DNA methylation, histone modification and the transcription factor *dE2F1* in *Drosophila*

Dissertation zur Erlangung des Grades

Doktor der Naturwissenschaften

Eingereicht am Fachbereich Biologie

der Johannes Gutenberg Universität Mainz

Erarbeitet im DAAD/MPG Labor

an der Chinesischen Akademie der Biologischen Wissenschaften,

Shanghai/China

Alexandra Weyrich

geboren am 20. März 1979 in Birkenfeld

Mainz, April 2007

Index

I. DNA methylation in the male germ line of *Drosophila*

II. Histone methylation in the male germ line of *Drosophila*

III. A transcript specific mutation of the *dE2F1* transcription factor in *Drosophila melanogaster*

Index of Figures

I. DNA methylation in the male germ line of *Drosophila*

II. Histone modification in the male germ line of *Drosophila*

III. A transcript specific mutation of the *dE2F1* transcription factor in *Drosophila melanogaster*

Index of Tables

I. DNA methylation in the male germ line of *Drosophila*

II. Histone modification in the male germ line of *Drosophila*

III. A transcript specific mutation of the *dE2F1* transcription factor in *Drosophila melanogaster*

Index of Abbreviations

AB	antibody
bp	base pair
cDNA	complementary/copy DNA
β2t	beta2-tubulin promoter
ORF	Open Reading Frame
DNA	Deoxy-ribo Nucleic Acid
Dnmt	DNA methyltransferase (nucleotide)
DNMT	DNA methyltransferase (amino acid)
dNTP	deoxy Nucleotide Tri-Phosphate
dE2F1	Drosophila melanogaster E2F transcription factor 1
dE2F1^{TF}	dE2F1 mutant found by **T**ang X. and **F**eng R.
ECFP	Enhanced Cyan Fluorescent Protein
EGFP	Enhanced Green Fluorescent Protein
EU	Euchromatin
Fig.	Figures
G1 phase	Gap phase 1 (cell cycle)
HET	Heterochromatin
HRP	Horse Radish Peroxidase
H3-K4me1	Histone 3 Lysine residue 4 mono-methylation
H3-K4me2	Histone 3 Lysine residue 4 di-methylation
H3-K9me1	Histone 3 Lysine residue 9 mono-methylation
H3-K9me2	Histone 3 Lysine residue 9 di-methylation
H3-K9me3	Histone 3 Lysine residue 9 tri-methylation
H3-K27me1	Histone 3 Lysine residue 27 mono-methylation
H3-K27me2	Histone 3 Lysine residue 27 di-methylation
H3-K27me3	Histone 3 Lysine residue 27 tri-methylation
H3-K36me2	Histone 3 Lysine residue 36 di-methylation
H3-K36me3	Histone 3 Lysine residue 36 tri-methylation
K3-K9ac	Histone 3 Lysine residue 9 acetylation
H3-S10p	Histone 3 Serine residue 10 phosphorylation
H4-K8ac	Histone 4 Lysine residue 8 acetylation

H4-K5ac	Histone 4 Lysine residue 5 acetylation
H4-K12ac	Histone 4 Lysine residue 5 acetylation
H4-K16ac	Histone 4 Lysine residue 16 acetylation
H4-K20me1	Histone 4 Lysine residue 20 mono-methylation
H4-K20me2	Histone 4 Lysine residue 20 di-methylation
H4-K20me3	Histone 4 Lysine residue 20 tri-methylation
IP	Immunoprecipitation
mRNA	messenger RNA
M phase	Mitosis phase (celly cycle)
MS	Mass Spectrometry
polyA	Polyadenylation
Rb	Retinoblastoma protein
RNA	Ribo Nucleic Acid
RNAi	RNA interference
Rp49	Ribosomal protein 49
R point	Restriction point (cell cycle)
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
S phase	Sythesis phase (cell cycle)
UTR	Un-Translated Region
2D	two dimensional
5mC	carbon-5-methylcytosine

I.

DNA methylation in the male germ line of *Drosophila*

1. Abstract

Drosophila melanogaster was reported to contain low but significant amounts of C5-methylcytosine. We have investigated testis DNA and found no evidence for the presence of significant amounts of methylated cytosine. The expression of two murine *de novo* DNA methyltransferases, *DNMT3A* and *DNMT3B*, in *Drosophila* testes under the control of the β2-tubulin promoter, does not show the expected increase in DNA methylation and has no effects on the fertility of the fly. Also the tissue-specific expression of *de novo* DNA methyltransferases with the help of the UAS/GAL4-system had no phenotypic effects. We conclude that DNA methylation has no functional relevance in the male germ line of *Drosophila*.

2. Introduction

DNA methylation and histone modifications play major epigenetic roles in mammals (BIRD 2002). In *Drosophila*, parental effects, like they are regularly observed in mammals, have only in exceptional cases been reported (REUTER and SPIERER 1992; LI *et al.* in preparation).

DNA methylation is important in gene silencing or for the periodical inactivation of certain parental alleles during early development (JAENISCH *et al.* 1982). So far it is still unclear how epigenetic regulation is established in *Drosophila melanogaster*. Although significant parts of the genome become inactive in a tissue-specific manner (MAGGERT and GOLIC 2002), methylation of DNA was only found at very low levels in *Drosophila* (FUJITA *et al.* 1999; GOWHER *et al.* 2000; KUNERT *et al.* 2003; SALZBERG *et al.* 2004; STACH *et al.* 2003). It is consistent with this, that the canonical *de novo* DNA methylases (*DNMT*s) are not represented in the *Drosophila* genome.

dDNMT2 is the only methyltransferase discovered in *Drosophila melanogaster* (HUNG *et al.* 1999; TWEEDIE *et al.* 1999; GOWHER *et al.* 2000). Its depletion does not show any apparent phenotypic effects, neither in *Drosophila melanogaster* (GOWHER *et al.* 2000) nor in mammalian stem cells (OKANO *et al.* 1998b) or *Arabidopsis thaliana* (GOLL *et al.* 2006). It has recently been recognized that *DNMT2* has a function as a RNA methyltransferase for aspartic acid tRNA in mammals, *Drosophila* and *Arabidopsis,* rather than a function as DNA methylase (GOLL *et al.* 2006).

However, Salzberg et al. (SALZBERG *et al.* 2004) suggested that *dDNMT2* can function as DNA methylase, mainly of retrotransposons or retrotransposon-related elements (like *rover*, *R1Dm*, *Pilger*) but also of repetitive DNA elements in heterochromatin, including centromeric repeats and a *dodeca* satellite. They conclude that *dDNMT2* works as a defence enzyme against transposons and can in this function also methylate DNA. The problem here, as in other data, is the fact that the levels of 5-carbon methylated cytosines are at the detection limit of the bisulfite sequencing technique employed to detected methylated bases. Moreover, Lyko et al. (LYKO *et al.* 2000b) have shown that

Dnmt2 mRNA expression in *Drosophila* is limited to nurse cells and early (blastoderm) embryos. This would exclude *DNMT2* as a source of methylation in any other cell type.

DNA-(cytosine-5) methyltransferases (encoded as *DNMT*s) are highly conserved during evolution (BESTOR 2000). These enzymes catalyse the transfer of a methyl group from S-adenosylmethionine to the carbon 5 of cytosines. Three families of *DNMT*s are known in mammals: *DNMT1*, *DNMT2* and *DNMT3* (Fig. 1). The main *de novo* DNA methylation activity is accredited to the three *DNMT3*-family members (OKANO *et al.* 1999; OKANO *et al.* 1998a), *DNMT3A* and *DNMT3B-1*, and one isoform, *DNMT3B-2*, while another isoform, *DNMT3B-3*, lacks enzymatic activity. All three active enzymes are targeting preferentially CpG-dinucleotides (MUND *et al.* 2004; PRADHAN and ESTEVE 2003).

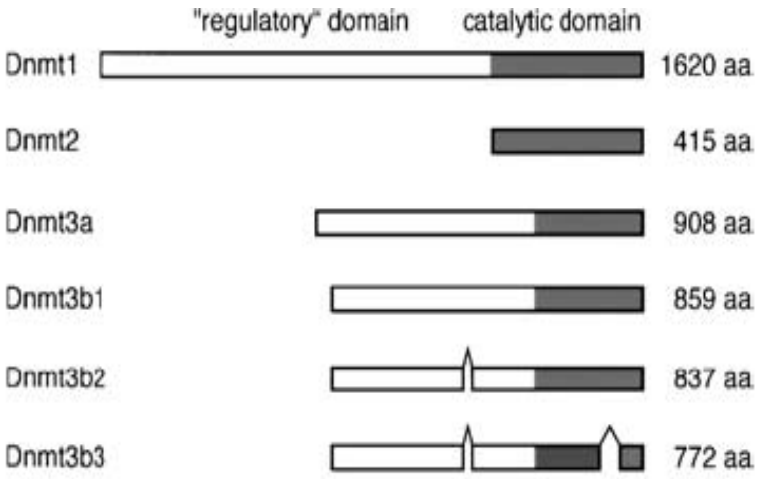


Fig. 1: The known murine DNA methyltransferases in a schematic overview (MUND *et al.* 2004; Fig. 1A).

Lyko and co-workers (LYKO *et al.* 1999) have shown that overexpression of the murine *Dnmt3a*, if transformed into the *Drosophila* genome, causes lethality in early embryogenesis. But regulatory functions of DNA methylation for gene expression in *D. melanogaster* have so far not been documented.

The mechanism used to control DNA methylation in specific target regions is not well understood. New evidence indicates that DNA methylation is at least partly regulated by the interaction with the histone modification machinery and small RNAs (MAISON *et al.* 2002; ALMEIDA and ALLSHIRE 2005). Histones are preferentially methylated on lysine residues in specific mono-, di-, or tri-methylation patterns. More generally, histone modifications can cause gene repression or activation (SIMS *et al.* 2003). In *Neurospora*

crassa and *Arabidopsis thaliana* DNA methylation requires prior histone methylation (TAMARU and SELKER 2001; JACKSON *et al.* 2002). Due to this, DNA methylation may be seen as a secondary epigenetic mechanism supporting the effects of histone methylation.

We have not been able to detect methylated DNA in the male germ line of *Drosophila*. The introduction of the mammalian *de novo* DNA methylase genes *Dnmt3a* and *Dnmt3b* does not increase the DNA methylation level, nor affects the male germ cell development or lead to sterility. The epigenetic mechanisms in *Drosophila* are probably mainly based on histone modification or other - unknown - mechanisms instead of DNA methylation.

3. Material & Methods

3.1 Subcloning and Microinjection

3.1.1 *β2t-Dnmt3a/3b*-constructs

The cDNA/ORF of the two mouse enzymes, *DNA cytosine-5 methyltransferase 3 alpha* (*Dnmt3a*) (Fig. 2) (Accession No. gi|6449467) (position: +1 to +2726) and *DNA cytosine-5 methyltransferase 3 beta* (*Dnmt3b*) (Fig. 3) (Accession No. gi|51556222) (position: +1 to +2579) (OKANO *et al*. 1998) were provided by Xu Guo-Liang (GE *et al.* 2004) in the mammalian expression vector pcDNA3-HA (a generous gift from Dr. Gang Pei). It includes an HA-tag sequence (positon: -1 to -39 bp) upstream of the *Dnmt* ORFs. We subcloned the HA-*Dnmt3* ORFs in several steps. PcDNA3-HA-*Dnmt3a* was cut with HindIII and XhoI, pcDNA3-HA-*Dnmt3b* with HindIII and EcoRI, and ligated into p-Blueskript II KS+ (Stratagene). HA-*Dnmt3a* was then cut with XhoI and XbaI, HA-*Dnmt3b* with NotI and KpnI, and cloned into the pSLfa1180fa-vector (kindly provided together with the pBac-vector by C. Horn and E. Wimmer) (HACKER *et al.* 2003; HORN and WIMMER 2000). This vector was modified from the original pSLfa1180-vector (Pharmacia) by adding the two restriction sites FseI and AscI to both ends of the multiple cloning site. Upstream of the HA-*Dnmt3*-ORFs we integrated the β2-tubulin promoter (position: -172 to -382) (MICHIELS *et al.* 1989) into the pSLfa1180fa-vector. The 3`UTR of histone 3.3B was added downstream of the *Dnmt3* ORF (*Dnmt3a*: +2811 to +4401, *Dnmt3b*: +2722 to +4313). Earlier research in our laboratory had established that this 3'UTR can be used to supply functional polyA-signals in *Drosophila* testis (AKHMANOVA *et al.* 1995; FENG *et al.* 2005). FseI and AscI restriction enzymes were used to recover the HA-*Dnmt3*-H3.3B fragments. They were ligated into the vector pBac[3*P3-EGFPafm], which includes the universal marker gene EGFP (enhanced green fluorescent protein) combined with an eye-specific promoter 3*P3 (PAPATSENKO *et al.* 1997). Restriction enzymes were obtained from MBI Fermentas.

The ORFs, 5'UTRs and 3'UTRs of the pBac-HA-β2t-*Dnmt3*-H3.3B constructs were verified by sequencing (WEYRICH 2003, Table 8 (*Dnmt3a*) and 9 (*Dnmt3*b)).

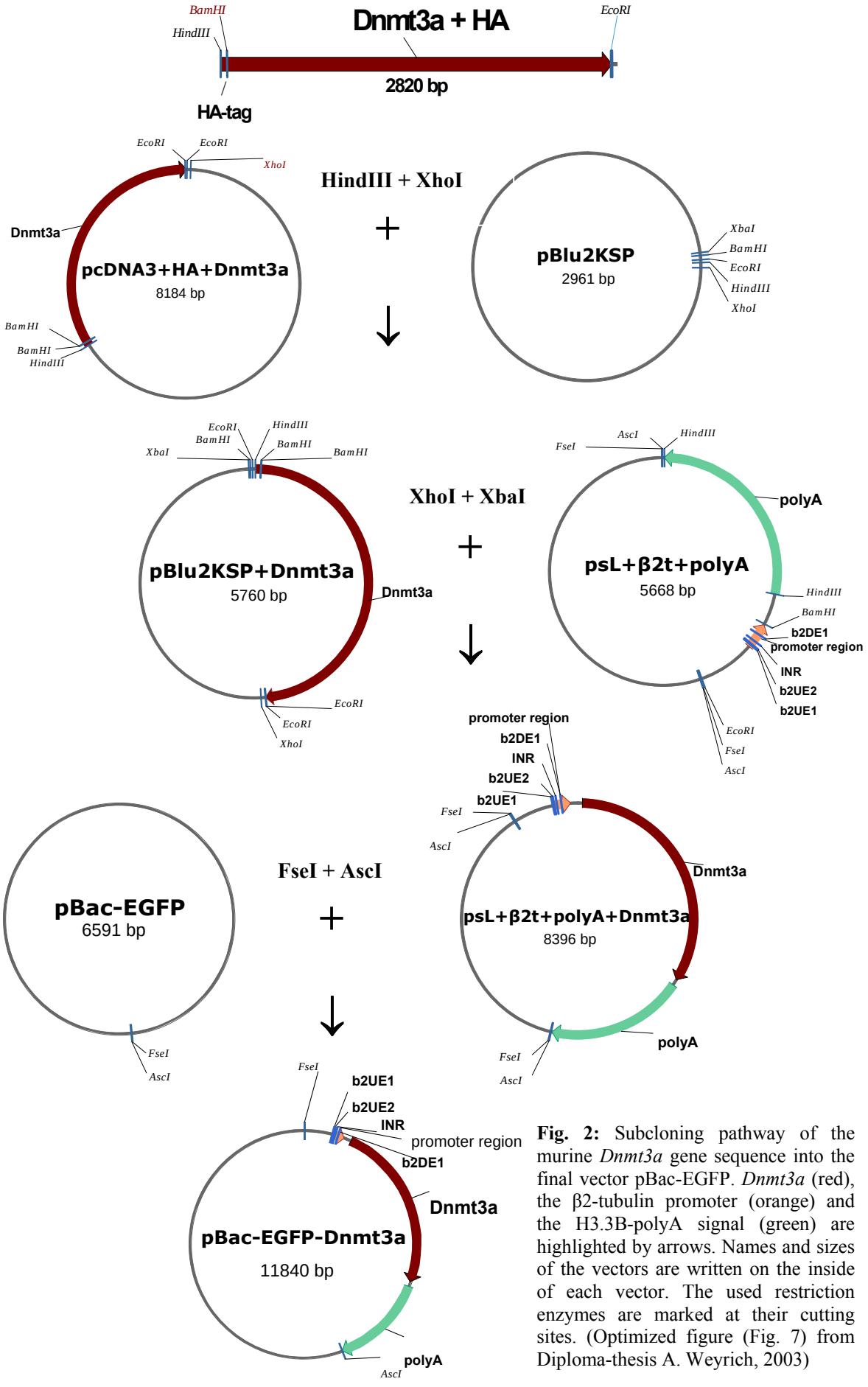


Fig. 2: Subcloning pathway of the murine *Dnmt3a* gene sequence into the final vector pBac-EGFP. *Dnmt3a* (red), the β2-tubulin promoter (orange) and the H3.3B-polyA signal (green) are highlighted by arrows. Names and sizes of the vectors are written on the inside of each vector. The used restriction enzymes are marked at their cutting sites. (Optimized figure (Fig. 7) from Diploma-thesis A. Weyrich, 2003)

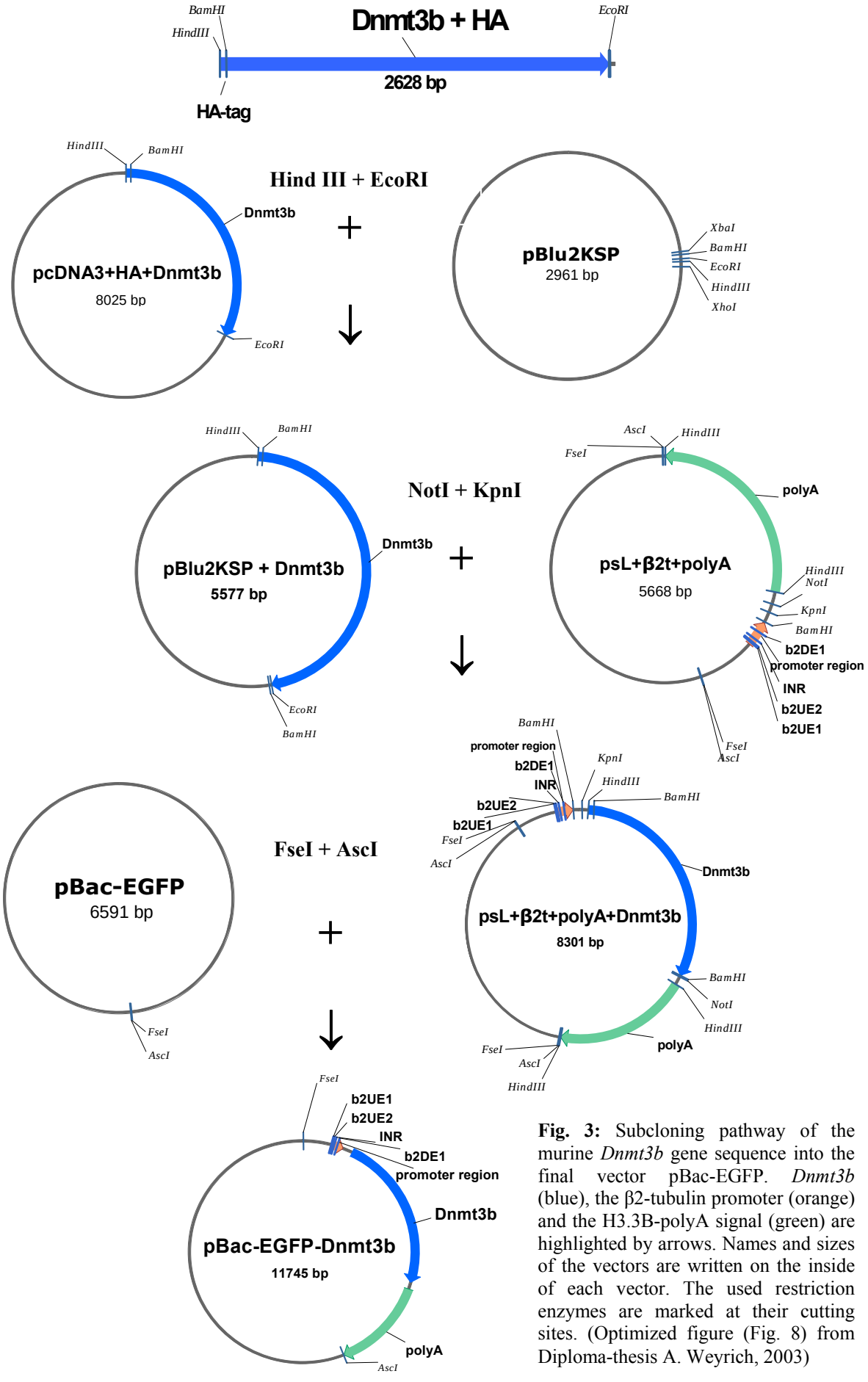


Fig. 3: Subcloning pathway of the murine *Dnmt3b* gene sequence into the final vector pBac-EGFP. *Dnmt3b* (blue), the β2-tubulin promoter (orange) and the H3.3B-polyA signal (green) are highlighted by arrows. Names and sizes of the vectors are written on the inside of each vector. The used restriction enzymes are marked at their cutting sites. (Optimized figure (Fig. 8) from Diploma-thesis A. Weyrich, 2003)

The DNAs of the final constructs were than purified (Macherey-Nagel; Application Kit NucleoBond ® PC 20; Cat.No. 740571) and injected, together with the phsp-transposase active helper plasmid, into the pole cell region of 1h *D. melanogaster white*^{1118} strain (Institute of Genetics, Johannes Gutenberg-University, Mainz/Germany) embryos. We recovered seven transformation strains of *β2t-Dnmt3a* and six strains of *β2t-Dnmt3b*.

3.1.2 *UAS-Dnmt3a/3b* constructs

pP[UAST]-vector constructs (kindly supplied by Institute for Genetic, Mainz) (BRAND and PERRIMON 1993; PHELPS and BRAND 1998) including the P3′ promoter, the UAS-sequence, an hsp70 heat shock promoter and a mini *white* gene, were digested with BglII downstream of these sequences and filled in with Klenow (Amersham Pharmacia Biotech) to produce blunt ends. The HA-*Dnmt3a* and HA-*Dnmt3b* fragments were cut out of the pBluescript II KS-HA-*Dnmt3a/3b* vector (see Fig. 2 and 3) construct with HindIII and EcoRI, filled in with Klenow and ligated into the pP[UAST]-vector. Sequencing primers were generated for the *Dnmt3a*-5`UTR (5′-TCTCCTCCTGTTCCTCTCCTTC-3′) and for the *Dnmt3b*-5`UTR (5`-GTGCAGATTGCCTCCTCCAAGACT-3′) which allowed the verification of complete insert sequence in the constructs. Afterwards the constructs were injected into *Drosophila* embryos. The flies with the most intensive red eye colour were used for further crossings.

3.2 GAL4 expression of *β2t-* and *UAS-Dnmt3a/3b*

Three testes pairs of adults of the *Dnmt3a/3b* strains (*β2t-Dnmt3a/3b* and *UAS-Dnmt3a/3b*), were dissected into lysis buffer (Roche) on ice. RT-PCR was performed using the mRNA Capture Kit of Roche (Cat.No. 1787896) in combination with the Titan one tube RT-PCR system (Roche; Cat.No. 1855476) and following primers (GenTech, Mainz/Germany) listed in Table 1.

Name	Direct.	Sequence	Position [bp]	Length [bp]	T_m [˚C]	Frag. size
Dnmt3a						
DM08	sense	AGTGACCACCAAGTCTCA	1053-1070	18	41.5	732bp
DM09b	anti sense	CATCCACCAAGACACAAT	1785-1767	18	42.2	
Dnmt3b						
DM02	sense	AGGCCGCAGGTCAAGCTC	1017-1034	18	55.1	691bp
DM03b	anti sense	CCAGAGCCACCAGTTTGTCA	1708-1685	23	53.3	

Table 1: Primers designed for RT-PCR of *Dnmt3a* and *Dnmt3b* transcripts. The melting temperature (T_m) was calculated according to the NTI vector program.

The cDNA fragments of *β2t-Dnmt3a/3b* strains were analysed on a 1% agarose gel and of the *UAS-Dnmt3a/3b* x GAL4 crosses on 1.5% gels. As size standard the GeneRuler™ 100bp DNA Ladder Plus (MBI Fermentas; SM0241) was loaded (Fig. 4). The expected fragment sizes were 732bp for *β2t-Dnmt3a* and 691bp for *β2t-Dnmt3b*.

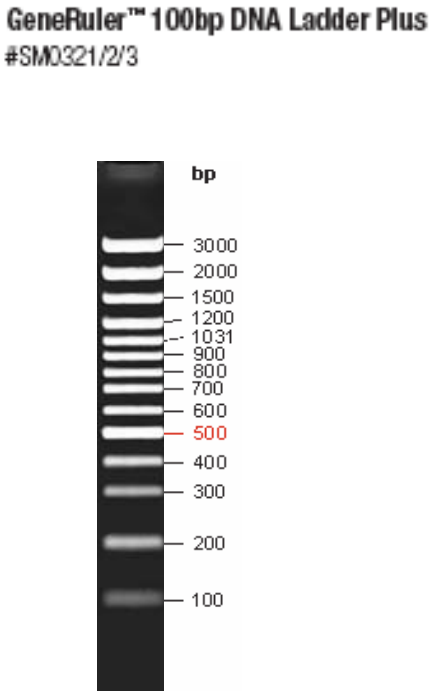


Fig. 4: 100bp DNA Ladder Plus

3.3 Western Blots

We isolated the total protein out of six testis pairs of the *β2t-Dnmt3b* strain 4 (Fig. 10, *β2t-Dnmt3b* lane 2) and strain 6 (Fig. 10, *β2t-Dnmt3b* lane 1), and of *white* testes as a control (Fig. 10, lane *w*). Purified *DNMT3B* enzyme, synthesized in mammalian cell culture, was used as a control (GE *et al.* 2004). The samples, together with a protein prestain marker

(Bio-Rad; Precision Protein Standards, prestain; Cat.No. 1610372), were applied to a 7% acrylamide gel, using the Mini Trans-Blot® Electrophoretic Transfer Cell from Bio-Rad (Cat.No. 170-3930, 170-3935). After blotting and blocking, we incubated the membrane with 1st AB rabbit anti-*DNMT3B* (GE *et al.* 2004) (1:1000) at 4°C o/n. As 2nd antibody we used donkey-anti-rabbit Fab fragments, coupled with HRP (horse radish peroxidase) (Amersham Biosciences; NA934V) (1:200.000). Incubation time was 2h at RT. After the ECL-reaction, the fluorescence was visualized with Kodak X-Omat BT Film (Kodak; XBT-1; Cat.No. 6535876).

3.4 *In Vitro* Methylation Assay

The method was carried out using standard procedures (ROTH and JELTSCH 2000). 60 testis pairs (about 300ng *DNMT3* protein) of *β2t-Dnmt3a*, *β2t-Dnmt3b* and *white* flies were dissected into methylation buffer (200mM Tris pH 7.5 (Sangon); 10mM EDTA (Roth)) including the two protease inhibitors PMSF (Amersco; Cat.No. 0754) (1:100) and Leupeptin (Sigma; L2023) (1:1000). The tissue was broken mechanically. The methylation reaction was carried out at 37°C o/n and the incorporated ^{3}H was measured by liquid scintillation counting (Beckman Instruments LS 6500 - Multi Purpose Scintillation Counter).

3.5 2D-Thin Layer Chromatography

DNA was extracted from 500 testis pairs of *white*, *β2t-Dnmt3a* (strain 5) and *β2t-Dnmt3b* (strain 4) flies. For 2D-Thin Layer Chromatography, also called Nearest Neighbour Analysis, the standard protocol of Methods in Molecular Biology (RAMSAHOYE 2002) was followed, using MboI as restriction enzyme and [α-^{32}P]dGTP (3000 Ci/mmol, Amersham Pharmacia Biotech) for labelling 5mCpGs. This method enables the separation of all single nucleotides thymine, adenine, guanine, cytosine and in addition methylcytosine on a chromatogram (see 4.2.2, Fig. 11) and their visualization by autoradiogram.

3.6 DAPI- and Immunostaining of Testis

The testes of one day old flies (*white*, *β2t-Dnmt3a*-5 and *β2t-Dnmt3b*-4 transformant strains) were squashed in testis buffer (183mM KCl; 47mM NaCl; 10mM Tris-HCl pH 6.8) with 4% formaldehyde (Fluka). After freezing in liquid nitrogen, the cover slips were removed and the slides inserted into methanol at -20°C for 5min, then transferred into methanol/acetone (1/1) for 5min at -20°C and finally into acetone for 5min at -20°C. Subsequently, slides were washed in PBS (137mM NaCl; 2.7mM KCl; Na_2HPO_4; 2mM KH_2PO_4; pH7.4) and twice in PBS+ 0.1% Triton X-100 for 5min. For 5-methylcytosine detection the testis DNA was denatured in 2M HCl for 30min. The incubation for 2h in 2M HCl as carried out by Kunert et al. (KUNERT *et al.* 2003) destroys the tissue completely. Moreover, no signal was detectable without HCl treatment. Alternatively, alkaline denaturation (0.1 N NaOH for 10min) and HCl treatment with 0.1M HCl for 10min did not deviate from the results obtained with 2M HCl. All slides were blocked for 15min in PBS with 4% bovine serum albumin (BSA) (Sigma), followed by an o/n incubation in a humid chamber at 4°C with 7µl of the first antibody anti 5-methylcytosine. The polyclonal antibody rabbit anti-5mC from Megabase Research Products (1:200, Cat.No. CP50250) (KUNERT *et al.* 2003) was the only one giving reaction. The two monoclonal antibodies mouse anti-5mC, one provided by Dr. T. Haaf (1:100) (REYNAUD *et al.* 1992) and one purchased from Eurogentec (1:50, Cat.No. MMS-900S-B) did not display any cytosine methylation signal in *Drosophila*. Positive reactions with all three antibodies were obtained with human lymphocyte metaphase chromosomes (kindly provided by Dr. T. Haaf).

Then slides were washed 3 times with PBS+ 0.1% Triton X-100. As a secondary antibody, 10µl DyLight™ 547 goat anti-rabbit (Pierce; 1:200; Cat.No. 35557) (for the two monoclonal antibodies mouse anti-5mC 2nd AB Alexa Fluor® 546 goat anti-mouse IgG (H+L) (Molecular Probes; 1:1000; Cat.No. A-11030)) with 1µl/ml DAPI (4´,6-diamidino-2-phenylindole) was incubated for 2h at RT. After washing in PBS+ 0.1% Triton X-100 three times for 5min, the tissue was covered with antifading solution (1% p-Phenylenediamine in PBS/Glycerol (1/1)) and studied in the epifluorescence microscope (Nikon Eclipse E600 epifluorescence microscope equipped with a Plan Apo 100x/1.40 Oil DIC H optics and fluorescence filter set G-2A).

3.7 Testis preparation for HPLC and MALDI-TOF

DNA was isolated out of 500 testis pairs of *Drosophila melanogaster white* flies and 500 pairs of *β2t-Dnmt3b-4* transformants. Testis were dissected into testis buffer on ice, centrifuged 3min at 14.000rpm and heated with 65°C water bath in lysis buffer (10mM Tris pH 8; 10 mM EDTA pH 8; 100mM NaCl). After 5min 3.5µl 10% SDS, 12µl beta-mercapto-ethanol, and 2.5µl RNaseA (10ug/ml) were added and incubated another 30min at 65°C. Then 48µl DTT (1M), and 12µl Proteinase K (20mg/ml) were added and kept at 65°C for 3h (after 1.5h additional 12µl Proteinase K was added). Phenol and SEVAG extraction were performed and DNA precipitated with 50µl NH_4OAc (7.5M), 50µl Glycogen, 800µl abs. ethanol. After washing in 70% ethanol, the DNA pellet (~3µg) was dissolved in 10µl ddH_2O for 5min at 65°C and digested with 2µl MboI at 37°C o/n in a final volume of 50µl. The reaction was stopped at 70°C for 20min and DNA fragments were precipitated by 5µl 3M Na-acetate and 100µl abs. ethanol. The pellet was air dried, dissolved in 5µl micrococcal digest buffer (10mM $CaCl_2$, 100mM Tris-HCl) 5min at 65°C and digested into single nucleotides with 30µl micrococcal nuclease (MBI; Cat.No. EN0181; 8000u (300u/µl)) at 37°C o/n for HPLC and MALDI analysis.

3.8 High Performance Liquid Chromatography (HPLC) analysis

Experiments were performed with a Waters Alliance 2695 HPLC system. Prior to HPLC analysis, 40% of each sample (~10µl) was diluted 1:10 in water. 50µl (~20% of the original sample) were applied to the HPLC-system.

The chromatographic separation was achieved using a Gemini C18 column (Phenomenex; 150×3mm I.D.; 5µm particles) at room temperature. Mobile phase A consisted of 20mM ammonium acetate, pH 4.5, in a 99:1 (v/v) water-acetonitrile mixture, and mobile phase B consisted of 20mM ammonium acetate, pH 4.5, in a 90:10 (v/v) water-acetonitrile mixture. After a 1min period with 100% mobile phase A, a concave gradient started from 0%B to 75%B in 5min, followed by a 8min period with 75%B. Finally, the column was re-equilibrated with the initial mobile phase, adding up to a total run time of 17min. A flow rate of 0.5ml/min was delivered throughout the entire procedure. The effluent was monitored by a photodiode array detector (Waters PDA 996). Assignment of compounds was done via retention time and specific absorption spectra (λ= 200-400nm). For

quantitation, area under the curve (AUC) values of the sample compound peaks at λ= 260nm were compared to the AUC values of defined amounts of the respective standard. (Proceeded by ProteoSys AG, Mainz)

3.9 Matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF) analysis

For mass spectrometry sample preparation, 10% of the original samples were evaporated to complete dryness in a vacuum centrifuge and reconstituted in 4µl 0.1% trifluoroacetic acid (TFA). 1µl was spotted on a 384 MTP ground steel target (Bruker-Daltonics) and allowed to air dry. As a control, 1µl of pure 0.1% TFA was spotted. 1µl of matrix solution (10mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) was applied to the dried samples and again allowed to air dry.

Mass spectra were obtained using an Ultraflex TOF/TOF (Bruker-Daltonics) in the fully automated reflectron TOF operation mode controlled by the FlexControl software. The mass spectrometer was equipped with a SCOUT-MALDI source for multisample handling, a pulsed UV laser, a two-stage gridless reflector, a 2 GHz digitizer, and multichannel-plate detectors for linear and reflector mode measurements. All measurements were carried out in positive ionization mode using a reflector voltage of 20kV. (Proceeded by ProteoSys AG, Mainz)

4. Results

4.1 Does DNA methylation exist in *Drosophila*?

Testes of *Drosophila* adults contain germ cells as well as somatic cells. This permits a comparative study of a possible contribution of DNA methylation to imprinting or gene silencing in *Drosophila* germ cells and somatic cells. Our experiments were carried out with a *white* strain of *D. melanogaster*, which was also used for transformation experiments (see below).

5-methylcytosine in DNA can be detected by different methods. A sensitive and precise method is immunocytology with antibodies directed against methylated cytosines at its carbon 5 sites. We have tested three different antibodies (see material and methods 3.6) but only one of them gave signals in germ line nuclei (Fig. 5-8). The label occurs in stained patches in spermatocytes (Fig. 5A) and elongated spermatids (Fig. 5B, C) as well as in the somatic cells of the testis envelope (Fig. 6A-C). Due to the bad conservation of the chromatin structure as a consequence of the HCl treatment (3.6), the assignment of the signal to particular chromosome regions is not possible in *D. melanogaster*.

