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Study of the Role of Casein Kinase I epsilon in Breast Cancer Using Drosophila as a Model

Master thesis

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Annotation

A particular allele of the Drosophila *discs overgrown* locus called dco^3 leads to hyperplastic overgrowth of imaginal wing discs. Since high rate of mutations in human homologue of the *dco* gene coding Casein kinase I epsilon was found in breast cancer tissue samples, I attempted to re-create the observed mutations in the Drosophila genome.

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Prohlašuji, že svoji diplomovou práci jsem vypracovala samostatně, pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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I owe great thanks to all my friends who had to put up with me talking about work all the time and with my delays when my „gel was running".

And last, but not least, I would like to thank my family and my almost husband for their support and keeping the faith.

Abbreviations

APC	adenomatous polyposis coli
BRCA1/2	breast cancer 1/2
CAM	cell adhesion molecule
CHEK2	CHK2 checkpoint homologue
CKI	casein kinase I
Cy	curly
dbt	double time
dco	discs overgrown
DEPC	diethyl pyrocarbonate
DIAP1	Drosophila inhibitor of apoptosis 1
DKK-1	dickkopf-1
ds	double strand
Dvl	dishevelled
FGF	fibroblast growth factor
FLP	flippase (FLP recombinase)
FRAT	frequently rearranged in advanced T-cell lymphomas
FZD	frizzled
GSK3β	glycogen synthase kinase 3β
HEK cells	human embryonic kidney cells
IR	inverted repeat
JNK	c-Jun N-terminal kinase
LEF	lymphoid enhancer factor
LRP	low-density lipoprotein receptor-related protein
mPER	mammalian PERIOD
NHEJ	non-homologous end joining
NLS	nuclear localization signal
PCP	planar cell polarity
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homologue on chromosome ten
Sb	stubble
Ser	serrate
shRNA	short hairpin RNA

SIPA1L1	signal-induced proliferation-associated protein 1 like 1
Tb	tubby
TCF	T-cell factor
yw	yellow white
ZF	zinc finger
ZFN	zinc finger nuclease

Contents

1. INTRODUCTION

1.1 BREAST CANCER

Breast cancer is by far the most frequent form of cancer diagnosed in European women, accounting for 429 900 cases in 2006 (28,9% of total) and ranking third when mortality among both sexes is considered /Ferlay et al., 2007/.

The tumours of the breast is a heterogeneous group of various proliferative diseases with well described morphology and histological grading. The vast majority of invasive breast carcinomas are believed to arise from epithelium of the terminal duct lobular unit and they are characterized by invasion of adjacent tissues and tendency to metastasise to distant sites /Tavassoli and Devilee, 2003/.

As for the molecular characterization, there is only little known about the origin and progress of the disease. As it has been known for long, there is strong predisposition in women with family history of breast cancer. The two best known genes associated with familial occurrence are *BRCA1* and *BRCA2*, first identified in 1994 and 1995 respectively /Miki et al., 1994; Wooster et al., 1995/. BRCA1 is known to play significant role in processes such as DNA repair, cell-cycle-checkpoint control, protein ubiquitination and chromatin remodelling, whereas BRCA2 is involved in homologous recombination /reviewed by Narod and Foulkes, 2004/. Alterations in some other genes have been identified to be linked with an increased risk of developing breast cancer, such as *TP53* (coding for the protein p53), *PTEN* (phosphatase and tensin homologue on chromosome ten) or *CHEK2* (coding for a serine/threonine kinase involved in cell-cycle control and DNA repair) /reviewed by Campeau et al., 2008/.

Still, familial occurrence accounts for only 5-10% of breast cancer cases and only one fourth of the familial breast cancer genes are known (Fig. 1. 1) /Balmain et al., 2003/. The development of all the other cases remains molecularly unexplained and many groups of researchers try to shed light into this issue.

1. 2 FROM *discs overgrown* TO CASEIN KINASE Iε

In 1990 a recessive lethal allele with the phenotype of overgrowing imaginal discs (Fig. 1. 2) was described in fruit fly *Drosophila melanogaster* and the locus was therefore named *discs overgrown* (*dco*) /Jursnich et al., 1990/. The gene was isolated and sequenced nine years later and it was discovered that it is identical to the previously described gene *double time* (*dbt*) /Zilian et al.,

