Laboratory for Molecular Virology and Molecular Medical Genetics
Studies on Epigenetics, 2016

Institute for Clinical and Molecular Virology, University Erlangen-Nürnberg Medical School and Institute of Genetics, University of Cologne

The Research Group: Dr. rer. nat. Anja Naumann (part time), Dr. rer. nat. Stefanie Weber,

Principal Investigator: Walter Doerfler (W.D.) is Professor *emeritus*, University of Cologne and Guest Professor (2002-), Institute for Virology, Erlangen University Medical School. After Medical School in Munich (LMU) and postdoctoral training at the Max-Planck-Institute for Biochemistry in Munich (1961-1963) and the Department of Biochemistry at Stanford University Medical School (1963-1966), W.D. held faculty positions at Rockefeller University in New York City, N.Y., USA (Assistant-, Associate-, and Adjunct-Professor, 1966-1978) and at the Institute of Genetics in Cologne (Professor, 1972-2002). He was guest professor at Uppsala (1971/72, 2002, 2006, 2007, 2009) as well as at Stanford (1978, 1993), Princeton (1986, 1999), and Vanderbilt (2006) Universities. W.D. was the speaker of SFB 74 (1978-1988) and of SFB 274 (1988-2000) of the DFG. In 1994, members of SFB's 274 and 288 in Cologne played an important role in the foundation of the Center for Molecular Medicine Cologne (CMMC). Since 2002, W.D. has continued basic research in molecular genetics as guest professor in the Institute of Clinical and Molecular Virology, University Erlangen-Nürnberg.

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The interests of the research group have been focused on the biological function of DNA methylation in the regulation of biological processes that are of interest in medicine and epigenetics. In these investigations, we have been using viral systems (adenovirus type 12 and HIV-1) and several human genes, currently the fragile X mental retardation gene 1. DNA methylation patterns in mammalian genomes can be cell-type specific, are sometimes conserved, but can also be altered, and play an important role in genome structure and function. We have shown in a number of biological systems that the insertion of foreign DNA into an established mammalian genome can lead to genome-wide alterations in DNA methylation and transcription patterns. These latter findings are thought to be of fundamental importance for the understanding of viral and general oncogenesis and in evolution.

Since July 2002, our laboratory has been located in the **Institute for Virology, Erlangen Medical School** and maintains close ties to the **Institute of Genetics, University of Cologne**.

Research Projects

1. **Structure and possible function of the methylation boundary in the upstream region of the FMR1 promoter**