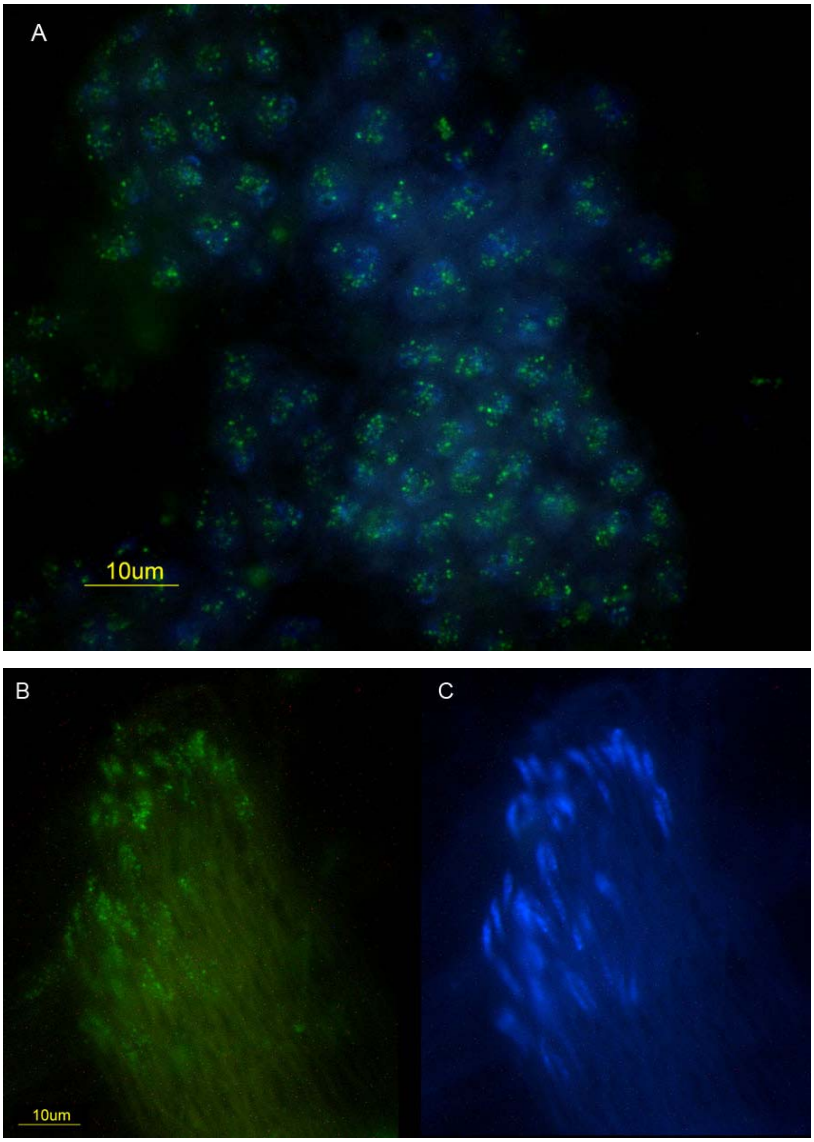


Fig. 5: 5mC-Immunocytology of *Drosophila melanogaster* germ cells. **A.** Merged DAPI and 5mC-AB staining in spermatocytes beside a great unmethylated area. **B.** 5mC-AB staining shows partial signal in the heads of the elongated spermatids and a weak signal in their tails. **C.** DAPI-staining of spermatids indicates the localization of DNA.

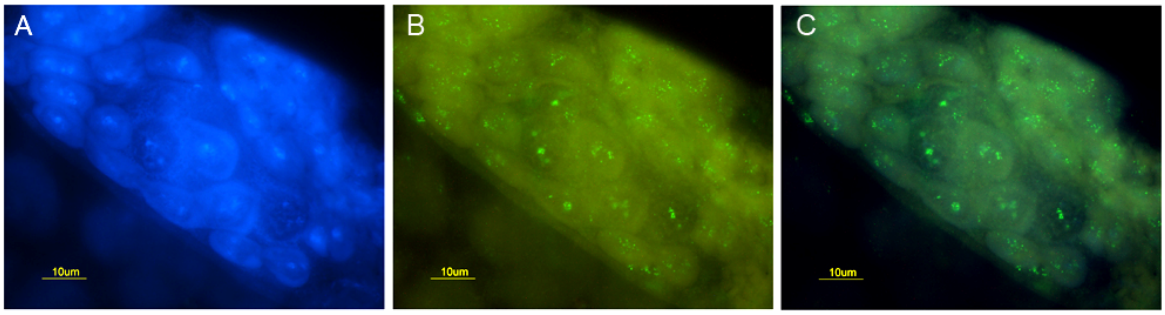


Fig. 6: Testis envelope cells stained with DAPI (**A**) and an antibody against 5mC (**B**). **C** displays a merged pictures of A and B.

Therefore, we have included the germ line of *D. hydei* in our study, as here the cytology permits clear conclusions. Signals were identified in primary spermatocytes (Fig. 7) and in nuclei of young round spermatids (Fig. 8).

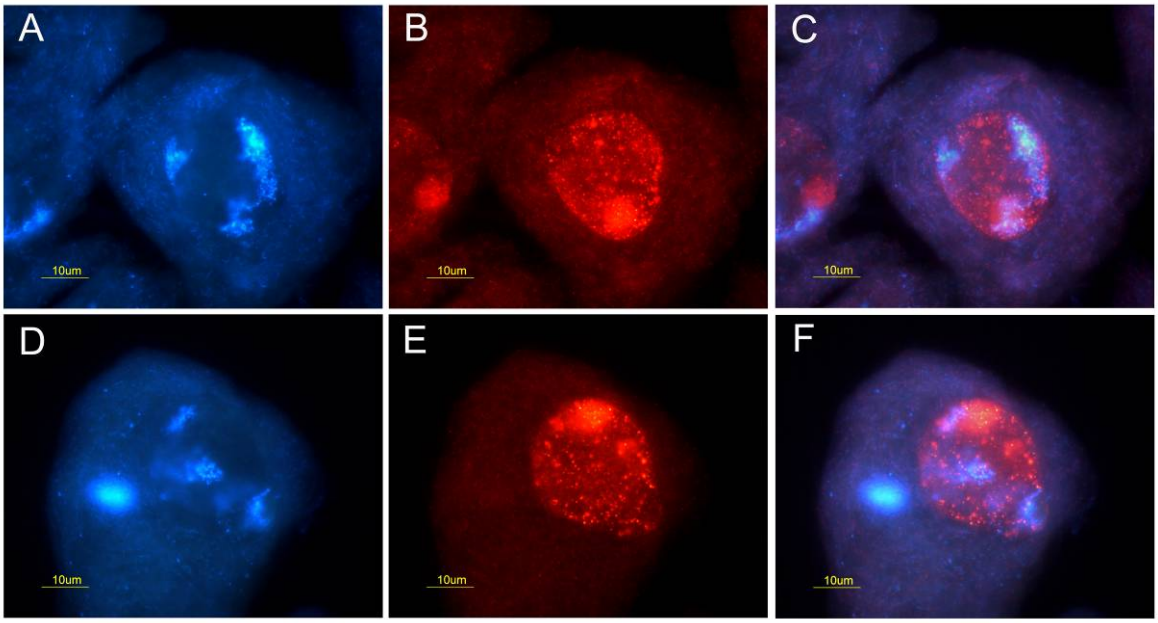


Fig. 7: Immunocytology of primary spermatocytes in *Drosophila hydei*. **A, D.** DAPI staining of chromosomes in the nuclei. **B, E.** 5mC-AB staining shows signal in primary spermatocytes not related to the DNA. **C, D.** Merged DAPI- and 5mC-AB labelling displays their different localizations.

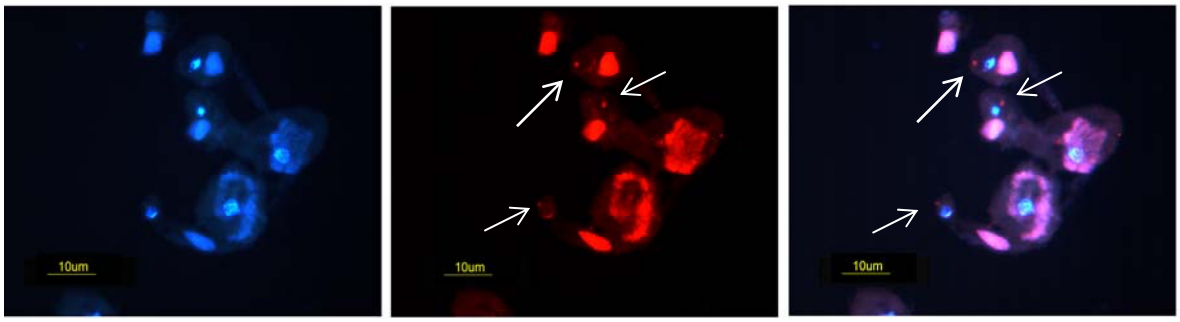


Fig. 8: Immunocytology of young round spermatids of *Drosophila hydei*. **A.** DAPI staining of the DNA in the nucleus and mitochondria. **B.** 5mC-AB staining displays labelling around the DNA structure inside the nuclei identified as protein bodies (arrows), as well as of the mitochondria. **C.** Merged DAPI- and 5mC-AB labelling displays the location of the chromatin (blue) and the staining of the protein body and the mictochondria by the 5mC antibody (red).

Both signals are, however, not compatible with the localization of DNA. In primary spermatocytes it is associated with a round structure, most likely the "pseudonucleolus" which contains mainly protein and RNA while it is surrounded by DNA (HENNIG 1967).

The signal in spermatids is restricted to a nuclear structure designated as protein body (GROND 1984; HENNIG and KREMER 1990). This protein body has been shown to contain RNA and basic proteins but no DNA. These observations imply that the antibody is not specific for 5-methylcytosine but recognizes other antigens, possibly mehyl-groups in RNA and/or proteins.

The results obtained by immunocytology indicate that the male germ cells of *Drosophila* contain no significant amounts of 5mC.

4.2 DNA methylation in *Dnmt*-transformant flies

It has been shown that transformed mammalian *Dnmt*s can methylate genomic DNA of *Drosophila* (LYKO *et al.* 1999). This induced us to investigate whether methylation of male germ cell DNA has effects on fertility or on the embryonic development of the following generation. We introduced the murine *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b-1* into the genome of *D. melanogaster white*. To restrict their expression to germ cells we placed them under the control of the β2-tubulin promoter, specific for transcriptional regulation in primary spermatocytes (MICHIELS *et al.* 1989).

4.2.1 *Dnmt3a*

In seven different transformation strains analysed for *Dnmt3a*, no phenotypic abnormalities or effects on fertility were observed. Since this could be caused by a failure of the expression of the transformed methylases, we carried out RT-PCR experiments to ascertain that the transformed gene was transcribed. We detected the expected cDNA fragment of *Dnmt3a* in testes (Fig. 9). This proves the transcription of the transformed *Dnmt3a* gene.

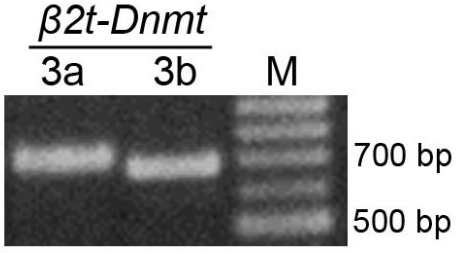


Fig. 9: RT-PCR analysis of *β2t-Dnmt3a* (strain 5) and *β2t-Dnmt3b* (strain 4) transformants proves transcriptional expression of both transformed methylases in testes under the tissue-specific control of the β2-tubulin promoter. The primers (material and methods 3.2, Table 1) give the expected cDNA-fragments of 732bp for *β2t-Dnmt3a* and of 691bp for *β2t-Dnmt3b* (for DNA marker see material and methods 3.2, Fig. 4).

Western blot experiments with several transformation strains of *Dnmt3a* did not give evidence for *DNMT3A* protein expression in testes, nor did the 2D-TLC show an additional methylcytosine spot on the autoradiogram for CpG-dinucleotides (Fig. 12B). Also *in vitro* assays for methylase activity did not give evidence for *DNMT* activity, even in the strain (*β2t-Dnmt3a* strain 5) with the highest GFP-fluorescence and proven transcriptional activity (Table 2).

In addition, 5mC-immunocytology with *Dnmt3a* transformant testes squashes did not show any differences to *white* eye flies. *Dnmt3a* is apparently not translated and consequently, no effects on the male germ cell development are expected.

4.2.2 *Dnmt3b*

For *Dnmt3b*, six transformation strains were generated and analysed. Like for *Dnmt3a*, we could not observe any phenotypic abnormalities or effects on fertility of the transformant flies. RT-PCR experiments prove transcriptional activity of the transgenes since they gave the expected cDNA fragments in testes (Fig. 9).

Contrary to *Dnmt3a*, the translation of the *Dnmt3b* mRNA could be documented by western blots using anti-Dnmt3b antibodies (Fig. 10). The *DNMT3B* protein extracted from the transformant strains (Fig. 10, *β2t-Dnmt3b* lane 1 and 2) has the same molecular mobility in gels as the control protein synthesized in mammalian cell cultures (GE *et al.* 2004) (Fig. 10, lane C).

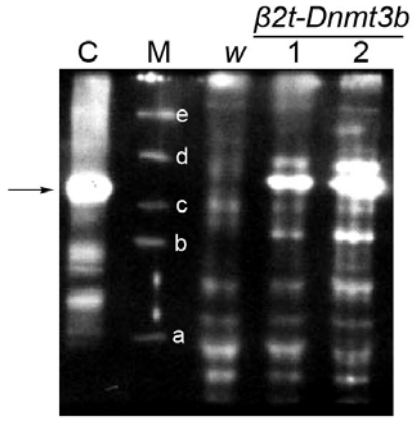


Fig. 10: Western blot of testis protein from *β2t-Dnmt3b* transformant strains. Positive signals in western blots were obtained from testes of two *β2t-Dnmt3b* transformation strains, but not from *β2t-Dnmt3a* transformation strains (data not shown). *DNMT3B* protein synthesised in mammalian cell culture was used as a positive control (C). A polyclonal anti-*Dnmt3b* antibody detects a protein fragment about 130kDa (arrow). Testes protein from strains 6 and 4 (lane 1 and 2) of *β2t-Dnmt3b* transformants show a protein fragment of the same size. Strain 4 was also used for RT-PCR analysis. In w*hite* fly testis (*w*), used as negative control, no *DNMT3B* protein was detectable. Marker sizes: a= 50 kDa; b= 75 kDa; c= 100 kDa; d= 150 kDa; e= 250 kDa

The strain, with the highest *DNMT3B* protein expression (*β2t-Dnmt3b* strain 4) in testes (see Fig. 10, *β2t-Dnmt3b* lane 2) was used for further experimentation.

For this strain the total CpG-methylation level of the *Drosophila* genome was investigated by 2D-TLC. The testis DNA of *white* flies and transformation strains of both methyltransferases were radioactive labelled at CpG, then digested into single nucleotides, separated and visualized by 2D-TLC. If 5-methylcytosine occurs in the male germ line it would be visible as an additional spot on the radiogram (Fig. 11).

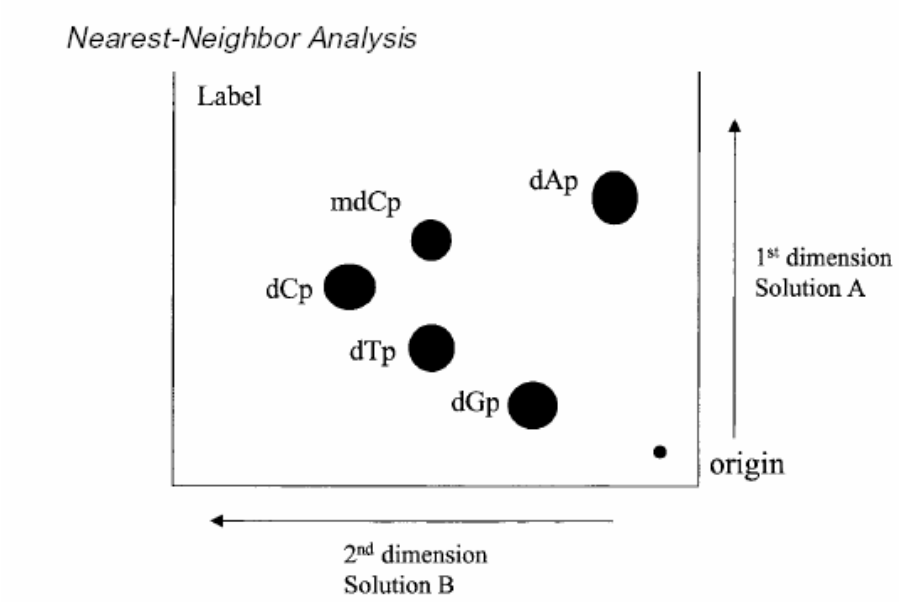


Fig. 11: The expected positions of nucleotide 3′-monophosphates after two-dimensional chromatography, illustrated in this schema. mdCp indicates the additional fifth spot by 5-methylcytosine occurrence (Methods in Molecular Biology).

As for *Dnmt3a* (Fig. 12B), no additional spot for methylated cytosines could be detected on the radiogram in testes of *Dnmt3b* transformant flies (Fig. 12C).

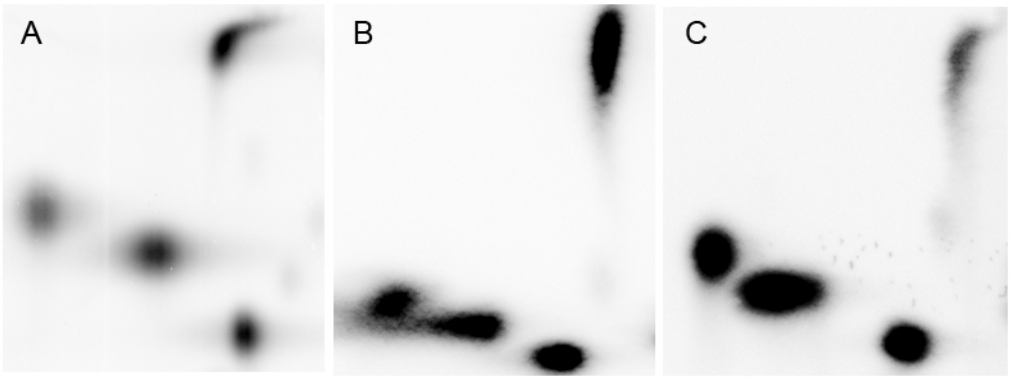


Fig. 12: The two-dimensional chromatography does not display the additional 5mC spot on the autoradiogram. 5mC was neither detected in the DNA isolated out of *white* flies (**A**), nor in the DNA deriving from the transformant strains of *β2t-Dnmt3a* (**B**) and *β2t-Dnmt3b* (**C**).

To see whether the *DNMT3B* protein in testes has enzymatic activity we applied an *in vitro* methylation assay (ROTH and JELTSCH 2000) to testes from the *β2t-Dnmt3b* (strain 4) and included testes of *white* and *β2t-Dnmt3a* (strain 5) respectively. The *in vitro* methylation activity was only slightly above background levels (Table 2), compared to the negative control where no reaction occurs. As positive control the prokaryotic DNA-methyltransferase M-HhaI (Bioloabs) was applied, proving the functional capability of the method.

Positive control	**Negative control**	***white***	***Dnmt3a***	***Dnmt3b***
2556.98 CPM	47.28 CPM	58.25 CPM	73.67 CPM	70.23 CPM

Table 2: Incorporated ^{3}H was measured by liquid scintillation counting for *white*, *Dnmt3a* (strain 5) and *Dnmt3b* (strain 4). Mean values were calculated in counts per minutes [CPM] after four radioactive measurements for 10min. M-HhaI was applied as positive control and for the negative control ddH_2O replaced the enzyme sample.

The low proportion of primary spermatocytes in whole testes may explain the low level of activity. A fractionation of spermatocytes is not feasible and the test system permits only the addition of a limited number of 60 testes pairs in the reaction volume. As a consequence we could not increase the sensitivity of detection in these experiments.

HPLC-UV and MALDI-TOF experiments may support the former results. Therefore the DNA from 500 testis pairs of *white* and *β2t-Dnmt3b* (strain 4) were isolated and digested into single nucleotides.

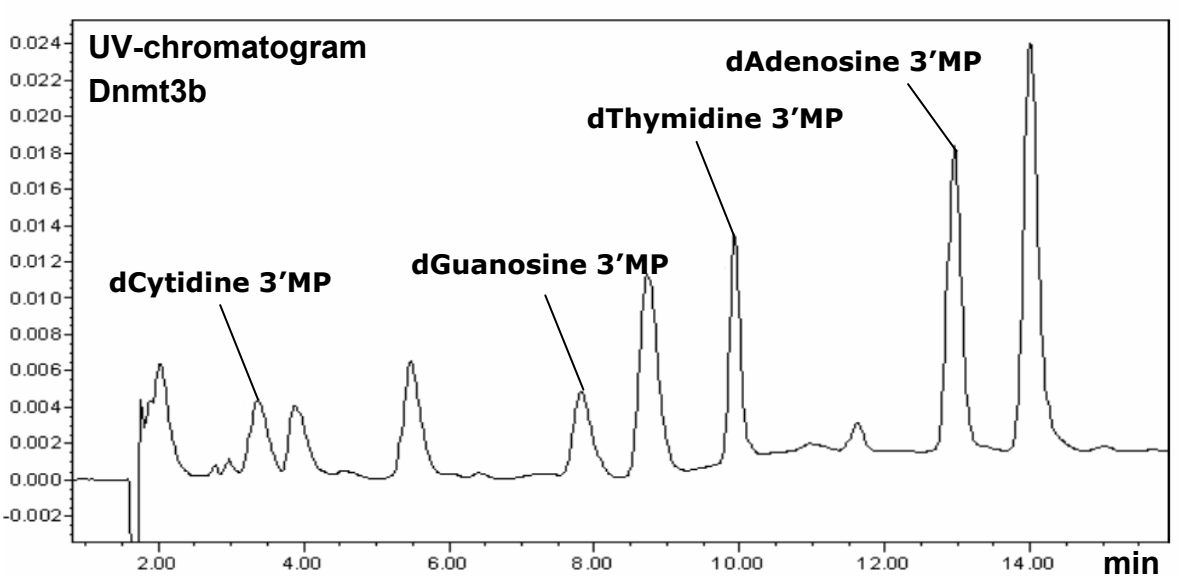


Fig. 13: HPLC analysis of the transformant strain *β2t-Dnmt3b* (strain 4). The UV-chromatogram (260 nm) displays the peaks for the four regular (deoxy-) nucleotide 3'-monophosphates with concentrations between 150- 200ng/nucleotide. Deoxy-methylcytidine 3'-monphosphate was not detectable (ProteoSys, Mainz).

By HPLC (Fig. 13) all regular nucleotides of testis DNA of the *β2t-Dnmt3b-4* transformant strain, as well as of *white* (data not shown), could be detected, but methylcytosine was only detectable in insignificant levels no additional d-methylcytidinmonophosphat, d-methylcytidin or methylcytosin.

In addition, MALDI-TOF analysis detected only marginal and insignificant signals of methylcytosine and d-methylcytidine (Table 3) for *Dnmt3b-4* transformant like for *white* DNA.

			Signal intensity -- samples		
	MH+		***white***	***Dnm3b***	**Control**
Cytosine	112,10		+	+	
Methylcytosine	126,12		(+)	(+)	
Thymine	127,11		++	++	++
Adenine	136,13		+	+++	
Guanine	152,13		+	+++	
dCytidine	228,09		(+)	+	(+)
dMethylcytidine	242,09				
dThymidine	243,09		(+)	+	
dAdenosine	252,10		+	+	(+)
dGuanosine	268,10			+	
dCMP	308,06		(+)	(+)	(+)
dmCMP	322,06		(+)	(+)	(+)
dTMP	323,06		+	+	+
dAMP	332,07		+	+++	+
dGMP	348,06		+	++	(+)
signal intensity (SI):	(+)	S/N ≤ 5			
	+	S/N 5 ≤ 25			
	++	S/N 25 ≤ 100			
	+++	S/N 100 ≤ 500			
	++++	S/N ≤ 500			

MH+: Monoisotopic mass (Dalton) of the protonated compound

Table 3: MALDI-TOF analysis of *white* and of the transformant strain *β2t-Dnmt3b-4*. All regular (deoxy-) nucleotide 3'-monophosphates could be identified in both samples, mostly on the nucleobase level. The amount of 5-methylcytosine is less than 10% (ProteoSys, Mainz).

We conclude that *DNMT3B* might have a low if any methylation activity in spermatocytes, although this does not seem to result in detectable DNA methylation. Like for *Dnmt3a*, immunocytology labelling 5mC in testis DNA showed no differences to *white* eye flies testis.

4.2.3 Tissue specific *Dnmt* expression

Our unsuccessful approach to induce methylation of DNA by the introduction of the methylating enzymes into the male germ line, induced us to make use of the UASxGAL4 system (PHELPS and BRAND 1998; BRAND and PERRIMON 1993; DUFFY 2002) to express the *de novo* DNA methylases in defined other tissues and developmental stages (Table 4). The germ line might have special mechanisms to suppress genes of foreign origin, but *DNMT* expression in tissues other than testes might cause abnormalities in the phenotype (LYKO *et al.* 1999; WEISSMANN *et al.* 2003).

We constructed *pUAS-Dnmt3a* and *pUAS-Dnmt3b* transformation strains and crossed them with selected GAL4 strains (Table 4), to induce the expression of the mammalian methyltransferases in distinct tissues or developmental stages of *Drosophila*. The expression of all GAL4 strains used in our experiments was checked by crosses with an UAS-LacZ strain that catalyses, induced by the expression of GAL4 protein, the reaction of β-Galactosidase with X-Gal. This reaction results in a blue staining of the respective tissues. The LacZ expression patterns obtained deviates partially from the patterns indicated in flybase for the respective strains (Table 4, fourth column).

GAL4 strains

Stock	ASCII Genotype	Expression Loci Bloomington	Expression Loci LacZ/X-Gal[(a)]
1854	w[*]; P{w[+mW.hs]=GawB}I-76-D, Ubx[9.22] e[1]/TM6B, Tb[+]	GAL4 in epidermal stripes in embryo (T. Kaufman)	Larva fatbody, brain, salivary glands; in adult fatbody, Malipighian tubules, male intestine
1878	w[*]; P{w[+mW.hs]=GawB}T80/CyO	Ubiquitous in third instar imaginal discs (K.M.)	Larva imaginal discs, brain, salivary glands
3741	P{w[+mW.hs]=GawB}167Y, w[1118]	GAL4 pattern in third instar larva: brain - neuroblasts in central brain and ventral ganglion, Bolwig's nerve, discs - spot in leg disc only, (L. Manseau)	Larva and adult fatbody, intestine, salivary glands, soma cells of testis in the area of mature spermatocytes
6870	w[1118]; P{w[+mC]=Sgs3-GAL4.PD}TP1	Expresses GAL4 in the salivary gland (L. Cherbas)	Larva salivary glands
6996	w[1118]; P{w[+mW.hs]=GawB}T98	GAL4 expressed in embryonic PNS and CNS, larval brain, wing and eye discs and salivary glands, adult male cyst cells and spermatocytes. May be segregating CyO (K.C.)	Larva brain, eye imaginal discs, intestine, Malphigian tubules, salivary glands, adult male cyst cells
7029	y[1] w[*]; P{w[+mW.hs]=GawB}60IIA	GAL4 expressed in the nervous system (S. Younger)	Larva brain, salivary glands, fatbody, wing imaginal discs; adult malpighian tubules, male intestine
6984	P{w[+mW.hs]=GawB}c754, w[1118]	GAL4 expressed in larval brain and fat body, (L. Manseau)	Larva brain, fat body

Table 4: Table of all GAL4 strains used for crossing with *UAS-Dnmt3a* and *UAS-Dnmt3b* respectively to induce methylase expression in distinct tissues. The left column represents the stock number of the Bloomington Stock Center; the second column gives its genotype. A description of the expression patterns obtained from the information of the Bloomington Stock Center is listed in the third column.

[(a)]The expression of GAL4 strains (Bloomington) has been checked by crosses with the UAS-LacZ strain (Bloomington Nummer: B-3955; UAS-LacZ nuclear; w[1118]; P{w[+mC]=UAS-lacZ.NZ}20b). The LacZ expression patterns obtained deviate partially from the patterns indicated in flybase for the respective strains. The deviating patterns are listed in the fourth column.

We confirmed the expression of the UAS regulated *Dnmt3* genes in all GAL4 crosses at the transcriptional level by RT-PCR. In all cases, RNA of *Dnmt3* was found (Fig. 14). The development, however, was normal in all crosses and no abnormal phenotypes were observed (see 8.1, Table 5-7). Also the F2 generation of the UAS/GAL4 flies displayed normal development, phenotypes and/or fertility.

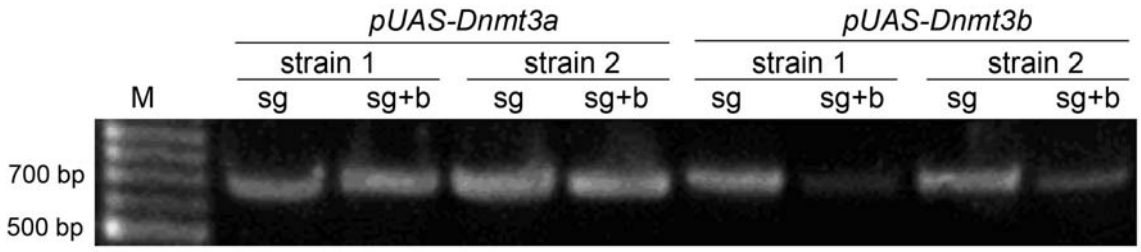


Fig. 14: RT-PCR products of *pUAS-Dnmt3a/3b* visualized on a 1.5% agarose gel are displaying the expected cDNA fragments in all samples analyzed. Sg: salivary glands, sg+b: salivary glands and larva brain.

RT-PCR of two strains of *pUAS-Dnmt3a* and *pUAS-Dnmt3b* (strain 1, strain 2) crossed with two distinct GAL4 strains (stock numbers underlined in the table 4) confirmed the transcriptional activity of the methylases. One GAL4 strain induces expression in larva salivary glands (sg; GAL4 strain 6870), the other in salivary glands and larva brain (sg+b; GAL4 strain 6996). The same primers were used as for *β2t-Dnmt3a/3b* RT-PCR and the expected size cDNA fragments were separated and visualized on a 1.5% agarose gel.

5. Discussion

While in mammals, gene silencing and epigenetic programming is closely connected with DNA methylation, the respective molecular mechanism in *Drosophila* and other insects is unclear.

DNA methylation in insects generally occurs only at a low level and is not likely to be used as a general tool for gene silencing (GOWHER *et al.* 2000), (FUJITA *et al.* 1999; URIELI-SHOVAL *et al.* 1982) although there might be species-specific differences (GARCIA *et al.* 2007). Our present study has shown that no significant amount of DNA methylation was detectable in the male germ line and associated somatic cells in *D. melanogaster* or *D. hydei*, even though a substantial proportion of inactivated DNA exists in somatic cells of males. The *Y* chromosome represents approximately 10% of the genomic DNA. Probably with the exception of its ribosomal RNA genes, the *Y* chromosome is inactive and heterochromatic in somatic tissues (BRIDGES 1916; MAGGERT and GOLIC 2002). In the male meiotic prophase it is however transcriptionally active (HENNIG 1968; HENNIG *et al.* 1974). If the somatic silencing was achieved by DNA methylation, the level of methylated DNA should be increased in soma cells. Even though the sensitivity of the TLC method is limited, a considerable proportion of the cytosines are expected to be methylated if this chromosome is entirely silenced in somatic cells, comparable to the inactive *X* chromosome in mammals. We could, however, not detect any increase in cytosine methylation in somatic cells compared to testes. Immunostaining experiments with an antibody against 5-methylcytosine did not give evidence for DNA methylation. Both results were confirmed by HPLC and MALDI analysis of testis DNA. It seems therefore, that DNA methylation plays no prevalent role in gene silencing in *Drosophila*.

5.1 DNA methylation in *Dnmt*-transformant flies

The experiments of Lyko et al. (LYKO *et al.* 1999) demonstrated that an expression of the transgenic mammalian *Dnmt3a* gene in *Drosophila melanogaster* can lead to lethality in embryogenesis and early pupal development. We therefore expected that males may become sterile if mammalian methylases are expressed during spermatogenesis.

The use of the testis specific β2-tubulin promoter (MICHIELS *et al.* 1989) permits a spermatocyte-speficic expression of genes, which therefore avoids potential lethality if expressed universaly in all cells. We used it in transformation experiments in combination with the mouse *Dnmt3a* and *Dnmt3b* genes. However, we were not able to recover transformant flies with enzymatically active DNA methylases. The *DNMT3B*-protein was expressed as western blots document, but *in vitro* methylation assays did not show significant increase in enzyme activity. The reason is unclear. Either an inhibitor suppressed the enzyme actively or the fly's germ cells do not provide additional components, necessary for an active DNA methylation pathway as has been suggested to be required by Pradhan for mammalian cells (PRADHAN and ESTEVE 2003).

Dnmt3a was not even expressed at the protein level, but transcripts could be discovered by RT-PCR. Either the synthesis of the protein is postranscriptionally inhibited or the protein is degraded immediately after synthesis.

Since a general high level expression of mammalian DNA methylases in all the somatic cells may be lethal (LYKO *et al.* 1999) we decided to carry out GAL4 experiments to specifically express the *DNMT*s in certain defined tissues. The transformants obtained from UAS promoter constructs also did not show any phenotypic effects in the respective tissues if combined with different GAL4 strains. Since RT-PCR demonstrates that the transformed genes are transcriptionally expressed, the function of the transformed genes must be prevented at a posttranscriptional level or otherwise their expression level is too low to show any effect. In these experiments we have not investigated whether the *DNMT* proteins are synthesized and whether DNA methylation has occurred. The results of these experiments contradict the results of Lyko and colleagues (LYKO *et al.* 1999, WEISSMANN *et al.* 2003). One possible cause might be a different level of expression of the mammalian enzymes in the different experiments. It is possible that only a strong over-expression affects the cellular differentiation.

From our experiments we conclude that DNA methylation has no significant role in epigenetic processes during the normal *Drosophila* development, neither in male germs nor in somatic cells. This conclusion is supported by the fact that the only methyltransferase found in *Drosophila*, *dDNMT2*, has been identified as an RNA methylating enzyme. Over

expressing *dDnmt2*, reducing its expression (KUNERT *et al.* 2003) or even knocking out the gene (GOLL *et al.* 2006) did not yield any phenotypic effects on the fly.
In addition, mammalian *DNMT*s have distinct target sequences when over-expressed in *Drosophila* (MUND *et al.* 2004), but interestingly no preferred methylation of promoters or other regulatory elements were detected in *Drosophila* as they characteristically occur in mammals (ANTEQUERA and BIRD 1999). Compared to mammals the amount of CpG (MUND *et al.* 2004) in the *Drosophila* genome is highly reduced. This low amount of clustered CpGs in the *Drosophila* genome could be one reason why DNA methylation mechanisms have been lost in evolution of this species. This would also explain why we found no different methylation pattern in between the *Dnmt*-transformant strains and the control strain (*white*) in our experiments. Cytosine methylation in *Drosophila* mainly occurs at CpT instead of CpG-sites (LYKO *et al.* 2000b). CpT's are not preferentially targeted by the mammalian *DNMT3A* and *DNMT3B*. High concentration of DNA methylase might overcome the need for additional components required as co-factors in the DNA methylation pathway which could explain the results of Lyko and associates. The use of a heat shock promoter might lead to a strong over expression of the enzymes causing lethal effects.