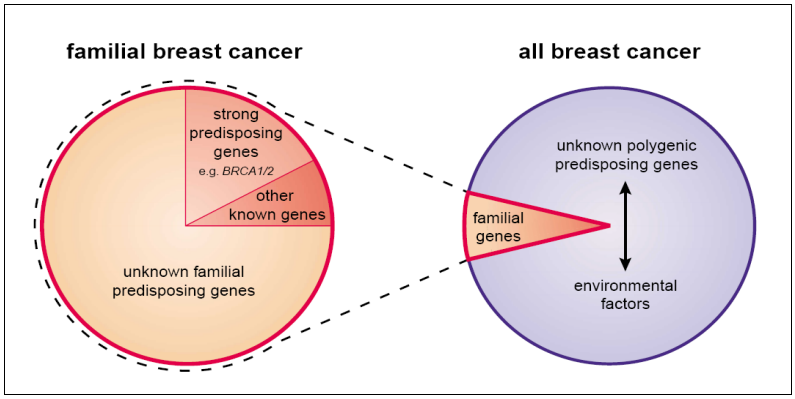

Fig. 1. 1 Proportion of known and unknown genes involved in the development of breast cancer. Sporadic cancer (shown in blue) accounts for about 90-95% of all breast cancer cases, and only about one fourth of the remaining familial occurrence (shown in red) has been proven to be linked with a known predisposing gene. Most of the genetic variants contributing to the development of sporadic breast cancer are unknown and may interact with environmental agents. (Figure taken from Balmain et al., 2003.)

1999/. Dbt was primarily found to play a significant role in controlling the period of the circadian rhythm and it was also shown to be a structural homologue of human casein kinase I epsilon (CKIε), with 86% of the amino acids composing the putative kinase domain of DBT identical to those composing the kinase domain of human CKIε /Kloss et al., 1998/.

The *dco*^{3} allele, if homozygous or in combination with deficiency in the *dco* gene, shows a phenotype of prolonged larval period (by many days), throughout which imaginal discs continue to grow to several times the wild type final size (Fig. 1. 2). The mutant discs retain their epithelial structure, hence the overgrowth should be classified as hyperplastic rather than neoplastic /Zilian et al., 1999/.

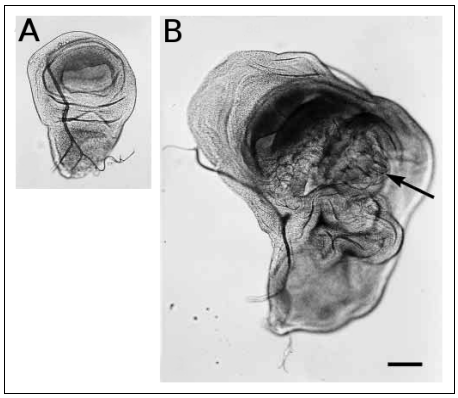

Fig. 1. 2 Overgrowth of imaginal wing disc in *dco*^{3} mutant. A/ Wild type imaginal disc, 5 days after oviposition. B/ Highly overgrown disc of a larva with heteroallelic combination of the *dco*^{3} mutation and *dco* deficiency Df(3R)PH3, 8 days after oviposition. Arrow shows the central area of highly folded epithelial layers. Scale bar corresponds to 100 μm. (Figure taken and adapted from Zilian et al., 1999.)

The overgrowing phenotype of the *dco*^{3} allele had led another research group to include the gene encoding CKIε in their investigation of somatic mutations found in human breast cancer /Fuja

et al., 2004/. To explore the potential role of three candidate tumour suppressor genes in mammary ductal carcinoma, they obtained DNA from 82 tumour samples and corresponding normal tissue samples by laser capture microdissection. Among others, they searched for somatic mutations within PCR-amplified fragments encoding critical regions of each protein using direct sequencing. As for the NH_2-terminal portion of the Ser/Thr kinase domain of CKIε, there were 19 nonsynonymous and no synonymous mutations revealed, whereof some were found repeatedly (up to 5 times, Fig. 1. 3, Tab. 1. 1) /Fuja et al., 2004/.

