

# Epigenetics Group, 2019

## *Genome-wide DNA Methylation Profiles*

Walter Doerfler, Stefanie Weber

**Institute for Clinical and Molecular Virology, Friedrich-Alexander University (FAU)  
Erlangen-Nürnberg and Institute of Genetics, University of Cologne**

**The Research Group:** Dr.rer.nat. **Stefanie Weber** has received her doctorate in 2013 from the University of Cologne for research performed in the Institute for Clinical and Molecular Virology, FAU Erlangen-Nürnberg. Since then, she has continued postdoctoral work in the same Institute on the role of DNA methylation in the genomes of viruses and mammalian cells. Phone: +49-9131-852-6416; E-mail: [siweber@gmx.de](mailto:siweber@gmx.de)

**Principal Investigator: Walter Doerfler** (W.D.) is Professor *emeritus*, University of Cologne and Guest Professor (2002- ) Institute for Clinical and Molecular Virology, FAU Erlangen-Nürnberg. After Medical School in Munich (LMU, MD 1959) and an internship at Mercer Hospital, Trenton, N.J., W.D. did postdoctoral work 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; Dr.med. h.c., 2018) 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 founding the Center for Molecular Medicine Cologne (CMMC). Since 2002, W.D. has continued basic research in molecular genetics as guest professor in the Institute for Clinical and Molecular Virology, University Erlangen-Nürnberg. **Phone:** +49-171-205-1587; **E-mail:** [walter.doerfler.extern@uk-erlangen.de](mailto:walter.doerfler.extern@uk-erlangen.de); [walter.doerfler@t-online.de](mailto:walter.doerfler@t-online.de)

**Institute for Clinical and Molecular Virology – FAU Erlangen-Nürnberg**



## Interests of the Research Group

The sequence-specific methylation of cytosine residues mainly, but not exclusively, of CpG dinucleotides in eukaryotic DNA is one of the powerful signals for the modification of genetic functions and genome stability, also in mammalian DNA. Our laboratory has been one of the first (1976-1983) to document the importance of promoter methylation in long-term gene silencing. Most likely, this effect on promoter function is enacted by altering specific DNA-protein interactions in the promoter region.

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

## Our concept

The human genome sequence contains about 28 million CpG pairs which are potential targets for the modification of cytosine- to 5-methyldeoxycytidine-residues (5-mC) by DNA methyltransferases. The distribution of 5-mC's across the human genome can vary with cell type. Depending on environmental conditions, CpG methylation patterns can be subject to change. Lacking a complete map of 5-mC locations in the human genome, how might one visualize these patterns which hold high functional significance for genomic stability and activity? The challenges raised by CpG methylation landscapes emerge from the large number of CpG's, and from the quest to decipher their functional meaning. By selecting the two following examples for more detailed inspection, we are fully aware that there will be many additional ones worth consideration.

(i) The presence of 5-mC residues in specific, functionally decisive positions of the genome is undoubtedly related to genetic activity.

(ii) Perhaps as importantly, the genome-inherent 5-mCpG versus CpG algorithms might be an important guardian of genomic stability. Perhaps similar as innate and acquired immunity respond to the intrusion of foreign, often pathogenic molecules or cells, the CpG arrays are thought to be highly sensitive to the invasion of foreign DNA into the cell. The CpG guardian might already be alerted by the contact of foreign nucleic acids with the cell surface or the mere application of techniques for gene transfer. This CpG alarm clock has probably developed early in evolution and, like other ancient biological defenses, has progressed and evolved over evolutionary times. This system is flexible and permits alterations, not always under strictly controlled conditions. Altered methylation patterns can be transmitted over cell generations, i. e. are at least in part inheritable.

The notion of a guardian for genome stability is caught between two contradicting, equally essential options. (i) Maintaining the inherited genome is the precondition for survival in the real world that abounds with a gamut of competing molecules and organisms. However, will defense of genome maintenance suffice as the major principle for survival? (ii) More realistically, the system requires the genetic and epigenetic potential to exploit competing organisms and their intruding foreign genetic information. Novel genetic and epigenetic information from foreign sources might be convenient to have around and could be constantly scanned for internal usefulness. Ubiquitous non-homologous recombination mechanisms enable the cell to incorporate newly-acquired foreign DNA into its own genome. Subsequently, this acquisition could be screened for internal advantage or might be eliminated from the cell's indigenous nucleotide

sequence if advantageous. Selection in a competitive environment would then determine survival of propitious acquisitions of foreign DNA sequences.

We have set out to study changes in the cellular CpG methylation profiles upon introducing foreign DNA into mammalian cells. As stress factors served the genomic integration of foreign (viral or bacterial plasmid) DNA, virus infections or the immortalization of cells with *Epstein Barr Virus (EBV)*. In several systems studied, alterations in cellular CpG methylation and transcription profiles were observed to different degrees. In the case of adenovirus DNA integration in adenovirus type 12 (Ad12)-transformed hamster cells, the extensive changes in cellular CpG methylation persisted even after the loss of the transgenomic Ad12 DNA. Hence, stress-induced alterations in CpG methylation can be inherited independent of the continued presence of the transgenome. Upon adenovirus infections, changes in cellular CpG methylation have not been observed. In *EBV* immortalized as compared to control cells, CpG hypermethylation in the far-upstream region of the human *FMR1* promoter decreased four-fold. In the wake of cellular stress due to foreign DNA entry, preexisting CpG methylation patterns were altered, possibly at specific CpG dinucleotides. Frequently, transcription patterns were also affected. As a caveat towards manipulations of cells with foreign DNA, such cells can no longer be considered identical to their un-manipulated counterparts.