One primary reason for the incompatibility of our observations with those of Lyko and associates might be found in the target tissue: We have concentrated on the male germ line. The male germ line may differ from other cell types with regard to the expression of factors required for DNA methylation. For example, Weismann and his colleagues (WEISSMANN *et al.* 2003) have shown that histone H3-K9 methylation is required for the induction of experimental DNA methylation by transgenic *DNMT*s. Moreover, experience in our laboratory also documents that the expression of transgenes in the male germ line of *Drosophila* is difficult or can be lost within a year after the successful functional introduction of a gene into the genome by transformation. The male germ line might therefore have mechanisms which prevent or eliminate the expression of foreign genes. In this context, the assumption becomes of interest that DNA methylation serves for protection of the cell against the activity of retroviruses (BESTOR and BOURC'HIS 2004; SALZBERG *et al.* 2004). If this holds true for *Drosophila* one should expect a high level of methylation in the testis, as we have shown earlier that retrotransposons are highly expressed in the male germ line (HUIJSER *et al.* 1988; LANKENAU and HENNIG 1990;

LANKENAU *et al.* 1988). Our present data do not support the assumption that DNA methylation is required for the suppression of retroviral genome components.

One difficulty to judge earlier conclusions on the level of DNA methylation are the low levels detected by bisulfite sequencing (LYKO *et al.* 2000a; LYKO *et al.* 1999; SALZBERG *et al.* 2004). The 5mC levels detected in these studies range around 0.4%. This, however, comes close to the values identified as experimental limit for the method. In several publications (CLARK *et al.* 1994; GRUNAU *et al.* 2001; WARNECKE *et al.* 2002) it has been pointed out that between 0.3 and 0.5% of the unmethylated cytosines do not become desaminated in the bisulfite reaction which will lead to the erroneous conclusion that they were methylated. Also repetitive DNA elements, which can rapidly renature, may be misinterpreted as double-stranded DNA, and are not accessible for desamination by the bisulfite reaction. In addition, protein contamination of the reacting DNA, most likely caused by packaging of heterochromatic chromosome regions, may prevent desamination of cytosine. In fact, preferentially transposable elements and repetitive DNA sequences have been identified as methylated DNA by bisulfite sequencing (SALZBERG *et al.* 2004).

Considering these limitations in the detection methods of 5mC and our failure to find or induce significant methylation in the male germ line, we doubt that DNA methylation in *Drosophila* exists or has substantial regulatory effects comparable to those in mammals.

7. References

AKHMANOVA, A. S., P. C. BINDELS, J. XU, K. MIEDEMA, H. KREMER *et al.* (1995) Structure and expression of histone H3.3 genes in Drosophila melanogaster and Drosophila hydei. Genome **38:** 586-600.

ALMEIDA, R., and R. C. ALLSHIRE (2005) RNA silencing and genome regulation. Trends Cell Biol **15:** 251-258.

ANTEQUERA, F., and A. BIRD (1999) CpG islands as genomic footprints of promoters that are associated with replication origins. Curr Biol **9:** R661-667.

BESTOR, T. H. (2000) The DNA methyltransferases of mammals. Human Molecular Genetics **9:** 2395-402.

BESTOR, T. H. and BOURC'HIS D. (2004) Transposon silencing and imprint establishment in mammalian germ cells. Cold Spring Harb Symp Quant Biol **69:** 381-387.

BIRD, A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev **16:** 6-21.

BRAND, A. H., and N. PERRIMON (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401-415.

BRIDGES, C. (1916) Non-disjunction as proof of the chromosome theory of heredity. Genetics **1:** 1-52 and 107-63.

CLARK, S. J., J. HARRISON, C. L. PAUL and M. FROMMER (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res **22:** 2990-2997.

DUFFY, J. B. (2002) GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis **34:** 1-15.

FENG, R., X. TANG, A. BECKER, A. BERGER, J. YE *et al.* (2005) Regulation of the expression of histone H3.3 by differential polyadenylation. Genome **48:** 503-510.

FUJITA, N., S. TAKEBAYASHI, K. OKUMURA, S. KUDO, T. CHIBA *et al.* (1999) Methylation-mediated transcriptional silencing in euchromatin by methyl-CpG binding protein MBD1 isoforms. Mol Cell Biol **19:** 6415-6426.

GARCIA, R. N., M. F. D'AVILA, L. J. ROBE, E. L. LORETO, Y. PANZERA *et al.* (2007) First evidence of methylation in the genome of Drosophila willistoni. Genetica.

GE, Y. Z., M. T. PU, H. GOWHER, H. P. WU, J. P. DING *et al.* (2004) Chromatin targeting of de novo DNA methyltransferases by the PWWP domain. J Biol Chem **279:** 25447-25454.

GOLL, M. G., F. KIRPEKAR, K. A. MAGGERT, J. A. YODER, C. L. HSIEH *et al.* (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science **311:** 395-398.

GOWHER, H., O. LEISMANN and A. JELTSCH (2000) DNA of Drosophila melanogaster contains 5-methylcytosine. Embo J **19:** 6918-6923.

GROND, C. (1984) Spermatogenesis of Drosophila hydei.

GRUNAU, C., S. J. CLARK and A. ROSENTHAL (2001) Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acids Res **29:** E65-65.

HACKER, U., S. NYSTEDT, M. P. BARMCHI, C. HORN and E. A. WIMMER (2003) piggyBac-based insertional mutagenesis in the presence of stably integrated P elements in Drosophila. Proc Natl Acad Sci U S A **100:** 7720-7725.

HENNIG, W. (1967) [Investigations of the structure and function of lampbrush-Y-chromosome in spermatogenesis in Drosophila]. Chromosoma **22:** 294-357.

HENNIG, W. (1968) Ribonucleic acid synthesis of the Y-chromosome of Drosophila hydei. J Mol Biol **38:** 227-239.

HENNIG, W. (1996) Spermatogenesis in Drosophila. Int J Dev Biol **40:** 167-176.

HENNIG, W., and KREMER, H. (1990) Spermatogenesis of Drosophila hydei. Int Rev Cytol **123:** 129-175.

HENNIG, W., G. F. MEYER, I. HENNIG and O. LEONCINI (1974) Structure and Function of the Y-Chromosome of Drosophila hydei. Cold Spring Harb Symp Quant Biol **38:** 673-683.

HORN, C., and E. A. WIMMER (2000) A versatile vector set for animal transgenesis. Dev Genes Evol **210:** 630-637.

HUIJSER, P., C. KIRCHHOFF, D. H. LANKENAU and W. HENNIG (1988) Retrotransposon-like sequences are expressed in Y chromosomal lampbrush loops of Drosophila hydei. J Mol Biol **203:** 689-697.

HUNG, M. S., N. KARTHIKEYAN, B. HUANG, H. C. KOO, J. KIGER *et al.* (1999) Drosophila proteins related to vertebrate DNA (5-cytosine) methyltransferases. Proc Natl Acad Sci U S A **96:** 11940-11945.

JACKSON, J. P., A. M. LINDROTH, X. CAO and S. E. JACOBSEN (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416:** 556-560.

JAENISCH, R., K. HARBERS, D. JAHNER, C. STEWART and H. STUHLMANN (1982) DNA methylation, retroviruses, and embryogenesis. J Cell Biochem **20:** 331-336.

KUNERT, N., J. MARHOLD, J. STANKE, D. STACH and F. LYKO (2003) A Dnmt2-like protein mediates DNA methylation in Drosophila. Development **130:** 5083-5090.

LANKENAU, D. H., and W. HENNIG (1990) Micropia-Dm2, the nucleotide sequence of a rearranged retrotransposon from Drosophila melanogaster. Nucleic Acids Res **18:** 4265-4266.

LANKENAU, D. H., P. HUIJSER, E. JANSEN, K. MIEDEMA and W. HENNIG (1988) Micropia: a retrotransposon of Drosophila combining structural features of DNA viruses, retroviruses and non-viral transposable elements. J Mol Biol **204:** 233-246.

LI, H., WEYRICH, A, FENG, R., TANG, X., CHEN, Y., HENNIG, W. (2007) RNAi-induced epigenetic effects of dE2F1-transcripts in Drosophila. in preparation.

LYKO, F., B. H. RAMSAHOYE and R. JAENISCH (2000a) DNA methylation in Drosophila melanogaster. Nature **408:** 538-540.

LYKO, F., B. H. RAMSAHOYE, H. KASHEVSKY, M. TUDOR, M. A. MASTRANGELO *et al.* (1999) Mammalian (cytosine-5) methyltransferases cause genomic DNA methylation and lethality in Drosophila. Nat Genet **23:** 363-366.

LYKO, F., A. J. WHITTAKER, T. L. ORR-WEAVER and R. JAENISCH (2000b) The putative Drosophila methyltransferase gene dDnmt2 is contained in a transposon-like element and is expressed specifically in ovaries. Mech Dev **95:** 215-217.

MAGGERT, K. A., and K. G. GOLIC (2002) The Y chromosome of Drosophila melanogaster exhibits chromosome-wide imprinting. Genetics **162:** 1245-1258.

MAISON, C., BAILLY, D., PETERS *et al.* (2002) Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. Nat. genet. **30**.

MICHIELS, F., A. GASCH, B. KALTSCHMIDT and R. RENKAWITZ-POHL (1989) A 14 bp promoter element directs the testis specificity of the Drosophila beta 2 tubulin gene. Embo J **8:** 1559-1565.

MUND, C., T. MUSCH, M. STRODICKE, B. ASSMANN, E. LI *et al.* (2004) Comparative analysis of DNA methylation patterns in transgenic Drosophila overexpressing mouse DNA methyltransferases. Biochem J **378:** 763-768.

OKANO, M., D. W. BELL, D. A. HABER and E. LI (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell **99:** 247-257.

OKANO, M., S. XIE and E. LI (1998a) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet **19:** 219-220.

OKANO, M., S. XIE and E. LI (1998b) Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res **26:** 2536-2540.

PAPATSENKO, D., G. SHENG and C. DESPLAN (1997) A new rhodopsin in R8 photoreceptors of Drosophila: evidence for coordinate expression with Rh3 in R7 cells. Development **124:** 1665-1673.

PHELPS, C. B., and A. H. BRAND (1998) Ectopic gene expression in Drosophila using GAL4 system. Methods **14:** 367-379.

PRADHAN, S., and P. O. ESTEVE (2003) Mammalian DNA (cytosine-5) methyltransferases and their expression. Clin Immunol **109:** 6-16.

RAMSAHOYE, B. H. (2002) Nearest Neighbour Analysis, pp. 9-15 in *Methods in Molecular Biology*, edited by K. I. A. R. MILLS, B.H. Humana Press Inc., Totowa, NJ, USA.

REUTER, G., and P. SPIERER (1992) Position effect variegation and chromatin proteins. Bioessays **14:** 605-612.

REYNAUD, C., C. BRUNO, P. BOULLANGER, J. GRANGE, S. BARBESTI *et al.* (1992) Monitoring of urinary excretion of modified nucleosides in cancer patients using a set of six monoclonal antibodies. Cancer Lett **61:** 255-262.

ROTH, M., and A. JELTSCH (2000) Biotin-avidin microplate assay for the quantitative analysis of enzymatic methylation of DNA by DNA methyltransferases. Biol Chem **381:** 269-272.

SALZBERG, A., O. FISHER, R. SIMAN-TOV and S. ANKRI (2004) Identification of methylated sequences in genomic DNA of adult Drosophila melanogaster. Biochem Biophys Res Commun **322:** 465-469.

SIMS, R. J., 3RD, K. NISHIOKA and D. REINBERG (2003) Histone lysine methylation: a signature for chromatin function. Trends Genet **19:** 629-639.

STACH, D., O. J. SCHMITZ, S. STILGENBAUER, A. BENNER, H. DOHNER *et al.* (2003) Capillary electrophoretic analysis of genomic DNA methylation levels. Nucleic Acids Res **31:** E2.

TAMARU, H., and E. U. SELKER (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature **414:** 277-283.

TWEEDIE, S., H. H. NG, A. L. BARLOW, B. M. TURNER, B. HENDRICH *et al.* (1999) Vestiges of a DNA methylation system in Drosophila melanogaster? Nat Genet **23:** 389-390.

URIELI-SHOVAL, S., Y. GRUENBAUM, J. SEDAT and A. RAZIN (1982) The absence of detectable methylated bases in Drosophila melanogaster DNA. FEBS Lett **146:** 148-152.

WARNECKE, P. M., C. STIRZAKER, J. SONG, C. GRUNAU, J. R. MELKI *et al.* (2002) Identification and resolution of artifacts in bisulfite sequencing. Methods **27:** 101-107.

WEISSMANN, F., I. MUYRERS-CHEN, T. MUSCH, D. STACH, M. WIESSLER *et al.* (2003) DNA hypermethylation in Drosophila melanogaster causes irregular chromosome condensation and dysregulation of epigenetic histone modifications. Mol Cell Biol **23:** 2577-2586.

WEYRICH, A. (2003) Expression menschlicher Methyltransferasen in der männlichen Keimbahn von *Drosophila melanogaster*. Diploma-thesis.

8. Appendix

8.1 *pUAS-Dnmt3* crossed with Gal4 strains

Two strains of *pUAS-Dnmt3a* and *pUAS-Dnmt3b* were crossed with flies of GAL4 strains listed in table 4 (see 4.2.3). The first strain of *pUAS-Dnmt3a/3b* was crossed with all of the GAL4 strains. The second strain was reduced to those checked by RT-PCR (Fig. 14). Each crossing was performed twice using one male and one female of the *pUAS-Dnmt* strain for mating with three GAL4 flies of the other sex. Flies were kept at 18°C. The development of the P and F1 generation were observed and documented in the following tables (Table 5 to 7).

None of the strains displayed unusual developmental behaviour or phenotypes. The developing time from the date of crossing (cross) until adult stage (some or many flies) was calculated in days.

P	1878	1854	6870	7029	3741	6984	6996
pUAS-Dnmt3a-1 ♀	Cross: 05-03-02 1st: 05-03-14 Empty: 05-03-18 → 16 days discard: 05-04-07	Cross: 05-03-04 3rd: 05-03-14 Empty: 05-03-14 Some flies: 05-04-01 → 28 days discard: 05-04-18	Cross: 05-03-01 2nd: 05-03-09 Pupa+flies: 05-03-14 Empty: 05-03-14 Many flies: 05-03-15 3rd: 05-03-25 Pupa: 05-04-01 → 14 days discard: 05-04-21	Cross: 05-03-01 Pupa: 05-03-14 Empty: 05-03-14 Many flies: 05-03-18 Empty: 05-04-01 → 17 days discard: 05-04-18	Cross: 05-03-02 2nd: 05-03-09 Pupa: 05-03-14 Empty: 05-03-14 Many flies: 05-03-15 Empty: 05-04-01 → 13 days discard: 05-04-18	Cross: 05-03-02 2nd: 05-03-09 Pupa: 05-03-14 Empty: 05-03-14 Many flies: 05-03-15 Add yeast: 05-03-15 Some flies+3rd: 05-04-01 → 13 days discard: 05-04-18	Cross: 05-02-28 2nd: 05-03-09 3rd: 05-03-14 Empty: 05-03-14 New flies: 05-03-18 → 17 days discard: 05-04-07
pUAS-Dnmt3a-1 ♂	Cross: 05-03-02 1st: 05-03-09 3rd: 05-03-14 Pupa: 05-03-18 Empty: 05-03-18 Many flies: 05-04-01 → 30 days discard: 05-04-07	Cross: 05-03-04 2nd: 05-03-14 3rd+pupa: 05-03-18 Empty: 05-03-18 Many flies: 05-04-01 → 28 days discard: 05-04-07	Cross: 05-03-07 2nd: 05-03-14 3rd: 05-03-18 New flies: 05-03-25 Empty: 05-04-01 → 25 days discard: 05-04-18	Cross: 05-03-07 2nd: 05-03-14 3rd+pupa: 05-03-18 Empty: 05-03-18 Empty: 05-04-01 → 25 days discard: 05-04-18	Cross: 05-02-28 2nd: 05-03-09 3rd+pupa: 05-03-14 Empty: 05-03-14 Many flies: 05-03-18 → 18 days discard: 05-04-07	Cross: 05-02-28 3rd: 05-03-09 Pupa+flies: 05-03-14 Empty: 05-03-14 Many flies: 05-03-15 → 17 days discard: 05-04-18	Cross: 05-03-02 1rst: 05-03-09 2nd: 05-03-14 3rd+pupa: 05-03-18 Empty: 05-04-01 → 30 days discard: 05-04-18
pUAS-Dnmt3b-1 ♀	Cross: 05-04-14 1st: 05-04-18 Pupa: 05-04-22 → P in new bottle 2 Many flies: 05-05-04 → 21 days discard: 05-05-09 2nd P bottle: 05-04-22 1st: 05-04-27 Pupa: 05-05-04 Empty: 05-05-04 Many flies: 05-05-09 → 18 days discard: 05-05-09	Cross: 05-03-04 3rd+pupa: 05-03-14 Many flies: 05-03-18 → 14 days discard: 05-04-13	Cross: 05-03-18 3rd: 05-04-01 Many flies: 05-04-07 Empty: 05-04-07 → 20 days discard: 05-04-18	Cross: 05-03-01 1st: 05-03-09 Pupa: 05-03-14 Empty: 05-03-14 → 13 days discard: 05-04-13 Cross: 05-03-07 3rd:+pupa: 05-03-14 Flies: 05-03-25 → 18 days discard: 05-04-13	Cross: 05-03-03 3rd+pupa: 05-03-14 Empty: 05-03-14 Many flies: 05-03-25 → 11 days discard: 05-04-18	Cross: 05-03-04 Pupa: 05-03-14 Empty: 05-03-14 Pupa+flies: 05-03-15 Some pupa: 05-04-01 → 11 days discard: 05-04-18	Cross: 05-03-04 Pupa: 05-03-14 Empty: 05-03-14 New flies: 05-04-01 → 10 days discard: 05-04-07
pUAS-Dnmt3b-1 ♂	Cross: 05-03-02 1st: 05-03-09 2nd+3rd: 05-03-14 Empty: 05-03-15 1fly: 05-03-18 Many flies: 05-04-01 → 16 days discard: 05-04-18	Cross: 05-03-02 2nd: 05-03-09 Pupa: 05-03-14 Many flies: 05-03-15 → 13 days discard: 05-04-13	Cross: 05-03-04 1st: 05-03-09 3rd+pupa: 05-03-14 Empty: 05-03-14 Flies: 05-04-01 → 28 days discard: 05-04-13	Cross: 05-03-02 2nd: 05-03-14 3rd+ flies: 05-03-18 Many flies: 05-04-01 → 16d days discard: 05-04-13	Cross: 05-03-03 1st: 05-03-09 2nd+3rd: 05-03-14 Empty: 05-03-14 Pupa+1fly: 05-03-18 Many flies: 05-03-25 → 15 days discard: 05-04-07	Cross:05-03-02 2nd: 05-03-09 Pupa: 05-03-14 Some flies: 05-03-14 → 12 days discard: 05-04-18	Cross: 05-03-03 2nd: 05-03-14 Many flies: 05-04-01 Empty: 05-04-01 → 28 days discard: 05-04-07

Table 5: Crossing data of parent (P) generation of *pUAS-Dnmt3a/3b* strain 1. Cross: day of crossing; 2nd bottle: parent flies are transferred into a second bottle; 1st: first instar larva; 2nd: second instar larva; 3rd: instar larva.

F1	1878	1854	6870	7029	3741	6984	6996
pUAS-Dnmt3a-1 ♀	Cross: 05-03-22 Normal phenotype 2nd+3rd: 05-04-01 Empty: 05-04-07 Lot of flies 05-04-13 → 16 days discard: 05-04-13	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 3rd: 05-04-01 Pupa: 05-04-07 Empty: 05-04-17 Many flies: 05-04-12 → 21 days discard: 05-04-13	Cross: 05-03-15 Normal phenotype Normal ♂/♀amount 1st-2nd: 05-03-22 3rd+pupa: 05-04-01 Empty: 05-04-01 Many flies: 05-04-07 → 23 days Discard: 05-04-07	Cross: 05-03-18 Normal phenotype ♂/♀= 4/12 2nd: 05-03-25 3rd+pupa: 05-04-01 Empty: 05-04-01 many pupa: 05-04-07 Many flies: 05-04-13 → 26 days discard: 05-04-13	Cross: 05-03-15 Normal phenotype ♂/♀= 10/21 2nd: 05-03-22 3rd: 05-03-25 Empty: 05-03-25 Many flies: 05-04-07 → 23 days discard: 05-04-07	Cross: 05-03-15 Normal phenotype Normal ♂/♀amount 2nd: 05-03-22 3rd: 05-03-25 Empty: 05-03-25 Many flies: 05-04-07 → 23 days discard: 05-04-07	Cross: 05-03-18 Normal phenotype Normal ♂/♀amount 2nd: 05-03-25 3rd: 05-04-01 Empty: 05-04-01 Many flies: 05-04-13 → 26 days discard: 05-04-07
pUAS-Dnmt3a-1 ♂	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 2nd: 05-04-01 Pupa: 05-04-07 Empty: 05-04-07 Many flies: 05-04-13 → 22 days discard: 05-04-13	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 2nd: 05-04-01 Pupa: 05-04-07 Empty: 05-04-07 Many flies: 05-04-13 → 22 days discard: 05-04-13	Cross: 05-03-25 Normal phenotype 3rd +pupa: 05-04-13 Empty: 05-04-07 Some flies: 05-04-18 → 24 days discard: 05-04-18	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 2nd + 3rd: 05-04-01 3rd+pupa: 05-04-07 Empty: 05-04-07 Many flies: 05-04-13 → 22 days discard: 05-04-18	Cross: 05-03-18 Normal phenotype ♂/♀= 8/18 1st+2nd: 05-03-25 2nd+3rd : 05-04-01 Empty: 05-04-07 Many flies: 05-04-13 → 26 days discard: 05-04-13	Cross: 05-03-15 Normal phenotype Normal ♂/♀amount 2nd: 05-03-22 3rd: 05-03-25 Empty: 05-03-25 Many flies: 05-04-01 → 17 days discard: 05-04-01	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 1st: 05-03-09 2nd+3rd: 05-04-01 Empty: 05-04-01 3rd+pupa: 05-04-07 Many flies: 05-04-13 → 22 days discard: 05-04-13
pUAS-Dnmt3b-1 ♀	Cross: 05-04-27 Show strange wings → Gal4-strain shows them as well 1st: 05-05-04 3rd+pupa: 05-05-09 discard: 05-05-09	Cross: 05-03-18 Normal phenotype Normal ♂/♀amount 1st+2nd: 05-03-25 Pupa:05-04-01 Empty: 05-04-01 Many flies: 05-04-07 → 20 days discard: 05-04-07	Cross: 05-04-01 Normal phenotype Normal ♂/♀amount Pupa: 05-04-13 Empty: 05-04-13 Many flies: 05-04-18 → 17 days discard: 05-04-18	Cross:05-03-18 Normal phenotype Normal ♂/♀amount 2nd:05-03-25 3rd: 05-04-01 Empty:05-04-01 many flies:05-04-13 → 26 days discard: 05-04-13	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 1st+2nd: 05-04-01 2nd+pupa: 05-04-07 Empty:05-04-07 Many flies: 05-04-13 → 22 days discard: 05-04-13	Cross: 05-04-07 Normal phenotype Normal ♂/♀amount 2nd: 05-04-13 3rd: 05-04-18 Empty: 05-04-18 Many flies:05-04-25 → 18 days discard: 05-04-25	Cross: 05-03-18 Normal phenotype Normal ♂/♀amount 2nd: 05-03-25 3rd+pupa: 05-04-01 Many flies: 05-04-01 → 11 days discard: 05-04-07
pUAS-Dnmt3b-1 ♂	Cross: 05-03-25 Normal phenotype Normal ♂/♀amount 1st: 05-04-01 3rd: 05-04-07 Empty: 05-04-07 Many flies: 05-04-18 → 24 days discard: 05-04-18	Cross: 05-03-18 Normal phenotype Normal ♂/♀amount 1st+2nd: 05-03-25 3rd+pupa: 05-04-01 Empty: 05-04-01 → 14 days discard: 05-04-07	Cross: 05-04-01 Normal phenotype Normal ♂/♀amount 1st: 05-04-07 3rd+pupa: 05-04-13 Empty: 05-04-13 Some flies: 05-04-18 → 17 days discard: 05-04-18	Cross: 05-03-25 Normal phenotype Normal ♂/♀amount 2nd+3rd: 05-04-07 Pupa: 05-04-13 Empty: 05-04-13 Many flies: 05-04-18 → 24 days discard: 05-04-18	Cross: 05-03-22 Normal phenotype Normal ♂/♀amount 2nd: 05-04-01 3rd+pupa: 05-04-07 Empty: 05-04-07 Many flies: 05-04-13 → 22 days discard: 05-04-13	Cross: 05-03-15 Normal phenotype Normal ♂/♀amount 1st: 05-03-22 3rd: 05-03-25 Empty: 05-03-25 Some flies: 05-04-01 → 17 days discard: 05-04-01	Cross: 05-03-25 Normal phenotype Normal ♂/♀amount 2nd: 05-04-01 3rd+pupa: 05-04-07 Empty: 05-04-07 Many flies: 05-04-13 → 19 days discard: 05-04-13

Table 6: Crossing data of first offspring (F1) generation of *pUAS-Dnmt3a/3b* strain 1. Cross: day of crossing; 2nd bottle: parent flies are transferred into a second bottle; 1st: first instar larva; 2nd: second instar larva; 3rd: instar larva.

P	6870	6996	2nd P	6870	6996	F1	6870	6996
pUAS-Dnmt3a-2 ♀	Cross: 05-04-04 Pupa: 05-04-13 → P in new bottle Many flies: 05-04-18 → 14 days discard: 05-04-28	Cross: 05-04-05 Pupa: 05-04-13 Many flies: 05-04-19 → 14 days discard: 05-04-20		Cross: 05-04-13 3rd: 05-04-18 Pupa: 05-04-19 Empty: 05-04-19 Many flies: 05-04-25 → 12 days discard: 05-05-09	**X**		Cross: 05-04-19 Normal phenotype Normal ♂/♀amount 2nd: 05-04-25 Pupa :05-04-28 → discard P Many flies: 05-05-09 → 20 days discard: 05-05-09	Cross: 05-04-19 Normal phenotype Normal ♂/♀amount 2nd: 05-04-25 3rd: 05-04-28 → discard P Many flies: 05-05-04 → some dark red eyes some dark → 15 days Discard: 05-05-09
pUAS-Dnmt3a-2 ♂	Cross: 05-04-04 1st: 05-04-13 pupa: 05-04-18 → P in new bottle many flies: 05-04-19 → 15 days discard: 05-04-27	Cross: 05-04-07 1st: 05-04-13 Pupa: 05-04-18 → P in new bottle Many flies :05-04-25 → 18 days discard: 05-04-28		**X**	Cross: 05-04-19 3rd: 05-04-25 Pupa: 05-04-28 Many flies: 05-05-04 → 15 days discard: 05-05-19		Cross: 05-04-19 Normal phenotype Normal ♂/♀amount 2nd: 05-04-25 pupa: 05-04-28 → discard P Many flies :05-05-09 → 20 days discard: 05-05-09	Cross: 05-04-22 Normal phenotype Normal ♂/♀amount 2nd: 05-04-28 2nd+3rd: 05-05-04 → discard P Many flies: 05-05-11 → 19 days discard: 05-05-09
pUAS-Dnmt3b-2 ♀	Cross: 05-04-04 1st: 05-04-13 pupa: 05-04-18 →P in new bottle many flies: 05-04-19 → all pupa close to the bottom→ possibly salivary gland effect → 15 days discard: 05-05-10	cross: 05-04-05 pupa: 05-04-13 → P in new bottle many flies: 05-04-18 → 15 days discard: 05-04-28		Cross :05-04-18 1st: 05-04-25 some pupa at the close to the food: 05-04-27 Some flies: 05-05-09 → 21 days discard: 05-05-17	Cross: 05-04-13 → ♀ died→ add new: 05-04-19 1st: 05-04-25 3rd: 05-04-27 Pupa: 05-04-28 Many flies: 05-05-04 → 21 days discard: 05-05-13		Cross: 05-04-19 Normal phenotype Normal ♂/♀amount 2nd: 05-04-25 3rd: 05-04-28 → discard P Many flies: 05-05-09 → 20 days discard: 05-05-09	Cross: 05-04-20 Normal phenotype Normal ♂/♀amount 2nd: 05-04-25 2nd: 05-04-28 → discard P Many flies: 05-05-09 → 19 days discard: 05-05-09
pUAS-Dnmt3b-2 ♂	Cross: 05-04-08 2nd: 05-04-13 → P in new bottle Many flies: 05-04-19 → 11 days discard: 05-04-28	Cross: 05-04-04 Many pupa: 05-04-13 → P in new bottle Many flies: 05-04-18 → 14 days discard: 05-04-28		Cross: 05-04-18 2nd: 05-04-25 3rd: 05-04-27 Many pupa: 05-04-28 Many flies: 05-05-04 → 16 days discard: 05-05-04	Cross: 05-04-13 2nd: 05-04-18 Pupa: 05-04-19 Many flies: 05-04-25 → 12 days discard: 05-05-04		Cross: 05-04-19 2nd: 05-04-18 3rd: 05-04-19 Pupa: 05-05-04 Many flies: 05-05-09 → 20 days discard: 05-05-09	Cross: 05-04-19 Normal phenotype Normal ♂/♀amount 2nd: 05-04-25 3rd: 05-04-28 Many flies: 05-05-09 → 20 days discard: 05-05-09

Table 7: Crossing data of P and F1 generation of *pUAS-Dnmt3a/3b* strain 2. Cross: day of crossing; 2nd P: parent flies are transferred into a second bottle; 1st: first instar larva; 2nd: second instar larva; 3rd: instar larva; X: the cross indicates.

II.

Histone modifications in the male germ line of *Drosophila*

1. Abstract

The histone modification patterns in testes of *D. melanogaster* and *D. hydei* were investigated. Substantial differences were found between the histone modification patterns of germ cells and somatic cells in the testis envelope. Both differ from the modification patterns typically expected for eu- and heterochromatin. Some modifications like H4-K16ac and H4-K5ac are not found in the meiotic prophase even though they are expected from their normal presence in active chromatin. Moreover, H4-K16ac is closely linked to dosage compensation, which may hence not take place during the meiotic prophase. Based on earlier studies of the histone H3 distribution in male germ cells, our observations permit the assignment of certain histone modifications to either the cell cycle-regulated histone H3.1 or to the variant H3.3. For some modifications the presence in cytoplasm of early germ line stages is indicated, while usually modifications were assumed to occur *in situ* in the chromatin.

2. Introduction

Epigenetic signaling and imprinting is generally assumed to be associated with the methylation of DNA. However, *Drosophila* has only low levels of methylated DNA if any. Its significance is far from beeing understood, especially as the canonical DNA methylases, except for *Dnmt2*, do not exist in *Drosophila*. *Dnmt2* is mainly considered as an RNA methylating enzyme (GOLL *et al.* 2006). Furthermore, *Dnmt3a* and *Dnmt3b*, known to have the main *de novo* DNA methylation function, are not identified in *Drosophila* (OKANO *et al.* 1999; OKANO *et al.* 1998a). Our investigation of *Drosophila* male germ line cells has given no evidence for DNA methylation (see part I; WEYRICH *et al.* in prep.). However, mechanisms for gene silencing and imprinting must exist in *Drosophila* just as in mammals. Furthermore, the *Y* chromosome is inactive in somatic tissues and gene silencing as regulatory mechanism has widely been demonstrated (for example: MAGGERT and GOLIC 2002). Observations of genetically transmitted imprinting phenomena are exceptional in *Drosophila*, but a few cases have been reported (LI *et al.* in prep.; REUTER and SPIERER 1992). The molecular mechanism of those is, however, unknown.

A wide range of experiments have documented that modifications of histones and other chromosomal proteins play an important role for gene regulation and, at least in some organisms, even for DNA methylation. Histone modifications occur preferentially but not exclusively at the accessible N-terminal tail of certain amino acid residues, mainly of histones 3 and 4. Methylation, acetylation and phosphorylation are the most common reactions connected with repressor and/or activator functions (Fig. 1).

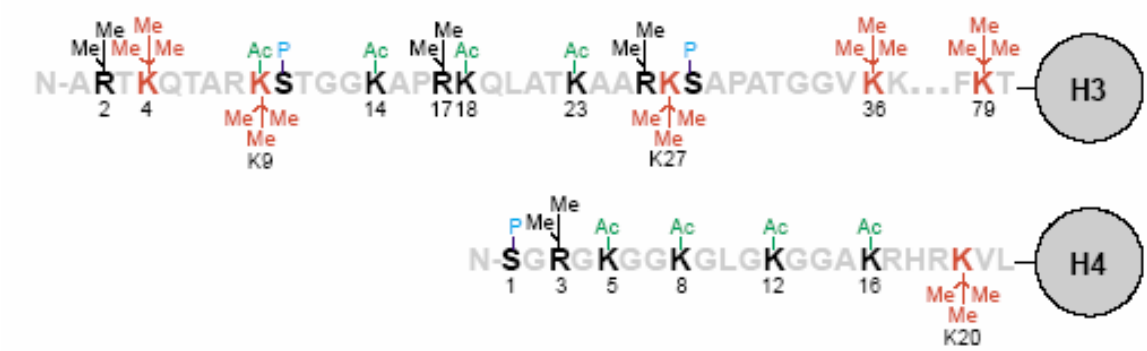


Fig. 1: Illustration of histone 3 and 4 modifications. Mono-, di- and tri- methylation, displayed in their highest possible modification level, are occurring in specific patterns. Me demonstrates methylation, Ac- acetylation and P- phosphorylation. Modifications written above the amino acid of the accessible N-terminal histone tail have an activation function, the ones underneath have a repression function in gene expression. Lysine (K), Arginine (R) and Serin (S) are the main modified residues (SIMS *et al.* 2003).

The absence of relevant amounts of DNA methylation, in addition to our observations on inherited imprinting effects mediated by RNAi (LI *et al.* in prep.), have induced us to investigate the histone modifications in the male germ line of *Dosophila*. Furthermore, the question arises whether changes in the modification patterns might be associated with the transmission of RNAi-induced phenotypes. While we could not detected major differences between *wild type* and the RNAi transformant strains, the histone modification patterns found deviate from those conventionally established in somatic cells. In particular, some histone modifications display an unexpected discrimination between autosomal and *X* chromosomal chromatin on one side, and *Y* chromosomal chromatin on the other. Also, the histone modification patterns change during the germ cell development – from spermatogonia to late spermatids – and some modifications are restricted to the first meiotic prophase or are specifically absent during the meiotic prophase. Our observations also support earlier data, that histones are maintained at least until very late elongation stages of the spermatids (AKHMANOVA *et al.* 1997). This is consistent with the absence of transition proteins and it even appears likely that histones persist in mature sperm.

3. Material & Methods

3.1 *Drosophila* strains

We used a *Drosophila melanogaster* CS strain and the *white*^{1118} strain from the collection of the Institute of Genetics, Johannes Gutenberg-University, Mainz/Germany. *Drosophila hydei* and the *wild type* strain belong to our own collection.