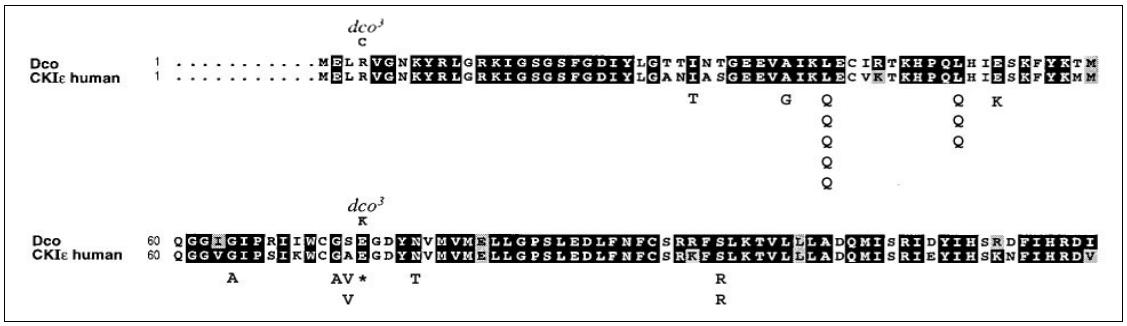

Fig. 1. 3 Amino acid sequence of N-terminal region of the Dco protein compared to the corresponding sequence of human CKIε. Alterations associated with the *dco³* allele are shown above, somatic mutations found in breast cancer tissue are shown below both sequences. Asterisk stands for mutation to a stop codon. (Figure compiled from Zilian et al., 1999 and Fuja et al., 2004.)

Patient number	Changes predicted by mutations
1	I29T, A36G, L39Q, S101R
2	E52K, G64A, A73V, E74*
3	L39Q, S101R
4	L39Q, L49Q, N78T
5	L39Q, L49Q, G72A, A73V
6	L39Q

Tab. 1. 1 Summary of the mutations in CKIε found in breast cancer samples of 6 patients out of 82 examined. The mutations are named after the original amino acid (standard one letter-code) with its sequence number and the amino acid created after the mutation is introduced /Fuja et al., 2004/.

The shown high frequency of somatic mutations may lead us to the suggestion that CKIε plays a role in the development of at least some cases of breast cancer. Unfortunately, these data are not sufficient enough to prove whether there is a causative relationship between the found mutations and development of cancer or whether they are mere passenger mutations due to the impaired DNA repair system in the tumorous cells. Therefore, subsequent functional studies, e.g. returning to the

Drosophila model, in order to estimate the actual role of the revealed mutations would be of great value.

1. 3 CASEIN KINASE Iε AND THE CELL REGULATION

Casein kinase I epsilon belongs to the CKI family of serine/threonine specific protein kinases that are evolutionary conserved and expressed in eukaryotic organisms from yeast to human. There are seven known family members, referred to as isoforms and encoded by distinct genes: CKIα, CKIβ, CKIγ1-3, CKIδ and CKIε /Fish et al., 1995; Rowles et al., 1991; Zhai et al., 1995/. CKIε is a protein of 416 amino acids with kinase domain (residues 9 to 293) of 53-98% identity to the kinase domains of other isoforms and is most closely related to the CKIδ /Fish et al., 1995/. Individual isoforms differ mainly in the amino acid sequence and length of their C-terminal non-catalytic domains as well as their N-terminal tails /Price, 2006/.

CKIε is involved in a large variety of cellular processes, such as circadian rhythms, cell proliferation and differentiation, programmed cell death or cell adhesion and its malfunction can be linked to many human diseases like sleep disorders, carcinogenesis or neurodegenerative diseases /for review, see Knippschild et al., 2005/.

1. 3. 1 CANONICAL WNT SIGNALLING PATHWAY

CKIε plays an important role in the Wnt signalling pathway that controls cell proliferation, planar polarity, cell adhesion and other processes. The most studied branch of the Wnt pathway is the canonical signalling mediated by the β-catenin dependent transcription activation of genes under control of TCF (T-cell factor)/LEF (lymphoid enhancer factor) family of transcription factors /Molenaar et al., 1996; Behrens et al., 1996/. In the absence of Wnt signalling, the level of cytosolic β-catenin is kept low by the degradation complex assembled around the scaffolding protein Axin, consisting of GSK3β (glycogen synthase kinase 3β), PP2A (protein phosphatase 2A), APC (adenomatous polyposis coli) and CKIε (Fig 1. 4) /Behrens et al., 1998; Hsu et al., 1999; Sakanaka, 2002/. GSK3β and CKIε are responsible for N-terminal phosphorylation of β-catenin (GSK3β requires the priming phosphorylation of β-catenin at serine 45 mediated by CKIε) /Ikeda et al., 1998; Sakanaka, 2002; Amit et al., 2002/, thus marking it for the ubiquitin-proteasome mediated degradation /Aberle et al., 1997/.