*Doerfler W, Weber S, Naumann A. (2018)* Inheritable epigenetic response towards foreign DNA entry by mammalian host cells: a guardian of genomic stability. Invited Review - *Epigenetics* 13, 1141–1153.

*Doerfler W. (2019)* Commentary – Epigenetic consequences of genome manipulations: caveats for human germline therapy and genetically modified organisms. *Epigenomics* 11, 247–250, 2019.

---

## Research Projects

- (i) We have adduced evidence in several biological systems (viral, human *FMR1* promoter region) that alterations in cellular DNA methylation and transcription profiles in mammalian genomes in the wake of insertions of foreign DNA are a general phenomenon. These alterations might play a role in (viral) oncogenesis and are possibly instrumental during evolution due to multiple retroviral DNA insertions into ancient genomes. Over evolutionary times, these alterations of transcription profiles might have led to novel phenotypes. Specific DNA methylation patterns in a genome might also contribute to its stability (Heller et al. 1995; Remus et al. 1999; Müller et al. 2001; Weber et al. 2015, 2016; Doerfler 2016, 2019; Doerfler et al. 2018).
- (ii) DNA viral genomes integrated into their host genome often become extensively *de novo* methylated. However, HIV1 proviral DNA in the genomes of peripheral mononuclear blood cells (PMBC's) in HIV1-infected individuals frequently remain unmethylated. Interestingly, we have identified one long-term HIV-infected non-progressor in whose PMBC's, which were studied over an 11-year period, varying levels of proviral CpG methylation were observed. Apparently, *de novo* methylation of HIV1 proviral genomes might be subject to complex and multiple regulatory mechanisms which remain to be investigated (Weber et al. 2014).

- (iii) We have also shown that the genome of African Swine Fever Virus does not become *de novo* methylated after the infection of monkey Vero cells. This study is part of a series of investigations on the methylation status of different DNA viruses in mammalian cells (Weber et al. 2018).
- (iv) We have identified a distinct DNA methylation boundary in the 5'-upstream region of the human FMR1 (fragile X mental retardation 1) gene promoter. This boundary region is capable of binding nuclear proteins whose nature we currently try to identify. Loss of the boundary and expansion of CpG methylation into the promoter region of the gene is associated with the fragile X syndrome (FXS), one of the most frequent causes of mental retardation in humans. In rare cases of FXS, in so called high functioning males, the boundary is preserved, in the presence of a full expansion of the CGG repeat which is conventionally thought to be the major cause for FXS. Hence CGG expansion and promoter methylation are not necessarily linked, an observation which argues for the overall importance of promoter methylation in FXS causation. We also report in section 4.2 on a loss of CpG methylation in the far upstream region of the promoter in cells which carry foreign DNA in the episomal (Epstein Barr Virus genomes) or integrated (telomerase gene) configuration. (Naumann et al. 2014).

## **1. Stability of genome-wide CpG methylation profiles**

### ***1.1 Destabilization of the human epigenome: consequences of foreign DNA insertions***

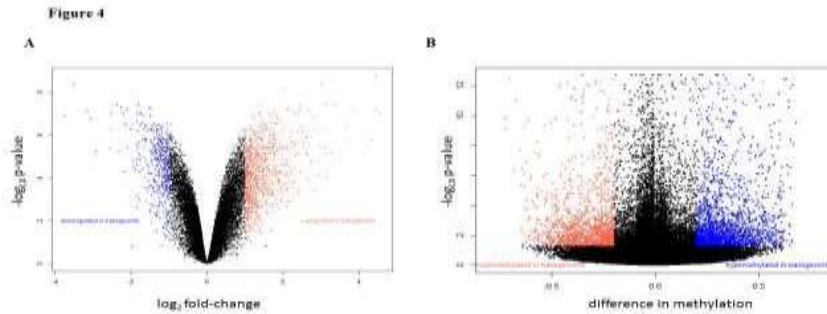
Based on the study of human adenovirus type 12 (Ad12) as an oncogenic DNA virus, the fate of foreign DNA in mammalian systems and the epigenetic consequences of foreign DNA insertions have been a long-term interest in this laboratory. Foreign DNA which emanates from a panoply of sources is ubiquitous 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) (Heller et al., 1995). Moreover, alterations of DNA methylation and transcription profiles were documented in Ad12 DNA- and in bacteriophage lambda DNA transgenomic cells.

- We have adduced evidence in several systems that epigenetic effects in mammalian genomes due to the insertion of foreign DNA are a general phenomenon. These alterations of methylation and transcription profiles might play a role in (viral) oncogenesis and are possibly instrumental during evolution as a consequence of multiple retroviral DNA insertions into ancient genomes.
- 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 different cell clones.

- In five non-transgenomic HCT116 control clones without the plasmid, transcription and methylation patterns proved similar, most frequently 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 1A). 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 361,983 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 1B).
- 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 have assumed a major role in molecular biology and medicine:
  - Epigenetic factors in (viral) oncogenesis;
  - Thoughts on epigenetics and evolution – epigenetic effects upon retrotransposon insertions;
  - Experimental approaches using genome manipulations;
  - Transgenic and transgenomic cells and organisms in many biological systems;
  - Gene therapeutic regimens;
  - Induced embryonic stem cells;
  - Knock-out or knock-in experiments;
  - Applications of the CRISPR-Cas9 technology.

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 and presently pursue more far-reaching questions by using the Ad12 system as a versatile model organism and guide.