3.2 Preparation of slides and Immunocytology

Testes were dissected in testis buffer (0.047M NaCl; 0.183M KCl; 10mM Tris pH6.8) (HENNIG 1967), squashed and fixed in testis buffer with 3.7% or 4% formaldehyde (Merck) and subsequently frozen in liquid nitrogen. After removal of the cover slip, slides were immersed in methanol at -20°C for at least 5min, transferred into 1/1 (v/v) methanol/acetone at -20°C for 5min and finally into acetone at -20°C for 5min. They were washed twice in PBS with 0.1% Triton-X100 at room temperature and fixed in PBS with 0.37% para-formaldehyde or 4% formaldehyde for 20min at room temperature. After washing twice for 10min in PBS the slides were blocked for at least 30min in PBS with 1% bovine serum albumine (BSA, Sigma) and incubated with 7µl of the respective primary antibody diluted in PBS, at 4°C in a humid chamber overnight. The next day slides were washed 3 times in PBS including 1% Triton-X100. Incubation with the secondary antibody diluted in PBS took place for 1h at room temperature in a humid chamber. Finally slides were washed 3 times in PBS with 0.1% Triton-X100 at room temperature and embedded with antifading solution (1% p-phenylenediamine in 50% glycerol in PBS). Inspection of the slides was done with a Zeiss Imager Z1 with a Plan-Neofluar 40x/1.3 Oil, a Plan Apochromat 63x/1.4 Oil or a Plan-Apochromat 100x/1.4 Oil, or with a Nikon Eclipse E600 epifluorescence microscopes with Plan Fluor 40x/0.75 and Plan Apo 100x/1.40 Oil DIC H optics.

Primary antibodies against the different methylated histones H3 and H4 were affinity-purified rabbit antibodies for the most part kindly provided by Dr. T. Jenuwein, IMP

Vienna (diluted 1:1000 in PBS). Additional antibodies against modified histones were H3-K4me1, sourced from Abcom (Cat.No. ab 8895), H3-K4me2 from Abcom (Cat.No. ab 7766), H3-K36me2 from UBI (Cat.No. 07-274) and H3-K9ac from UBI (Cat.No. 06-942). As secondary antibodies, the Alexa 488 – labeled ″Goat-Anti-Mouse″ - F(ab')2 fragment of IgG (H+L) (Molecular Probes; Cat.No. #A11017) or the Dylight-547 or the Dylight-549 – conjugated Goat-Anti-Rabbit IgG (H+L) (Pierce; Cat.No. #31020) – were applied in a dilution 1:200 in PBS.

4. Results

We have studied the localization of modified histones H3 and H4 in the male germ line of *Drosophila melanogaster* (Table 1). Of the 18 antibodies directed against specific histone modifications, two (anti-H3-K4me1 and anti-H4-K8ac) did not show significant reactions, neither in germ line nor in somatic cells of testes. Specific reactions were found for antisera: anti-H3-K9me2 reacts preferentially in germ cells, but only slightly in heterochromatin of somatic cells of the testis. Anti-H3-K36me3 was only observed in meiotic prophase germ line cells, while anti-H4-K16ac and anti-H4-K5ac as well as H3-S10p do not react in meiotic prophase cells. The strongest reactions were found with anti-H3-K9me3, anti-H3-K27me2 and –me3, and with anti-H4-K12ac antibodies. Those four react in most of the cell types studied. An exception is the anti-H3-K9me3 serum, which was not reactive in spermatogonia and postmeiotic cells. Occasionally after extended methanol and acetone treatments some weak reactions in postmeiotic nuclei were oberved with anti-H3-K9me3, anti-H3-K9ac and anti-H4-K20me2. Several of the remaining antisera show no reaction in spermatogonia. In addition, the patterns obtained in the different cell types vary for several of the antisera as will be described subsequently. All results are summarized in the following table (Table 1).

Antiserum	***Germline***					***Soma (Testis)***			***Comments***
	Gonia	Spermatocytes	Meiosis	Spermatids		polytene	diploid		
H3-K4me2						early stages			
H3-K9me1	0								
H3-K9me2						0	HET		
H3-K9me3	0			??		HET	HET		
K3-K9ac		Cytopl./Diffuse		??					
H3-K27me1									
H3-K27me2						**EU**	**EU+HET**		
H3-K27me3	0								
H3-K36me2	0					0			
H3-K36me3	0					0	0		only in germ line
H4-K20me1									cytoplasm in gonia
H4-K20me2	0	Cytopl./Diffuse		??					
H4-K20me3	0						**EU+HET**		
H4-K5ac		0	0						no meiotic prophase
H4-K12ac							restricted EU+HET		
H4-K16ac		0	0						no meiotic prophase

Table 1: Summarizing all antibodies used for immunostaining experiments targeting histone modifications in *Drosophila melanogaster* and displaying their specific reaction signal in different cell types. Brown: positive antibody reaction. Orange: the antibody anti-H3K4me2 only showed reaction in early polytene cells. Yellow: no antibody reaction. Green: Antibody reacts only in the cytoplasm. Blue: antibody does not react in prophase of the cell cycle. 0: no signal detected; ??: signal weak and unclear; EU: euchromatin; HET: heterochromatin.

4.1 Germ cells

Remarkable differences in the labelling patterns for different antisera were observed in primary spermatocytes. They are summarized in Fig. 2. Differences were found not only in the amount of a specific histone modification, which may change with increasing age of the spermatocyte, but also in the intranuclear labelling patterns. This indicates a differential representation of certain histone modifications within distinct chromatin areas during development.

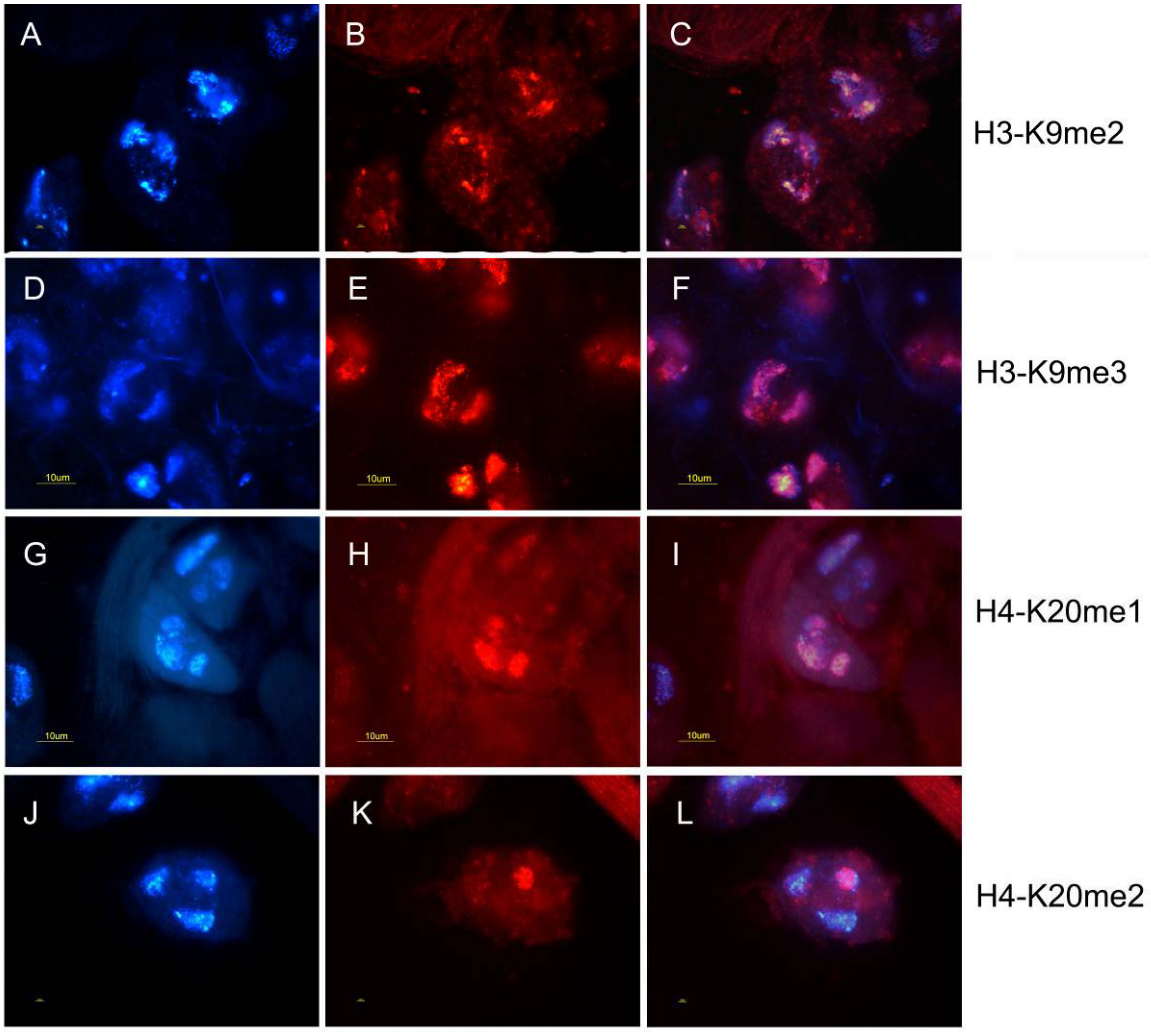


Fig. 2: Specific labelling of the different antibodies in *D. melanogaster* spermatocytes. **A-C:** H3-K9me2 show AB signal in condensed heterochromatin as well as in the looser euchromatin. **D-F:** H3-K9me3 as well as **G-I:** H4-K20me1 showed reaction in eu- and heterochromatin. **J-L:** H4-K20me2 shows staining in a diffuse pattern all over the nuclei. (A, D, G, J, M: DAPI staining; B, E, H, K, N: antibody staining; C, F, I, L, O: merged picture of DAPI and antibody.)

The most obvious differential distribution was observed between the H4-K20me2 and the H3-K9ac patterns and the patterns of most of the other modified histones. While the majority of the modifications are preferentially or exclusively associated with the autosomal and *X* chromosomal chromatin, histones H4-K20me2 (Fig. 2J-L) and H3-K9ac (Fig. 3D-E) are preferentially found in a diffuse pattern all over the nuclei. Earlier studies have shown that this chromatin is mainly - if not exclusively - assigned to the *Y* chromosomal lampbrush loops which are specifically formed during the primary spermatocyte stage (review: HENNIG 1996). In *D. melanogaster* this assignment by cytology is more difficult. Therefore we investigated the reaction of the anti-H4-K20me2 serum also in spermatocytes of *D. hydei*, where the location in the *Y* chromosomal chromatin can be more easily seen. The patterns obtained with H4-K20-me2 antiserum (Fig. 2J-L) are in striking contrast to those obtained with either H4-K20me1 (Fig. 2G-I) or H4-K20-me3. The former diplays a strong reaction with *Y* chromosomal chromatin, while the latter react in a fine granular pattern all over the nucleus. A similar diffuse localization pattern as with H4-K20me2 (Fig. 2J-L) was observed with anti-H3-K9ac (Fig. 3D-F); even though at a lower level. These histone modifications are mainly associated with the dispersed *Y* chromosomal chromatin. Furthermore, the antibody anti-H3-K9ac reacts only at a low level in the earliest spermatids and not at all in later postmeiotic nuclei.

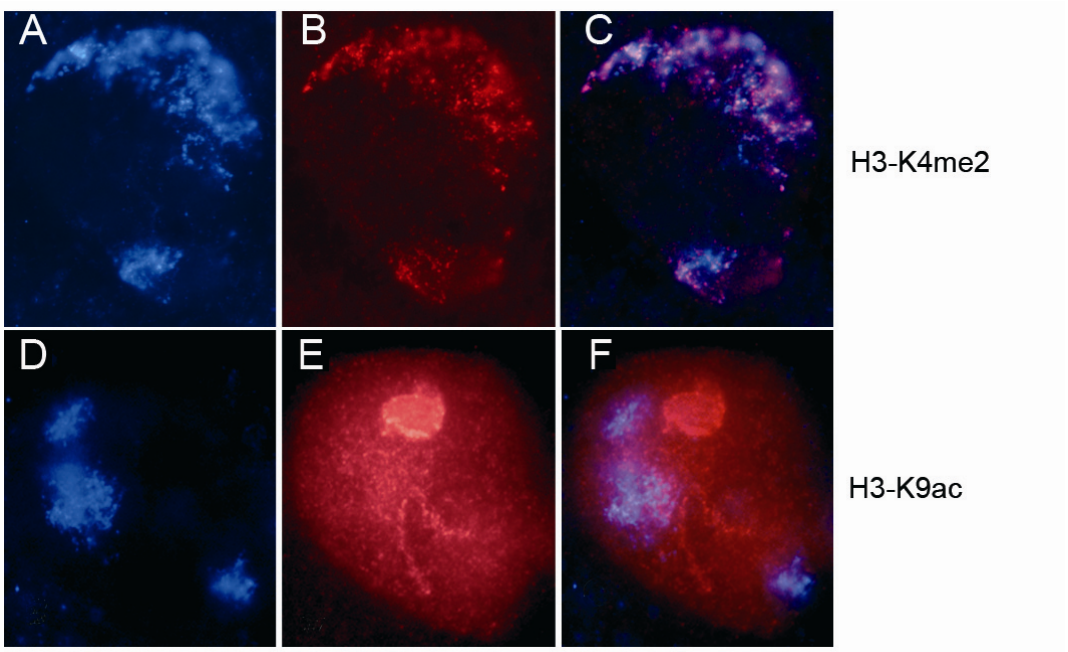


Fig. 3: Staining with antibody anti-H3-K9ac and H3-K4me2 in spermatocytes of *D. hydei*. **A-C:** H3-K4me2 displays signals in the cytoplasm and a diffuse pattern all over the nuclei. **D-F:** H3-K9ac shows staining of the *Y* chromosomal lampbrush loop structure. (A, D: DAPI staining; B, E: antibody staining; C, F: merged picture of DAPI and antibody.)

Another modification-specific localization was observed in spermatogonia with anti-H3-K9ac and anti-H3-K9me2 sera. Both types of modifications are found in the cytoplasm of spermatogonia, the H3-K9ac only in cytoplasm while H3-K9me2 is also found in condensed chromatin of spermatogonia.

Several other antisera also gave a slight cytoplasmic signal (H3-K27me1 and H3-K27me3, H4-K20me1 and H4-K20me3) in spermatogonia or young spermatocytes. The significance of these reactions may be questionable because it cannot be excluded that the low signals reflect an unspecific binding. A cytoplasmic location of modified histone H3-K9 has earlier been described (LOYOLA *et al.* 2006). We have observed that histone H3.3 antibodies react strongly in the cytoplasm of spermatogonia but not in the nuclei (AKHMANOVA *et al.* 1997). Moreover, in primary spermatocytes, histone H3.3-specific antibodies react in a pattern similar to that of the anti-H3-K9me2 and anti-H3-K9ac antisera (see Fig. 7 in AKHMANOVA *et al.* 1997), which reflect a *Y* chromosomal association. It is therefore likely that these histone modifications are primarily in the histone H3.3 variant protein, while the other histone modifications may preferentially be associated with histone H3.1.

4.2 Somatic cells

Most of the histone modifications are also observed in somatic cells, with some differences between testis tube cells and polytene cells from fat body (such cells are usually attached to testes tubes if testis from young males are isolated) (Table 1). In addition, the testis envelope has a few nuclei of low polyteny.

The patterns in somatic cells of the testis envelope differ only in some cases. Usually the dispersed chromatin is labeled while the chromocenter remains excluded (Fig. 4).

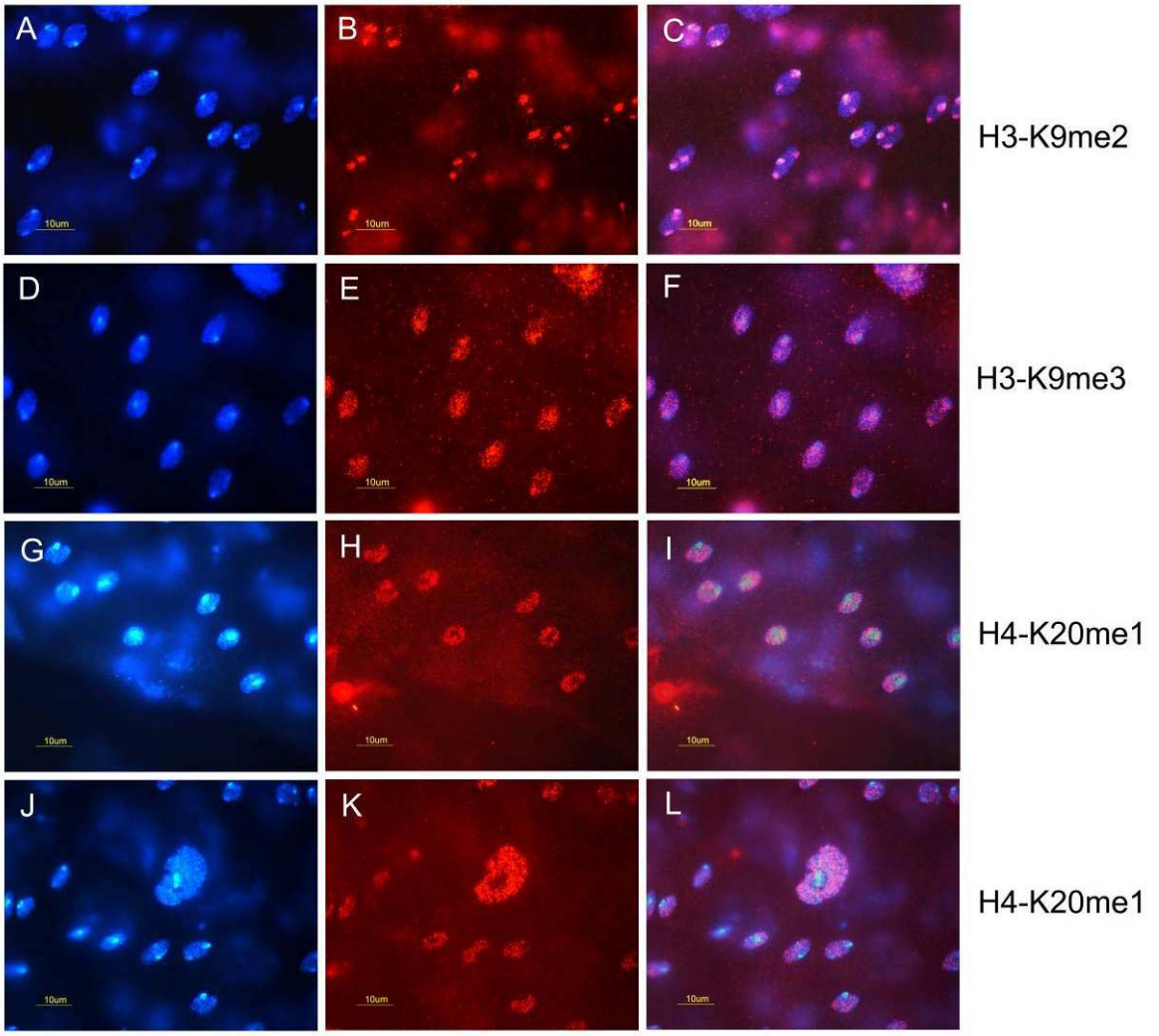


Fig. 4: Signals in the somatic cells of testis envelope in *D. melanogaster*. **A-C:** Heterochromatin is labeled by the anti-H3-K9me2 antibody in diploid and polytene somatic cells. **D-F:** H3-K9me3 shows reaction in eu- and heterochromatin of diploid somatic cells. **G-I:** H4-K20me1 shows signals in the euchromatin of the diploid cells of the testis envelope. **J-L:** Additional euchromatin label of H4-K20me1 in low polytene cells and somatic cells (A, D, G, J: DAPI staining; B, E, H, K: antibody staining; C, F, I, L: merged picture of DAPI and antibody.)

The entire chromatin including the chromocenter reacts only with anti-H3-K27me2 and anti-H4-K20me3. With anti-H3-K9me2 (Fig. 4A-C) and anti-H3-K9me3 (Fig. 4D-F) only the chromocenter reacts. Another exception is H4-K12ac. This modification was found only in somatic cells of a restricted region of the vas deferens. Here histones in eu- and heterochromatin are strongly acetylated, while in other regions of the testis the H4-K12 acetylation is low if present at all. The cells in the region of acetylated histones have secretory functions which explain their high transcriptional activity.

In polytene fat body cells a similar situation was observed. While most modifications are found in the polytene chromosomes except in the chromocenter, H3-K9me3 was only

found in the chromocenter. In contrast to somatic cells in the testis envelope, H3-K20me2 and H3-K27me2 do not react in the chromocenter. H3-K4me2 was only found in lower polyteny stages but disappears at higher polyteny.

5. Discussion

During the meiotic prophase, the chromatin of male germ cells in *Drosophila* displays remarkable differences to the chromatin in mitotic prophase cells. Typically, prophase chromatin successively condenses into cytologically visible chromosomes, which in their fully condensed state are seen during the metaphase. In most organisms this also holds true for the male meiotic prophase which passes through a characteristic sequence of chromosome stages connected with chromosome pairing and recombination. In *Drosophila* males, such meiotic prophase stages are missing as is recombination. Instead the chromatin passes through a stage of extreme decondensation until it rapidly condenses at prometaphase (KREMER *et al.* 1986).

Our investigation of histone modifications has shown that the chromatin during the meiotic prophase is subject to changes in the modifications of the chromatin. These modifications do, in part, not agree with the modifications expected from the conventional "histone code" (RICE and ALLIS 2001; TURNER 2005). This histone code distinguishes characteristic modifications of inactive heterochromatin – essentially methylation of H3-K9 and H3-K27 (NAKAYAMA *et al.* 2001; PETERS *et al.* 2002) – and of actively transcribed chromatin. The later is characterized by histone H3 modifications, in particular H3-K4me, H3K36me and H3-K9ac. It has however recently been shown that histone patterns are more complex and are far from beeing understood in their specific relevance for gene regulation (JENUWEIN 2006; LOYOLA *et al.* 2006). Earlier studies had already revealed that H3-K9-methylation, which is supposed to be related to inactive chromatin in pericentromeric heterochromatin, still permits transcription (LENHERTZ *et al.* 2003).

A remarkable observation is the absence of H4-K16 acetylation during the meiotic prophase. H4-K16 acetylation is important for the dosage compensation mechanism in *Dosophila* males. MOF, MSL and MSL1-3 proteins form an RNP complex with roX1 and roX2 RNA. MOF is a histone methylating enzyme and induces a high level of H4-K16 acetylation (DENG and MELLER 2006) in the dosage compensated *X* chromosome (MENDJAN and AKHTAR 2006). The absence of H4-K16 acetylation during the meiotic prophase may indicate that during male meiosis no dosage compensation takes place. This

conclusion is supported by preliminary studies with MOF and MLE antibodies (HENNIG, unpublished).

The modifications observed for histone H3 can be related to our earlier studies which gave evidence for a strong expression of the histone H3.3 variant in the male germ line (AKHMANOVA *et al.* 1995, 1997). Immunocytology demonstrated that histone H3.3 is not present in the nuclei of spermatogonia but is present in primary spermatocytes and postmeiotic chromatin (AKHMANOVA *et al.* 1997, Fig. 6 to 10). Histone H3.3 appears as a major chromatin component of these stages. In spermatocytes, histone H3.3 was particularly found in *Y* chromosomal chromatin. Moreover, histone H3.3-specific immunofluorescence was observed in the cytoplasm of spermatogonia (Fig. 6a in AKHMANOVA *et al.* 1997). These observations, which correlate certain patterns of the histone modifications, are related to modifications of the variant histone H3.3 rather than to modifications of the cell-cycle regulated histone H3.1. In particular the distribution patterns of H3-K9ac and H4-K20me2 can be correlated with the distribution of the histone H3.3 variant. H3-K9ac is found in the cytoplasm of spermatogonia and in the *Y* chromsomal chromatin (Fig. 3D-E). H4-K20me2 is found preferentially (if not exclusively) in *Y* chromsomal chromatin in spermatocytes. Considering the typical association of these modifications with transcribed chromatin, these observations are not unexpected as the *Y* chromosome is highly transcriptionally active (HENNIG 1968; HENNIG *et al.* 1974). In contrast, in our earlier studies, histone H3.1 signals in immunocytology could preferentially be assigned to autosomes and the *X* chromosome (AKHMANOVA *et al.* 1997, Fig. 6e, f). This location relates to immunofluorescent signals obtained with methylated H3-K27 and H4-K20 antibodies. Especially strong signals were obtained with H3-K27me2 and H4-K20me1, lower signals with H3-K27me3 and H3-K27me1. Some weaker reactions of the autosomes and the *X* chromosome were also found with antibodies against methylated H3-K9. The intensity of the signal is generally not very strong and decreases with the age of the spermatocyte. Such an age-dependent decreasing signal intensity was also found for H3-K27me1. This might be related to an increasing level of H3-K27me2 modification on account of the monomethylated state.

The low level of H3-K9-methylation is not unexpected as H3-K9-methylation is generally assumed to be associated with transcriptionally inactive chromatin, while H3-K4 methylation is associated with transcriptionally active genome regions (SIMS *et al.* 2003).

However, an exclusive location in inactive chromatin has recently been contradicted by studies of Vakoc et al. (VAKOC *et al.* 2005) and Loyola et al. (LOYOLA *et al.* 2006). Their conclusions are supported by our observations. The highly active state of the meiotic prophase chromatin in *Drosophila* as documented by autoradiography and hybridization data (HENNIG 1967; HENNIG *et al.* 1974) also contradicts the conventional idea that not only methylation of H4-K20, but also of H3-K27 correlate directly with large-range repression of gene activity. On the other hand, the typical mark for active chromatin, H4-K16ac, was not found in meiotic prophase cells. The absence of this mark in postmeiotic cells, however, which in *Drosophila* are transcriptionally inactive, was expected.

Loyola et al. (LOYOLA *et al.* 2006) have recently analyzed the posttranslational modifications (PMTs) in non-nucleosomal and nucleosomal histones. They found hyperacetylated H4 preferentially associated with H3.3. Such H4 molecules were di-acetylated in K8 and K16. We found acetylated H4-K16, but have not studied the H4-K8ac location. According to Loyola et al. (LOYOLA *et al.* 2006) histone H3.3-containing nucleosomes are also acetylated in K9. This agrees with our observations and the conclusion that histone H3.3 is mainly found in the *Y* chromosomal chromatin. Histone H4 associated with H3.1-containing nucleosomes was mainly acetylated at K12 and K16. H4-K16ac was not found in meiotic prophase cells while H4-K12ac reacts mainly with autosomes and the *X* chromosome. This suggests that - in agreement with our conclusions drawn from the location of methylated H3-K27 and H4-K20 histones - histone H3.1 is deposited here preferentially. Of interest is, that opposite to the pattern determined by Loyola et al. (LOYOLA *et al.* 2006) no H4-K16ac was found during the prophase, while it reacts in spermatogonia. Spermatogonia contain mainly histone H3.1 as discussed before.

In conclusion, our observations on histone H3 and H4 modifications in the male germ line contribute to the recent view that the “histone code” is more complex than initially assumed. Even within actively transcribed chromatin the modification patterns appear quite variable dependent on the specific location within the genome and the cell type studied. Germ cells, in addition, appear to have specific patterns different from those of somatic cells.

7. References

AKHMANOVA, A.S., BINDELS, P.C., XU, J., MIEDEMA, K., KREMER, H., HENNIG, W. (1995) Structure and expression of histone H3.3 genes in Drosophila melanogaster and Drosophila hydei. Genome **38:** 586-600.

AKHMANOVA, A., MIEDEMA, K., WAND, Y., VAN BRUGGEN, M., BERDEN, J.H., MOUDRIANAKIS, E.N., HENNG, W. (1997) The localization of histone H3.3 in germ line chromatin of Drosophila males as established with a histone H3.3-specific antiserum. Chromosoma **106:** 335-347.

DENG, X. and MELLER, V.H. (2006) roX RNAs are required for increased expression of X-linked genes in Drosophila melanogaster males. Genetics. **174:** 1859-66.

GOLL, M. G., F. KIRPEKAR, K. A. MAGGERT, J. A. YODER, C. L. HSIEH *et al.* (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science **311:** 395-398.

HENNIG, W. (1967) [Investigations of the structure and function of lampbrush-Y-chromosome in spermatogenesis in Drosophila]. Chromosoma **22:** 294-357.

HENNIG, W. (1968) Ribonucleic acid synthesis of the Y-chromosome of Drosophila hydei. J Mol Biol **38:** 227-239.

HENNIG, W. (1996) Spermatogenesis in Drosophila. Int J Dev Biol **40:** 167-176.

HENNIG, W., MEYER, G.F., HENNIG, I., LEONCINI, O. (1974) Structure and function of the Y chromosome of Drosophila hydei. Cold Spring Harb Symp Quant Biol **38:** 673-683.

JENUWEIN, T. (2006) The epigenetic magic of histone lysine methylation. Febs J **273:** 3121-3135.

KREMER, H., HENNIG, W., DIJKHOF, R. (1986) Chromatin organization in the male germ line of Drosophila. Chromosoma **94:** 147-161.

LENHERTZ, B., UEDA, Y., DERIJCK, A.A., BRAUNSCHWEIG, U., PEREZ-BURGOS, L., KUBICEK, S., CHEN, T., LI, E., JENUWEIN, T., PETERS, A.H. (2003) Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. Curr Biol **13:** 1192-1200.

LI, H., WEYRICH, A., FENG, R., TANG, X., CHEN, Y., HENNIG, W. (2007) RNAi-induced epigenetic effects of dEf1-transcripts in Drosophila. in preparation.

LOYOLA, A., BONALDI, T., ROCHE D, IMHOF, A., ALMOUZNI, G. (2006) PTMs on H3 Variants before Chromatin Assembly Potentiate Their Final Epigenetic State. Mol Cell **24:** 309-316.

MAGGERT, K. A., and K. G. GOLIC (2002) The Y chromosome of Drosophila melanogaster exhibits chromosome-wide imprinting. Genetics **162:** 1245-1258.

MENDJAN, S., AKHTAR, A. (2006) The right dose for every sex. Chromosoma.

NAKAYAMA, J., RICE, J.C., STRAHL, B.D., ALLIS, C.D., GREWAL, S.I. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science **292:** 110-113.

OKANO, M., XIE, S., LI, E. (1998a) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet **19:** 219-220.

OKANO, M., D. W. BELL, D. A. HABER and E. LI (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell **99:** 247-257.

PETERS, A.H., MERMOUD, J.E., O'CARROLL, D., PAGANI, M., SCHWEIZER, D., BROCKDORFF, N., JENUWEIN, T. (2002) Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. Nat Genet **30:** 77-80.

REUTER, G. and SPIERER, P. (1992) Position effect variegation and chromatin proteins. Bioessays **14:** 605-612.

RICE, J.C. and ALLIS, C.D. (2001) Code of silence. Nature **414:** 258-261.

SIMS, R. J., 3RD, K. NISHIOKA and D. REINBERG (2003) Histone lysine methylation: a signature for chromatin function. Trends Genet **19:** 629-639.

TURNER, B.M. (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol **12:** 110-112.

VAKOC, C.R., MANDAT, S.A., OLENCHOCK, B.A., BLOBEL, G.A. (2005) Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol Cell 19: 381-391.

III.

A transcript specific mutation of the *dE2F1* transcription factor in *Drosophila melanogaster*

1. Abstract

We recovered a new *dE2F1* mutant in *Drosophila melanogaster* which was generated by transposable element insertion inside one of the three *E2F* transcripts, *dE2F1-RB*. The mutation causes failure in embryo development of homozygous females, demonstrating a typical maternal effect. Even though homozygosity was proven by PCR experiments, the mutation was not fully penetrant. 2D-gel electrophoresis and immunoprecipitation with further mass spectrometric analysis could not detect any *dE2F* isoform. Real time PCR experiments were established to study the expression levels of the three transcripts (*dE2F1-RA, -RB, -RC*). Recent results display tissue specific expression which differs for control flies and mutant. The different transcripts are likely to contribute to the quantitative regulation of the *E2F1* expression in different cell types.

2. Introduction

2.1 The *E2F* transcription factor

The transcription factor *E2F* plays a key regulatory role in cell cycle progression, DNA-repair, proliferation and oncogenesis, tumor suppression and apoptosis, mainly achieved by its transcription activation of a quantity of down-regulated *E2F* target genes with positive and negative functions.

E2F functions in a heterodimer formed with the *DP* protein and is controlled by the retinoblastoma pathway (*pRb*). The retinoblastoma protein (*Rb)* binds to *E2F* and blocks its trans-activation domain. Cyclin and the *cyclin-dependent kinase* (*CDK*) phosphorylate the *Rb* protein in the *E2F/DP/Rb* complex. The hyperphosphorylated *pRb* releases and thus activates *E2F* (Fig. 1).

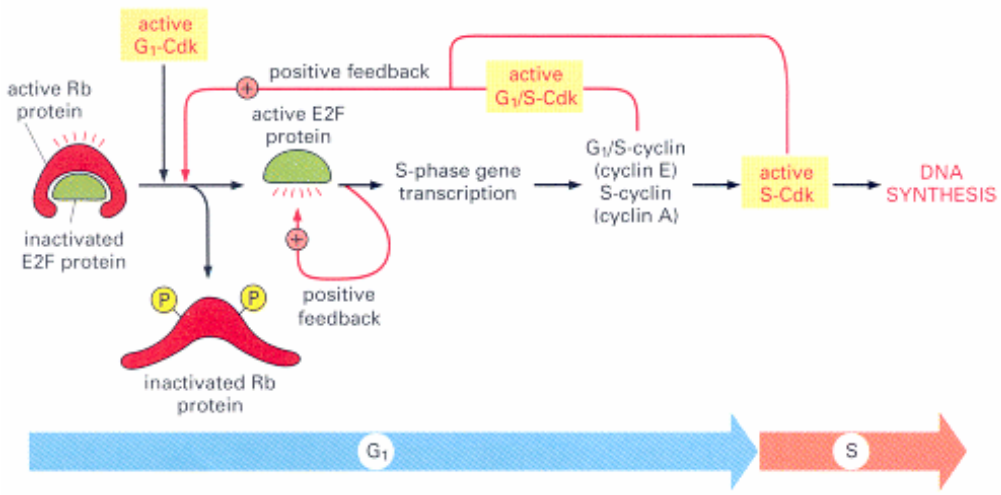


Fig. 1: During the G1 phase of the cell cycle *E2F* triggers the entry into the S-phase. The *Rb* protein in the *E2F* complex becomes phosphorylated by G1-Cdk activity (*cyclin D-Cdk4*) and *E2F* is released. *E2F* then initiates the transcription of S-phase genes, including the genes for a G1/S-specific cyclin (*cyclin E*) and S-cyclin (*cyclin A*). G1/S-Cdk and S-Cdk stimulate a positive feedback loop, which enhances *Rb* phosphorylation and thus stimulates the transcription of *E2F* target genes, forming an additional positive feedback loop (ALBERTS et al. 2002, Molecular Biology of the Cell 4th edition, Fig. 17-30).

The evolutionary highly conserved *E2F* is required in G1 (gap phase 1) to overcome the restriction point (R) to enter the S (synthesis) phase and allow chromosome duplication (OTHANI and NEVINS 1993; TRIMARCHI and LEES 2002; ATTWOOLL *et al.* 2004). When *E2F* is over-expressed, quiescent cells are induced to enter DNA synthesis (ATTWOOLL *et*

al. 2004). Moreover, the G1 phase is shortened when *Rb* protein is repressed (WU *et al.* 2001).