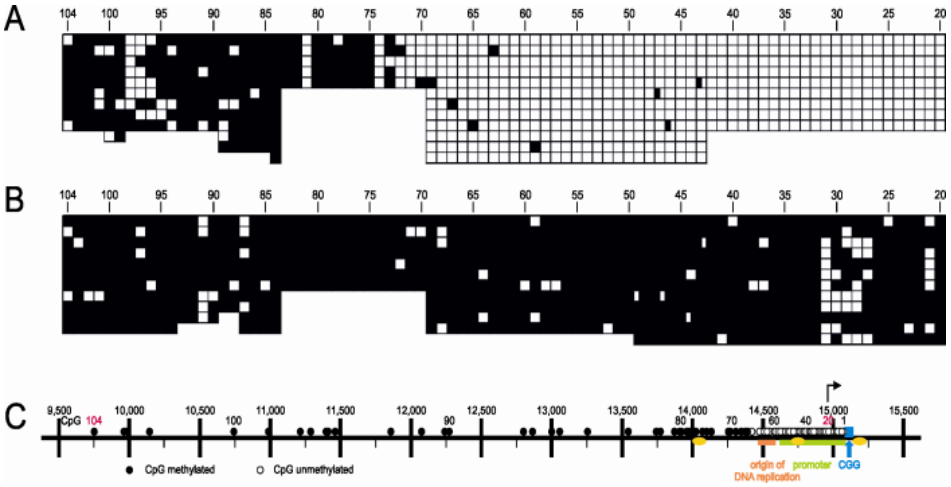

Figure 1 - The CpG methylation boundary 5'-upstream from the *FMR1* promoter. DNA samples were extracted from (A) telomerase gene-transformed fibroblasts of a non-FXS male individual, (B) non-transformed PBMC's from an FXS patient. These data document the methylation boundary and its loss in FXS individuals. In (A) and (B), the CpG dinucleotide positions were not depicted according to scale as in (C) but were compressed and immediately juxtaposed to each other. (C) Map of the 5'-upstream region of the *FMR1* gene drawn to scale. The nucleotide numbers 9,500 to 15,500 refer to NC_000023: 146.786,201 to 146,840,303 *Homo sapiens FMR1* region. The numbers 1 to 104 designate the CpG dinucleotides in the region - ○/□ unmethylated, ●/■ methylated. Other symbols are as follows: Arrow – site of transcriptional initiation; blue - CGG repeat; yellow - CTCF binding sequences; green - FMR1 promoter; orange – origin of DNA replication. On this map, the boundary has been located to CpG pair 65. The range of CpG's determined by bisulfite sequencing was demarcated by red numbering. This figure was taken from Naumann et al., 2014.

1.1 Safeguard against methylation spreading into the promoter area

The human genome segment upstream of the FMR1 (fragile X mental retardation 1) gene (Xq27.3) contains several genetic signals (see legend to Figure 1), among them a DNA methylation boundary which is located 65 to 70 CpG's upstream of the CGG repeat. In fragile X syndrome (FXS), the boundary is lost, and the promoter is inactivated by methylation spreading. We have documented boundary stability in spite of critical expansions of the CGG trinucleotide repeat in male or female premutation, in female full mutation carriers and in high functioning males (HFMs) (Figure 2). HFMs carry a full CGG repeat expansion but exhibit an unmethylated promoter and lack the FXS phenotype. The boundary is also stable in Turner (45, X) females. A CTCF-binding site is located slightly upstream of the methylation boundary and carries a unique G to A polymorphism (SNP) which occurs 3.6 times more frequently in genomes with CGG expansions. In CGG expansions the CTCF-site does not harbor additional mutations. In FXS individuals and often in **cells transgenomic for foreign DNA,** the large number of previously methylated CpG's in the far upstream region of the boundary is about fourfold decreased. A methylation boundary is also present in the human genome segment upstream of the huntingtin (HTT) promoter (4p16.3) and is stable both in normal and Huntington disease chromosomes. Hence, the vicinity of an expanded repeat does not *per se* compromise methylation boundaries. Methylation boundaries might have an important function as promoter safeguards. The increased frequency of an SNP in the boundary of genomes with CGG expansions could have functional significance.

Naumann A, Hochstein N, Weber S, Fanning E, Doerfler W. (2009). A distinct DNA methylation boundary in the 5´-upstream sequence of the FMR1 promoter binds nuclear proteins and is lost in fragile X syndrome. ***American Journal of Human Genetics* 85,** 606-616.

Naumann A, Kraus C, Hoogeveen A, Ramirez CM, Doerfler W. (2014). Stable DNA methylation boundaries and expanded trinucleotide repeats: Role of DNA insertions. ***Journal of Molecular Biology* 426,** 2554–2566.