**Figure 1 - Alterations in patterns of transcription (A) and methylation (B) in pC1-5.6 transgenic HCT116 cell clones as compared to non-transgenic cells.** (A) Volcano plot displays non-standardized signals (log<sub>2</sub> fold-change) on the x-axis against standardized signals (-log<sub>10</sub> FDR-adjusted p-value) on the y-axis for the comparison of five non-transgenic against seven transgenic cell clones of all 28,869 gene segments analyzed. Up-regulated genes in transgenic cell clones were displayed in red and down-regulated genes in blue (FC  $\pm 2$ , adjusted p-values < 0.05; n=1343 genes). (B) Volcano plot displays differences in methylation on the x-axis against standardized methylation (-log<sub>10</sub> FDR-adjusted p-value) on the y-axis for the comparison of four non-transgenic against five pC1-5.6 transgenic cell clones of all 361,983 CpG's interrogated. All p-values were corrected for multiple testing by using the Benjamin-Hochberg method (*J. R. Stat. Soc.* 289:289–300, 1995). Hyper-methylated CpG's in transgenic cell clones were displayed in red and hypo-methylated CpG's in blue ( $\Delta\beta$  value  $\geq 0.2$ , adjusted p-value < 0.05; n=3,791 CpG's). This Figure and its legends were taken from Weber et al., 2015.

As a corollary to these studies, 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 transgenic HCT116 cell clones which had exhibited epigenetic alterations in other parts of the human genome (Weber et al. 2015). In the cell clones selected for this investigation, the HERV and LINE sequences did not show altered methylation profiles upon 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.

### ***1.2 Thoughts on epigenetics & evolution.***

During evolution, both genome stability and flexibility must have been decisive factors which were in constant competition with each other. Since foreign DNA is readily inserted into established genomes, this competition continues to be relevant in today's biology. The presence of ancient retroviral and retrotransposon elements in the human genome attests to the long evolutionary history of retroviral insertions into genomes at evolutionary times. The ancient, now degenerate retroviral genomes, continue to be transcribed with unknown function; active viral genomes are not produced. Each insertion in evolutionary times added novel genetic information. However, more importantly, each impact upon the insertion of foreign DNA into an ancestral genome had epigenetic consequences in that it altered methylation and transcription profiles and

could have led to the generation of completely new cell types. Depending on the environmental conditions then prevalent, the novel cell types were either eliminated or had gained an evolutionary advantage, survived and contributed to the development of present day (human) genomes. Hence, the most significant contribution of the insertion of ancient foreign genomes would have been the generation of new (epi)-genetic profiles and the rise of new cell types.

*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, Jung S, Doerfler W.* (2016a) DNA methylation and transcription in HERV (K, W, E) and LINE sequences remain unchanged upon foreign DNA insertions. *Epigenomics* 8, 157-165.

*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. Edited by W. Doerfler & P. Böhm, Springer Verlag Cham, Heidelberg, New York, Dordrecht, London, pages 123-143.

*Doerfler W.* (2016) Beware of manipulations on the genome: epigenetic destabilization through (foreign) DNA insertions. Invited Commentary - *Epigenomics* 8, 587-591.

*Doerfler W, Weber S, Naumann A.* (2018) Inheritable epigenetic response towards foreign DNA entry by mammalian host cells: a guardian of genomic stability. Invited Review - *Epigenetics* 13, 1141–1153.

---

### ***1.3 Epigenetic Changes in Viral and Host Cellular DNA upon Virus Infections?***

In our current work, we have asked the question, whether the infection of mammalian cells with DNA viruses, like adenovirus type 12 (Ad12) or African Swine Fever Virus (ASFV) can lead to alterations in the CpG methylation status of (i) the intruding viral genome or (ii) the genomes of the recipient cells. The data so far available are the following. (i) In the course of the productive infection of human HCT116 cells with Ad12 or of monkey cells with ASFV, the viral genomes do not become de novo methylated. For the Ad12 genome, it had been shown earlier that the viral DNA inside virions is not CpG methylated (Günthert et al. 1976). (ii) Preliminary findings suggest that there are no changes in the analyzed CpG's of human cellular DNA between 12 and 48 h after Ad12 infection. For ASFV-infected monkey cells, such investigations have not yet been done.

*S. Weber, A. Hakobyan, H. Zakaryan, A., W. Doerfler.* Intracellular African Swine Fever Virus DNA remains unmethylated in infected Vero cells. *Epigenomics* 10, 289-299, 2018.

*S. Weber, D. Conn, S. Herms, P. Hoffmann, C. Ramirez, W. Doerfler.* Unpublished data.