In *Drosophila* two genes were identified encoding *E2F* proteins (SAWADO *et al.* 1998). In mammals eight *E2F* genes, *E2F1* to *E2F8,* were discovered. The functions of the individual *E2F* are in part overlapping with other members of the *E2F* family and have not yet been determined in detail. Each individual *E2F* can function to activate or repress transcription, promote or impede cell cycle progression and enhance or inhibit cell death (DEGREGORI and JOHNSON 2006).

Potent activators of transcription are *E2F1 to 3* while *E2F4* to *8* act as repressors (ATTWOOLL *et al.* 2004; CHRISTENSEN *et al.* 2005). If the cells lack *E2F1-3* the initiation of the S phase is suppressed (WU *et al.* 2001). *E2F3* alone is necessary for S phase in the growing cell (KONG *et al.* 2007). When *E2F-4* and *E2F-5* are missing, the cell is unable to repress some cell cycle signals that prevent the induction of cell cycle exit and cell differentiation (LINDEMAN *et al.* 1998; ATTWOOLL *et al.* 2004). *E2F6-8* are considered as transcriptional repressors, but their precise roles are still unclear (MORKEL *et al.* 1997; TRIMARCHI and LEES 2002; DISTEFANO *et al.* 2003). A mutation of the retinoblastoma protein *Rb* results in high expression of *E2F*. This causes an uncontrolled cell proliferation, a misregulation leading to the majority of human cancers (CLASSON and HARLOW 2002), and demonstrating the high importance of *Rb* proteins in tumor suppression. Experiments with mutants of *E2F* and *DP* in flies show that the mutations affect the DNA binding affinity, reduce the replication rate, and cause lethality in late larval or pupal stages (DYNLACHT *et al.* 1994; ROYZMAN *et al.* 1997).

In the endo-cell cycle, omitting cell division (M phase), *E2F* has additional regulatory functions. This cycle takes place in polytene cells, like salivary glands and diploid imaginal discs in *Drosophila* (ROYZMAN *et al.* 1997). *Drosophila* uses the endo-cycle extensively during the development to enhance metabolism by the synthesis of large amounts of gene products. The mechanisms of the cell cycle and the endo-cell cycle are not fully understood and need further research (ROYZMAN and ORR-WEAVER 1998).

Due to the complexity of the *E2F* family and their functions the question arises if the low number of *E2F* genes in *Drosophila* is substituting the large number of *E2F* genes in mammals. This could be achieved with products of alternative splicing which can greatly enhance the complexity of gene expression. The recovery of a maternal effect mutant in

dE2F1 which eliminates one of the multiple transcripts of the *dE2F1* gene (FENG 2005), suggests that alternative splicing or alternative translation initiation sites might create different *E2F1* proteins.

2.2 The *dE2F1^{TF}* mutation

The *dE2F1* gene (CG6376; http://flybase.bio.indiana.edu/reports/FBgn0011766.html) encodes three transcripts: *dE2F1-RA*, *-RB* and *-RC*. All three transcripts contain the same ORF sequence encoding for one known protein of 805aa. In contrast, the 5'UTR differs for each transcript. This could be caused by different transcription initiation sites and/or alternative splicing.

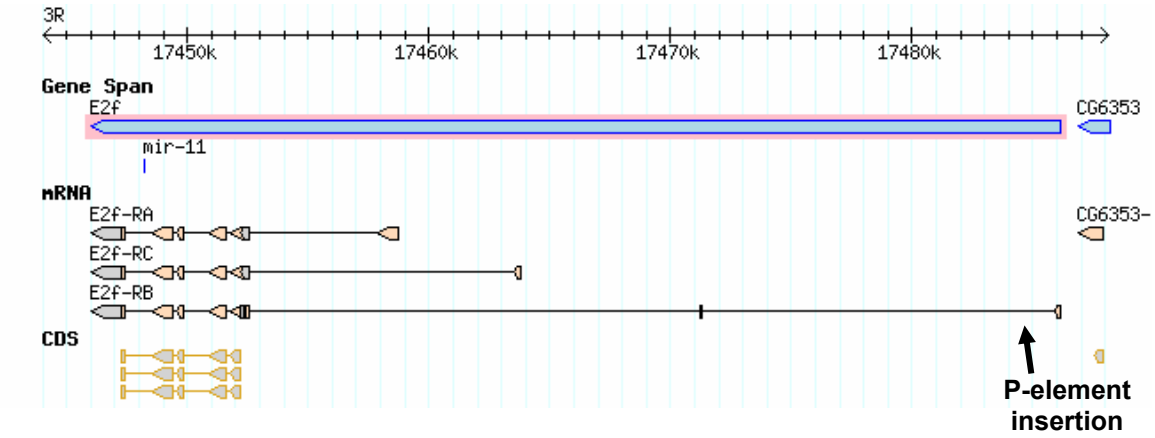


Fig. 2: The *dE2F1* gene and its three transcripts *E2F-RA*, *-RB* and *-RC*. The arrow indicates the P-element insertion site into the transcript *E2F-RB* (http://flybase.bio.indiana.edu/reports/FBgn0011766.html).

In our laboratory a mutation, *dE2F1^{TF}*, was generated by P-element transformation experiments (FENG 2005). The transformation construct inserted into the *dE2F1* gene of *Drosophila melanogaster* (position inside the *dE2F1* gene: 9036bp) (for details see appendix 8.1). It caused an aberration in the 5'UTR, by adding itself into the first intron of *dE2F1-RB* transcript (indicated by the arrow in Fig. 2). RT-PCR proves the absence of this transcript. As a result homozygous female flies do not produce viable embryos. Early embryos turned brown and occasionally formed melanotic pseudotumors (FENG 2005). Homozygous mutant males are not only viable as females but they are also fertile. The mutation is therefore a typical maternal effect mutation as it affects early embryogenesis.

The investigation of the *dE2F1*^{TF} mutant revealed that the three transcripts *dE2F1-RA*, *-RB* and *–RC* are differentially expressed in different tissues and during different developmental stages (FENG 2005).

To verify the lethality of the offspring of homozygous females, a series of single crosses of homozygous *dE2F1* mutant females with *white* males were carried out and checked for offspring. In addition, the homozygosity of the mutation in the mothers was checked by PCR. It emerges that a low proportion of embryos can develop into adults.

The existence of three transcripts posed the question whether more than one protein is encoded. Therefore we raised antibodies, established 2D-gel electrophoresis and immunoprecipitation methods, and searched for additional *E2F* protein components by Mass Spectrometry (kindly carried out by PD Dr. André Schrattenholz, CSO and his co-worker Dr. Gerhard Schwall (ProteoSys, Mainz) and Prof. Dr. Dieter Oesterhelt and his co-worker Dr. Frank Siedler of the (Max Planck Institute of Biochemistry in Martinsried)).
In order to study the different expression levels and to achieve a better insight into the possible functions of the three transcripts we developed and performed real time PCR, which offers a more accurate method than the semi-quantitative PCR experiments (FENG 2005).

3. Material & Methods

3.1 Crossings of balanced *dE2F1* mutant flies

The balancer system is a unique system to maintain homozygous lethal, semi-lethal or homozygous sterile mutations in *Drosophila*, which would easily get lost in an unbalanced situation within a few generations. By including a balancer chromosome homologous to the chromosome carrying the mutation, this mutation can be stabilized in a heterozygous condition. Balancer chromosomes carry a dominant marker for selection, a complex inversion to eliminate recombinants, and a lethality factor that only allows Balancer-heterozygous flies to survive.

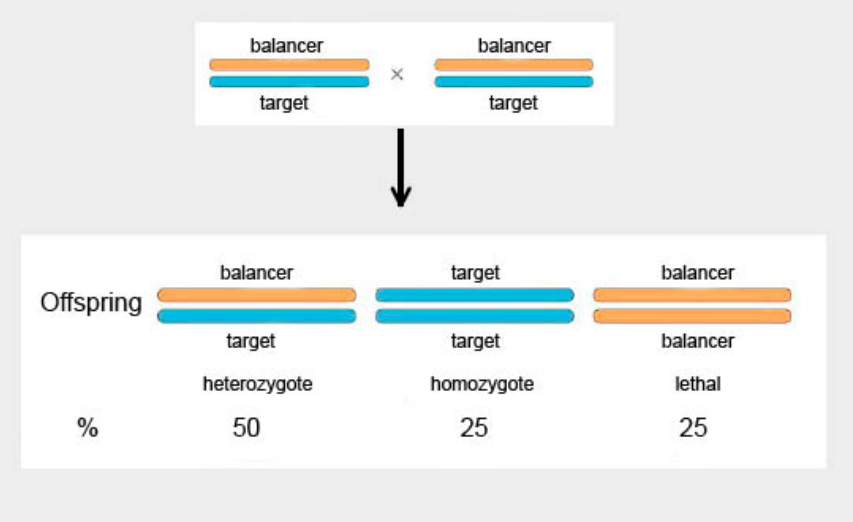


Fig. 3: The balancer system.

The *dE2F1*^{TF} mutant includes as a marker gene the *ECFP* (*Enhanced Cyan Fluorescent Protein*) which is the cyan mutant of the green fluorescent protein *EGFP* (*Enhanced Green Fluorescent Protein*) derived from the jellyfish *Aequorea victoria.* The ECFP marker appears as cyano (blue) colour fluorescence (instead of green as *EGFP* does) when exposed to UV light.

In our constructs the *ECFG* maker gene is under the control of an eye specific promoter 3*P3 (PAPATSENKO *et al.* 1997). Therefore flies showing cyano fluorescent eyes if exposed to UV light include the insertion and thus the mutation.

The balancer is *TM(3)Ser* and is, as the P-element insertion, located on chromosome *3*. The *Serrate* (*Ser*) gene serves as the dominant marker gene for the balancer chromosome and shows frayed wings. Hence, flies showing both markers, *Ser* and cyan fluorescence, are heterozygote ($dE2F1^{TF}/TM3_{Ser}$), those showing only *ECFP* are homozygote for the mutation ($dE2F1^{TF}/ dE2F1^{TF}$).

For all crosses virgin female flies were used. To assure virginity bottles were emptied completely in the evening and checked early the following morning. Females usually eclose earlier than males, and were kept separately until used for crossings. Crosses were kept at 24°C.

3.2 PCR for *dE2F1* homozygosity check

3.2.1 DNA-isolation from single flies

The homozygous flies obtained by the homozygous crossings were stored in 1.5ml punctured Eppendorf tubes, shock frozen in liquid nitrogen and stored at -80°C.

For DNA isolation flies were homogenized individually in 100µl Homogenization Buffer (10mM Tris-HCL pH7.5; 60mM NaCl; 10mM EDTA pH8.0; 0.3mM spermidine; 5% sucrose) in a 1.5ml Eppendorf tube. For cells` lysis 100µl lysis buffer (300mM Tris-HCl pH9.0; 100mM EDTA pH8.0; 0.625% SDS, 5% sucrose) was added and incubated at 65°C for 30min. After cooling to room temperature, 30µl 8M KOAc was added and placed on ice for 30min. Centrifugation was performed 30sec at 13,900rpm and supernatant was carefully pipetted into a new Eppendorf tube, avoiding contamination with the lipid surface or pellet. DNA was pelleted by adding 2vol. absolute ethanol, incubation for 5min at room temperature and centrifugation 15min at 13,900rpm. Solution was removed and the pellet was washed in 70% ethanol. After centrifugation for 5min at 13,900rpm, the pellet was air dried and then dissolved in 10µl ddH_2O.

3.2.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) offers a method to amplify certain defined DNA sequences by the use of specific primer pairs encircling the target region. The Taq-DNA polymerase synthesizes the desired DNA region starting from one of the annealed primers (see appendix 8.1.3 for *dE2F1* sequence with primer positions). We confirmed the

homozygosity of each single female in the test crosses by a PCR reaction. Heterozygous females yield two PCR fragments, different in length.

Name	Direct.	Sequence	Position [bp]	Length [bp]	Tm [˚C]	Frag. size
Inside *E2F* gene						
FRR41	sense	CAACCAATTGACAGGTGACG ATCT	67-90	24	56.8	1354bp
AW18	anti sense	CGCTTCGACGAGCTTTGCTG	1420-1401	20	58.5	
Overlapping the insertion pBac 3432-3456						
FRR41	sense	CAACCAATTGACAGGTGACG ATCT	67-90	24	56.8	1176bp
FRR37	anti sense	CAGTGACACTTACCGCATTGA CAAG	3432-3456 (in pBac)	25	57.6	

Table 1: PCR primer for homozygous check. The T_m was calculated by the NTI.

With each extracted 10µl DNA (see 3.2.1) two PCR reactions were performed using the two respective primer pairs (Table 1).

PCR

DNA (from *dE2F1* homozygous fly)	5 µl
Primer 1 (FRR41) [10µM]	1 µl
Primer 2 (AW18 or FRR37) [10µM]	1 µl
10x PCR reaction buffer	2 µl
$MgCl^{2+}$ [25mM]	1.2 µl
dNTP`s [10mM/each]	0.4 µl
Taq-DNA polymerase	1 µl
ddH_2O	8.4 µl
Final volume	20 µl

The Taq-DNA polymerase was prepared in our laboratory.

Step	Temp.	Time	Function
1	94 °C	2 min	Denaturation
3	94 °C	30 sec	Denaturation
4	55°C	45 sec	Annealing
5	72 °C	90 sec	Elongation
6	go to step 2 → 30 cycles		
7	72 °C	5min	Long elongation
8	10 °C	for ever	Cooling

PCR products were then separated on a 1.5% agarose gel at 60V for 1.5h. As marker standard we utilized the GeneRuler™ 100bp DNA Ladder Plus (MBI Fermentas; #SM0241) displayed in Fig. 6C.

3.3 2-Dimensional Polyacrylamid Gel Electrophoresis (2D-PAGE)

The two-dimensional gel electrophoresis was developed by O´Farrell and Klose in 1975 independently. In the first dimension the protein mixtures are separated by isoelectric focussing (IEF) using carrier ampholytes. In the second dimension the proteins are separated according to their molecular weight like in one-dimensional SDS-PAGE. To avoid protein degradation a cool surrounding is required. For MS all solutions including Urea, DDT or protease inhibitor have to be freshly prepared.

3.3.1 Protein sample preparation of *Drosophila* ovaries

For sample preparation (HUNZINGER *et al.* 2006) the ovaries were homogenized (50 *white* ovary pairs and 25 pairs of $dE2F1^{TF}$ mutant ovaries (each for two IPG strips)) in 200µl solubilization buffer I (SB I) (0.3% SDS; 200mM DDT (Biosynth); 28mM Tris-HCl (Sangon); 22mM Tris-Base (Promega)) + 2µl Protease Inhibitor Mix [10 µl/ml] (EDTA free Protease Inhibitor Mix; Amersham; Cat.No. 80650123). 25µl of SB II (24mM Tris-Base; 476mM Tris-HCl; 50mM $MgCl_2$; 4-6U/µl DNaseI (Promega); 250µg/ml RNaseA) was added, the mixture was incubated on ice for 10min and centrifuged (Eppendorf

centrifuge) at 4°C for 10min at 12,000rpm. For acetone-precipitation the supernatant was mixed with 800µl ice-cold acetone and incubated on ice for 30min. After centrifugation for 10min, at 14,000rpm and 4°C the pellet was air dried for 5min. Then the pellet was resuspended in 300µl of SB III (9M Urea (Roth); 4% CHAPS (Amersham); 1% Triton X-100; 20mM Tris-Base; 0.8% IPG buffer including the Pharmalyte for pH3-11 NL (Amersham); 12µl/ml DeStreak (Amersham); EDTA free Protease Inhibitor Mix) and 6µl of filtered bromphenol-blue solution (0.5% bromphenol-blue in Resolving Buffer) was added. The pharmalyte in the IPG buffer carries the amino and carbon groups.

3.3.2 Passive rehydration and IEF 1st dimension

For passive rehydration the Immobiline DryStrips 3-11NL (non-linear) (Amersham Pharmacia Biotech; Cat.No. 17-6003-77) placed in the IPGphor strip holders (Hofer; Amersham Biosciences) were incubated for 1h with the 300µl protein solution in SB III solution. For rehydration o/n, strips were then covered with IPG-overlay-oil (Amersham Pharmacia Biotech) and a lid to avoid drying.
Running of IEF first-dimension took place the next day in the IPG-Dalt (Hofer; Amersham Biosciences).

Step	Voltage	Time
S1	150 V	30 min
S2	300 V	60 min
S3	600 V	60 min
S4	1,500 V	60 min
S5	3,500 V	13 h 40 min
S6	3,500 V	2 h
S7	1000 V	10 h

Table 2: Running conditions for IEF (isoelectric focusing) 1st dimension.

3.3.3 Casting and run of the 2nd-Dimension

The gels for the 2nd dimension measure 200x250mm and 1.0mm thick. For casting the 12% gels for the 2nd dimension ten gels were prepared in the Casting Chamber (Hofer; Amersham Biosciences). Therefore filtered acrylamid solution (30% Acrylamid (Promega); 4% Bisacrylamid (Fluka)) with resolving gel buffer pH8.8 (Laemmli, 1970:

0.4% SDS; 1.5M Tris-Base) was deaerated for 1h to avoid inhibition of the polymerization and radical oxygenic reactions. Such a reaction could affect the proteins and influence mass spectrometry data. To start the polymerization reaction 0.3% APS (Merck) and 0.05% TEMED (Roth) was added shortly before casting. About 200ml Displacing Solution (1.5M Tris-HCl pH8.8; 50% Glycerol; 0.01% bromphenol-blue solution) were filled in the casting chamber to push the gel solution up the glass plates. Overlay buffer (2:3 (v/v) resolving gel buffer:n-Butanol) was added on top of each gel to sharpen the gel surface and prevent drying. Polymerization took place o/n.

Each IPG strip was equilibrated in 30ml Equilibration Buffer (12.5M Tris-HCl pH6.8; 6M Urea (Roth); 30% Glycerol; 2% SDS) two times for 15min. In the first incubation 1% dithiothreitol (DDT) was added into the buffer to fill open cysteins, and 4.5% iodoacetamide and 10µl bromphenol-blue solution in the second equilibration incubation.

Then the 1st dimension strips were loaded onto the gels for the 2nd dimension and fixed with low melting agarose (Roche). An extra pocket was made for 15µl protein marker (MBI Fermentas; Protein ladder 10-200kDa; Cat.No. #SM0661) illustrated in Fig. 4.

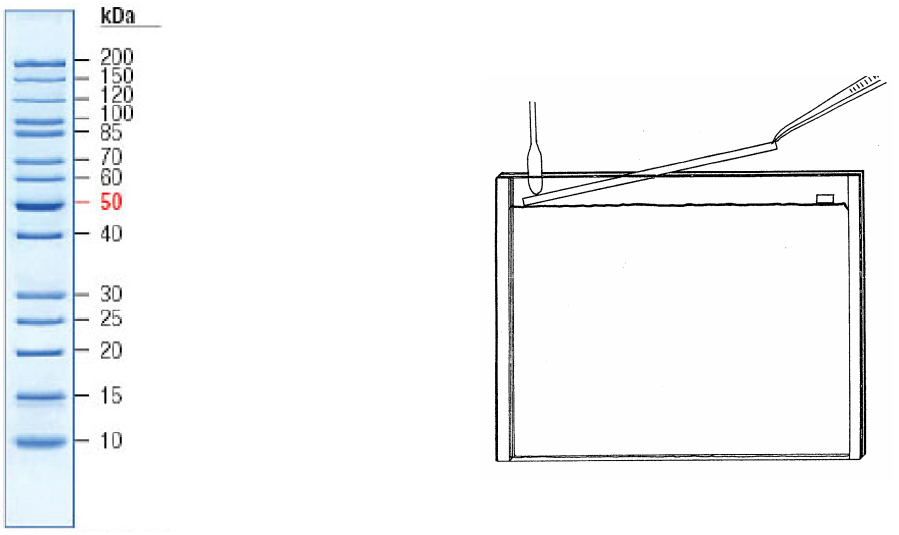


Fig. 4: Left: PageRuler™ Unstained Protein Ladder. Right: Loading of IPG-strip onto the second dimension polyagrylamid-gel (GÖRG *et al.* 1988, http://www.weihenstephan.de/blm/deg).

The 2D-gels were run in the DALT tank (Hofer; Amersham Biosciences) in electrode running buffer (Laemmli, 1970: 250mM Tris-base; 2M Glycine; 1% SDS) pre-tempered to 20°C.

	Time	**Voltage**	**Current**	**Power**	**Temp.**
Setting	25 h	65 V	400 mA	40 W	20 °C
Final data	17h15min	65 V	196 mA	13 W	20 °C

Table 3: Running conditions for 2nd dimension, including the final data of our run.

3.3.4 Blotting and Silver Staining

After the run, gel regions of interest were semi-blotted for 15min in the Bio-Rad sandwich blot machine to the Hybond nitrocellulose membrane. Normal western blot procedure (see material and methods I, 3.3) was performed using as 1st antibody the polyclonal antibody from chicken against the C´terminal end of *dE2F1* (*C´dE2F1*), synthesized in our laboratory (1:2800). The antibody was affinity-purified and checked by western blot. As 2nd antibody we applied the peroxidase-conjugated affinity purified rabbit anti-chicken (Jackson ImmunoResearch Laboratories, Inc.; Cat. No. 303036003).

After blotting, the remaining proteins were fixed and the gel stained with the silver staining protocol according to Shevchenko and colleagues (SHEVCHENKO *et al.* 1996), which allows subsequent MS.

Step	**Reagent**	**Duration time**
Fixing	50% ethanol / 5% acetic acid	20min – 1h
Wash	50% ethanol	10min
Wash	ddH_2O	> 2h (overnight)
Sensitizing	0.02% $Na_2S_2O_3$ x 5 H_2O	1min
Wash	ddH_2O	2 x 1min
Silver	0.1% Silver Nitrate	20min at 4°C
Change tray!		
Wash	ddH_2O	1 min
Development	Developer	3 x 5min
Watch the colour and change solution when the developer turns yellow!		
Stop 5% acetic acid		3 x 5min
Storage 1% acetic acid (4°C)		up to several weeks

Table 4: Silver Staining procedure by Shevchenko *et al.* (1996) compatible for mass spectrometry.

3.4 Immunoprecipitation of *dE2F1*

In the immunoprecipitation (IP) technique a specific antibody (here: anti-*dE2F1*) is attached to a sedimentable matrix (Sepharose beads) to which an antigen binds and thus can be isolated together with its complex forming partners (proteins, RNAs, DNAs). Antigen for Immunoprecipitation was derived from ovaries of *Drosophila melanogaster white* flies (BONIFACINO *et al.* 1999; Current Protocols in Molecular Biology; 10.16 Immunoprecipitation).

3.4.1 Dialysis of the antibody

All solutions used for mass spectrometry were sterile filtrated before usage to avoid contamination with external particles. The in rabbit newly synthesized and by Sulfo Link Kit (Pierce; Cat.No. 44895) purified antibody against the entire whole protein of *dE2F1* was dialyzed at 4°C o/n to remove all small molecules containing free amino or sulfhydryl groups. The dialysis buffer (0.1M $NaHCO_3$; 0.5M NaCl) was changed three times. Aggregates were removed by centrifugation for 1h at 100,000rcf at 4˚C. Then the antibody concentration was measured at 280nm against BSA standards to adjust the coupling concentration to 1-30 mg/ml.

3.4.2 CNBr-Sepharose activation

The CNBr-Sepharose (Amersham; CNBr-activated Sepharose 4 Fast Flow; Cat.No. 17098101) (0.02g for 100µl final volume) was activated by adding 1mM HCl and swelling for 30min. The HCl was discarded after centrifugation for 1min at 16,000rcf. After washing in 750µl coupling buffer (0.1M $NaHCO_3$; 0.5M NaCl) and centrifugation at 16,000rcf for 1min, the sepharose beads were ready to become covalently and irreversibly linked to the antibody with the help of cyanogen bromide.

3.4.3 Antibody coupling

For coupling the antibody, 1:1 (v/v) CNBr-Sepharose and antibody were stirred at 4°C o/n. The percentage of coupling was measured at 280nm by comparing to the value with the one before coupling. To saturate the remaining reactive groups, incubation for 2-4h at 4°C

with 0.05M glycine pH8.0 was performed. After centrifugation the sepharose was stored in TSA solution (10mM Tris/HCl pH8.0; 140mM NaCl; 0.025% NaN_3).

3.4.4 Sample preparation

For sample preparation 100 ovary pairs were dissected from three to five days old females in Ringer buffer (182mM KCl; 46mM NaCl; 3mM $CaCl_2$; 10mM Tris-HCl pH7.2) + Complete Mini Protease Inhibitor Cocktail Tablets (Roche; Cat.No. 11836153001) on ice. Ovaries were homogenized mechanically and incubated for 3h on ice in 100µl IP buffer (100mM NaCl; 20mM Tris-HCl pH8.0; 2mM EDTA (Roth), 0.5% Triton X-100, Protease Inhibitor Complete Mini). After centrifugation at 13,900rpm for 1min at 4˚C, the supernatant was diluted 1:5 (v/v) in IP buffer.

3.4.5 Antigen binding

Then the sepharose in TSA solution was washed and equilibrated in IP buffer + Complete Mini Protease Inhibitor and incubated with the sample while shaking 3h at 4˚C. Antigen within its complex was linking to the beads.

3.4.6 Elution of the protein complexes

Before eluting the complexes from the beads, beads were washed four times for 5min in ice-cold wash buffer (300mM NaCl; 20mM Tris-HCl pH8.0; 2mM EDTA; 0.5% Triton X-100; Complete Mini Protease Inhibitor) and once in ice-cold PBS. Supernatant was discarded after centrifugation at 16,000rcf for 1min. 15µl 2x protein loading dye (2% SDS; 10% Glycerol; 50mM Tris-HCl pH6.8; 0.6% β-mercapto-ethanol; 0.1% bromophenol-blue) were added to the beads, resuspended by pipetting up and down and boiled for 5min at 95°C. 20µl of the samples were then loaded on 10% SDS-PAGE with a 4% stacking gel and run at 180V for 1h, together with 7µl of the 10-180kDa pre-stained Protein Ladder (MBI Fermentas, #SM0671). After running the gel was silver stained so that it would be compatible for MS (see 3.3.4, Table 4).

3.5 MALDI-Mass Spectrometry (MS)

Mass Spectrometry (MS) is a technique to analyse masses of single molecules in a mixture. Proteins out of silver or Coomassie stained gels can be partially digested enzymatically and the resulting peptides sequenced by MS.

Matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF) analysis was kindly performed by PD Dr. André Schrattenholz, CSO and his co-worker Dr. Gerhard Schwall (ProteoSys AG, Mainz) and by Prof. Dr. Dieter Oesterhelt and his co-worker Dr. Frank Siedler of the Max Planck Institute of Biochemistry in Martinsried, using the MALDI peptide mass fingerprint method for protein identification (TEBBE *et al.* 2005). Protein samples were cut by hand and the MSDB database used for further investigations (http://www.matrixscience.com/help/database_help.html).

3.6 Real Time PCR

Based on the PCR method (described in 3.2.2), in real time PCR primers and a Taq-DNA polymerase work together to amplify certain nucleic acid fragments. The real time PCR machine (LightCycler 480, Roche) can monitor this reaction in real time by either incorporating fluorescent dyes (e.g. SYBR Green I) into each amplified DNA fragment and measuring the fluorescent signals, or by binding a specific fluorescent probe sequence onto each amplified DNA fragment and measuring the signals.
Reference genes, ideally expressed at relatively constant copy numbers in all tissues to be analyzed, are necessary for comparing the expression level of the target transcripts (*dE2F1-RA*, *-RB*, *-RC*) to ascertain the experimental basis and to eliminate experimental errors. We isolated the mRNA of several *Drosophila* tissues and found it necessary to pre-amplify its cDNA to increase the amount of material analyzed by real time PCR.

3.6.1 Tissue dissection

All tissues were dissected into tubes kept in liquid nitrogen and stored at –80°C.

Tissue	Dissected amount	Tissues per reaction	Notes
Salivary Glands	18 pairs	3.6 pairs	
Larvae brains	18 pairs	3.6 pairs	
Wing discs	18 pairs	3.6 pairs	
Testis	12 pairs	2.4 pairs	
Ovaries	12 pairs	2.4 pairs	
Adult brains	12 pieces	2.4	
Embryos	18 embryos	3.6	*white*: 1h lay, 2h wait; *dE2F1*-mutant: 7h lay, 2h wait
Malpighian tubules	30 larvae	6	
Whole *w* flies	20 flies	2	For standards

Table 5: Dissected tissues for four reactions each kind.

3.6.2 mRNA isolation and cDNA pre-amplification

For mRNA isolation and cDNA amplification we used the mRNA Capture Kit of Roche (Cat.No. 1787896) in combination with the Titan one tube RT-PCR system (Roche; Cat.No. 1855476). DEPC-H_2O was utilized throughout all steps.

For four primer reactions (*Rp49*, *dE2F1-RA*, *dE2F1-RB*, *dE2F1-RC*), 400µl lysis buffer (mRNA Capture Kit, Roche) was added onto the tissues. The 1.5ml Eppendorf tube was then vortexed to destroy the texture of the tissue. After centrifugation at 13,900rpm for 1min the supernatant was taken out and about 350µl transferred into a new tube. 4µl of biotin-labeled Oligo $(dT)_{15}$ (20µM) was added, mixed equally and incubated 5min on ice to label the mRNA at its polyA tail with biotin. Afterwards each tissue sample was separated into four streptavidin tubes and incubated for 3min at room temperature. The streptavidin binds the biotin-labeled dT-A hybrids and thus immobilizes the mRNA. Unbound contaminants were washed off in three washing steps with wash buffer (Roche, mRNA Capture Kit). Then the entire reaction of reverse transcription and cDNA amplification takes place in the single streptavidin tube using the Titan One Tube Kit (Roche).

dNTPs [10mM each]	1 µl
primer 1	1 µl
primer 2	1 µl
DTT	2.5 µl
5x RT-buffer	10 µl
enzyme mix	0.2 µl
ddH_2O	34 µl
Final volume	50 µl

Antisense and sense primers were designed for all three *dE2F1* transcripts as well as for *Rp49*. The ribosomal protein (CG7939-RB; http://flybase.bio.indiana.edu/cgi-bin/getseq.html?source=dmel&id=FBgn0002626&chr=3R&dump=PrecompiledFasta&targetset=transcript) was used as reference gene. Unfortunately 8bp of the original 19bp sense primer AW30 were by mistake located in the intron sequence.

Name	Direct.	Sequence	Position [bp]	Length [bp]	T_m [˚C]
RP49					
AW30	sense	*TCCTTGCA*GCACAAATGGC	125-135	19	45.4
AW31	antisense	CGGGTGCGCTTGTTCGAT	229-212	18	56.8
Fragment size			**104bp**		
dE2F1-PA					
FRR43	sense	ATTCAAGTCGCTGAGTAGCAATAGC	428-452	25	55.3
AW24	antisense	AGCAGTTCGAGCATTTGTTG	540-521	20	50.5
Fragment size			**112 bp**		
dE2F1-PB					
FRR41	sense	CAACCAATTGACAGGTGACGATCT	67-90	24	56.8
AW22	antisense	TCCATTCCGCCTTCGTATG	181-163	19	53.3
Fragment size			**114 bp**		
dE2F1-PC					
FRR42	sense	TGTTCTGCAACTTATTACTCCAGTTCT	95-121	27	54.1
AW23	antisense	CGTTACTTTGCTTTAAAAGAATTTGGCT	187-160	28	58.0
Fragment size			**92 bp**		

Table 6: All primers for real time PCR were adjusted to 10µM. The primers were designed to enclose fragments of about 100bp obtaining optimal conditions. All primer pairs were checked by RT-PCR first. Listed melting temperatures (T_m) were calculated by the Vector NTI program 9.0.0 and chosen to be in a similar range.

Step	Temp.	Time	Function
1	55 °C	30 min	Activation RT-enzyme
2	94 °C	2 min	Denaturation
3	94 °C	30 sec	Denaturation
4	55 °C	30 sec	Annealing
5	68 °C	45 sec	Elongation
6	go to step 3 → 10 cycles		Amplification
11	68 °C	7 min	Long elongation
12	10 °C	for ever	Cooling

3.6.3 cDNA concentration measured by photometer

After ten amplification cycles for each tissue the concentration of the cDNA of only one primer pair was measured by photometer (Biophotometer, Eppendorf) for double stranded DNA at 260nm (Table 7). This value was used to calculate the pipetting volume needed for an end concentration of 1ng per reaction. Equal volumes were adopted for all four cDNA samples of each tissue calculated by the one measured, ensuring to keep the expression rates in equal proportions. The concentration of 1ng was tested to be optimal for the reaction in earlier experiments. Measurements were used as reference value for further experimentations and do not provide absolute data, since no purification step was performed after the PCR reaction.

Tissue	**Sample**	**A_{260} (1:50)**	**Amount**
1 - salivary glands (SG)	*w-1*	9.9	495 ng/µl
	dE2F1^{TF}-1	15.8	790 ng/µl
2 – larvae brain (LB)	*w-2*	11.3	565 ng/µl
	dE2F1^{TF}-2	13.7	685 ng/µl
3 – wing discs (WD)	*w-3*	13.2	660 ng/µl
	dE2F1^{TF}-3	12.9	645 ng/µl
4 – testis pairs (T)	*w-4*	10.1	505 ng/µl
	dE2F1^{TF}-4	9.1	455 ng/µl
5 – ovary pairs (O)	*w-5*	8.5	425 ng/µl
	dE2F1^{TF}-5	10.2	510 ng/µl
6 – adult brain (B)	*w-6*	10.1	505 ng/µl
	dE2F1^{TF}-6	10.4	520 ng/µl
7 – embryos (E)	*w-7*	10.1	505 ng/µl
	dE2F1^{TF}-7	8.0	400 ng/µl
8 – Malphigian tubules (MT)	*w-8*	8.8	440 ng/µl
	dE2F1^{TF}-8	12.5	625 ng/µl

Table 7: Photometric measuring of the amplified cDNA samples for real time PCR at 260nm.

3.6.4 Standards

Standards are a series of dilutions, containing target and/or reference nucleic acids in known concentrations. They are essential to calculate the standard curve, to quantify the unknown concentrations (e.g. copies or ng/µl) of the samples and to determine the efficiencies of each real time PCR experiment. We made use of so called "external standards" that are amplified in the same real time PCR run as the target, but not in the same well.

For the standards, mRNA was isolated out of 20 *white* flies using the mRNA Capture Kit (Roche). The mRNA was distributed onto ten tubes and two reactions were performed for each primer with a final volume of 50µl to avoid over-saturation of the streptavidin coated tube. After reverse transcription and cDNA amplification for ten cycles using the Titan One Tube Kit (Roche) as described before (see 3.6.2), the two reactions of the same primer pair were pooled (final volume 100µl) and the absorbance of 1:50 dilution of each sample was measured by photometer (Biophotometer, Eppendorf) at 260nm.

Primer	**A_{260} (1:50)**	**Amount**
Rp49	17.3	865 ng/µl
dE2F1-RA	15.3	765 ng/µl
dE2F1-RB	15.5	775 ng/µl
dE2F1-RC	15.7	785 ng/µl

Table 8: Photometric measuring of the amplified cDNA standards of all four primer pairs used for real time PCR.

A dilution serial of 10ng/µl, 1ng/µl, 100pg/µl, 10pg/µl was prepared of each primer and matched to the expected concentration range of the target, important for high accuracy.