Stimulation of the pathway arises from binding of Wnt ligands to the Frizzled (FZD) family receptors /Bhanot et al., 1996/ and LRP5/LRP6 co-receptor (low-density lipoprotein receptor-related protein) /Pinson et al., 2000/ that pass the signal on to Dishevelled (Dvl) which negatively

affects the activity of the Axin-based destruction complex /Kishida et al., 1999/. Consequently, the cytosolic β-catenin is stabilised and relocated to the nucleus /Tolwinski and Wieschaus, 2004/, where it co-activates transcription of genes like c-MYC, FGF20 (fibroblast growth factor 20) or DKK1 (dickkopf-1) /Chamorro et al., 2005; He et al., 1998/.

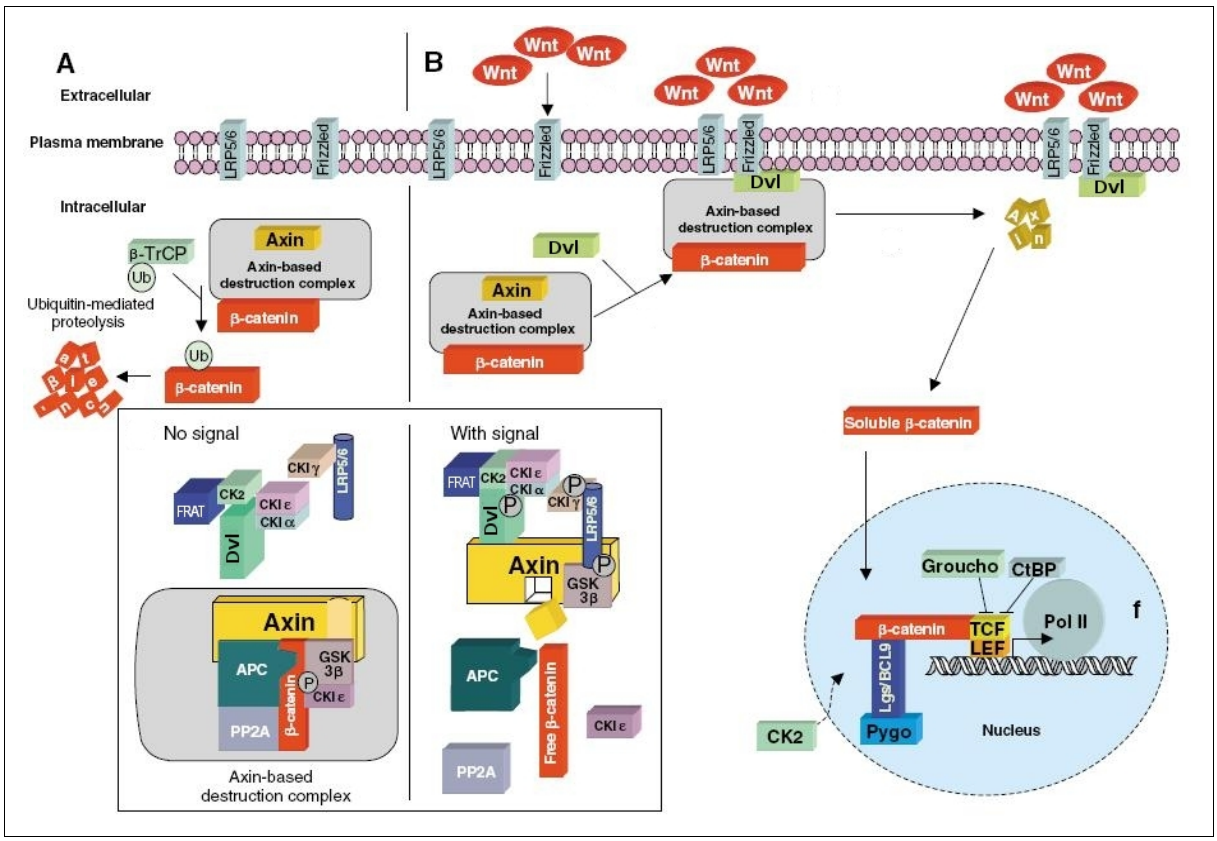

Fig. 1. 4 Scheme of the core mechanism of canonical Wnt signalling. A/ In the absence of ligand, destruction complex assembles around the scaffolding protein Axin that binds and subsequently labels β-catenin for proteolysis. B/ Wnt binds to Frizzled and triggers signalling through LRP5/6 co-receptor and Dishevelled, resulting in recruitment of Axin-based destruction complex to the membrane, where it disassembles. Consequently, the hypophosphorylated cytosolic β-catenin enters the nucleus and serves as a transcription co-activator for the TCF/LEF family of transcription factors. Black box shows detailed structure of the destruction complex and other participants of the pathway with and without present signal. (Figure adapted from Hayward et al., 2008.)