---

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

### ***Unmethylated HIV-1 proviral genomes in HIV-1 infected individuals and an exception***

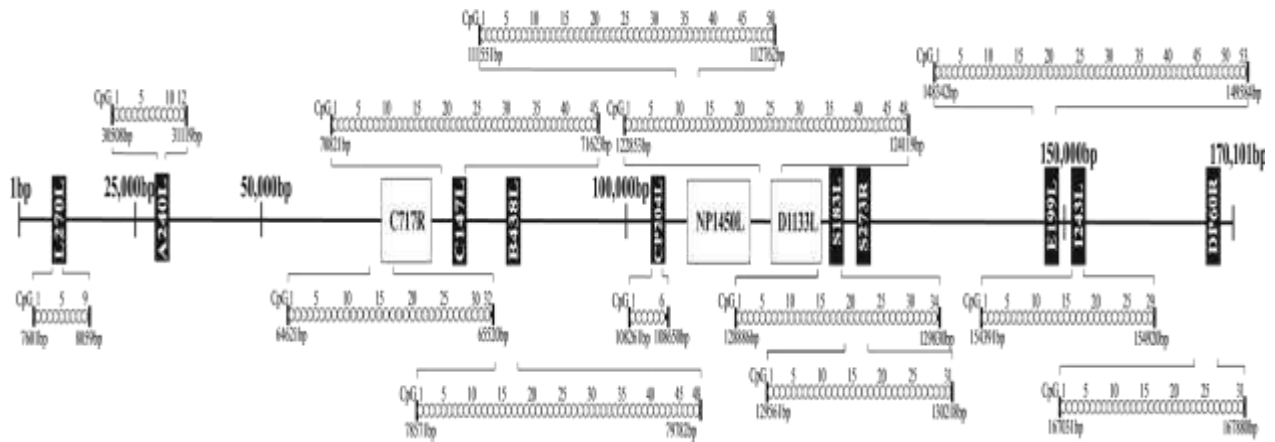
Integrated DNA from various viruses often becomes methylated *de novo* and transcriptionally inactivated. We therefore investigated CpG methylation profiles in 55 of the 94 CpG's (58.5%) in HIV-1 proviral genomes including ten CpG's in each LTR and additional CpG's in portions of the *gag*, *env*, *nef*, *rev*, and *tat* genes. We analyzed 33 DNA samples from PBMC's of 23 subjects representing a broad spectrum of HIV-1 disease or infection. 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 likely not a major factor in regulating HIV-1 proviral activity in PBMC's of infected individuals.

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

### **3. Intracellular African swine fever virus DNA remains un-methylated in infected Vero cells**

African swine fever virus (ASFV) is an important animal pathogen in many countries. In basic research on numerous different viruses, profound analyses of the molecular genetics of the virus have proved of paramount importance to understand its biological and medical characteristics. In this report, we describe the analysis of the methylation status of ASFV DNA after the infection of monkey Vero cells in culture. By applying the bisulfite sequencing technique, the gold standard in work on DNA methylation, no evidence was found for the presence of methylated cytosine residues in the BA71V strain of the ASFV genome in Vero cells. A selection of genome sites spread across the entire genome was analyzed; 5-methylcytosine (5-mC) residues were not found. The graph (Figure 2) presents a map of the 170,101 base pair genome of the BA71V strain in which the bisulfite-analyzed genome segments and the viral genes therein have been indicated. The strings of open symbols ( $\diamond$ ) lined up by brackets to the investigated genome sections symbolically depict unmethylated cytosine residues. These genome segments have remained unmethylated throughout virus infection. We have analyzed 7.25% of all the methylation receptive CpG sites and 8.55% of the genes in the ASFV genome. We cannot rule out the possibility that pockets of methylated sequences might exist somewhere in the genome.

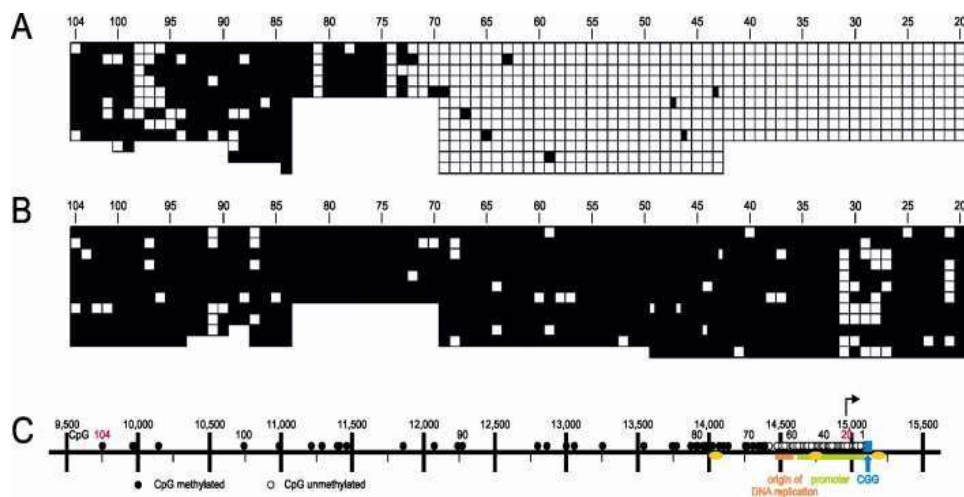




**Figure 2. African Swine Fever Virus (ASFV) DNA is not de novo methylated in monkey Vero cells.** ASFV BA71V DNA in Vero cells, DNA methylation profiles at 2, 6, 14, 24 h p. i. in selected parts of the viral genome as determined by the bisulfite sequencing technique. The map of the BA71V genome variant of ASFV locates all viral genes which were analyzed for methylation or the lack thereof in this study. In the CpG profiles, each left to right line of CpG's represents one DNA molecule; each column one CpG dinucleotide pair. We interpret the data to demonstrate the lack of CpG methylation in all viral segments investigated between 2 and 24 h after infection.

*Weber S, Hakobyan A, Zakaryan H, Doerfler W.* Intracellular African Swine Fever Virus DNA remains unmethylated in infected Vero cells. *Epigenomics* 10, 289-299, 2018.

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



**Figure 3. 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 with the boundary located at CpG 75. (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 75. The limits of the range of CpG's determined by bisulfite sequencing were demarcated by red numbering (20 to 104). This figure was taken from Naumann et al., 2014.