3.6.5 No template control (NTCs)

Running NTCs (no template controls) provides a good quality control for the PCR reaction. In here, instead of standard or tissue sample cDNA DEPC-treated RNase-free water was added. The NTCs are useful to identify primer dimers which are most likely formed in the NTCs due to low template concentrations. Products driving from this reaction are therefore artefacts of non- target amplification products, caused by homologies within primers.

3.6.6 SYBR Green reaction mix

Real time PCR reaction was performed using the LightCycler System from Roche and the QuantiTec® SYBR® Green RT-PCR-Kit (Qiagen; Cat.No. 204143) for quantitative real-time one-step RT-PCR.
We followed the protocol "Using the LightCycler System" of the Qiagen handbook.
First the primer/SYBR Green mix was prepared for all primer pairs and all samples by adding 2µl (10µM) of each primer to 10µl 2x QuantiTec SYBR Green and then distributing the mixtures onto the 96-well plates (Roche; Cat. No. 04640268001).

Only one primer pair was applied to each plate to avoid contamination. The mixture was prepared for 63 samples:

8 tissues
x 2 (*white* & *dE2F1*^{TF} mutant strain)
x 3 replicates
+ 3 NTCs
+ 4 dilutions for standards
x 3 replicates

= 63 samples

Then 6µl (1ng final concentration) of the cDNA derived from the tissue samples or the standards was mixed specifically with the primer/SYBR Green mix inside the single wells, resulting in a final reaction volume of 20µl.

3.6.7 Plate design

To evidence accuracy of the method and pipetting, three replicates of each tissue sample were added. To avoid primer contamination, only one primer pair was used on each plate. Homologous standards, having the identical amplicon due to amplification with the same primer pair, were included for calculation of the standard curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	*w1*	*E2F1*	*w2*	*E2F2*	*w3*	*E2F3*	*w4*	*E2F4*	*w5*	*E2F5*	*w6*	*E2F6*
B	*w1*	*E2F1*	*w2*	*E2F2*	*w3*	*E2F3*	*w4*	*E2F4*	*w5*	*E2F5*	*w6*	*E2F6*
C	*w1*	*E2F1*	*w2*	*E2F2*	*W3*	*E2F3*	*w4*	*E2F4*	*w5*	*E2F5*	*w6*	*E2F6*
D												
E	10ng	1ng	100pg	10pg		NTC			*w7*	*E2F7*	*w8*	*E2F8*
F	10ng	1ng	100pg	10pg		NTC			*w7*	*E2F7*	*w8*	*E2F8*
G	10ng	1ng	100pg	10pg		NTC			*w7*	*E2F7*	*w8*	*E2F8*
H												

Table 9: The 96 well plate (Roche) was designed and used for all tissue samples in three replicates. In total four plates for the four transcript specific primer pairs were accomplished. 10ng to 10pg are the standard mRNA amplified with the same primers as for the material of interest. NTC are the positions of the null template control.

Before starting the LightCycler 480 the plate was sealed with an adhesive foil (Roche).

3.6.8 Program conditions

Samples and standards were applied to the Light Cycler®480 software release 1.2.0.0625 (Roche). For calculation of the standard curve, concentrations of standard samples had to be inputted into the program.

The real time PCR run started with an initial (pre-) incubation at 95°C for 15min to activate the HotStarTaq-DNA polymerase (Qiagen). After completion of 40 cycling reactions, a melting curve step was included to check primer specificity as well as for primer dimers and by-products.

Program Name	**Cycles**	**Analysis**	**Target (°C)**	**Acquisition Mode**	**Hold**
Pre-incubation	1	None	95°C	none	15min
Amplification	40	Quantification	95°C	none	15sec
			55°C	single	30sec
			72°C	none	30sec
Melting Curve	1	Melting Curves	95°C	none	1
			65°C	none	10sec
			95°C	Continuous	
Cooling	1	None	40°C	none	1sec

Table 10: Program settings for PCR run.

3.6.9 Final calculation - Relative quantification with external standards

This relative quantification approach first calculates the absolute value of the target under investigation and the value of the reference gene in the same sample. To obtain the concentration of these two parameters an external standard curve is used. The Mean-concentration (C) of the three replicates of each sample is calculated.

Then the absolute concentration of the target is divided by the absolute concentration of the reference gene.

$$\mathbf{Ratio = C_{Target} / C_{Reference}}$$

The resulting target/reference ratio expresses the amount of target now normalized to the level of an endogenous reference gene within each unknown sample.

This method is probably the most common used for quantitative PCR in biological research for all applications where the determination of relative changes are important.

4. Results

4.1 Crossings for *dE2F1^{TF}* homozygosity check

4.1.1 Single crosses with homozygous *dE2F1^{TF}* mutant flies

Embryos of homozygous females die during early development. This prevents the maintenance of the mutation in a homozygous condition. We, therefore, stabilized the strain using the balancer chromosome *TM(3) Ser*. The lethal phenotype was checked by single crosses using a homozygous virgin female crossed with a homozygous male.

$$dE2F1^{TF}/\,dE2F1^{TF} \quad x \quad dE2F1^{TF}/\,dE2F1^{TF}$$
$$\text{♀} \quad x \quad \text{♂}$$

Four of eight single crosses did not produce any viable offspring. As observed in earlier studies (Feng 2005), embryos fail in development and three day-old-eggs turned brown. In the four remaining crosses a low number of offspring developed up to later stages, alongside many brown eggs that were arrested in their development.

In cross 1: three flies developed to the adult stage.

In cross 2: one fly developed to adult stage and three reached the pupal stage.

In cross 3: one fly developed to adult stage and one reached pupal stage.

In cross 4: two flies developed to adult stage and two reached pupal stage.

All F1 flies showed cyan fluorescent eyes and no balancer marker. Thus they are phenotypical homozygous. Those flies were frozen in liquid nitrogen and stored at -80°C for further experimentation.

Single Cross	P generation	P generation in fresh food bottle
1	After 28 days - no offspring - food bad - bottle was discarded	
2	After 28 days - no offspring - food bad - bottle was discarded	After 47 days - no offspring - male died - bottle was discarded
3	After 32 days - no offspring - food bad - bottle was discarded	After 47 days - no offspring - both P died - bottle was discarded
4	After 29 days - no offspring - food bad - bottle was discarded	After 47 days - no offspring - both P died - bottle was discarded
5	After 22 days - 1 ♂ fly After 26 days - 1 ♀ and 1 ♂ fly - food bad - bottle was discarded	
6	After 29 days - 3 pupa and 1 ♂ fly After 43 days - bottle was discarded	After 27 - no offspring - no larvae - bottle was discarded
7	After 27 days - 1pupa and 1 ♂ fly - food bad - bottle was discarded	After 27 days - no offspring - both P died - bottle was discarded
8	After 27 days - 2 pupa and 2 ♂ fly After 42 days - bottle was discarded	After 28 days - 1 pupa and 1 ♀ fly After 48 days - bottle was discarded

Table 11: Monitoring of the data of homozygous *dE2F1*^{TF} single crosses. In four of eight crosses a low amount of offspring managed to develop until the adult stage.

4.1.2 Crosses with heterozygous *dE2F1^{TF}* mutant flies

We also carried out mass crosses using five virgin homozygous females (*dE2F1^{TF}/ dE2F1^{TF}*) crossed with five heterozygous males (*dE2F1^{TF}/TM3$_{Ser}$*) and the reciprocal crosses to see whether the better breeding conditions might enhance survival of offspring from homozygous mothers.

Heterozygous cross 1: crossing 5 homozygous females with 5 heterozygous males

$$dE2F1^{TF}/\,dE2F1^{TF} \quad x \quad dE2F1^{TF}/\,TM3_{Ser}$$
$$\text{♀} \quad x \quad \text{♂}$$

Heterozygous cross 2: crossing 5 heterozygous females with 5 homozygous males

$$dE2F1^{TF}/\,TM3_{Ser} \quad x \quad dE2F1^{TF}/\,dE2F1^{TF}$$
$$\text{♀} \quad x \quad \text{♂}$$

Flies in heterozygous cross 2 developed much faster than in heterozygous cross 1 where heterozygous females were used for mating. A large F1 population of homozygous and heterozygous flies could be obtained in cross 2, while only two flies developed up to adult stage in cross 1. These results confirmed the maternal effect occurring in the homozygous *dE2F1^{TF}* mutant females!

	dE2F1^{TF}/ dE2F1^{TF}x dE2F1^{TF}/ TM3$_{Ser}$ ♀ ***x*** ♂	***dE2F1^{TF}/ dE2F1^{TF}x dE2F1^{TF}/ TM3$_{Ser}$*** ♀ ***x*** ♂
Day 1	1st instar larvae, food wet	Food not wet
Day 6		Some pupa
Day 10	1 pupa, no larva	Many pupa Discard parents
Day 13	2 pupa close to the food Discard parents	Many hetero- and homozygous flies
Day 21	1 homozygous ♂; 1 heterozygous ♂	Many flies
Day 27		Discard bottle
Day 40	Discard bottle	

Table 12: Crossing homozygous females with heterozygous males (left column) and heterozygous females with heterozygous males (right column) displays the maternal effect, caused by the *dE2F1^{TF}* mutation and the developmental delay in the offspring of homozygous flies.

4.2 PCR for *dE2F1*^{TF} homozygosity check

After checking the homozygosity with classical genetics by following the marker conditions, we used PCR to prove the homozygosity on a molecular level as fluorescent markers may be misleading due to low signal strength.

We isolated the DNA out of all single flies collected from the crossing experiments (see table 11). A *white* fly was used as control. One primer pair was chosen inside the *dE2F1* gene and one additional primer inside the P-element (pBac-ECFP-cPrm2-H3.3B (9399bp)) which had caused the mutation of the *dE2F1-PB* transcript (Fig. 5). Performing a PCR using the primer FRR41 and AW18 on *white* fly DNA and hence without P-element insertion, a DNA fragment of 1354bp length should be obtained. If the P-element insertion had occurred the fragment would be 10,753bp (1354bp + 9399bp). A fragment of > 2kb in size cannot be achieved using the normal Taq-enzyme and is therefore not visible in our experiment. When applying primer FRR41 and FRR37, only hetero- and homozygous *dE2F1*^{TF} mutant fly DNA, can generate a fragment of 1176bp in length. If both bands are visible on the agarose gel using DNA isolated out of the same fly, the fly is heterozygote.

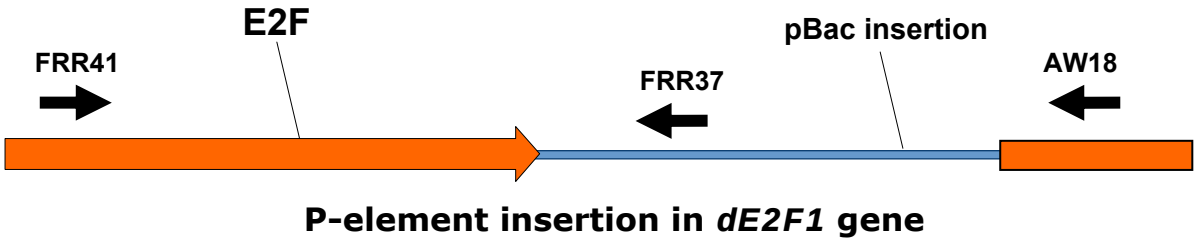


Fig. 5: P-element insertion into the *dE2F1* gene located on chromosome *3*. Black arrows indicate PCR primers.

The flies collected from the single crosses were checked by PCR using both primer pairs in two individual PCR reactions. PCR products were then separated on a 1.5% agarose gel.

Six of the DNA samples isolated out of the collected flies (five are shown in Fig. 6B; samples A-E) showed the 1176bp fragment generated by the primer FRR41 and FRR37 positioned within the insertion, but not the 1354bp fragment where primers were located outside the insert, but inside the *dE2F1* gene.

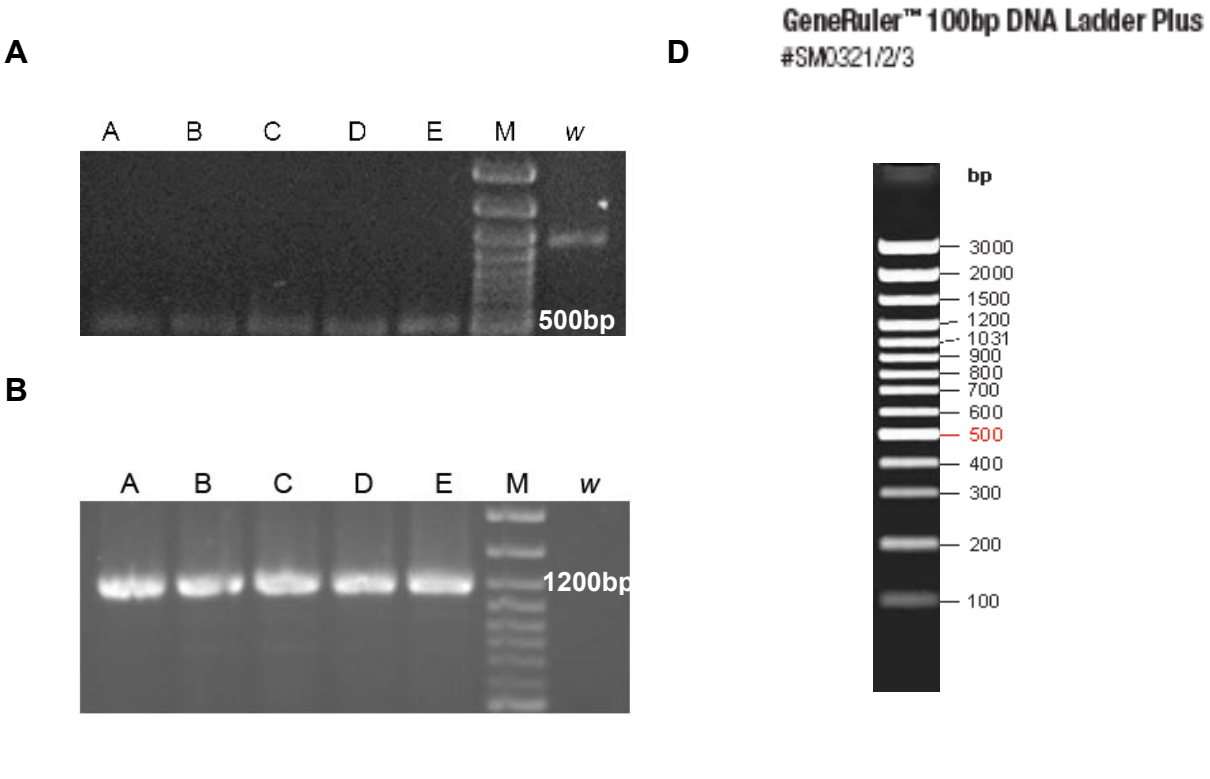


Fig. 6: Separation of PCR products by agarose gel electrophoresis (1.5%). **A.** Primers FRR41/AW18 inside the *dE2F1* gene in the DNA samples A-E did not synthesize a fragment of 1354bp, evidencing that the *dE2F1* gene is interrupted by the P-element insertion. In contrast, the *white* (*w*) DNA, used as control, showed this PCR product. The 500bp fragment was produced by additional primer binding. **B.** Using the primer FRR41/FRR37 overlapping the insertion, a fragment of 1176bp in length was detected for the same samples, proving the presence of the mutation. **C.** Sample F, which was phenotypically affirmed to be homozygous displayed the 1354bp fragment when using AW18/FRR41. Thus this fly was heterozygous. **D.** The Gene Ruler 100bp DNA ladder was applied as molecular size marker.

For one fly (sample F), which was selected to be homozygous, the 1354bp fragment was produced when using the primer pair AW18/FRR41 (Fig. 6C). This evidenced the heterozygosity of this fly and demonstrates the necessity of the molecular conformation by PCR.

The 500bp fragment was sequenced by a student in our laboratory (Alexandra Brecht) and indicated an extra unspecific primer binding site inside the third chromosome at position 3R:1..27905053. For detailed information see sequencing and BLAST results in appendix 8.1.4. This additional primer binding site is not closely located to the *dE2F1* gene which is at 3R: 17446026...17486127.

Summarizing, the flies were on a molecular level proven to be homozygous. It confirms that the marker genes *ECFP* and *Ser* can reliably be used to distinguish homozygous from heterozygous flies.

4.3 *dE2F1* protein sequencing by Mass Spectrometry

The occurrence of the three transcripts arises the question whether more than one *dE2F1* protein, as is presently assumed, is the consequence of potential alternative splicing. There is also the possibility that downstream translation initiation sites are used for the different transcripts.

```
1       MSKFFVNVAPINNSNSSSSHTTTSSNTQRHQQHQQHYGGSGTTGHTMVAR
51      RLNYDLHGGTTSINNNNNIVIKNESVDLDYDHVLSSSDSNSNGGVAAHLR
101     DHVYISLDKGHNTGAVATAAAAATAGHTQQQLQQQHHHQNQQQRKATGKS
151     NDITNYYKVKRRPHAVSDEIHPKKQAKQSAHHQTVYQKHTASSAPQQLRH
201     SHHQLRHDADAELDEDVVERVAKPASHHPFSLSTPQQQLAASVASSSSSG
251     DRNRADTSLGILTKKFVDLLQESPDGVVDLNEASNRLHVQKRRIYDITNV
301     LEGINILEKKSKNNIQWRCGQSMVSQERSRHIEADSLRLEQQENELNKAI
351     DLMRENLAEISQEVENSGGMAYVTQNDLLNVDLFKDQIVIVIKAPPEAKL
401     VLPNTKLPREIYVKAENSGEINVFLCHDTSPENSPIAPGAGYVGAPGAGC
451     VRTATSTRLHPLTNQRLNDPLFNNIDAMSTKGLFQTPYRSARNLSKSIEE
501     AAKQSQPEYNNICDIAMGQHHNLNQQQQQQQQQLLQQPEEDDVDVELNQL
551     VPTLTNPVVRTHQFQQHQQPSIQELFSSLTESSPPTPTKRRREAAAAAIA
601     AGSSTTATTTLNSHNNRNHSNHSNHSNHSSSNNSKSQPPTIGYGSSQRRS
651     DVPMYNCAMEGATTTSATADTTAATSRSAAASSLQMQFAAVAESNNGSSS
701     GGGGGGGGYGSIAGAGANADPHQPYSHDRNSLPPGVADCDANSNSSSVTL
751     QGLDALFNDIGSDYFSNDIAFVSINPPDDNDYPYALNANEGIDRLFDFGS
801      DAYGP
```

The *dE2F1* protein sequence has a length of 805aa. The methionines for possible translation origins are shaded in red http://www.flybase.org/cgi-bin/getseq.html?source=dmel&id=E2f-PB&chr=3R&dump=PrecompiledFasta&targetset=translation).

Performing several 1D and 2D protein PAGEs, varying results were obtained with different antibodies. By comparing those results and considering the ATG start codons in the *dE2F1* nucleotide sequence encoded as methionines (M) in its amino acid sequence (marked in red in the sequence), we derived the possible molecular weights for *dE2F1* isoforms in the range between 40 and 85kDa. The Isoelectric Points (IPs) were calculated to be in between 4.8 to 6.16.

Amino acids	Size	IPs
805aa	87.5kDa	6.16
370aa	46.5kDa	5.08
478aa	34.8kDa	4.97
517aa	30.5kDa	4.80

Table 13: Possible *dE2F1* proteins detected by western blot experiments earlier.

The intention of the experiments was to identify possible *dE2F1* variants by MS.
As tissues delivering the proteins to be analysed we first chose ovaries due to the phenotype of our $dE2F^{TF}$ mutant strain. We focused on the situation in *white* flies to detect *dE2F1* isoforms of *E2F* in the *wild type* situation.
MS analysis was performed three times. Candidate bands or spots were cut out of one-dimensional (1D)- or two–dimensional (2D)-gels, applying different sample preparations. For detection and immunoprecipitation we used different *dE2F1* antibodies, synthesized in our laboratory in chicken and in rabbit respectively. None of these antibodies proved to be highly specific for *dE2F1*.
None of the experiments gave convincing evidence that isoforms of the *dE2F1* protein exist in *Drosophila melanogaster*, even though minor fractions of *E2F* peptides were found in a 50kDa spot from the 2D-gel.

4.3.1 Two Dimensional Poly Acrylamid Gel Electrophoresis (2D-PAGE)

2D-gel electrophoresis presents the combination of isoelectric focussing (IEF) and SDS-PAGE. Because it is less likely that two different proteins will have the same properties in both IEF and molecular weight, they can be reliably distinguished in 2D-electrophoresis. Especially in a range of 50-55kDa, where a major portion of the proteins are located, the separation by 2D electrophoresis is essential.

Since the size and IP of possible *E2F* variants were unknown we choose a wide pH range of 3-11 NL (non-linear) for the first dimension. The bromphenol-blue added to solubilization buffer III works as front marker for the run of the first dimension, displaying a yellow band at the positive pole of the IPGphor chamber. The band was visible when we stopped the run after 24h 13min and 38,609Vhrs.

The gel of the 2nd dimension was then blotted for a short time to transfer approximately half of the proteins onto a PVDF membrane for sensitive immunostaining with the antibody against the C´terminal end of *dE2F1* protein synthesized in chicken. The gel was then silver stained, giving a sensitive method providing a detection limit between 1 to 10ng. Arising spots were compared to the ones on the immunostained blot. Spots at the same location were identified as potential *dE2F1* protein variant, cut out and send to MS analysis (Fig. 7). The same procedure was performed for samples of the $dE2F^{TF}$ mutant. Here six instead of five samples were further studied.

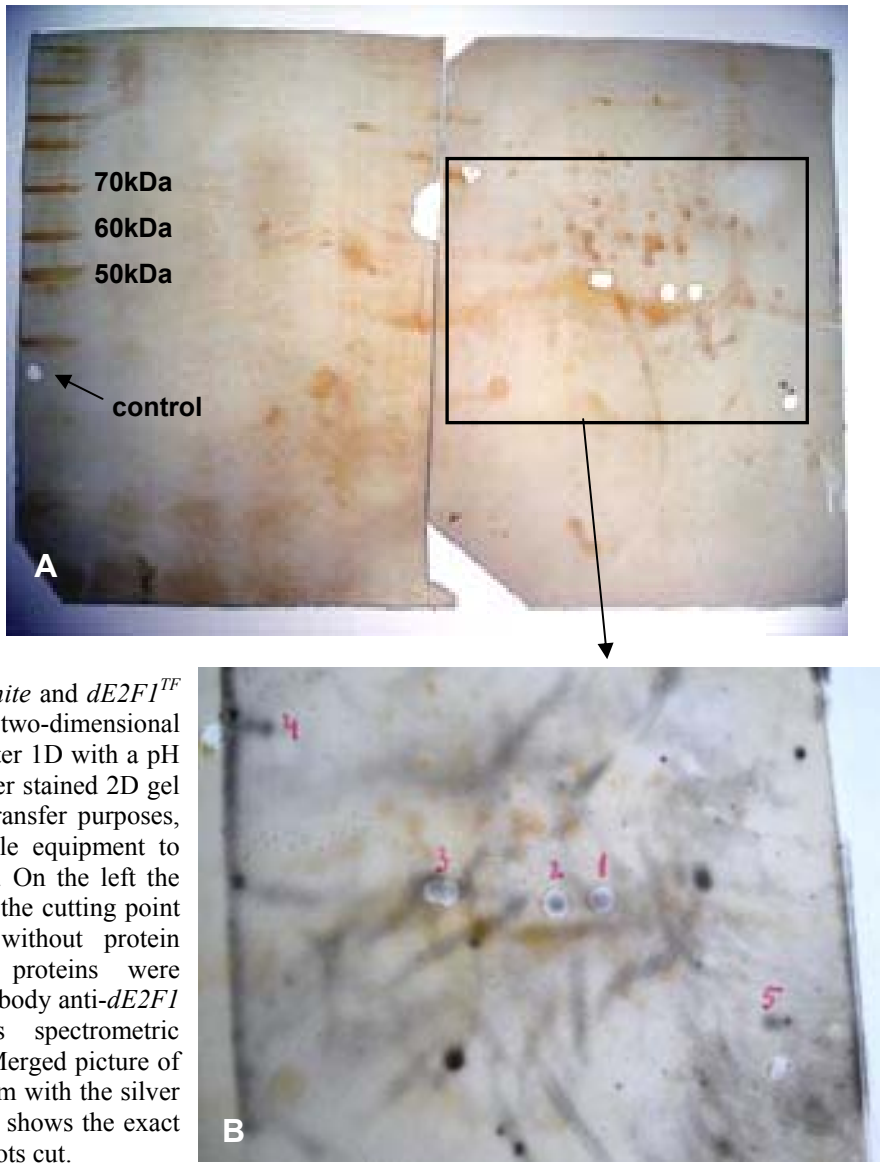


Fig. 7: Proteins of *white* and $dE2F1^{TF}$ ovaries separated by two-dimensional gel electrophoresis after 1D with a pH 3-11NL strip. **A.** Silver stained 2D gel was cut in half for transfer purposes, as we had no suitable equipment to handle the large gels. On the left the marker is visible and the cutting point of a control spot without protein (background). The proteins were identified with an antibody anti-*dE2F1* and cut for mass spectrometric analysis (frame). **B.** Merged picture of western blot X-ray film with the silver stained 2D-gel on top shows the exact position of the five spots cut.

C. Six protein spots of *dE2F1*^{TF} ovaries were cut out of the silver stained 2D gel for mass spectrometry using the same procedure as *for white*. (For protein marker (MBI), Fig. 4.)

After MS analysis neither the full length *dE2F1* protein nor any possible isoforms, nor any other transcription factor could be detected. Instead the high abundance larval serum protein 1 gamma and the nearly identical CG6821-PA of *D. melanogaster* were identified. The overlapping of this protein with our protein of interest that is likely to be of low abundance could be the reason for the failure of *dE2F1* detection.

4.3.2 Immunoprecipitation

By immunoprecipitation the *dE2F1* protein in its complex was linked to the rabbit *dE2F1*-antibody (A_{280} = 3.4817mg/ml) covalently bound to CNBr-Sepharose beads. The extraction was then separated on a 10% polyacrylamid gel and silver stained. Many fragments were visible (Fig. 8). The gel was then sent to the Max Planck Institute of Biochemistry, Martinsried, for MS analysis. The bands cut out and analysed are marked with red squares and the numbers 1 to 14 in the gel picture. Sample 15 and 16 are providing negative controls located in a gel region without sample.

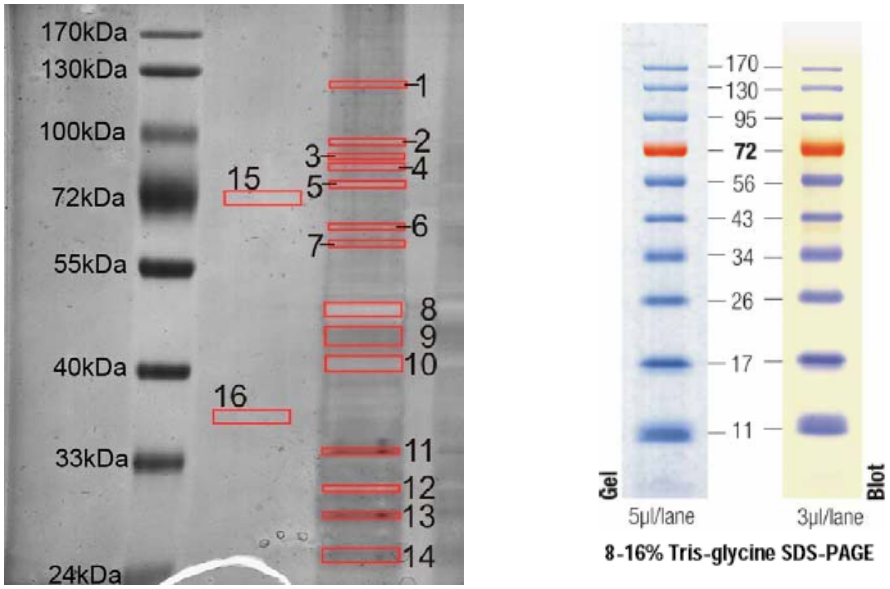


Fig. 8: Left: The silver stained gel after IP with marker band, control sample positions and the samples. Right: 10-180kDa Prestained Protein Ladder (MBI Fermentas, #SM0671).

The MALDI results were BLAST against the MSDB database with and without taxonomic limitation for *Drosophila*. Even though stringent working conditions (sterile filtration of all solutions used, wearing mask, changing cloves, etc.) were applied, human keratin-contamination was found in all samples as well as in sample 15 and 16. This shows that the conditions in our Chinese laboratory are inadequate for this sensitive kind of technique. Several other *Drosophila* proteins could be identified, but again not the *dE2F1* protein. These results do not prove the absence of the transcription factor *dE2F1*, but signals may be completely repressed due to keratin contamination.

4.4 The expression levels of the *dE2F1* transcripts

4.4.1 Semi-quantitative RT-PCR

After analysis of *dE2F1* proteins, and since additional protein isoforms could not be detected, we investigated in the mRNA expression level to achieve further understanding of the occurrence of the three *E2F* transcripts and their functions.

The expression level of the three *dE2F1* transcripts d*E2F1-RA*, *-RB* and *–RC* had been observed by semi-quantitative RT-PCR in earlier experiments (FENG 2005). Feng isolated mRNA from different developmental stages and tissues of *D. melanogaster*, using the

mRNA Capture Kit and Titan One Tube Kit (Roche) (as described in material and methods 3.6.2) and amplified specific fragments for the three *dE2F1* transcripts in 40 cycles. Distinct primers (see 8.2, Table 16) were located inside the 5'UTR of the certain transcript and one antisense primer inside the *dE2F1* common sequence (Fig. 9).

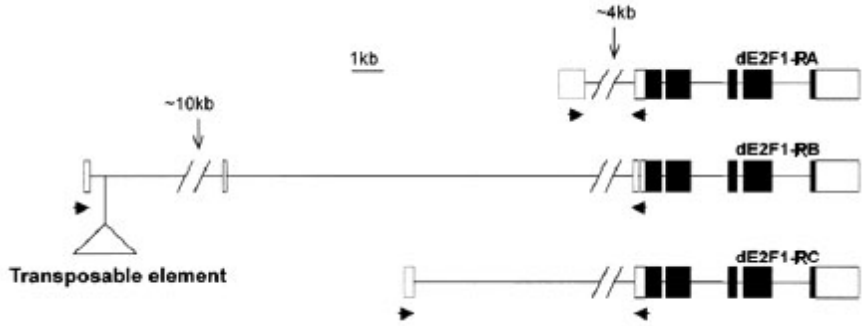


Fig. 9: Map of the three *dE2F1* transcripts and primer locations (black arrows) for RT-PCR in earlier experiments (FENG 2005).

The cDNA fragments were separated on agarose gels, followed by the incorporation of ethidium bromide and visualization by UV light. Transcript expression of interest was semi-quantified by comparison with the expression of the *ribosomal protein gene Rp49* as a reference gene (Fig. 10)**.**

As a result the three transcripts were expressed in distinct individual patterns. *dE2F1-RA* was transcribed in all tissues and developmental stages. It is the only one traced in Malpighian tubules, which are polytene but replicative inactive and adult brains, which are mitotically essentially inactive. All transcripts were found in wing discs and larvae brains, both undergoing rapid cell divisions. *dE2F1-RB* could not be detected in testis and in the polytene salivary glands but showed strong expression in ovaries and somewhat less in embryos, larvae and pupae. As expected, *Rp49* was expressed in all developmental stages and tissues.

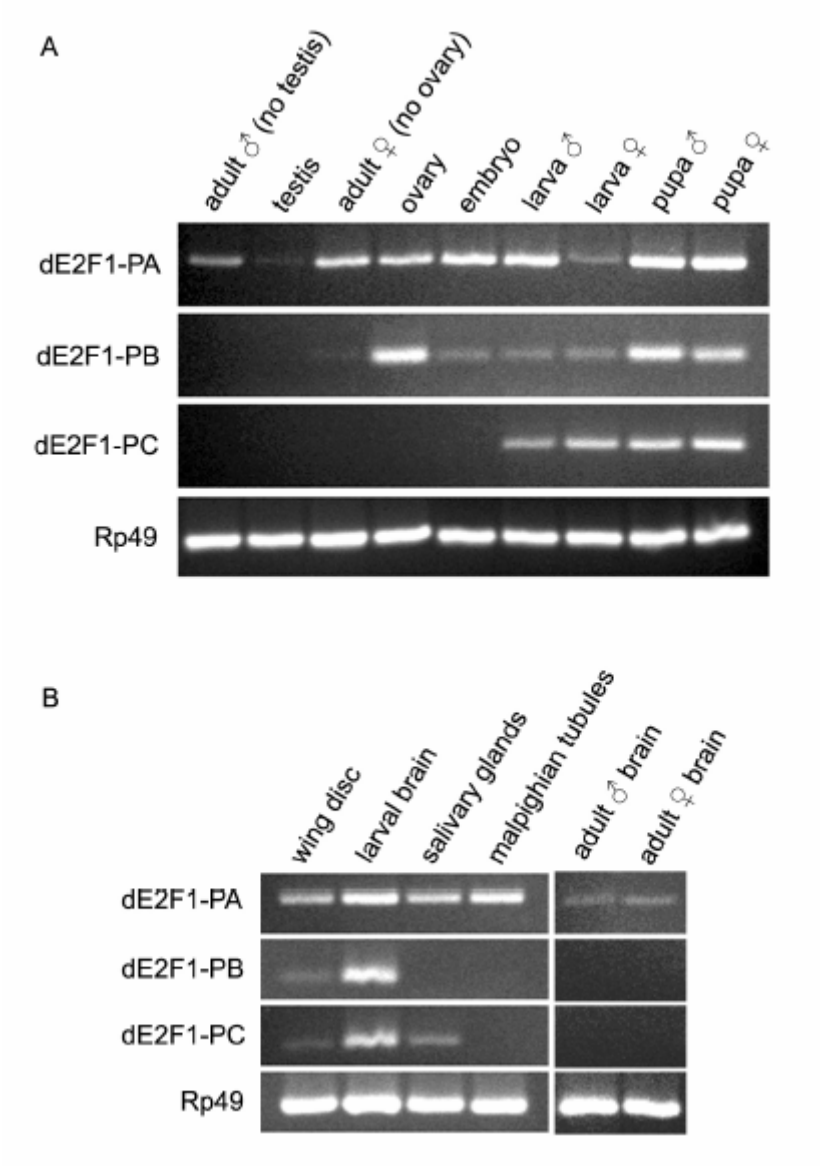


Fig. 10: Semi-quantitative RT-PCR was performed to determine the expression of the three *dE2F1* transcripts in different developmental stages (A) and tissues (B) of *D. melanogaster white* using *Rp49* as a reference gene. By visualizing on a agarose gel (1%), spatial and temporal specificity in the expression pattern of *dE2F1* transcripts was demonstrated (Fig. from PHD-thesis FENG 2005)

4.4.2 Quantitative Real Time PCR

The prior results induced us to study the different expression levels more accurately by the investigation in real time PCR experiments.

Due to the limited amount of material available, we reverse transcribed the mRNA and pre-amplified its cDNA for ten cycles by RT-PCR (3.6.2) to increase the working material. We focused on tissue specific expression levels and excluded, in contrast to the earlier experiments, whole flies and larvae.

The principle of the quantification is straight forward: the more copies of the target there are at the beginning of the assay, the fewer cycles of amplification are necessary to generate the number of amplicons to detect. Out of the three replicates the mean Cp (crossing point) values were calculated and their mean concentrations were determined by the standard curve generated by the in run homologous standards.