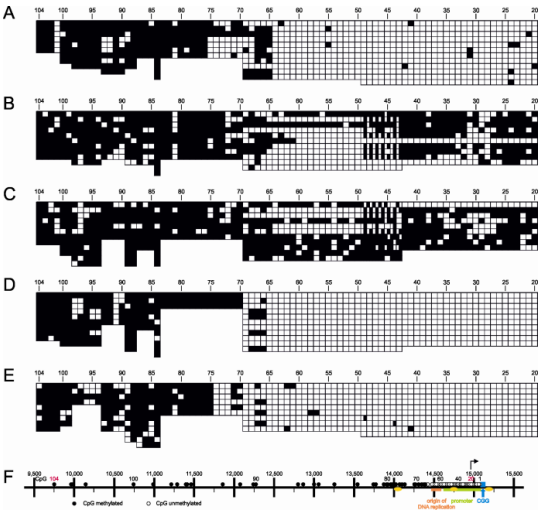

Figure 2 - The CpG methylation boundary in the 5'-upstream *FMR1* region remains **intact in borderline cases of CGG trinucleotide repeat expansions**. DNA samples were analyzed by bisulfite sequencing and were derived from (A) un-transformed PBMC's from a premutation male (n = 113 +/-1). (B) DNA from un-transformed PBMC's of a premutation female (n = 109 +/-1). Between CpG's 20 and 65, 45 % of the CpG's were methylated in this female premutation genome. (C) DNA from un-transformed PBMC's from a full mutation female (n = > 200) with 59 % of the CpG's methylated; (D) DNA from *EBV*-transformed PBMC's from a high functioning male (n = ~ 400); (E) DNA from *telomerase* gene-transformed fibroblasts from a high functioning male (n = ~ 330). (F) Map as described in the legend to Figure 1C. This figure was taken from Naumann et al., 2014.

2. Genetic and epigenetic studies on the HIV-1 proviral genome in HIV-1 infected individuals with a wide spectrum of infection modes

Unmethylated HIV-1 proviral genomes in HIV-1 infected individuals

Integrated DNA from various viruses often becomes methylated *de novo* and transcriptionally inactivated. We therefore investigated CpG methylation profiles in 55 of 94 CpG's (58.5%) in HIV-1 proviral genomes including ten CpG's in each LTR and additional CpG's in portions of the *gag, env, nef, rev,* and *tat* genes. We analyzed 33 DNA samples from PBMC's of 23 subjects representing a broad spectrum of HIV-1 disease. In 22 of 23 HIV-1-infected individuals, there were only unmethylated CpG's regardless of infection status. In one long term non-progressor, however, methylation of proviral DNA varied between 0 and 75% over an 11-year period although the CD4+ counts of this individual remained stable. Hence levels of proviral DNA methylation can fluctuate. The preponderance of unmethylated CpG's suggests that proviral methylation is not a major factor in regulating HIV-1 proviral activity in PBMC's. Unmethylated CpG's may play a role in HIV-1 immunopathogenesis.

Weber S, Weiser B, Kemal KS, Burger H, Ramirez CM, Korn K, Anastos K, Kaul R, Kovacs C, Doerfler W. (2014). Epigenetic analysis of HIV-1 proviral genomes from infected individuals: Predominance of unmethylated CpG's. ***Virology*** 449, 181-189.

3. Destabilization of the human epigenome: consequences of foreign DNA insertions

Based on the study of human adenovirus type 12 (Ad12) as an oncogenic DNA virus, the fate of foreign DNA in mammalian systems and the epigenetic consequences of foreign DNA insertions have been a long-term interest in my laboratory. Foreign DNA which emanates from a panoply of sources is ubiquitous and abundant in our environment. Research about the fate of this very stable and biologically potent molecule in the environment is a medically highly relevant topic. How can DNA interact with and be taken up by living cells, how frequently is it integrated in the invaded cell's genome, and what are the consequences of these interactions for cell survival and

genetic integrity? In studies on the integrated state of Ad12 DNA in Ad12-transformed hamster cells, we discovered that the CpG methylation profiles in some of their endogenous retrotransposon sequences and in several cellular genes were increased. This augmented methylation persisted in revertants of the transformed cells that had lost all Ad12 genomes ("hit and run" mechanism). Moreover, alterations of DNA methylation and transcription profiles were documented in Ad12 DNA- and in bacteriophage λ DNA-transgenomic cells.

• We previously hypothesized that epigenetic effects in mammalian genomes due to the insertion of foreign DNA are a general phenomenon. These alterations might play a role in (viral) oncogenesis and are possibly instrumental during evolution as a consequence of multiple retroviral DNA insertions into ancient genomes. Over evolutionary times, these alterations of transcription profiles might have led to novel phenotypes that were then selected for or against depending on environmental conditions during evolution.

• To examine the general significance of these observations, we designed a model system for proof of principle assessment. Human cells from cell line HCT116 were rendered transgenomic by transfecting a 5.6 kbp bacterial plasmid and selecting cell clones with foreign plasmids stably integrated, most likely at different genomic sites.

• In five non-transgenomic HCT116 control clones without the plasmid, transcription and methylation patterns proved similar, if not identical, among individual cell clones. This finding opened the possibility for comparisons of these patterns between non-transgenomic and transgenomic clones.