CKIε is involved in both positive and negative regulation of the canonical pathway. Its negative role consists in direct phosphorylation of β-catenin when incorporated into the degradation complex /Amit et al., 2002; Sakanaka, 2002/ as well as in Axin-dependent phosphorylation of APC with subsequent downregulation of β-catenin /Rubinfeld et al., 2001/. Moreover, CKIε has been recently shown to phosphorylate LRP5/6, resulting in its decreased ability to recruit Axin to the

membrane and therefore to contribute to the stabilisation of β-catenin /Swiatek et al., 2006/.

On the other hand, CKIε obviously regulates the pathway positively upon Wnt signalling /Sakanaka et al., 1999; Peters et al., 1999/. CKIε acts as a positive regulator downstream of Wnt and upstream of Dvl, apparently through phosphorylation of Dvl at positive regulatory sites in response to Wnt signalling, which results in its enhanced affinity to FRAT (an GSK3-binding protein) and consequently in stabilisation of β-catenin (Fig. 1. 4) /Hino et al., 2003; Klimowski et al., 2006/.

The combined role of CKIε has also been shown using genetic mutations, RNAi, dominant-negative kinase and overexpression on *Drosophila* limb development, confirming its both positive and negative roles at multiple levels /Zhang et al., 2006/.

The mechanism mediating the double-agent role of CKIε remains unclear, but it has been assumed that its negative regulatory activity may depend on the presence of active GSK3β, as CKIε alone is not able to trigger degradation of β-catenin /Rubinfeld et al., 2001/.

1. 3. 2 Non-canonical Wnt Signalling Pathways

So-called non-canonical Wnt pathways are characteristic by the absence of β-catenin as a part of the signal-transduction. One of them regulates the establishment of epithelial planar cell polarity (PCP) that is responsible for the uniform polarity of single cells or multicellular units within the plane of epithelium and plays an essential role during morphogenesis. As a typical example for PCP, I may mention the organisation of scales in fish or bristles in fly, where a single trichome emerges from the apical surface of each wing cell, pointing into the given direction.

At the beginning of the PCP pathway, Wnt signal is received by a Frizzled receptor, which leads to the accumulation of a protein complex including Dishevelled at the plasma membrane. At this level, two independent pathways lead to the activation of the small GTPases Rho and Rac. Rho finally mediates cytoskeletal re-organisation, while Rac leads to stimulation of JNK (c-Jun N-terminal kinase) activity /reviewed by Wallingford and Habas, 2005/.

CKIε was first shown to act negatively in the PCP pathway using cell-culture assays, which had led the group to the suggestion that CKIε might be the molecular switch between the canonical Wnt signalling and the PCP pathway /Cong et al., 2004/. Nevertheless, later *in vivo* experiments have shown that CKIε positively regulates the PCP and whereas its kinase activity is required for its function in the canonical signalling, its action in the PCP regulation possibly happens through a kinase-independent mechanism /Klein et al., 2006; Strutt et al., 2006/. Tsai et al. have recently identified an additional small GTPase Rap-1 acting alongside Rho and Rac downstream of non-canonical Wnt, whose activity is negatively regulated by the GTPase activating protein SIPA1L1.

According to their investigation, CKIε binds to, phosphorylates and stimulates degradation of SIPA1L1, thus positively regulating Wnt-induced morphogenesis /Tsai et al., 2007/.

One of the non-canonical Wnts, namely Wnt-5a, has been shown to induce the differentiation of dopaminergic progenitors /Castello-Branco et al., 2003/. Using in vitro gain-of-function and loss-of-function approaches, Bryja et al. have shown that CKI δ and ε are crucial for Dvl phosphorylation in response to the Wnt-5a /Bryja et al., 2007/. A later study conducted by this group has even suggested CKI together with a protein called β-arrestin to act as a molecular switch between non-canonical signalling pathways /Bryja et al., 2008/.