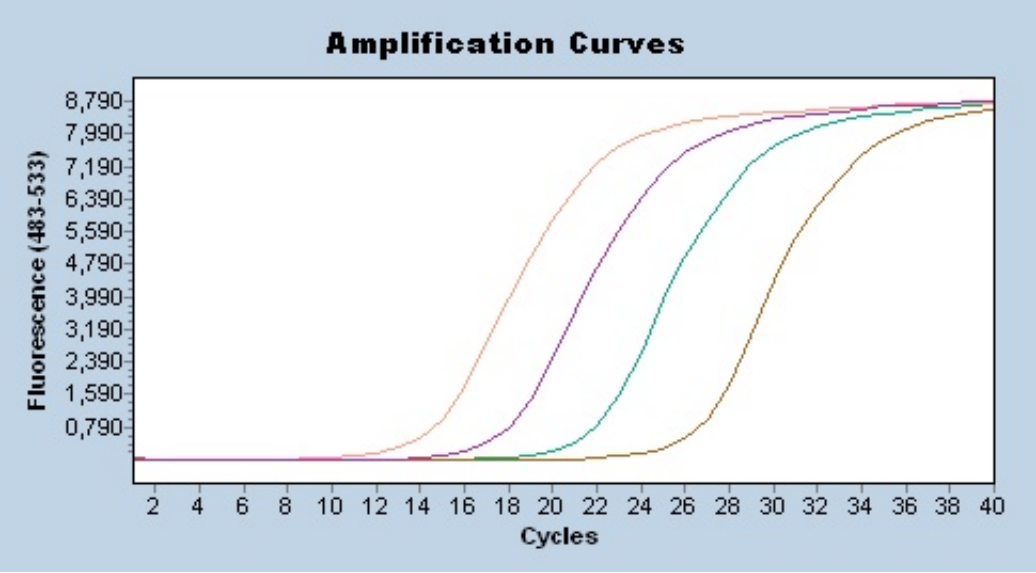


Fig. 11: Amplification curve of *Rp49* standards. The LightCycler 480 software calculated crossing points (Cp) automatically. From left to right: the Cp was reached at 14.4 cycles for 10ng dilution; 17.7 cycles for 1ng dilution; 21.6 cycles for 100pg and at 26.3 cycles for 10pg.

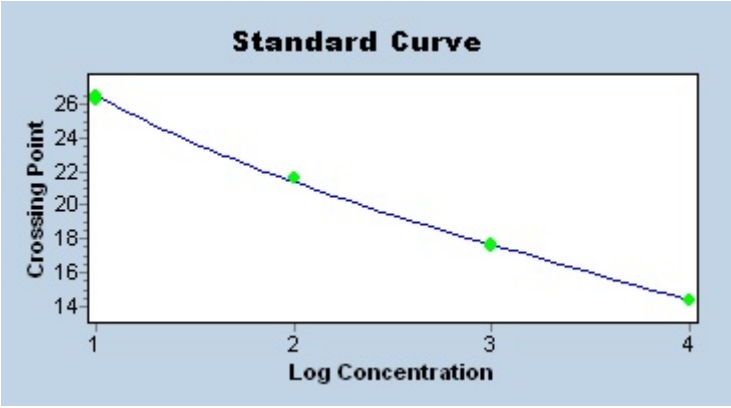


Fig. 12: On each primer specific 96-well plate a standard curve was calculated by a known dilution series of 10ng/µl, 1ng/µl, 100pg/µl and 10pg/µl concentration. This is the standard curve of the *Rp49* plate run using the primer for *Rp49*. (Error: 0,0141; Efficiency: 2,003).

The standard curve depends on the amplification efficiency of the standard samples and therefore describes the running efficiency for all samples. In this case (Fig. 12) the efficiency was calculated as 2.003 which is optimal. The error coefficient of 0.0141 is very low and proves reliability of the running conditions. Compare to *Rp49*, the efficiencies of *dE2F1-RA*, *-RB* and *–RC* were detected to be rather low (Table 14) displaying similar values. The error coefficient remained low for all samples.

Primer for	Efficiency	Error
Rp49	2.003	0.0141
dE2F-RA	1.753	0.0323
dE2F-RB	1.674	0.0105
dE2F-RC	1.626	0.0196

Table 14: Efficiencies and error coefficient of the four real time reaction plates.

By including a melting curve analysis at the end of each run, specific PCR products as well as unspecific by-products and primer dimers can be identified by their differences in melting peaks.

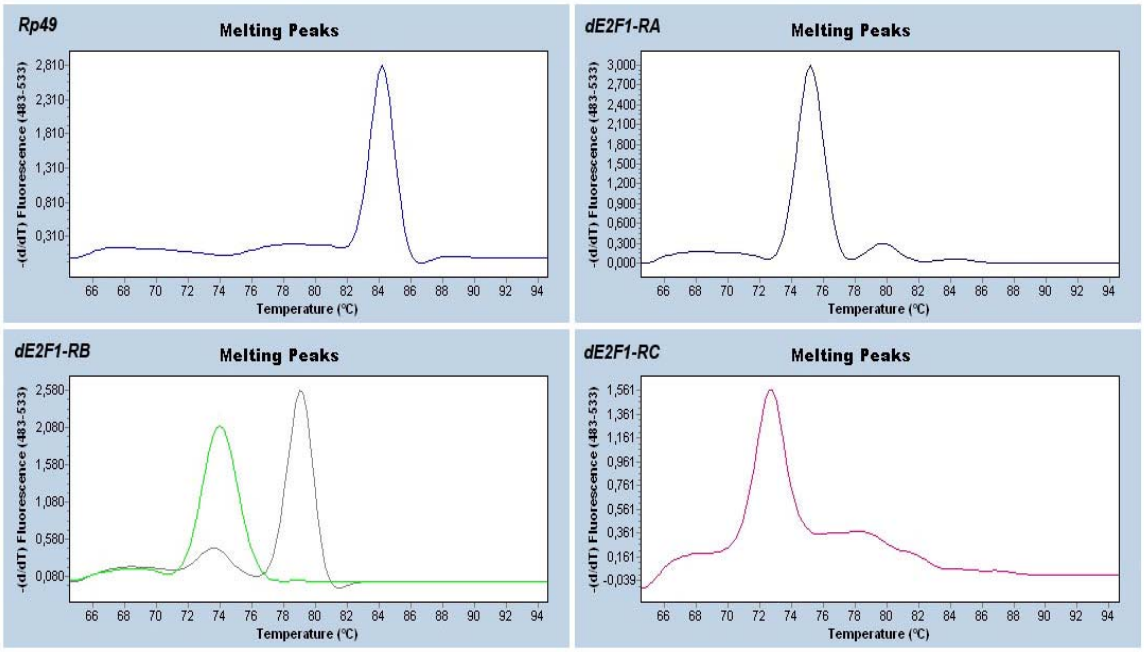


Fig. 13: Melting peaks of the four primer pairs in real time PCR reaction. For *Rp4*9 (84°C), *dE2F1-RA* (75°C) and *dE2F1-RC* (72.8°C) one main melting peak was detected. For *dE2F1-RB* primers two different melting peaks at 74°C and 79°C were observed, displaying synthesis of a by-product.

The primers of *Rp49* and *dE2F1-RA* showed low primer dimer and no by-product could be detected. The broad melting range of *dE2F1-RC* primer may display the low production of extra products. Besides *dE2F1-RB* displayed two independent melting peaks. The one with the higher temperature (79°C) demonstrates a product caused by unspecific binding of the primer in the genome.

The mean concentrations of all four transcripts are demonstrated in the following diagram (Fig. 15). *Rp49* as a ribosomal protein is assumed to be constantly and highly expressed independently of the cell type and developmental stage. It is a commonly used reference gene (ALONSO and AKAM 2003; MEE *et al.* 2004; ZHAO *et al.* 2006). A reliably constant, but unexpectedly low abundance of the *Rp49* transcripts compared to all three *dE2F1* products can be explained by the inappropriate location of the *Rp49* sense primer. Here, 8bp of a whole length of 19bp sense primer AW30 (Table 6) are located inside the intron sequence. To check whether the expected fragment of 104bp, deriving from the mRNA sequence, was amplified, real time reactions of the *Rp49* plate were run on a 2% agarose gel.

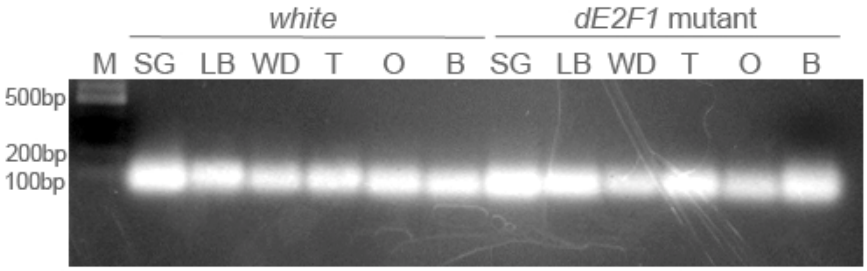
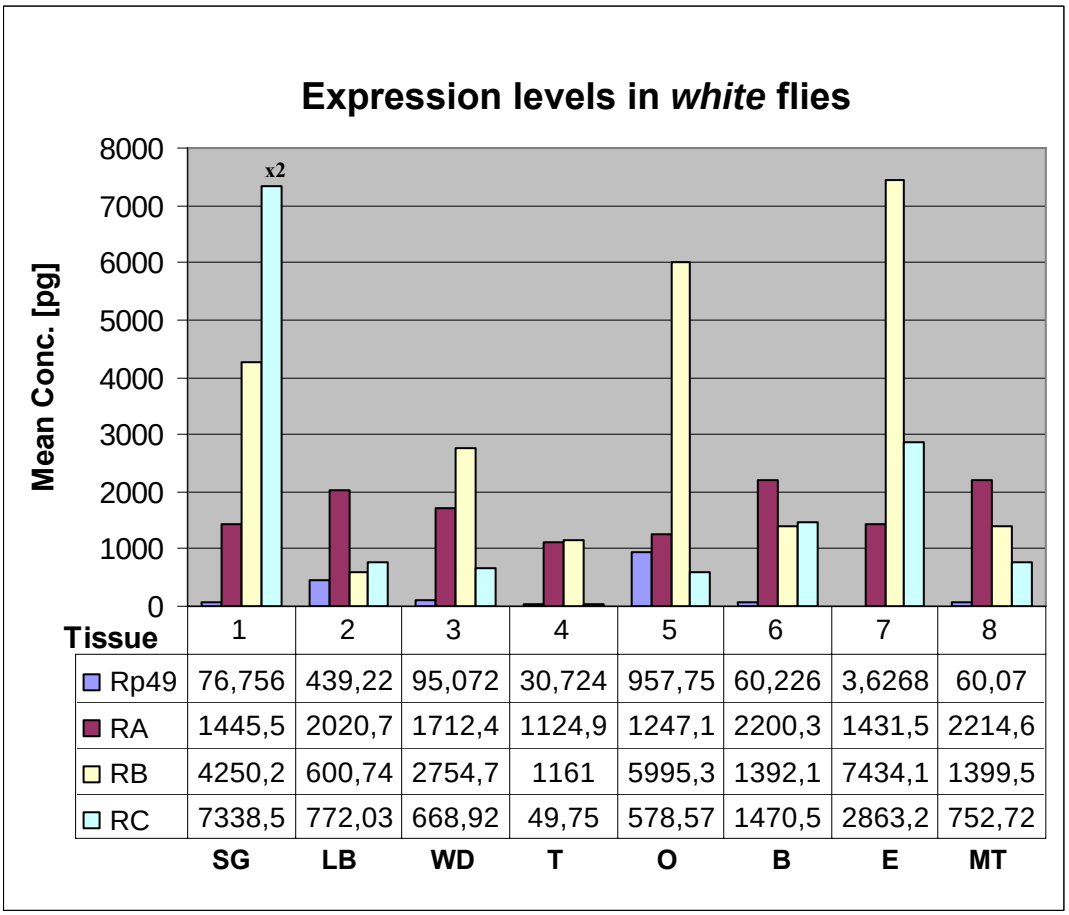


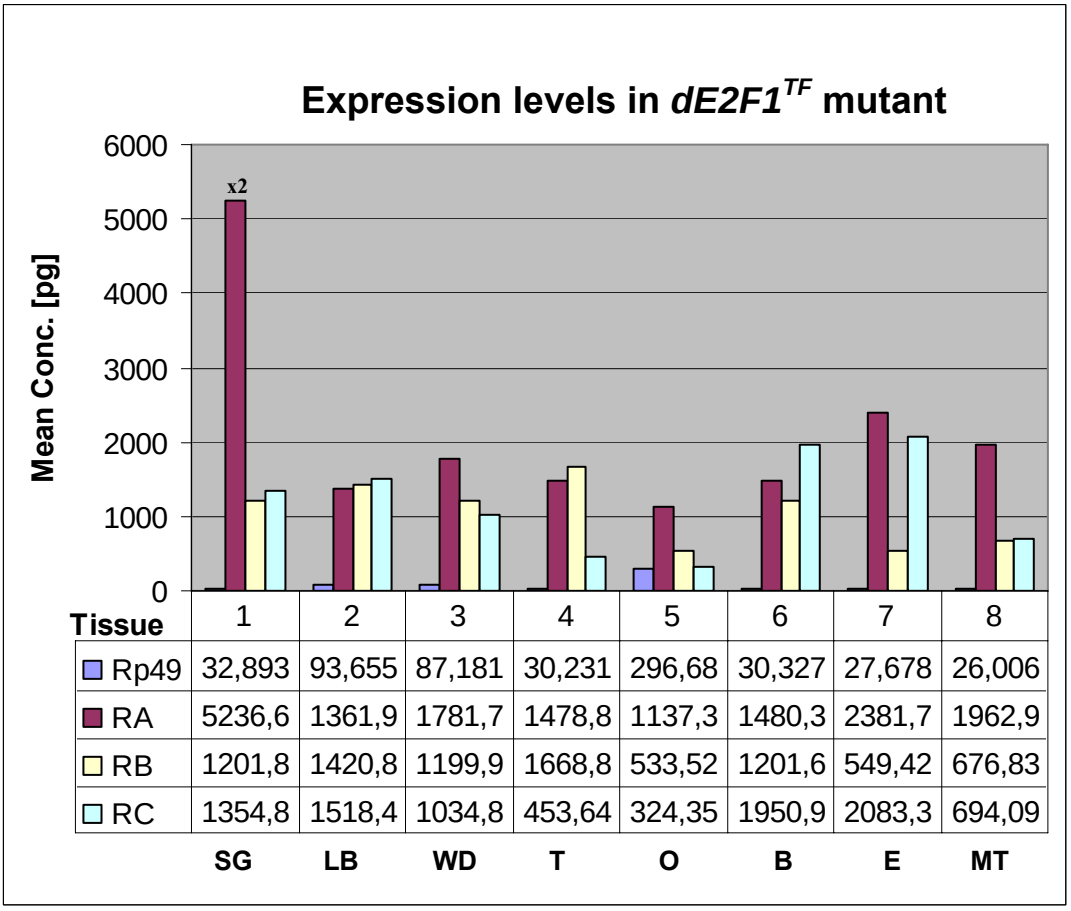
Fig. 14: After real time PCR the *Rp49* products were run on a 2% gel. In six samples of *white* flies and *dE2F1* mutant strain the 104bp fragment was amplified from the mRNA sequence. As marker (M) the Gene Ruler 100bp DNA ladder (Fig. 6C) was run in the left lane.

Rp49 was expressed in all tissues and the expected 104bp fragment was produced.

Fig. 15 on the next page: The diagram shows the mean concentrations (calculated by the three replicates) of the reference gene *Rp49* and the three mRNAs of interest *E2F-RA*, *-RB* and *–RC* in all eight tissues isolated from *D. melanogaster*. The upper diagram shows values for the *white* strain and the diagram underneath for the $dE2F1^{TF}$ mutant strain. Due to the outstanding high expression value for *dE2F-RC* in salivary glands of 14677pg in the *white* strain, it was divided with factor 2 for better visualisation of all data. The same conversion was performed in the mutant strain for the *dE2F1-RA* value again in salivary glands with original value of 10473pg. 1= SG= salivary glands; 2= LB= larvae brain; 3= WD= wing disc; 4= T= testis; 5= O= ovary; 6= B= brain; 7= E= embryo; 8= MT= Malpighian tubules.



Tissue	1	2	3	4	5	6	7	8
Rp49	76,756	439,22	95,072	30,724	957,75	60,226	3,6268	60,07
RA	1445,5	2020,7	1712,4	1124,9	1247,1	2200,3	1431,5	2214,6
RB	4250,2	600,74	2754,7	1161	5995,3	1392,1	7434,1	1399,5
RC	7338,5	772,03	668,92	49,75	578,57	1470,5	2863,2	752,72
	SG	LB	WD	T	O	B	E	MT



Tissue	1	2	3	4	5	6	7	8
Rp49	32,893	93,655	87,181	30,231	296,68	30,327	27,678	26,006
RA	5236,6	1361,9	1781,7	1478,8	1137,3	1480,3	2381,7	1962,9
RB	1201,8	1420,8	1199,9	1668,8	533,52	1201,6	549,42	676,83
RC	1354,8	1518,4	1034,8	453,64	324,35	1950,9	2083,3	694,09
	SG	LB	WD	T	O	B	E	MT

The transcripts were detected in all tissues dissected from both *D. melanogaster white* and *dE2F1*^{TF} mutant strain. Looking at the situation in the *white* strain widely different patterns were observed, illustrated by the mean concentrations in the upper diagram (Fig. 15). *dE2F1-RA* is constantly expressed in all tissues. In Malpighian tubules (MT) and the adult brain (B) it is expressed higher than *dE2F1-RB* and *dE2F1-RC*, supporting the earlier data (FENG 2005) and demonstrating its importance in these tissues. As well, *dE2F1-RA* is present in larvae brain (LB) and shows major contribution to *dE2F1* transcripts. In testis (T), beside *dE2F1-RA* and contrary to earlier results, also *dE2F1-RB* is found at a similar level. It is not clear if this expression belongs to the *dE2F1-RB* transcript or the unspecific by-product. After running the real time PCR reactions on an agarose gel, in mutant embryos an additional fragment was visible. The by-product seems to be of similar size.

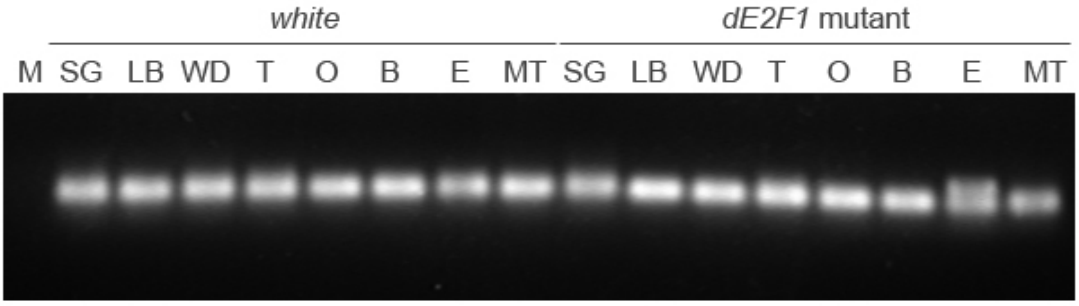


Fig. 16: The *dE2F1-RB* products after real time PCR run o a 2% gel displayed in all samples of *white* strain and *dE2F1* mutant strain show one significant fragment. In embryo of *dE2F1*^{TF} mutant a second fragment is visible.

The same case is demonstrated in salivary glands (SG), where *dE2F1-RB* showed even higher expression than *dE2F1-RA*, contrary to former results where no expression could be detected. Nevertheless the expression level of *dE2F1-RB* observed in embryos (E) and ovaries (O) are in significant amounts, supporting the earlier data (FENG 2005). *dE2F1-RB* is also highly expressed in wing discs (WD).

dE2F1-RC showed its highest transcript concentration in salivary glands, in full agreement with the earlier data (FENG 2005).

By looking at the mean concentrations of *dE2F1-RA, -RB* and *–RC* in the *dE2F1*^{TF} mutant strain (lower diagram), the expression signal varies widely and displays a completely different pattern to that of *white*. All *dE2F1* transcripts are expressed in all tissues, but, in general, at lower levels. Instead of 14.6ng as the highest concentration in *white,* only 10.5ng was produced in *dE2F1*^{TF} using the same starting amount of 1ng (see 3.6.3) for the

real time PCR reactions. *dE2F1-RA* is still constantly expressed throughout all tissues. In contrary to former results (Fig. 10; FENG 2005), *dE2F1-RB* expression is detected in low but significant amounts throughout all tissues, again indicating the production of a by-product. But especially in embryos and ovaries a significant decrease is remarkable.

4.4.3 Relative quantification

For the comparison of *white* eye flies to $dE2F1^{TF}$ mutant flies, the concentration of each sample was calculated relative to the non-regulated reference gene. For each unknown the result is expressed as a relative ratio of the contraction of the target divided by the concentration of the reference gene.

$$\mathbf{Ratio = C_{Target} / C_{Reference}}$$

Rp49 was used for the normalization of sample-to-sample differences. All relative quantification data and expression level changes are listed in the table and demonstrated in the following diagram (Fig. 17).

	Ratio with *Rp49*	SG	LB	WD	T	O	B	E	MT
dE2F1	***w***	18,8	4,6	18,01	36,6	1,3	36,5	394,7	36,9
-RA	***dE2F1^{TF}***	318,4	14,5	20,4	48,9	3,8	48,8	86,0	75,5
	Fold	**16,9** ↑	**3,16** ↑	1,13 ↑	1,34 ↑	**3,0** ↑	1,34 ↑	**4,59** ↓	2,1 ↑
dE2F1	***W***	55,4	1,4	29,0	37,8	6,3	23,1	2049,8	23,3
-RB	***dE2F1^{TF}***	36,5	15,2	13,8	55,2	1,8	39,6	19,9	26,0
	Fold	1,5 ↓	**11,1** ↑	2,1 ↓	1,5 ↑	**3,5** ↓	1,7 ↑	**103,3** ↓	1,1 ↑
dE2F1	***w***	191,2	1,8	7,0	1,6	0,6	24,4	789,5	12,5
-RC	***dE2F1^{TF}***	41,2	16,2	11,9	15,0	1,1	64,3	75,3	26,7
	Fold	**4,6** ↓	**9,0** ↑	1,7 ↑	**9,4** ↑	1,8 ↑	2,6 ↑	**10,5** ↓	2,1 ↓

Table 15: The Ratios of the three *dE2F1* transcripts normalized to *Rp49* are demonstrated. *dE2F1^{TF}* data are shaded in grey. Arrows indicate *dE2F1^{TF}* up- or down-regulation of the *dE2F1-RA*, *-RB* and *–RC* transcripts in folds compare to *white*. Main changes are marked in bold. SG = salivary glands; LB = larvae brain; WD = wing disc; T = testis; O = ovary; B = brain; E = embryo; MT = Malpighian tubules.

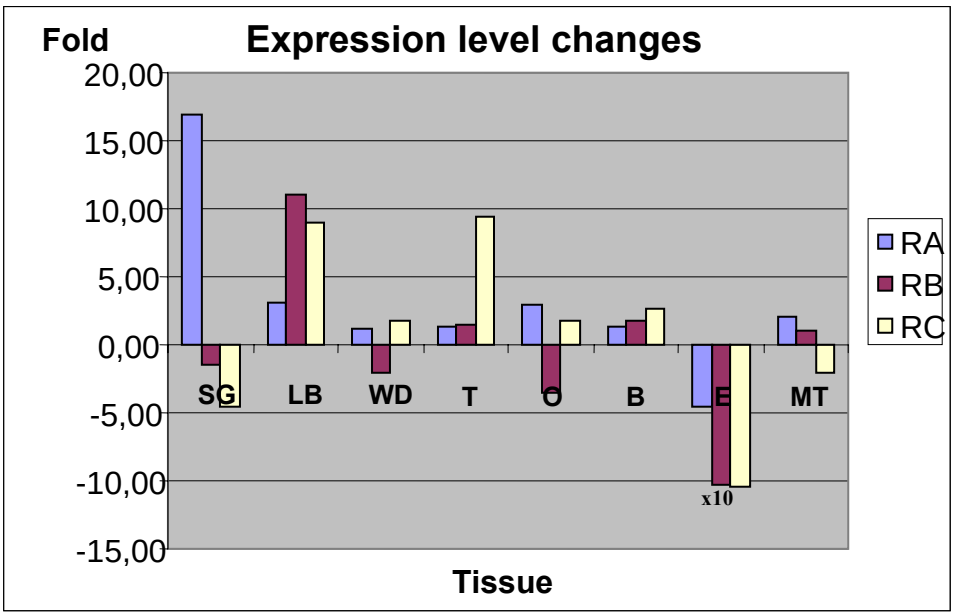


Fig. 17: The fold changes of the three transcripts of *dE2F^{TF}* to *white* are illustrated in this diagram. The value of *dE2F1-RB* was divided by factor 10 for better illustration. SG = salivary glands; LB = larvae brain; WD = wing disc; T = testis; O = ovary; B = brain; E = embryo; MT = Malpighian tubules.

The biggest change from *dE2F1 wild type* situation in *white* eye flies to mutant samples is displayed in embryos, where *dE2F1-RB* was decreased 103 folds in the mutant. This high fold is probably achieved by the produced additional extra product, shown in the agarose gel (Fig. 16). In ovaries a decrease in expression of 3.5 fold was detected. Those changes

indicate the former observed importance of *dE2F1-RB* gene product in embryogenesis and oogenesis. *dE2F-RC* also shows a 10.5 fold down-regulation in embryos of *dE2F1* mutant flies compared to *white* eye flies.

In the polytene salivary glands *dE2F1-RA* is 16.9 fold up-regulated in salivary glands, coming along with a down-regulation of *dE2F1-RC* of 4.6 fold. Those expression changes signalize the impact of the mutation of *dE2F1-RB* on the other transcripts occurring due to its misregulation. In testis and the larvae brain, undergoing rapid cell divisions, all transcripts were expressed at higher levels. The basic mechanism is unclear and needs further investigation. These experiments can only be considered as prelimiary study as they have only been carried out once. In addition, the presence of two components in the *dE2F1-RB* probes and the broad T_m range of *dE2F1-RC* need further ivestigations and the use of different primer pairs.

5. Discussion

In the mutant generated in our laboratory one specific *E2F* transcript (*dE2F1-RB*) is affected. This distinguishes the mutation from previous research on *dE2F1* null mutations which affect the open reading frame and lead to the arrest of G1-S transition combined with late development lethality (ASANO *et al.* 1996; ROYZMAN *et al.* 1997, 1999; DURONIO and O'FARRELL 1995a; BOSCO *et al.* 2001). The lethality of embryos from homozygous mutant females demonstrates that the function of the *dE2F1-RB* transcript cannot be compensated for the other two transcripts (*dE2F1-RA* and *dE2F1-RC*). Furthermore, this effect exposes the underlying specialization of the three different gene products of *dE2F1* and thus exemplifies the general importance of the transcriptome in addition to genome and proteome research. Whether this effect is based on a qualitative difference or simply reflects quantitatively insufficient production of *dE2F1* protein cannot be derived from our experiments.

5.1 The *dE2F1* protein(s)

In mammals multiple genes of the *E2F* family perform different functions which are not fully understood, but are possibly able to compensate each other partly (ATTWOOLL *et al.* 2004; KONG *et al.* 2007). Our approach on the proteomic level, to see whether multiple transcripts can compensate for the small quantity of *E2F* genes in *Drosophila* due to the formation of different translation products, could not prove the suspected existence of different protein components.

In mass spectrometry the low abundance of the transcription factor, may explain that the protein is untraceable. *E2F* is mainly expressed in the G1 phase of the cells to trigger the initiation of the S phase during cell cycle (ASANO *et al.* 1996; DURONIO *et al.* 1995b). After the entrance into the S phase, its amount falls from very high to low levels. Thus *E2F* is present in significant amounts only during a short period of cell development. In 2D-gel electrophoresis other proteins with the same or very similar isoelectric points and molecular sizes, occurring in higher abundance, could therefore easily cover the *E2F* transcription factor. Hence MALDI-TOF could not detect significant signals.

Our approach to separate *E2F* within its complex by immunoprecipitation, thereby increasing its concentration to enable the study of its protein binding partners could not be achieved by following mass spectrometry. Even though the process of immunoprecipitation appeared to be successful and several fragments could be observed (Fig. 8), *E2F* was not identified by MS. Several antibodies directed against the *dE2F1* protein were tested in western blots. Besides the sera synthesized in our laboratory, two additional antibodies were used, which were kindly provided by Terry Orr-Weaver (BOSCO *et al.* 2001; DU *et al.* 1996) and Asano (ASANO *et al.* 1996; OTHANI and NEVINS 1994) (data not shown). We obtained different immunostaining patterns using the different antisera. Those differences can be explained by differences in the target sequence of each antibody and its affinity to this epitope. Furthermore multiple reactions were detectable, revoking specificity due to cross reaction and/or signalling proteolysis. These results display the difficulty of *E2F* antibody synthesis which is not unexpected considering *E2F* as an essential and universally present protein. Antibody formation might hence induce autoimmunoreactions and might be lethal if highly specific. Difficulties in *E2F* protein research were early encountered. The *dE2F1* protein suggested by Othani and Nevins (OTHANI and NEVINS 1994) with a size of 805aa and 87.5kDa could not be detected by Asano and his colleagues (ASANO *et al.* 1996). Instead a fragment of about 100kDa as well as additional protein fragments and artefacts were detected.

The high keratin contamination in our experiments shows that working conditions in our laboratory in China are not adequately clean due to the high level of pollution for accomplishing such a sensitive experiment. The contamination occurred even though the working conditions were kept as stringent as possible. Those keratins generate additional tryptic peptides which contaminate and thus could quench the signal of interest. Finally, and even though we could not detect the protein of interest, we cannot exclude the presence of *E2F* in the samples analysed.

5.2 The *dE2F1* transcripts

The alternative splicing mechanism could not reveal potential cellular functions on the protein level. Semi-quantitative experiments have shown expression specificity of the single transcripts for certain tissues and developmental stages (FENG 2005). The

investigation in real time PCR analysis confirmed specialization, and different expression levels were measured among the transcripts. The comparison of *dE2F1*^{TF} mutant with *white* eye flies displays over-all changes in the expression levels of all transcripts of interest although only one splicing product is directly affected in the mutant. This general impact caused by the aberration of one splicing product strongly suggests co-dependency between the *dE2F1* transcripts.

Inspection of DAPI-stained embryos in our laboratory from homozygous mutant females shows a delay in development compared to *white* eye flies. Irregularities occur in the distribution of the nuclei in the syncytial blastoderm (divisions 10-13). Additionally, an effect on the microtubule structure in metaphase chromosomes emerged (FENG 2005). In some embryos development ceases during the blastoderm, in others gastrulation occurs and development leads up to early segmentation stages. Further development is arrested. This effect is most likely generated by the depletion of maternally derived *dE2F1-RB* mRNA in the embryo. Duronio and his colleagues (DURONIO *et al.* 1995b) found that flies complete their early cell cycle but DNA synthesis falls after stage 17 in embryos of *E2F* null mutants, caused by an insufficient amount of the transcription factor.
The crossing of homozygous female mutant flies with heterozygous males resulted in low offspring amounts, whereas homozygous males crossed with heterozygous females reproduced normally (see 4.1.2). This observation again proved the maternal effect of *dE2F1-RB*. These results together with the expression level changes of *dE2F1-RB* achieved by real time experiments evidenced the importance of this certain transcript for oogenesis and embryogenesis in the fly, including cells with high mitotic activity.

Nevertheless and even though homozygousity was ensured by PCR experiments (see 4.2), a small number of offspring was able to emerge up to adult stages in homozygous single crosses. The maternal *dE2F1-RB* mRNA somehow must have been in amount high enough to enable to the flies to develop. A delay in development is remarkable which was earlier found to occur in *E2F* null mutants (ROYZMAN *et al.* 1997).

dE2F1-RA is universally present in all cells, in accordance with earlier results and probably represents the canonical *E2F* factor involved in cell cycle regulation. Therefore *dE2F1-RA* seems to play an important role for all kinds of cell-cycles in the different tissues, e.g. in the testis, in the adult brain (which is known to be mitotically essentially inactive), and in

the Malpighian tubules (which are polytene, but replicative inactive). *dE2F1-RC* is mainly needed in the polytene salivary glands, undergoing high replication rates without mitosis. Recent observations, based on RNAi induced inhibition of *dE2F1-RC*, however suggest an important role of this transcript for eye and wing development, which is already established in the early embryo (LI *et al.* in prep.).

Thus different pathways seem to regulate the transcription and/or the alternative splicing of the gene products. Regulatory elements involved might be found in the *cyclin* family. *Cyclin E* for example is a known down-regulated gene of *E2F* and was shown to be of essential role during S phase in late embryogenesis (ROYZMAN *et al.* 1997). The interruption of its transcription surely affects development. Anyway, an aberration, caused by e.g. a mutation, misleads the underlying pathway and cannot immediately be stabilized in the early development.

We can conclude that the *E2F1* gene of *Drosophila melanogaster* includes different functions of *E2F*, comparable to the acquired in mammals by multiple gene products. Further studies of the interaction of three transcripts will uncover a deeper understanding of the *E2F* pathway and its role in cell cycle progression.

Due to the complexity and sensitivity of the real time PCR technique further development and optimization needs to proceed to ensure higher accuracy and reproducibility. Thus the results shown here do not display absolute data for real time PCR, but initial methodological steps for its establishment.

5.2.1 Improvements of future Real Time PCR

In mutant flies the usage of the primers for *dE2F1-RB* resulted in unexpected expression amplitude (Fig. 15 lower diagram). Since in semi-quantitative RT-PCR *dE2F1-RB* was never detectable and thus proved the absence of the transcript in mutant flies (Fig. 10; FENG 2005), its derivation in real time PCR can rather be explained by the produced by-product. Even though it was not significantly demonstrable on an agarose gel, this extra product is indicated by the additional melting peak that resulted from the melting curve analysis (Fig. 13, *dE2F1-RB*). To achieve clear data new primers will have to be designed and tested. It can also, at present, not be excluded that DNA contaminants exist, although

the biotin-binding reaction based on oligo-dT primers should remove other nucleic acid contaminants. Nevertheless, the high sensitivity of the PCR reaction can detect even small amounts or single contaminating molecules.

Rp49, the ribosomal protein chosen as reference gene, displayed low expression levels compared with the *E2F* transcripts. Since ribosomal proteins are highly expressed throughout all developmental stages and all cell types, this protein is a common used gene of reference (ALONSO and AKAM 2003; MEE *et al.* 2004; ZHAO *et al.* 2006). Due to 8bp of 19bp of the sense primer for *Rp49* being located inside the intron sequence, the efficiency to bind to its mRNA and amplify the specific product was repressed. Because identical conditions for samples and standards, as well as the same *Rp49* primers were applied for *white* and *dE2F1* mutant strains, *Rp49* can be used for normalization of all values (see 4.4.3). For future experiments, new primer will be designed spanning an intron region, but located inside the coding sequence.

Amplification curves were very accurate and confirmed a well running throughput. Still, improvements of the reaction conditions have to be proceeded, due to the low efficiencies of *dE2F1-RA*, *-RB* and *–RC* which were lower than 1.9 (Table 14). The adjustment of salt concentration ($MgCl_2$) and annealing temperatures are possible factors for enhancing the method.

7. References

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS K., WALTER, P. (2002) Molecular Biology of the Cell 4th edition, New York and London: Garland Science; c2002.

ALONSO, C.R. and AKAM, M. (2003) A Hox gene mutation that triggers nonsense-mediated RNA decay and affects alternative splicing during Drosophila development. Nucleic Acids Res. **31:** 3873-80.