• In 4.7% of the 28,869 human gene segments analyzed, the transcriptional activities were upregulated (907 genes) or downregulated (436 genes) in plasmid-transgenomic cell clones in comparison to control clones (Figure 3A). A significant gene set enrichment was found in 43 canonical pathways. Frequent upregulations were noted in small nucleolar RNA genes that regulate RNA metabolism and in genes involved in signaling pathways.

• Genome-wide methylation profiling was performed for >480,000 CpG sites. In comparisons of methylation levels in five transgenomic versus four non-transgenomic cell clones, 3791 CpG's were differentially methylated, 1504 CpG's were hyper- and 2287 were hypo-methylated (Figure 3B).

• Thus, the epigenetic effects in the wake of foreign DNA integration events can be considered a general effect also in human cells. We still lack insights into the role of transgenome size, gene or CG content or copy number. The mechanism(s) underlying the observed epigenetic alterations are unknown. Extent and location of alterations in genome activities and CpG methylation might depend on the site(s) of foreign DNA insertion.

• We note that genome manipulations in general – work with transgenomic or knocked cells and organisms – have assumed a major role in molecular biology and medicine. The consequences of cellular genome manipulations for epigenetic stability have so far received unwarrantedly limited attention. Before drawing far-reaching conclusions from work with cells or organisms with manipulated genomes, critical considerations for and careful analyses of their epigenomic stability will prove prudent. With previous and current research described here, we have barely scratched the surface of the problem but are now poised to ask more precise questions. The Ad12 system has been a very reliable guide to this approach which has, however, been extended also to other types of foreign DNA molecules. We will now pursue more far-reaching questions and again use the Ad12 system as a versatile model organism and guide.

Weber S, Hofmann A, Herms S, Hoffmann P, Doerfler W (2015) Destabilization of the human epigenome: consequences of foreign DNA insertions. ***Epigenomics*** 7:745-755

Weber S, Hofmann A, Naumann A, Hoffmann P, Doerfler W (2016b) Epigenetic alterations by inserting foreign DNA into mammalian genomes: oncogenesis and evolution. In: Epigenetics – a Different Way of Looking at Genetics, The Fifth Weissenburg Symposium, 2014. Springer Verlag Heidelberg, Berlin, New York. W. Doerfler & P. Boehm, editors. In press.

4. Methylation and transcription profiles in the human HERV and LINE-1 sequences

As a corollary to the earlier study (section 3 - Weber et al. 2015), we have investigated whether the alterations in transcriptional and methylation profiles had affected also repetitive genome elements like the HERV and LINE-1.2 sequences in the same transgenomic HCT116 cell clones which had exhibited epigenetic alterations in other parts of the human genome. Such differences were not found. Apparently, in the cell clones selected for this investigation the HERV and LINE elements had not responded to foreign DNA insertions. In addition, our present study provided a survey of the CpG modifications in the human endogenous viral sequences HERV-K, HERV-W, HERV-E and in LINE-1.2 whose methylation levels ranged between 60 and 98%. At least some of these elements were transcribed into RNA as determined by reverse transcription and PCR. Obviously, there are enough unmethylated control sequences to facilitate transcription of at least some of the tested elements into RNA.

Weber S, Jung S, Doerfler W (2016a) DNA Methylation in HERV (K, W, E) and LINE sequences and their transcription remain unchanged by foreign DNA insertions. ***Epigenomics*** 8, 000-000, in press. 2015 December 04: [Epub ahead of print].