1. 3. 3 OTHER ROLES OF CKIε

In multicellular organisms, most individual cells are connected to each other via cell-to-cell adhesions using cell-surface proteins called cell adhesion molecules (CAMs). Cadherins belong to the Ca^{2+}-dependent CAMs and are the most crucial membrane proteins for the formation of cell-to-cell adhesion as well as its dynamic alterations during diverse physiological and pathological processes. The regulation of cadherin-based cell-to-cell contacts is not fully understood, yet Dupre-Crochet et al. have revealed the negative regulatory role of CKI in this process. They have shown that a specific inhibitor of CKIδ/ε called IC261 stabilizes cadherin-based cell-to-cell contacts, whereas the overexpression of either CKIα or CKIε acts to the contrary. Moreover, their data show that CKI phosphorylates E-cadherin at the cytoplasmic domain, upon which the endocytosis-mediated downregulation of E-cadherin is enhanced /Dupre-Crochet et al., 2007/. Yet E-cadherin is not the only one CKI-phosphorylated transmembrane adhesion protein. Occludin belongs to integral-membrane proteins associated with tight junctions and CKIε was identified as its binding partner using yeast two-hybrid system and co-imunoprecipitation from human endothelial cells. Occludin was also shown to be phosphorylated by CKIε at the C-terminal domain *in vitro*, although the biological implications of these interactions remain unclear /McKenzie et al., 2006/.

CKIδ/ε isoforms have also been shown to participate in regulation of cell division, chromosome segregation and DNA repair through phosphorylation and consequent stabilization of p53 /Knippschild et al., 1997/, phosphorylation of topoisomerase IIα at serine-1106 enhancing its activity and sensitivity to topoisomerase II-targeted drugs /Grozav et al., 2009; Chikamori et al., 2003/, and through its interaction with centrosome that, if the kinase activity is inhibited by IC261, leads to drastic amplification of spindle poles with randomly spread, fully condensed chromosomes /Behrend et al., 2000; Sillibourne et al., 2002/. A further study from *Drosophila* suggests a negative role for CKIδ/ε in programmed cell death via posttranscriptional activation of

DIAP1 expression, a protein belonging to the family of apoptosis inhibitors /Guan et al., 2007/.

The Akt signalling pathway represents another regulatory mechanism of the cell, responding to different signals such as cytokines, growth factors and oncoproteins and its misregulation has great implication for carcinogenesis. The core protein of this pathway is the serine/threonine kinase Akt that can be activated by the phosphatidylinositol 3-kinase that in turn can be negatively regulated by the phosphatase and tensin homologue on chromosome ten (PTEN) /reviewed by Crowell et al., 2007/. CKIε has been found to promote PTEN activity in hematopoietic cells upon genotoxic stress, thus downregulating the Akt pathway /Okamura et al., 2006/. Conversely, Modak and Bryant have shown CKIε to act positively in the Akt signalling in breast cancer cell lines, up-regulating the pathway in a PTEN-independent manner /Modak and Bryant, 2008/.

Finally, the already mentioned role of CKIε/*dco* in regulation of the length of circadian period (see 1. 2) consists mainly in 1/ phosphorylation of the protein mPER2 (mammalian PERIOD2), causing it to be ubiquitinated and degraded /Eide et al., 2005/; and 2/ mediation of the cytoplasmic retention of the protein mPER1 (mammalian PERIOD1) through its binding and kinase-dependent masking of the nuclear localization signal /Vielhaber et al., 2000/.

1. 4 CKIε AND CANCER DEVELOPMENT

With regard to the pleiotropic roles of CKIε described above, it comes as no surprise that this kinase has been already linked to many types of diseases with no exception of cancer development /reviewed by Knippschild, 2005/.

As shown by Umar et al. on the animal model of transmissible murine colonic hyperplasia, epithelial hyperproliferation was associated with increased CKIε abundance and nuclear translocation as well as significant CKIε/β-catenin interaction, whereas GSK-3β was found to be increasingly phosphorylated and therefore less active /Umar et al., 2007/.

Using RNAi on sarcoma cell lines and human fibroblast-derived isogenic cell lines, Yang and Stockwell have found that short hairpin RNAs (shRNAs) targeting CKIε-coding mRNA can induce selective growth inhibition in engineered tumour cells and that treatment with the inhibitor IC261 showed similar effect. Moreover, concurrent treatment by IC261 and shRNA targeted to clock proteins revealed that downregulation of PER2 suppressed IC261-induced growth inhibition /Yang and Stockwell, 2008/. The role of PER2 in tumorigenesis has already been suggested, as its deletion in mice causes increased tumour development upon gamma-irradiation /Fu et al., 2002/.

Yang and Stockwell have also implied that high level of CKIε may cause growth or survival

advantage during tumorigenesis, as cancer cells have high level of CKIε compared to normal cells, regardless of the tissue origin /Yang and Stockwell, 2008/.