#### **4.1 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 3), among them a DNA methylation boundary which is located at about 75 CpG's upstream of the CGG repeat. In fragile X syndrome (FXS), the boundary is lost, and the promoter is inactivated by methylation spreading (Figure 3B). We have documented boundary stability in spite of critical expansions of the CGG trinucleotide repeat in male or female pre-mutation, in female full mutation carriers and in infrequently found high functioning males (HFMs). These data were not shown here (see Naumann et al. 2014). HFMs carry a full CGG repeat expansion but exhibit an un-methylated 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 (Figure 3C) 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. 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 which might have an important function as promoter safeguards (Naumann et al. 2014).

#### **4.2 Foreign DNA in human HCT116 cells alters the methylation profile far upstream of the methylation boundary in the FMR1 promoter region**

In FXS individuals with an expanded CGG repeat, a signal akin to foreign DNA, and often in **cells transgenomic for foreign DNA**, like the episomal Epstein-Barr Virus (EBV) genome or the chromosomally integrated transgenomic telomerase gene, the large number of previously methylated CpG's in the far upstream region of the boundary is about fourfold decreased (Naumann et al., 2014). This finding (Figure 4) is an additional case in point for the alterations of DNA methylation profiles in trans upon the introduction of foreign DNA into mammalian cells (see section 1).

#### **4.3 Proteins binding to the boundary region of the FMR1 upstream promoter region**

We have set out to isolate the proteins which can bind to the methylation boundary in the upstream region of the FMR1 promoter. In collaboration with Marcus Krüger's laboratory, CECAD Research Center University of Cologne, quantitative high-resolution mass spectrometry will be employed to identify these proteins and their function.

Naumann A, Hochstein N, Weber S, Fanning E, Doerfler W. 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.

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

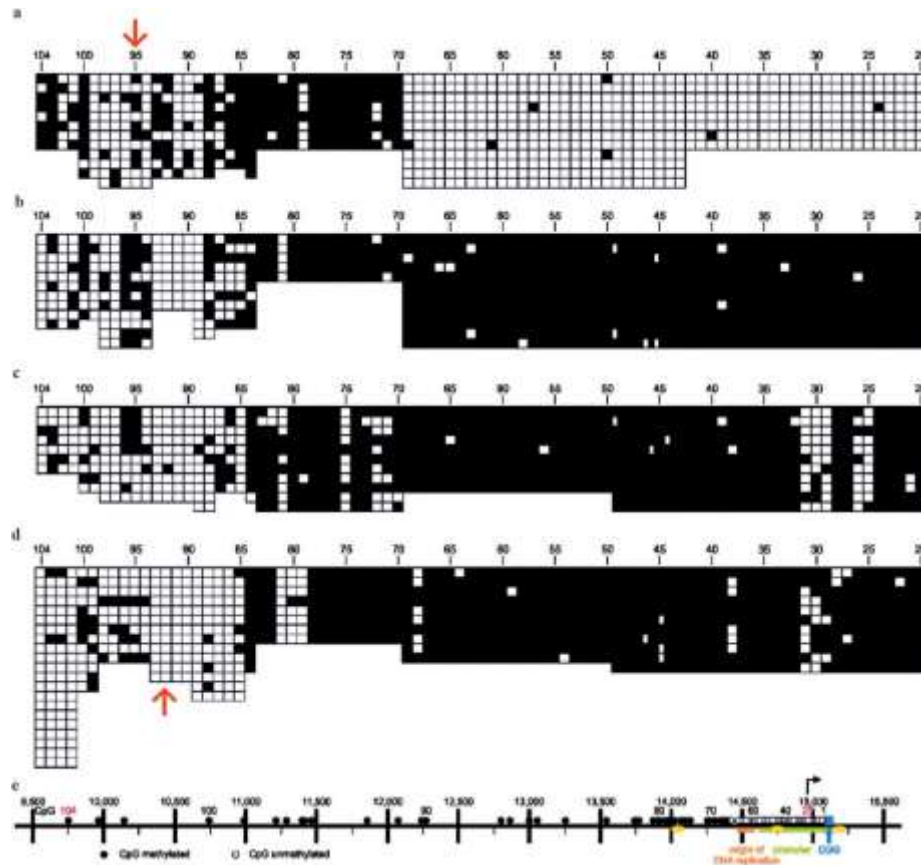


Figure 4. Extensive changes of methylation profiles in the far-upstream region of the FMR1 promoter boundary (red arrows). In human cells, which have been immortalized by EBV infection or by transfection with the human telomerase gene, methylation in the region approximately between CpG's 80 and 104 far-upstream from the methylation boundary has been four-fold decreased. Figure design is similar to the one in Figure 3. The analyzed DNA preparations have been taken from: (a) EBV-immortalized PBMC's (peripheral blood mononuclear cells) of a non-FXS individual with an intact methylation boundary. (b) EBV-immortalized PBMC's of an FXS male. (c) Similar to (b), except that the PBMC's had been immortalized by trans-formation with the human telomerase gene. (d) Cultured fibroblasts from a 22-week-old male FXS fetus (ATCC number GMO7072). In the DNA from FXS individuals (b) to (d), the methylation boundary was lost. (e) Map of the region as described in the legend to Figure 3. This figure was taken from Naumann et al. 2014.

## Collaborations

Harold **Burger** and Barbara **Weiser**, (formerly) University of California, Davis School of Medicine and Sacramento Veterans Administration Medical Center, Sacramento, California, 95655, USA.