ASANO, M., NEVINS, J.R., WHARTON, R.P. (1996) Ectopic E2F expression induces S phase and apoptosis in Drosophila imaginal discs. Genes Dev **10:** 1422-1432.

ATTWOOLL, C., DENCHI, E.L., HELIN, K. (2004) The E2F family: specific functions and overlapping interests. EMBO J. **23:** 4709–4716.

BONIFACINO, J.S., DELL'ANGELICA, E.C., SPRINGER, T.A. (1999) Current Protocols in Molecular Biology, Chapter **10:** Analysis of Proteins, 10.16 Immunoprecipitation. John Wiley & Sons, Inc. 10.16.1-10.16.29.

BOSCO, G., DU, W., ORR-WEAVER T.L. 2001 DNA replication control through interaction of E2F-RB and the origin recognition complex. Nat Cell Biol **3:**289-95.

CLASSON, M. and HARLOW, E. (2002) The retinoblastoma tumour suppressor in development and cancer. Nature Rev. Cancer **2:** 910–917.

CHRISTENSEN, J., CLOOS, P., TOFTEGAARD, U., KLINKENBERG, D., BRACKEN, A.P., TRINH, E., HEERAN, M., DISTEFANO, L., HELIN, K. (2005) Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription. Nucleic Acids Res. **33:** 5458-70.

DEGREGORI, J. and JOHNSON, D.G. (2006) Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis, Curr Mol Med. **6:**739-48.

DISTEFANO, L., JENSEN, M.R. and HELIN, K. (2003) E2F7, a novel E2F featuring DP independent repression of a subset of E2F-regulated genes. EMBO J. **22:** 6289–6298.

DURONIO, R.J. and O'FARRELL (1995a) Developmental control of the G1 to S transition in Drosophila: cyclin E is a limiting downstream target of E2F. Genes Dev. **9:** 1456-68.

DURONIO, R.J., O'FARRELL P.H., XIE, J.E., BROOK, A., DYSON, N. (1995b) The transcription factor E2F is required for S phase during Drosophila embryogenesis. Genes Dev. **9:** 1445-1455.

DU, W., VIDAL M., XIE J.E., DYSON, N. (1996) RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in Drosophila. Genes Dev. **10:**1206-18.

DYNLACHT, B.D., BROOK, A., DEMBSKI, M., YENUSH, L., DYSON, N. (1994) DNA-binding and trans-activation properties of Drosophila E2F and DP proteins. Proc. Natl. Acad. Sci. USA **14:** 6359-6363.

FENG, R. (2005) A maternal Effect Mutation of dE2F1 gene in Drosophila. PHD-thesis.

GÖRG, A., BOGUTH, G., HARDER, A., OBERMEIER, C., SCHEIBE, B., WILDGRUBER, R., WEISS, W. (1998) Two-Dimensional Electrophoresis of Proteins using Immobilized pH Gradients. Technical University Munich.

HORN, C. and WIMMER, E.A. (2000) A versatile vector set for animal transgenesis. Dev Genes Evol **210:** 630-637.

HUNZINGER, C., WOZNY, W., SCHWALL, G.P., POZNANOVIC, S., STEGMANN, W., ZENGERLING, H., SCHOEPF, R., GROEBE, K., CAHILL, M.A. *et al.* (2006) Comparative Profiling of the Mammalian Mitochondrial Proteome: Multiple Aconitase-2 isoforms Including N-formylkynurenine Modifications as Part of a Protein Biomarker Signature for Reactive Oxidative Species. J. Proteome Res. **5:** 625- 633.

KONG, L.-J., CHANG, J.T., BILD, A.H., NEVINS, J.R. (2007) Compensation and specificity of function within the E2F family. Oncogene **26:** 321–327.

LINDEMAN G.J., DAGNINO, L., GAUBATZ, S., XU, Y., BRONSON R.T., WARREN, H.B., LIVINGSTON D.M. (1998) A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. Genes Dev. **12:**1092-8.

LI, H., WEYRICH, A, FENG, R., TANG, X., CHEN, Y., HENNIG, W. (2007) RNAi-induced epigenetic effects of dE2F1-transcripts in Drosophila. in preparation.

MEE, C.J., PYM, E.C., MOFFAT, K.G., BAINES, R.A. (2004) Regulation of neuronal excitability through pumilio-dependent control of a sodium channel gene. J Neurosci **24:** 8695-703.

MICHIELS, F., GASCH, A., KALTSCHMIDT, B. and RENKAWITZ-POHL, R. (1989) A 14 bp promoter element directs the testis specificity of the Drosophila beta 2 tubulin gene. Embo J **8:** 1559-1565.

MORKEL, M., WENKEL, J., BANNISTER, A.J., KOUZARIDES, T., HAGENMEIER, C. (1997) An E2F-like repressor of transcription. Nature **11:** 390(6660):567-8.

OHTANI, K. and NEVINS, J.R. (1994) Functional properties of a Drosophila homolog of the E2F1 gene. Mol Cell Biol. **14:** 1603–1612.

PAPATSENKO, D., SHENG, G. and DESPLAN, C. (1997) A new rhodopsin in R8 photoreceptors of Drosophila: evidence for coordinate expression with Rh3 in R7 cells. Development **124:** 1665-1673.

ROYZMAN, I., WHITTAKER, A.J., ORR-WEAVER, T.L. (1997) Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program. Genes Dev **11:** 1999-2011.

ROYZMAN, I. and ORR-WEAVER, T.L. (1998) S phase and differential DNA replication during Drosophila oogenesis. Genes Cells **3:** 767-776.

ROYZMAN, I., AUSTIN, R.J., BOSCO, G., BELL, S.P., ORR-WEAVER, T.L. (1999) ORC localization in Drosophila follicle cells and the effects of mutations in dE2F and dDP.
Genes Dev. **13:** 827-40.

SAWADO, T., YAMAGUCHI, M., NISHIMOTO, Y., OHNO, K., SAKAGUCHI, K., *et al.* (1998) dE2F2, a novel E2F-family transcription factor in Drosophila melanogaster. Biochem Biophys Res Commun **251:** 409-415.

SHEVCHENKO, A., WILM, M., VORM, O., MANN, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. **68:** 850-8.

TEBBE, A., KLEIN C., BISLE, B., SIEDLER, F., SCHEFFER, B., GARCIA-RIZO, C., WOLFERTZ, J., HICKMANN, V., PFEIFFER, F., OESTERHELT, D. (2005) Analysis of the cytosolic proteome of Halobacterium salinarum and its implication for genome annotation. Proteomics **5:**168-79.

TRIMARCHI, J.M. and LEES, J.A. (2002) Sibling rivalry in the E2F family. Nature Rev. Mol. Cell. Biol. **3:** 11–20.

WU, L., TIMMERS, C., MAITI, B., SAAVEDRA, H.I., SANG, L., CHONG, G.T., NUCKOLLS, F., GIANGRANDE, P., WRIGHT, F.A., FIELD, S.J. *et al.* (2001) The E2F1–3 transcription factors are essential for cellular proliferation. Nature **414:** 457–462.

ZHAO, Y.M., CHEN, X., SUN, H., YUAN, Z.G., REN, G.L., LI, X.X., LU, J., HUANG, B.Q. (2006) Effects of histone deacetylase inhibitors on transcriptional regulation of the hsp70 gene in Drosophila. Cell Res. **16:** 566-76.

8. Appendix

8.1 The P-element insertion into the *dE2F1* sequence

8.1.1 P-element transformation vector

Transformation Vector: pBac[3xP3-ECFPafm]

- pBac = piggy bac

- ECFP = enhanced cyano fluorescence protein

- **af** = restriction enzymes: AscI, FseI

Promoter: beta2tubulin

cPrm2 = cDNA (of ORF) of human protamine 2

ORF: human protamine 2

3'UTR: histone variant 3.3B (H3.3B)

H3.3B = polyA sequence of histone 3.3 variant

pBac-ECFP-cPrm2-H3.3B (9399bp):

The sequence of the pBac vector form Carsten Horn and Ernst Wimmer (HORN and WIMMER 2000) is not fully known. The construct including the β2-tubulin promoter, the *human Protamine 2* ORF sequence (GeneID: 5620; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=5620) and the H3.3B– 3'UTR was cloned between the restriction sites AscI and FseI and has a total length of 2,822bp. The ECFG maker gene has an eye specific promoter 3*P3 (PAPATSENKO *et al.* 1997) which causes cyano fluorescence eyes when exposed to UV light. The exact sequence of the flanking sites is unknown. The 5'UTR flanking site involves an EcoRV restriction site and is about 700bp in size. The 3'UTR flanking site is

200bp in length and ends on the restriction enzyme sequences of: SalI, XbaI, BamHI and EcoRI.

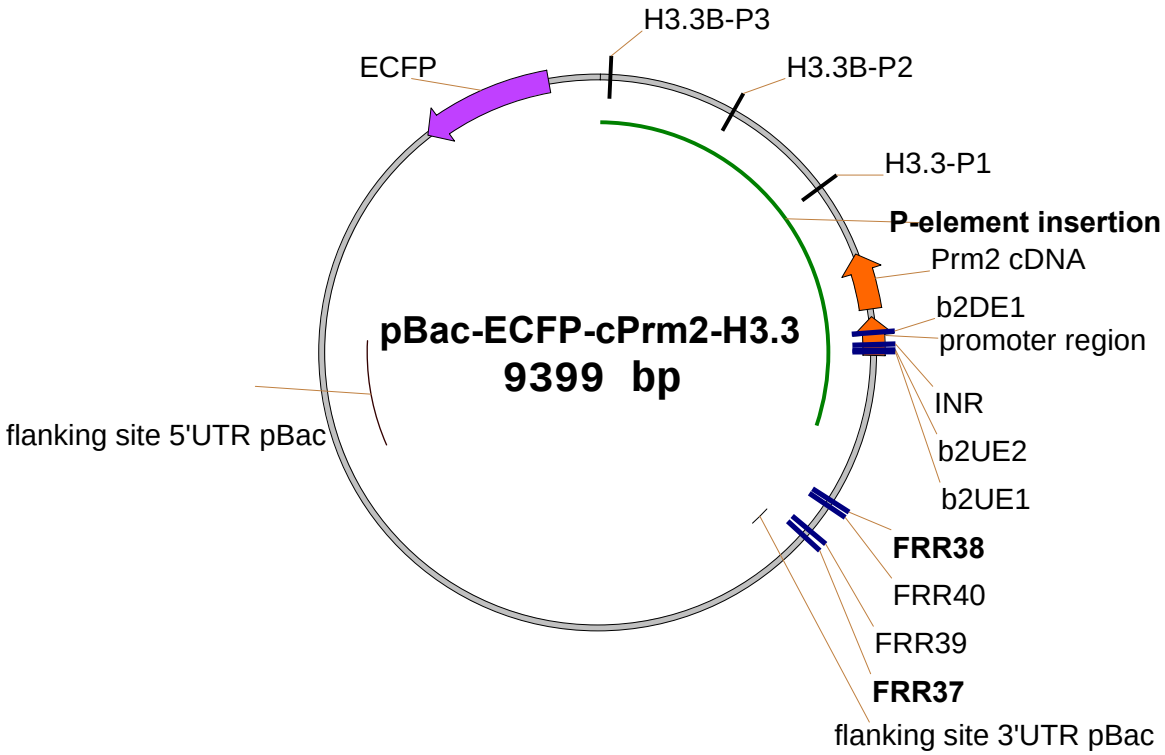


Fig. 18: pBac vector construct including the P-element and its components. The primers FRR37 and FRR38 used for localization of the P-element are marked in bold.

For localization of the P-element insertion site in the *Drosophila* genome two primers (FRR37 and FRR38) were designed reading out of the P-element inside the flanking sequence *dE2F1*. The fragments arising were then sequenced and the results blasted with the *Drosophila* genome. I later confirmed the position of the insertion point (see 4.2).

8.1.2 BLAST for P-element localization

The sequence derived with the primer FRR37 was used for blasting to identify the insertion site of the P-element.

```
  1    GTCAGCGCTG TGTTTATCAG TCGCGACACA AATATTATGC AACGATAAAG
       TTACTGATGC TAAGAAATCA AATGAGTTGG AAATAATACG TTGCTATTTC
       CAGTCGCGAC ACAAATAGTC AGCGCTGTGT TTATAATACG TTGCTATTTC
       AATGACTACG ATTCTTTAGT TTACTCAACC TTTATTATGC AACGATAAAG
 101   AATGACTACG ATTCTTTAGT TTACTCAACC TTTATGCCTA TTATATTTTT
       ATAGCCACCG ACAATTGTGG ATAAAAACTT TTGTACGGAT AATATAAAAA
       TTACTGATGC TAAGAAATCA AATGAGTTGG AAATACGGAT AATATAAAAA
       TATCGGTGGC TGTTAACACC TATTTTTGAA AACATGCCTA TTATATTTTT
 201   TATCGGTGGC TGTTAACACC TATTTTTGAA AACAAAACAG AAAAATGTGG
       ATAAAATACT ACATTTGTKT GCTTAATATA TTTATTTGTC TTTTTACACC
       ATAGCCACCG ACAATTGTGG ATAAAAACTT TTGTTTTGTC TTTTTACACC
       TATTTTATGA TGTAAACAMA CGAATTATAT AAATAAACAG AAAAATGTGG
 301   TATTTTATGA TGTAAACAKA CGAATTATAT AAATTGAATT TAAACGATAG
       TAATATCTGA CATAAAAAAA ATATGGGGTT TTTAACTTAA ATTTGCTATC
       ATAAAATACT ACATTTGTMT GCTTAATATA TTTAACTTAA ATTTGCTATC
       ATTATAGACT GTATTTTTTT TATACCCCAA AAATTGAATT TAAACGATAG
 401   ATTATAGACT GTATTTTTTT TATACCCCAA AAATCACATT ACTAACAATT
       AAGTACTGGG CGACCTTTTA GGAAAGAGCA CTGTGTGTAA TGATTGTTAA
       TAATATCTGA CATAAAAAAA ATATGGGGTT TTTAGTGTAA TGATTGTTAA
       TTCATGACCC GCTGGAAAAT CCTTTCTCGT GACACACATT ACTAACAATT
 501   TTCATGACCC GCTGGAAAAT CCTTTCTCGT GACAAGTTTT TGATTGAAAT
       CTAGAGAGAT GTGGGCATTT TTATATTCTT AGAATCAAAA ACTAACTTTA
       AAGTACTGGG CGACCTTTTA GGAAAGAGCA CTGTTCAAAA ACTAACTTTA
       GATCTCTCTA CACCCGTAAA AATATAAGAA TCTTAGTTTT TGATTGAAAT
 601   GATCTCTCTA CACCCGTAAA AATATAAGAA TCTTCATATG AAATTTTTAA
       ACCAATTGAA ACCAATCACT TTTTCTTGCT TTTGGTATAC TTTAAAAATT
       CTAGAGAGAT GTGGGCATTT TTATATTCTT AGAAGTATAC TTTAAAAATT
       TGGTTAACTT TGGTTAGTGA AAAAGAACGA AAACCATATG AAATTTTTAA
 701   TGGTTAACTT TGGTTAGTGA AAAAGAACGA AAACGGGTGC TCACTGTAGT
       CTAAAACTCG ACTTTACACT TGAGATAAAT CAATCCCACG AGTGACATCA
       ACCAATTGAA ACCAATCACT TTTTCTTGCT TTTGCCCACG AGTGACATCA
       GATTTTGAGC TGAAATGTGA ACTCTATTTA GTTAGGGTGC TCACTGTAGT
 801   GATTTTGAGC TGAAATGTGA ACTCTATTTA GTTATGAAAT GTTTGCAAAT
       GAAATCTTAG TGACGTCAGC SCCGAGATCT TATAACTTTA CAAACGTTTA
       CTAAAACTCG ACTTTACACT TGAGATAAAT CAATACTTTA CAAACGTTTA
       CTTTAGAATC ACTGCAGTCG SGGCTCTAGA ATATTGAAAT GTTTGCAAAT
 901   CTTTAGAATC ACTGCAGTCG SGGCTCTAGA ATATTTTCAC TGAAAGTGAC
       GAAATCTTAG TGACGTCAGC SCCGAGATCT TATAAAAGTG ACTTTCACTG
```

Sequence produced with FRR37 primer reading outside the p-element inside the genomic sequence.

Blast results:

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query = 608 letters

Database: dmel-assembly; dmelh-assembly

gnl|dmel|3R type=chromosome; loc=3R:1..27905053; ID=3R; release=r4.3; species=dmel; Length = 27905053

Score = 826 bits (416), Expect = 0.0
Identities = 450/462 (97%), Gaps = 2/462 (0%)
Strand = Plus / Plus

```
Query: 135      tcagcgctgtgtttatttatgcaacgataaagttactgatgctaagaaatcaaatgagtt 194
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 17485048 tcagcgctgtgtttatttatgcaacgataaagttactgatgctaagaaatcaaatgagtt 17485107
```

Identified as:

gene="E2f"
locus_tag="Dmel_CG6376"
note="CG6376 gene product from transcript CG6376-RB"
(http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=55380579&db=Nucleotide&from=17447291&to=17452219&view=gbwithparts)

8.1.3 Mutation of *dE2F1* gene

Gene name: *Drosophila melanogaster E2F transcription factor (dE2F1)*

Annotation symbol (ID): CG6376

Sequence location: Chromosome 3 R (17446026...17486127)

Full gene sequence in the flybase database:

http://flybase.bio.indiana.edu/cgi-bin/getseq.html?source=dmel&id=FBgn0011766&chr=3R&dump=PrecompiledFasta&targetset=gene

Insertion point of P-element inside the *dE2F1* gene: 9036bp

The *dE2F1* sequence used to check homozygosity by PCR (gene region 1-1599)**:**

```
   1  AGCACATTGT GCGCTAGTTG TTGCCGTTTT ACACCCAGAA ATCGCGGAGA
      TCGTGTAACA CGCGATCAAC AACGGCAAAA TGTGGGTCTT TAGCGCCTCT
                               FRR41→
  51  TATCGCCCTG AAAGCCCAAC CAATTGACAG GTGACGATCT CACCTATAAT
      ATAGCGGGAC TTTCGGGTTG GTTAACTGTC CACTGCTAGA GTGGATATTA
 101  TTGCGATCAA AAACACAAAG CTAACCCAAG GGGAACCTAG TGAAAAACGT
      AACGCTAGTT TTTGTGTTTC GATTGGGTTC CCCTTGGATC ACTTTTTGCA
 151  GGCCGAAAAA TCCATACGAA GGCGGAATGG AAAAAATGTG GTAGGTAGTA
      CCGGCTTTTT AGGTATGCTT CCGCCTTACC TTTTTTACAC CATCCATCAT
 201  CTAGAAGTAG AGAATGTGAC ATGTGTGTGT GTGTGCATGG TTGTGCGTGT
      GATCTTCATC TCTTACACTG TACACACACA CACACGTACC AACACGCACA
 251  GTGTGGGGGG TGCTGCGCTG GGCATTTTCT TTATAACAGC GGGCCGCAAG
      CACACCCCCC ACGACGCGAC CCGTAAAAGA AATATTGTCG CCCGGCGTTC
 301  CGAAACAAAA AACAACTGTT TTTGGGCGTG CGCCCAAAAC GCCGGCTAAA
      GCTTTGTTTT TTGTTGACAA AAACCCGCAC GCGGGTTTTG CGGCCGATTT
 351  AAAGTACATA TGCAAAAAAT TGCATGCCGG TCCAGCTGAC ATATCACCTG
      TTTCATGTAT ACGTTTTTTA ACGTACGGCC AGGTCGACTG TATAGTGGAC
 401  CATCAAGAAT AAGACGTATG ATGTGAGTAA AGTGTCGCGG GCGGGGGGAA
      GTAGTTCTTA TTCTGCATAC TACACTCATT TCACAGCGCC CGCCCCCCTT
 451  GAGACAACCA AGGGTGCTGT ATGATAATGC GCACTTTATA TAATTGCAGC
      CTCTGTTGGT TCCCACGACA TACTATTACG CGTGAAATAT ATTAACGTCG
 501  TACCTGCCAT CTCTAATGTT TCGTGCTTCT ATCTGTATGC GTATATGGGT
      ATGGACGGTA GAGATTACAA AGCACGAAGA TAGACATACG CATATACCCA
 551  GTGTGGGTGT GCGGGTGTGC AGGTGGGTGG CAAATGGCTA ACGGACTTCT
      CACACCCACA CGCCCACACG TCCACCCACC GTTTACCGAT TGCCTGAAGA
 601  GTCAAATTCC AATTCATGGG ATCTCCGCGC TGACGTCACT AAGATTTCAT
      CAGTTTAAGG TTAAGTACCC TAGAGGCGCG ACTGCAGTGA TTCTAAAGTA
 651  TTGCAAACAT TTCAATTTGA TTTATCTCAA GTGTAAAGTC GAGTTTTAGA
      AACGTTTGTA AAGTTAAACT AAATAGAGTT CACATTTCAG CTCAAAATCT
 701  CTACAGTGAG CACCCCAAAA GCAAGAAAAA GTGATTGGTT TCAATTGGTT
      GATGTCACTC GTGGGGTTTT CGTTCTTTTT CACTAACCAA AGTTAACCAA
 751  TAAAAATTTC ATATGTTCTA AGAATATAAA AATGCCCACA TCTCTCTAGA
      ATTTTTAAAG TATACAAGAT TCTTATATTT TTACGGGTGT AGAGAGATCT
 801  TTTCAATCAA AAACTACAGT GCTCTTTCCT AAAAGGTCGC CCAGTACTTA
```

```
       AAAGTTAGTT TTTGATGTCA CGAGAAAGGA TTTTCCAGCG GGTCATGAAT
 851   ATTGTTAGTA ATGTGTAAAA ACCCCATATT TTTTTTATGT CAGATATTAC
       TAACAATCAT TACACATTTT TGGGGTATAA AAAAAATACA GTCTATAATG
 901   TATCGTTTAA ATTCATAAAT ATATTAAGCA ACAAATGTAG TATTTTATCC
       ATAGCAAATT TAAGTATTTA TATAATTCGT TGTTTACATC ATAAAATAGG
 951   ACATTTTTCT GTTTACAAAA GTTTTTATCC ACAATTGTCG GTGGCTATAA
       TGTAAAAAGA CAAATGTTTT CAAAAATAGG TGTTAACAGC CACCGATATT
1001   AAATATAATA GGCAATTTCC AACTCATTTG ATTTCTTAGC ATCAGTAACT
       TTTATATTAT CCGTTAAAGG TTGAGTAAAC TAAAGAATCG TAGTCATTGA
                           Insertion site 1080
1051   TTATCGTTGC ATAAATAAAC ACAGCGCTGA|TTAAACAAGA GAGAACGCTT
       AATAGCAACG TATTTATTTG TGTCGCGACT|AATTTGTTCT CTCTTGCGAA
1101   CGAACGTCGA GTCACTCGAC TATTAGATAC CCCGTTACTT AGCTATTGGA
       GCTTGCAGCT CAGTGAGCTG ATAATCTATG GGGCAATGAA TCGATAACCT
1151   AGAGAGGGGA AGAAATTTCA TTTCCCTTAA CTGCACTTCA ACGATTATAG
       TCTCTCCCCT TCTTTAAAGT AAAGGGAATT GACGTGAAGT TGCTAATATC
1201   TATACCCTTT TACTCCACAA GTAACGGGTA TAAAACTAGC TTGGTTGTTG
       ATATGGGAAA ATGAGGTGTT CATTGCCCAT ATTTTGATCG AACCAACAAC
1251   ATTCATATTA CTTCTCTGAT AGCCCCAATA AAGTTCAGAC TTCTGGCAGA
       TAAGTATAAT GAAGAGACTA TCGGGGTTAT TTCAAGTCTG AAGACCGTCT
1301   CCACTATATT TTTATATTTA TATGTTGGCG TGTTGCATTT ACCGAGAAAT
       GGTGATATAA AAATATAAAT ATACAACCGC ACAACGTAAA TGGCTCTTTA
1351   AATGTTTGCC GCTAGGGAAT CGAAATCAGT GATTAGATTT CGAATTGGAG
       TTACAAACGG CGATCCCTTA GCTTTAGTCA CTAATCTAAA GCTTAACCTC
1401   CAGCAAAGCT CGTCGAAGCG TTGCCAGACT GTCCACAAGG GATGCTGTGA
                ← AW18
       GTCGTTTCGA GCAGCTTCGC AACGGTCTGA CAGGTGTTCC CTACGACACT
1451   CTGTGCACGC GATCACCTCG CCTCAGATTG GGCCAGAGAC TACTGCCAAG
       GACACGTGCG CTAGTGGAGC GGAGTCTAAC CCGGTCTCTG ATGACGGTTC
1501   GGTGTGGTTT TTATAGTGCC GTAATTGTGA TGGAAACACC ATCTAAAACC
       CCACACCAAA AATATCACGG CATTAACACT ACCTTTGTGG TAGATTTTGG
1551   TGGCGCATGA TAGCTGCACA ACCTTATCGG CGTATCAATT AGCCCAGATA
       ACCGCGTACT ATCGACGTGT TGGAATAGCC GCATAGTTAA TCGGGTCTAT
```

Sequence of *dE2F1* gene (1-2000bp). The insertion point at 1080bp is marked in blue. Primers for checking the homozygosity by PCR are marked in red, arrows indictae their direction.

8.1.4 Blast result of unspecific 500bp fragment

The 500bp fragment found by PCR (4.2, Fig. 6A) was isolated and sequenced by Alexandra Brecht. The resulting sequence does not have any known function and is so far not annotated in flybase.

file:///D:/A%20Study/E2F/HomoPCR/sequenyierung%20E2F%20500bp%20fragment/SequenzAB01.htm

```
gnl|dmel|3R type=chromosome; loc=3R:1..27905053; ID=3R; release=r4.3;
species=dmel; Length = 27905053
```

```
Score = 753 bits (380), Expect = 0.0
Identities = 472/487 (96%), Gaps = 13/487 (2%)
Strand = Plus / Plus


Query: 80       tgacgatctcattgcggcgcatatcggaggcggtcagagcacgatgaagctccacgggca 139
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 23523925 tgacgatctcattgcggcgcatatcggaggcggtcagagcacgatgaagctccacgggca
23523984


Query: 140      ctaggctgctgaatatatccttaaaattttcagccaaggggacagcaatgggcagtggct 199
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 23523985 ctaggctgctgaatatatccttaaaattttcagccaaggggacagcaatgggcagtggct
23524044


Query: 200      ttgccaactgggcttttccaggagaggtcaaggtgctcagctctggtatgatctcgttgt 259
                 | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 23524045 tggccaactgggcttttccaggagaggtcaaggtgctcagctctggtatgatctcgttgt
23524104


Query: 260      agatgaactcgttgtccttggtggactcggtcaagttgcgcttggcg-ggctgaagtatt 318
                 |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
Sbjct: 23524105 agatgaactcgttgtccttggtggactcggtcaagttgcgcttggcgcggctgaagtatt
23524164


Query: 319      catccaagtaggtctcattgccgctacg-gtctgagcc-ccttgaaaag-tcaat-gcgt 374
                 |||||||||||||||||| |||||||| ||||||||| |||||||||| ||||| ||||
Sbjct: 23524165 catccaagtaggtctcatttccgctacgcgtctgagccgccttgaaaagatcaatggcgt
23524224


Query: 375      tgcgcaa-cgagcaatctcctcgccaatcttcttggcggcacggcacaccagactctggt 433
                 ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 23524225 tgcgcaagcgagcaatctcctcgccaatcttcttggcggcacggcacaccagactctggt
23524284


Query: 434      acaactgggtgagggcatgg-agccgg-ctgctttccagcaatagtaggtatcc-ctcct 490
                 |||||||||||||||||| |||||| |||||||||||||||||||||||| |||||
Sbjct: 23524285 acaactgggtgagggcatggaagccggcctgctttccagcaatagtaggtatccactcct
23524344


Query: 491      tct-ccacaggctgcgcacagactccttttgcatagcgcgcag--catcggcgtaag-ct 546
                 ||| |||||||||||||||||||||||||||||||||||||  |||||||||||| ||
Sbjct: 23524345 tctcccacaggctgcgcacagactccttttgcatagcgcgcagcacatcggcgtaagact
23524404


Query: 547      cctccgc 553
                 |||||||
Sbjct: 23524405 cctccgc 23524411
```

8.2 Primers for semi-quantitative RT-PCR

Name	**Direct.**	**Sequence**	**Position [bp]**	**Length [bp]**	**Tm [˚C]**
Rp49 (accession Number: NT_033777)					
FRR63	sense	CCAAGGGTATCGACAACAGAG T	142-163	22	52.4
FRR64	antisense	CGACCACGTTACAAGAACTCTC A	472-450	23	53.6
Fragment size			**330 bp**		
dE2F1-PA (accession Number: NM_079713)					
FRR43	sense	ATTCAAGTCGCTGAGTAGCAAT AGC	428-452	25	55.3
FRR48	antisense	AATTAGCACCTAAGGATTCTTC ACAT	916-891	26	53.2
Fragment size			**488 bp**		
dE2F1-PB (accession Number: NM_169961)					
FRR41	sense	CAACCAATTGACAGGTGACGAT CT	67-90	24	56.8
AW48	antisense	AATTAGCACCTAAGGATTCTTC ACAT	244-219	26	53.2
Fragment size			**177 bp**		
dE2F1-PC (accession Number: NM_169962)					
FRR42	sense	TGTTCTGCAACTTATTACTCCA GTTCT	95-121	27	54.1
AW48	antisense	AATTAGCACCTAAGGATTCTTC ACAT	379-354	26	53.2
Fragment size			**284 bp**		

Table 16: Primer information of primers used for reverse transcriptase PCR by R. Feng (2005).

Zusammenfassung

Drosophila melanogaster enthält eine geringe, aber signifikante Menge an 5-methyl-Cytosin. Die von uns untersuchte männlichen Keimbahn von Drosophila weißt jedoch keine signifikanten Mengen an DNA-Methylierung auf. Eine künstliche Expression von der beiden murinen Methyltransferasen *DNMT3A* und *DNMT3B* in den Fliegenhoden, führte nicht zu der erwarteten Methylierungszunahme und hatte keinen Effekt auf die Fruchtbarkeit der Männchen. Auch die gewebespezifische Expression unter der Verwendung des UAS/GAL4-Systems zeigte keine phenotypischen Veränderungen auf.

Hingegen fanden wir auf der Protein-Ebene des Chromatins von *D. melanogaster* und *D. hydei* spezifische Modifikationsmuster der Histone H3 und H4 in der Keimbahn, wie auch in den somatischen Zellen des Hodenschlauches. Die Modifikationsmuster der beiden Zelltypen unterscheiden sich grundlegend und weichen zudem von dem für Eu- und Heterochromatin erwarteten ab. So war z.B. die als euchromatischer Marker angesehene Acetylierung von H4-K16 und H4–K5 nicht in der transkriptionell aktiven meiotischen Prophase auffindbar. Einige Histon-Modifikationen wurden nicht, wie nach anderen Untersuchungen erwartet, ausschließlich im Zellkern, sondern auch im Zytoplasma der Zelle gefunden. Diese Ergebnisse zeigen, dass der „Histon-Code“ größere Komplexität besitzt als bisher erwartet.

Folglich liegt eine epigentische Information in *Drosophila* voraussichtlich auf der Proteinebene (Histon-Code), wohingegen DNA-Methylierung keine - oder nur eine geringe - funktionelle Relevanz besitzt. In *Drosophila* würde Transkription somit durch die Struktur des Chromatins reguliert und nicht durch direktes Gen-Silencing durch Methylierung von Promoterregionen, wie diese im Säuger-Genom üblich ist.

Es wurde gezeigt, dass der Transkriptionsfaktor *E2F*, der eine Schlüsselfunktion im Zellzyklus hat, durch unterschiedliche Transkripte offenbar quantitativ reguliert wird. Diese Nachforschungen ergaben, dass die drei *E2F1* Genprodukte in *Drosophila* neben ihrer Zelltypspezifität auch in unterschiedlichen Expressionsniveaus auftreten, was die Annahme einer quantitativen Regulation unterstützt. Die verschiedenen Funktionen der multiplen Gene im Säuger könnten so funktionell kompensiert werden. Die durch die Expression der drei *dE2F1*-Transkripte mögliche Synthese verschiedener Proteine konnte bisher nicht bewiesen werden.

- CURRICULUM VITAE -

ALEXANDRA WEYRICH

PERSONAL INFORMATION:

Nationality: German
Date of birth: 20. March 1979
Place of birth: Birkenfeld/Germany

E-Mail address: alexandraweyrich@gmx.de

Home address: Gartenstrasse 2, 55758 Herborn
Laboratory address: Shanghai Institutes for Biological Sciences, MPG/DAAD-Laboratory, 320 Yue Yang Lu, Shanghai 200031, China

EDUCATION:

1985 - 1989 **Primary School**, Idar-Oberstein/Germany
1989 - 1995 **Secondary School**, Idar-Oberstein/Germany
1995 - 1998 **Economic Secondary School**, Idar-Oberstein/Germany

SCHOOL GRADUATION:

1998 **Abitur** (School-leaving examination, qualifying for University)

STUDIES:

Oct. 1998 - Dec. 2003 **Studies in Biology**
Johannes Gutenberg University, Mainz/Germany

Main Subjects: **Genetics, Botany, Zoology**

DIPLOMA & PHD:

Dec. 2002 - Aug. 2003 **Diploma thesis**
Academy for Biological Sciences, Shanghai/China

Topic: **Expression of mammalian methylases in the male germ line of *Drosophila melanogaster***

Final mark: **-very good-**

Jan. 2004 –May 2007 **PHD thesis**
Chinese Academy of Sciences, Shanghai/China

Topic 1:	**DNA methylation in the male germ line of *Drosophila melanogaster***
Topic 2:	**Histone modification in the male germ line of *Drosophila***
Topic 3:	**A transcript specific mutation of the *dE2F1* transcription factor in *Drosophila melanogaster***
Scholarship:	**Max Planck Gesellschaft**
Final Mark:	**-magna cum laude-**

OTHER ACTIVITIES:

1998	Temporary assistant Animal Hospital, Birkenfeld/Germany
2002 - 2003	**Scientific assistant of Plant Physiology** Johannes Gutenberg University of Mainz

WORKSHOPS:

Pavia, Italy - Wilhelm Bernhard Workshop - Sep.2003 (presentation)

Shanghai, China - SIBS Bioscience DAAD Alumni Meeting - May 2004

Wuhan, China - EMBO-Workshop on proteomics and human diseases - Sep. 2004 (assistant)

Asilomar, USA West Coast Chromatin & Chromosomes Meeting Dec. 2004 (presentation)

Mahabaleshwar, India - Evolution of Developmental Mechanisms - Jan. 2005

Shanghai, China - Computational Biology 2005-Challenges and Visions - Oct. 2005

Shanghai, China - Symposium "Stories DNA tells" - Dec. 2005

Manila, Philippines - EMBO-Workshop Gene Expression Analysis in Diagnostic Medicine - Sept. 2006 (presentation)

Shanghai, China - PICB, EMBO Manila-follow-up Workshop on Gene Expression Analysis in Diagnostic Medicine, Data analysis - Nov. 2006

PUBLICATIONS:

Weyrich A., Dong M., Tang X., Xu G., Schrattenholz A., Hunzinger C., Hennig W. (2007) Absence of DNA methylation in the male germ line of *Drosophila*. in preparation.

Li H., Weyrich A., Feng R., Tang X., Chen Y., Hennig W. (2007) RNAi-induced epigenetic effects of dEf1-transcripts in *Drosophila*. in preparation.