The positive role of CKIε in carcinogenesis has also been proposed by Boehm et al., because the enforced expression of myristoylated CKIε induced the transformation of non-tumorigenic HEK cell line into tumorigenic colony-forming cells /Boehm et al., 2007/.

The mechanism(s) by which CKIε participates in tumorigenesis remains elusive, but with regard to the fact that many of its interacting counterparts mentioned above are known tumour-suppressors or oncogenes, its role in the process seems plausible.

1. 5 *Drosophila melanogaster* as a Model Organism

On the verge of the twentieth century, *Drosophila melanogaster* started its way up into the glare of popularity of biologists. At that time, Thomas Morgan identified the recessive *white* eye pigment mutation that was linked to the X chromosome and he found for the first time an evidence for the theory that genes are physically arrayed on chromosomes /Morgan, 1910/. Since then, *Drosophila* has evolved into a powerful tool for investigation of almost every biological process from gene arrangement over developmental biology to behaviour and disease including all the aspects in between.

Several facts make *D. melanogaster* such a powerful tool: the easiness of keeping the fly stocks together with short generation time, presence of only 4 chromosomes, sequenced genome /Adams et al., 2000/ and a variety of molecular tools facilitating experiments such as tissue-specific expression or directed mutagenesis. Since most of the signalling pathways and developmental mechanisms in vertebrates and invertebrates remained largely conserved during evolution, *Drosophila* has been successfully used for answering questions about the function of genes involved in human diseases such as developmental disorders, neurological disorders or cancer /reviewed by Bier, 2005/.

1. 5. 1 Balancer Chromosomes

The invention of balancer chromosomes was surely the most important event that has set *Drosophila* apart the other model organisms. Balancer chromosomes are multiply inverted chromosomes that are highly unlikely to undergo exchange with their normal homologs, thus simplifying segregation analysis and facilitating the establishment of “balanced” stocks and even true breeding stocks with lethal mutations. Balancers commonly carry dominant marker mutations, allowing to track the balancer during segregation by scoring the given phenotype such as curled

wings (*Curly*, *Cy*), shortened larval/pupal body (*Tubby*, *Tb*), shorter and thicker bristles (*Stubble*, *Sb*) or wings with notched margin (*Serrate*, *Ser*). They also usually carry recessive lethal mutations (often the marker mutations themselves) that prevent the balancer from taking over the stock.

For more on fly-basics, see Greenspan, 2004.

1. 5. 2 P ELEMENT-MEDIATED GERMLINE TRANSFORMATION

One of the powerful tools in *Drosophila* is P element-mediated germline transformation that allows us to introduce cloned DNA into a germline chromosome and/or disrupt a gene by insertion of the P element into its sequence.

P elements belong to transposons that are able to move within genome using the enzyme transposase. The length of P elements is variable, but they constantly contain a perfect 31-bp terminal inverted repeat (IR) and an 11-bp subterminal inverted repeat that are essential, though not sufficient for the transposition /Mullins et al., 1989/. So called autonomous P elements carry a transposase gene and are therefore able to mobilize themselves, whereas non-autonomous P elements are only capable of transposition if the enzyme is expressed ectopically /Rio et al., 1986/. These findings have been used to develop a wide range of vectors carrying IR that facilitate insertion when co-injected with another vector carrying the transposase gene. As the second "helper" vector is not able to integrate and will be therefore lost, the insertion of the construct of interest is stable and heritable /Rubin and Spradling, 1983/. In order to allow an easy screening for transgenic flies, the construct usually carries a short version of the white gene that accounts for reddening eyes of the originally white-eyed stock after insertion /Klemenz et al., 1987/.

1. 5. 3 TARGETED MUTAGENESIS USING ENDS-IN HOMOLOGOUS RECOMBINATION

In 2000, a novel method for gene targeting by homologous recombination in *D. melanogaster* was developed. It exploits the endogenous machinery for DNA reparation in response to introduction of double strand (ds) break on exogenous DNA. The method takes advantage of three P element-introduced compounds: 1/ heat shock-inducible expression of the enzyme flippase; 2/ heat shock inducible expression of the endonuclease I-SceI and 3/ donor construct bearing recognition sites for both enzymes and DNA from the locus to be targeted. Flies with all three parts are generated by crossing and after the heat-shock treatment, extrachromosomal donor DNA molecule with ds break is produced, inducing homologous recombination at the targeted site (Fig. 1. 5) /Rong and Golic, 2000/.