Ellen **Fanning** (deceased), Department of Biology, Vanderbilt University in Nashville, TN, USA.

Stefan **Herms**, Institute of Human Genetics, Forschungszentrum Life & Brain, Bonn University, D-53127 Bonn, Germany and Division of Medical Genetics, University Hospital Basel, CH-4055 Basel, Switzerland.

Per **Hoffmann**, Institute of Human Genetics, Forschungszentrum Life & Brain, Bonn University, D-53127 Bonn, Germany and Division of Medical Genetics, University Hospital Basel, CH-4055 Basel, Switzerland.

Andrea **Hofmann**, (formerly) Institute of Human Genetics, Forschungszentrum Life & Brain, Bonn University, D-53127 Bonn, Germany.

Susan **Jung**, Pediatric Research Center, University Erlangen-Nürnberg, 91052 Erlangen, Germany.

Marcus **Krüger**, CECAD Research Center, University of Cologne, 50931 Cologne, Germany.

Christina **Ramirez** and Daniel **Conn**, Department of Biostatistics, UCLA School of Public Health, Los Angeles, CA 90095-1772, USA.

Hovakim **Zakaryan** and Astghik **Hokobyan**, Institute of Molecular Biology of the National Academy of Sciences, 0014, Yerevan, Armenia.

### *DNA samples were contributed by:*

Katryn **Anastos**, Director WIHS (Women's Interagency HIV Studies), Montefiore Hospital, Albert Einstein College of Medicine, Bronx, NY 10461, USA.

André **Hoogeveen**, Department of Clinical Genetics Erasmus University Medical School, 3000 DR Rotterdam, the Netherlands.

Rupert **Kaul**, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

Klaus **Korn**, Institute for Virology, Erlangen University Medical School, D-91054 Erlangen, Germany.

Colin **Kovacs**, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

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

## **Present and Recent Funding**

1. Institute for Clinical and Molecular Virology, FAU Erlangen-Nürnberg, Medical School, 2002 - .
2. Dr. Robert Pflieger Stiftung, Bamberg – 2019 - .
3. Staedtler Stiftung, Nürnberg, 2016-2018 (WW/eh 01/15).
4. NIH grant # 2 UOI AI035004-2, subcontract from WIHS – Erlangen University, 2013.
5. Fritz Thyssen Foundation, Cologne, Az. 10.07.2, 2010-2012.
6. Fritz Thyssen Foundation, research fellowship (Az. 40.12.0.029) for Dr. Anja Naumann, 2012/2013.
7. Fritz Thyssen Foundation – The Fifth Weissenburg Symposium, Az.: 30.14.0.033, 2014.
8. Deutsche Forschungsgemeinschaft, GZ: DO 165/28-1, 2010-2012.
9. Rotary Club Weissenburg in Bayern, short-term stipend to Dr. Stefanie Weber, 2016.
10. Nationale Akademie der Wissenschaften Leopoldina, Fourth Weissenburg Symposium 2011.

## **Selected Publications 2007 – 2019**

**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.

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

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

**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 and transcription in HERV (K, W, E) and LINE sequences remain unchanged upon foreign DNA insertions. **Epigenomics** 8, 157-165, 2016.

**S. Weber, A. Hofmann, A. Naumann, P. Hoffmann, and W. Doerfler.** Epigenetic alterations upon the insertion of foreign DNA into mammalian genomes: oncogenesis and evolution. In *Epigenetics – a Different Way of Looking at Genetics, Fifth Weissenburg Symposium*. Edited by W. Doerfler & P. Böhm, **Springer Verlag** Cham, Heidelberg, New York, Dordrecht, London, 2016; pages 123-143.

**W. Doerfler.** Beware of manipulations on the genome: epigenetic destabilization through (foreign) DNA insertions. *Invited Commentary* - **Epigenomics** 8, 587-591, 2016.

**W. Doerfler.** Discoveries in molecular genetics with the adenovirus 12 system: Integration of viral DNA and epigenetic consequences. In: *Epigenetics of Infectious Diseases*. W. Doerfler, J. Casadesús (Eds.). **Springer Verlag** Cham, Heidelberg, New York, Dordrecht, London, 2017; pages 47-63.

**W. Doerfler.** Zum Diskurs zwischen Theologie und Naturwissenschaft - Perzeptionen aus dem Universum? In: „SAGEN, WAS SACHE IST“, VERSUCHE EXPLORATIVER ETHIK. Festgabe zu Ehren von Hans G. Ulrich, Hg. von G. den Hertog, S. Heuser, M. Hofheinz, B. Wannewetsch. **Evangelische Verlagsanstalt, Leipzig**, 2017, pages 273-290.

**S. Weber, A. Hakobyan, H. Zakaryan, A. and W. Doerfler.** Intracellular African Swine Fever Virus DNA remains unmethylated in infected Vero cells. **Epigenomics** 10, 289-299, 2018.

**W. Doerfler, S. Weber, and A. Naumann.** Inheritable epigenetic response towards foreign DNA entry by mammalian host cells: a guardian of genomic stability. *Invited Review*. **Epigenetics** 13, 1141–1153.

**W. Doerfler.** Commentary – Epigenetic consequences of genome manipulations: Caveats for human germline therapy and genetically modified organisms. **Epigenomics** 11, 247–250, 2019

## **Editor (Co-Editor) of Books**

Epigenetics. In: Reference Module in Biomedical Sciences. Section editor, W. Doerfler. Elsevier, Ltd., San Diego, 2014.