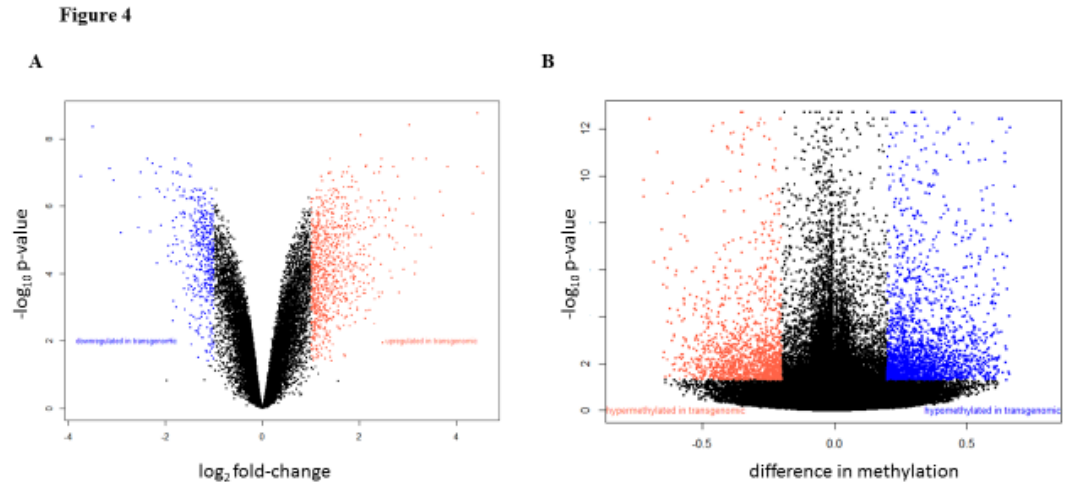

Figure 3 - Alterations in patterns of transcription (A) and methylation (B) in pC1-5.6 transgenomic HCT116 cell clones as compared to non-transgenomic cells. (A) Volcano plot displays non-standardized signals (log2 fold-change) on the x-axis against standardized signals (-log10 FDR-adjusted p-value) on the y-axis for the comparison of five non-transgenomic against seven transgenomic cell clones of all 28,869 genes analyzed. Up-regulated genes in transgenomic cell clones were displayed in red and down-regulated genes in blue (FC ±2, adjusted p-values < 0.05; n=1343 genes). **(B)** Volcano plot displays differences in methylation on the x-axis against standardized methylation (-log10 FDR-adjusted p-value) on the y-axis for the comparison of four non-transgenomic against five pC1-5.6 transgenomic cell clones of all 361,983 CpG's interrogated. Hyper-methylated CpG's in transgenomic cell clones were displayed in red and hypo-methylated CpG's in blue (Δβ value ≥0.2, adjusted p-value < 0.05; n=3,791 CpG's). This Figure and its legends were taken with permission from Weber et al., 2015.

Between **1967 and 2013**, Walter Doerfler has guided **81 doctoral students** at different times at Rockefeller University in New York City (1967-1976), at the Institute of Genetics in Köln (1972-2002), and at the Institute for Clinical and Molecular Virology (2002-2013).

Collaborations

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Cornelia **Kraus**, Institute for Human Genetics, Erlangen University Medical School, D-91054 Erlangen, Germany.

Present and Recent Funding

1. Fritz Thyssen Foundation, Cologne, Az. 10.07.2, 2010-2012.
2. Fritz Thyssen Foundation, research fellowship (Az. 40.12.0.029) for Dr. Anja Naumann, 2012/2013.
3. Fritz Thyssen Foundation – The Fifth Weissenburg Symposium, Az.: 30.14.0.033.
4. Deutsche Forschungsgemeinschaft, GZ: DO 165/28-1, 2010-2012.
5. NIH grant # 2 UOI AI035004-2, subcontract from WIHS – Erlangen University, 2013.
6. Institute for Clinical and Molecular Virology, Erlangen University Medical School.
7. Staedtler Stiftung, Nürnberg
8. Rotary Club Weißenburg in Bayern, stipend to Dr. Stefanie Weber
9. Nationale Akademie der Wissenschaften Leopoldina, Fourth Weissenburg Symposium 2011.

Publications

Selected Publications 2007 – 2016

S.J. Gray, J. Gerhardt, W. Doerfler, L.E. Small, and E. Fanning. An origin of DNA replication in the promoter region of the human fragile X mental retardation (FMR1) gene. **Molecular and Cellular Biology 27**, 426-437, 2007.

N. Hochstein, I. Muiznieks, L. Mangel, H. Brondke, and W. Doerfler. The epigenetic status of an adenovirus transgenome upon long-term cultivation in hamster cells. **Journal of Virology 81**, 5349-5361, 2007.

N. Hochstein, D. Webb, M. Hösel, W. Seidel, S. Auerochs, and W. Doerfler. Human CAR gene expression in non-permissive hamster cells boosts entry of type 12 adenovirus and nuclear import of viral DNA. **Journal of Virology 82**, 4159-4163, 2008.

