–12514; *Angew. Chem.* **2015**, *127*, 12691–12695.
- [26] Y. F. He, B. Z. Li, Z. Li, P. Liu, Y. Wang, Q. Tang, J. Ding, Y. Jia, Z. Chen, L. Li, Y. Sun, X. Li, Q. Dai, C. X. Song, K. Zhang, C. He, G. L. Xu, *Science* **2011**, *333*, 1303–1307.
- [27] F. R. Traube, S. Schiffers, K. Iwan, S. Kellner, F. Spada, M. Müller, T. Carell, *Nat. Protoc.* **2019**, *14*, 283–312.
- [28] F. Yuan, Y. Bi, J.-Y. Zhang, Y.-L. Zhou, X.-X. Zhang, C.-X. Song, *RSC Adv.* **2019**, *9*, 29010–29014.
- [29] V. Busskamp, N. E. Lewis, P. Guye, A. H. Ng, S. L. Shipman, S. M. Byrne, N. E. Sanjana, J. Murn, Y. Li, S. Li, M. Stadler, R. Weiss, G. M. Church, *Mol. Syst. Biol.* **2014**, *10*, 760.
- [30] M. Y. Liu, J. E. DeNizio, R. M. Kohli, *Methods in Enzymology*, Vol. 573, Ed.: R. Marmorstein, Academic Press, **2016**, pp. 365–385.
- [31] K. Hochedlinger, R. Jaenisch, *Cold Spring Harbor Perspect. Biol.* **2015**, *7*.
- [32] Y. Gao, J. Chen, K. Li, T. Wu, B. Huang, W. Liu, X. Kou, Y. Zhang, H. Huang, Y. Jiang, C. Yao, X. Liu, Z. Lu, Z. Xu, L. Kang, J. Chen, H. Wang, T. Cai, S. Gao, *Cell Stem Cell* **2013**, *12*, 453–469.
- [33] Y. Atlasi, H. G. Stunnenberg, *Nat. Rev. Genet.* **2017**, *18*, 643–658.
- [34] Y.-F. He, B.-Z. Li, Z. Li, P. Liu, Y. Wang, Q. Tang, J. Ding, Y. Jia, Z. Chen, L. Li, Y. Sun, X. Li, Q. Dai, C.-X. Song, K. Zhang, C. He, G.-L. Xu, *Science* **2011**, *333*, 1303–1307.
- [35] D. Cortázar, C. Kunz, J. Selfridge, T. Lettieri, Y. Saito, E. MacDougall, A. Wirz, D. Schuermann, A. L. Jacobs, F. Siegrist, R. Steinacher, J. Jiricny, A. Bird, P. Schär, *Nature* **2011**, *470*, 419–423.
- [36] H. M. Hassan, B. Kolendowski, M. Isovich, K. Bose, H. J. Dranse, A. V. Sampaio, T. M. Underhill, J. Torchia, *Cell Rep.* **2017**, *19*, 1685–1697.
- [37] L. S. Pidugu, Q. Dai, S. S. Malik, E. Pozharski, A. C. Drohat, *J. Am. Chem. Soc.* **2019**, *141*, 18851–18861.
- [38] E.-A. Raiber, D. Beraldi, G. Ficiz, H. E. Burgess, M. R. Branco, P. Murat, D. Oxley, M. J. Booth, W. Reik, S. Balasubramanian, *Genome Biol.* **2012**, *13*, R69.
- [39] M. Bachman, S. Uribe-Lewis, X. Yang, H. E. Burgess, M. Iurlaro, W. Reik, A. Murrell, S. Balasubramanian, *Nat. Chem. Biol.* **2015**, *11*, 555–557.

Manuscript received: February 12, 2021

Accepted manuscript online: March 26, 2021

Version of record online: ■■■, ■■■■

COMMUNICATION



*F. Schelter, Dr. A. Kirchner, Dr. F. R. Traube, Dr. M. Müller, Prof. Dr. W. Steglich, Prof. Dr. T. Carell**

1 – 6

5-Hydroxymethyl-, 5-Formyl- and 5-Carboxydeoxycytidines as Oxidative Lesions and Epigenetic Marks



Exact UHPLC-MS² quantifications were used to determine the oxidative lesion and epigenetically induced

levels of the non-canonical bases hmdC, fdC and cadC and of the levels of base excision repair intermediates.