Epigenetics – a Different Way of Looking at Genetics, Fifth Weissenburg Symposium. Edited by W. Doerfler & P. Böhm. Springer Verlag: Cham, Heidelberg, New York, Dordrecht, London, 2016. DOI 10.1007/978-3-319-27186-6.

Epigenetics of Infectious Diseases. Edited by W. Doerfler & J. Casadesús. Springer Verlag: Cham, Heidelberg, New York, Dordrecht, London, 2017. DOI 10.1007/978-3-319-55021-3.

---

## **Recent Abstracts**

- March 16, 2017. Tele-Conference Framingham, MA, USA – „One Health Epigenomics and Microbiomes” Abstract: W. Doerfler - Epigenetics of virus infections and viral oncogenesis.
- April 27-29, 2017. The 2nd Tore Nilson/Karolinska Institutet Conference, Nobel Forum, Karolinska Institutet, Stockholm “More Epigenetics in Clinical Medicine”. Abstract: W. Doerfler, S. Weber, A. Naumann - Epigenetic consequences of foreign DNA insertions: A mechanism in oncogenesis?
- June 06, 2017. Institute of Molecular Biology, National Academy of Sciences, Yerevan, Armenia – Epigenetics Conference. Abstract: W. Doerfler, S. Weber, A. Naumann - Epigenetic consequences of foreign DNA insertions: Beware of genome manipulations.
- June 25, 2017 American Society for Virology, 36<sup>th</sup> Annual Meeting, Madison, WI, USA. Abstract: S. Weber, H. Zakaryan, A. Hakobyan, W. Doerfler – Epigenetics of virus infections: Viral and global cellular methylation profiles.
- January 24, 2018 Biomedicum Uppsala Universitet, Sweden, Uppsala Universitet, medicinsk fakultet, Dr. med. h.c. to Walter Doerfler. Abstract: W. Doerfler, S. Weber, A. Naumann - Epigenetic re-programming upon foreign (viral) DNA invasion into cells.
- March 19, 2018 Shrimp Epigenome Project, 119<sup>th</sup> National Shellfisheries Association Annual Meeting, Seattle, WA, USA (Video Conference from Erlangen). Abstract: W. Doerfler, S. Weber, A. Naumann - Epigenetic re-programming of the host genome upon foreign (viral) DNA invasion into cells.
- May 29, 2018 Fakultäten-Club, Universität Erlangen – Epigenetik. Abstract: W. Doerfler- Epigenetik – ein etwas anderer Blick auf die Genetik.
- October 14, 2018 Ventnor Foundation Alumni, Emden, Hilton S. Read Lecture – Abstract: W. Doerfler - Genetically manipulated organisms (GMO’s) – Epigenetic consequences of foreign DNA insertions.

## **Dissertations (Dr. rer. nat.)**

- Between **1967 and 2013**, W.D. has personally guided **81 graduate students: two at Rockefeller University in New York City (1967-1976), 73 in Cologne (Institute of Genetics, 1972-2002), and 6 in Erlangen (Institute for Virology, 2002-2013)**. Many of the graduate students were from Germany; several came from abroad, namely from USA (4), Israel (2), Indonesia (1), Turkey (1), Nederland (1), France (1), China (1), Poland (1), Iran (1), Brazil (1),
- **About 35 postdoctoral researchers** from Australia, Brazil, Canada, China, France, Germany, Hungary, Irak, Israel, Japan, Latvia, Poland, Portugal, Russia, Spain, Sweden and USA worked in W.D.'s laboratory.

## **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.

**U. Günthert, M. Schweiger, M. Stupp, and W. Doerfler.** DNA methylation in adenovirus, adenovirus-transformed cells, and host cells. **Proc. Natl. Acad. Sci. USA** **73**, 3923-3927, 1976.

**J. Groneberg, Y. Chardonnet, and W. Doerfler.** Integrated viral sequences in adenovirus type 12-transformed hamster cells. **Cell** **10**, 101-111, 1977.

**D. Sutter, M. Westphal, and W. Doerfler.** Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. **Cell** **14**, 569-585, 1978.

**D. Sutter, and W. Doerfler.** Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. **Proc. Natl. Acad. Sci. USA** **77**, 253-256, 1980.

**L. Vardimon, R. Neumann, I. Kuhlmann, D. Sutter, and W. Doerfler.** DNA methylation and viral gene expression in adenovirus-transformed and -infected cells. **Nucleic Acids Res.** **8**, 2461-2473, 1980.

**R. Deuring, G. Klotz, and W. Doerfler.** An unusual symmetric recombinant between adenovirus type 12 DNA and human cell DNA. **Proc. Natl. Acad. Sci. USA** **78**, 3142-3146, 1981.

**R. Deuring, U. Winterhoff, F. Tamanoi, S. Stabel, and W. Doerfler.** Site of linkage between adenovirus type 12 and cell DNAs in hamster tumour line CLAC3. **Nature** **293**, 81-84, 1981.



**L. Vardimon, A. Kressmann, H. Cedar, M. Maechler, and W. Doerfler.** Expression of a cloned adenovirus gene is inhibited by *in vitro* methylation. **Proc. Natl. Acad. Sci. USA** **79**, 1073-1077, 1982.

**Kuhlmann I., S. Achten, R. Rudolph, and W. Doerfler.** Tumor induction by human adenovirus type 12 in hamsters: loss of the viral genome from adenovirus type 12-induced tumor cells is compatible with tumor formation. **EMBO J.** **1**, 79-86, 1982.