A. Naumann, N. Hochstein, S. Weber E. Fanning, and W. Doerfler. A distinct DNA methylation boundary in the 5´-upstream sequence of the FMR1 promoter binds nuclear proteins and is lost in fragile X syndrome **American Journal of Human Genetics 85,** 606-616, 2009.

W. Doerfler. DNA – a molecule in search of additional functions: recipient of cosmic wave emissions? - A hypothesis. **Medical Hypotheses 75,** 291-293, 2010.

W. Doerfler. Epigenetic consequences of foreign DNA integration: Global alterations of methylation and transcription patterns in recipient genomes. **Reviews in Medical Virology 21**, 336-346, 2011.

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S. Weber, B. Weiser, K.S. Kemal, H. Burger, C.M. Ramirez, K. Korn, K. Anastos, R. Kaul, C. Kovacs, and W. Doerfler. (**2014**). Epigenetic analysis of HIV-1 proviral genomes from infected individuals: Predominance of unmethylated CpG's. **Virology** 449, 181-189.

A. Naumann, C. Kraus, A. Hoogeveen, C.M. Ramirez, and W. Doerfler. (**2014**). Stable DNA methylation boundaries and expanded trinucleotide repeats: Role of DNA insertions. **Journal of Molecular Biology 426,** 2554–2566.

S. Weber, A. Hofmann, S. Herms, P. Hoffmann, and W. Doerfler. Destabilization of the human epigenome: consequences of foreign DNA insertions. **Epigenomics 7**, 745-755, 2015.

S. Weber, S. Jung, and W. Doerfler. DNA Methylation in HERV (K, W, E) and LINE sequences and their transcription remain unchanged by foreign DNA insertions. **Epigenomics 8**, 000-000, 2016, in press. Epigenomics. 2015 Dec 2. [Epub ahead of print]

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W. Doerfler. Discoveries with the adenovirus 12 system: Integration of viral DNA and epigenetic consequences. In *Epigenetics and Infectious Diseases.* Edited by W. Doerfler, J. Casadesús, M. Noyer-Weidner, Springer Verlag Heidelberg, Berlin, New York, 2016.

Selected Publications 1968 to 1999: Integration of Foreign DNA and DNA Methylation

W. Doerfler. The fate of the DNA of adenovirus type 12 in baby hamster kidney cells. **Proc. Natl. Acad. Sci. USA 60**, 636-643, 1968.

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Organization of International Symposia on DNA Methylation and Epigenetics, 1981, 2001 – 2014, Weissenburg Symposia

Cologne Spring Meeting 1981: *DNA Methylation and Gene Activity*

Weissenburg Symposia (Weissenburg in Bayern, Germany**)**

Weissenburg Symposium 2001: *Medicine and Molecular Biology*

Second Weissenburg Symposium 2004: *DNA Methylation, an Important Genetic Signal*

Third Weissenburg Symposium 2007: *Medicine at the Interface between Science and Ethics*

Fourth Weissenburg Symposium 2011: *Epigenetics and the Control of Genetic Activity.*

Fifth Weissenburg Symposium 2014: *Epigenetics – a Different Way of Looking at Genetics.* September 14-17, 2014. Program, see **Homepage Institute of Genetics**.

Organizer and Speaker at the **Annual Meeting of the American Association for the Advancement of Science (AAAS)**, 12-16 February, 2009 in **Chicago, IL**, **USA**: *Epigenetics: Mechanisms and Impact on Biomedicine***.**

Organizer and Speaker at the **Annual Meeting of the American Association for the Advancement of Science (AAAS**), 18-22 February, 2010 in **San Diego, CA, USA**: *Science and Divinity – Genetics and Ethics***.**

Co-organizer with Andrew Feinberg of Symposium on *Epigenetics: Methylating the Mind.* **Annual Meeting of the American Society of Human Genetics (ASHG),** 02-06 November 2010 in **Washington, DC, USA.**

From right to left Indrikis Muiznieks, Stefanie Weber, Norbert Hochstein, Anja Naumann, W.D. - in front of the Institute of Clinical and Molecular Virology in Erlangen

Datenschutzerklärung: hier