**I. Kruczek, and W. Doerfler.** Expression of the chloramphenicol acetyltransferase gene in mammalian cells under the control of adenovirus type 12 promoters: effect of promoter methylation on gene expression. **Proc. Natl. Acad. Sci. USA** **80**, 7586-7590, 1983.

**W. Doerfler.** DNA methylation and gene activity. **Ann. Rev. Biochem.** **52**, 93-124, 1983.

**K.-D. Langner, L. Vardimon, D. Renz, and W. Doerfler.** DNA methylation of three 5' C-C-G-G 3' sites in the promoter and 5' region inactivates the E2a gene of adenovirus type 2. **Proc. Natl. Acad. Sci. USA** **81**, 2950-2954, 1984.

**K.-D. Langner, U. Weyer, and W. Doerfler.** Trans-effect of the E1 region of adenoviruses on the expression of a prokaryotic gene in mammalian cells: resistance to 5'-CCGG-3' methylation. **Proc. Natl. Acad. Sci. USA** **83**, 1598-1602, 1986.

**R. Jessberger, D. Heuss, and W. Doerfler.** Recombination in hamster cell nuclear extracts between adenovirus type 12 DNA and two hamster preinsertion sequences. **EMBO J.** **8**, 869-878, 1989.

**M. Toth, U. Lichtenberg, and W. Doerfler.** Genomic sequencing reveals a 5-methylcytosine free domain in active promoters and the spreading of pre-imposed methylation patterns. **Proc. Natl. Acad. Sci. USA.** **86**, 3728-3732, 1989.

**S. Kochanek, M. Toth, A. Dehmel, D. Renz, and W. Doerfler.** Inter-individual concordance of methylation profiles in human genes for tumor necrosis factors  $\alpha$  and  $\beta$ . **Proc. Natl. Acad. Sci. USA** **87**, 8830-8834, 1990.

**S. Kochanek, A. Radbruch, H. Tesch, D. Renz, and W. Doerfler.** DNA methylation profiles in the human genes for tumor necrosis factors  $\alpha$  and  $\beta$  in subpopulations of leukocytes and in leukemias. **Proc. Natl. Acad. Sci. USA** **88**, 5759-5763, 1991.

**S. Kochanek, D. Renz, and W. Doerfler.** DNA methylation in the Alu sequences of diploid and haploid primary human cells. **EMBO J.** **12**, 1141-1151, 1993.

**H. Heller, C. Kämmer, P. Wilgenbus, and W. Doerfler.** Chromosomal insertion of foreign (adenovirus type 12, plasmid, or bacteriophage lambda) DNA is associated with enhanced methylation of cellular DNA segments. **Proc. Natl. Acad. Sci. USA** **92**, 5515-5519, 1995.

**H. Deissler, A. Behn-Krappa, and W. Doerfler.** Purification of nuclear proteins from human HeLa cells that bind specifically to the unstable tandem repeat (CGG)<sub>n</sub> in the human FMR1 gene. **J. Biol. Chem.** **271**, 4327-4334, 1996.

**H. Deissler, M. Wilm, B. Genç, B. Schmitz, T. Ternes, F. Naumann, M. Mann, and W. Doerfler.** Rapid protein sequencing by tandem mass spectrometry and cDNA cloning of p20 CGGBP. **J. Biol. Chem.** **272**, 16761-16768, 1997.

**R. Schubbert, D. Renz, B. Schmitz, and W. Doerfler.** Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. **Proc. Natl. Acad. Sci. USA** **94**, 961-966, 1997.

**M. Zeschnigk, B. Schmitz, B. Dittrich, K. Buiting, B. Horsthemke, and W. Doerfler.** Imprinted segments in the human genome: different DNA methylation patterns in the PraderWilli/Angelman syndrome region as determined by the genomic sequencing method. **Hum. Molec. Genet.** **6**, 387-395, 1997.

**S. Schwemmler, E. de Graaff, H. Deissler, D. Gläser, D. Wöhrle, I. Kennerknecht, W. Just, B.A. Oostra, W. Doerfler, W. Vogel, and P. Steinbach.** Characterization of FMR1 promoter elements by *in vivo*-foot printing analysis. **Americ. J. Hum. Genet.** **60**, 1354-1362, 1997.

**A. Schumacher, K. Buiting, M. Zeschnigk, W. Doerfler & B. Horsthemke.** Methylation analysis of the PWS/AS region does not support an enhancer competition model of genomic imprinting on human chromosome 15. **Nature Genet.** **19**, 324-325, 1998.

**J. Hertz, G. Schell, and W. Doerfler.** Factors affecting *de novo* methylation of foreign DNA in mouse embryonic stem cells. **J. Biol. Chem.** **274**, 24232-24240, 1999.

**K. Müller, H. Heller, and W. Doerfler.** Foreign DNA integration: Genome-wide perturbations of methylation and transcription in the recipient genomes. **J. Biol. Chem.** **276**, 14271-14278, 2001.

## **Organization of International Symposia on DNA Methylation and Epigenetics, 1981, 2001 – 2020, Weissenburg Symposia (2001-2020)**

**Cologne Spring Meeting 1981: DNA Methylation and Gene Activity – First International Meeting on DNA Methylation.**

**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.

**Sixth Weissenburg Symposium 2020:** *Epigenetics – Genome-wide Methylation Profiles.*  
September 02-05, 2020 – *planning stage.*

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.**

Co-organizer **34<sup>th</sup> Ernst-Klenk Symposium in Molecular Medicine – Epigenetics: Basic principles and clinical applications.** 04-06 October 2018, **Cologne, Germany.**



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