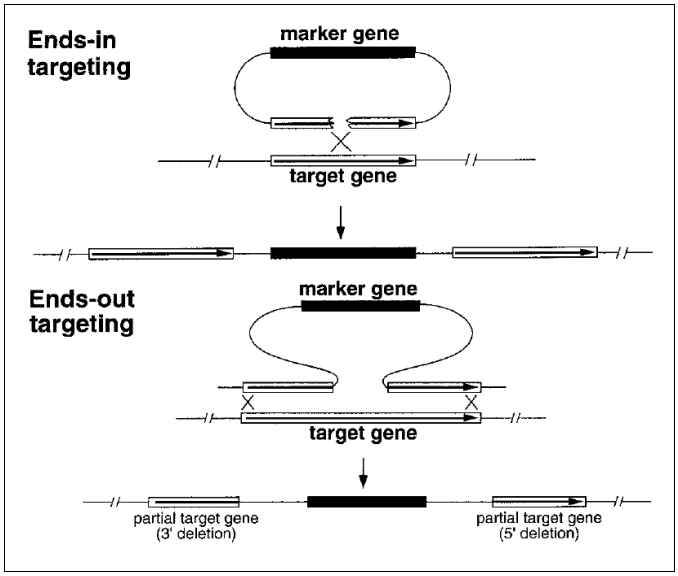

Fig. 1. 5 Diagram of the gene-targeting configurations. After the construct has been mobilized by flippase and cut by I-SceI, it initiates the homologous recombination with the target locus. Two typical forms of gene-targeting constructs are shown, with results of the recombination. If the outer parts of a gene are involved in the ends-in strategy, a duplication of the gene is produced. (Figure taken from Rong and Golic, 2000.)

The method has been later extended by an additional step in order to precisely substitute an engineered mutant allele for the endogenous wild-type allele, without leaving any other exogenous DNA behind. An additional I-CreI recognition site was introduced into the construct and after the duplication of the target gene is created, the transgenic fly is crossed with a stock bearing heat shock-inducible I-CreI endonuclease. The following double strand break of the chromosome induces homologous recombination between the two copies, resulting in reduction to only the engineered version of the gene (Fig. 1. 6) /Rong et al., 2002/.

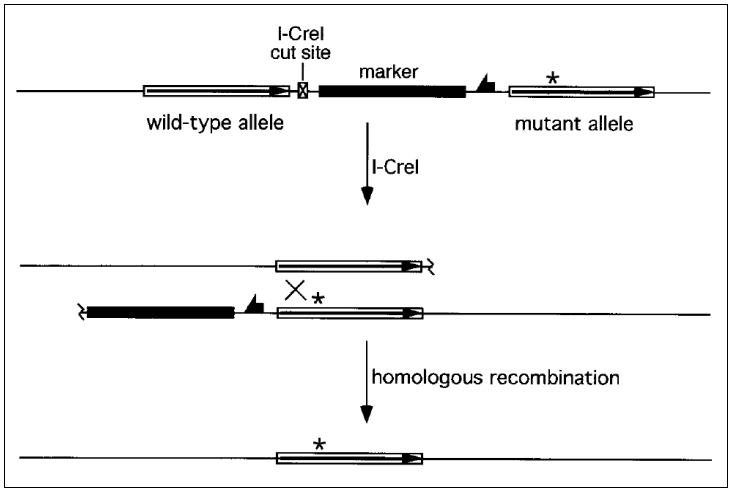

Fig. 1. 6 Diagram of the I-CreI induced reduction of the duplicated target gene. The asterisk stands for the mutation to be introduced. Depending on the particular site where the recombination occurs, the mutation will be established or lost. (Figure taken from Rong et al., 2002.)

The method has been successfully used for site-specific mutagenesis in several cases /summarized by Maggert et al, 2008/, although some groups report considerable difficulty or even impossibility in carrying out this approach when targeting some other genes /personal communication, unpublished data/. It seems likely that the extrachromosomal double strand break is

not sufficient enough to stimulate the homologous recombination with some particular genes.

1. 5. 4 TARGETED MUTAGENESIS USING ZINC FINGER NUCLEASES

Another method for introducing targeted mutations into *Drosophila* genome has been developed only recently. It is based on creation of a double strand break directly on the endogenous chromosome at the locus of interest using recombinant proteins called zinc finger nucleases (ZFNs). ZFNs combine the cleavage domain of FokI endonuclease with zinc finger proteins, thus ensuring site-specific DNA cleavage (Fig. 1. 7) /Kim et al., 1996/.

Zinc fingers (ZFs) are known DNA binding proteins mediating the sequence specificity of a wide range of transcription factors. The C_2H_2 zinc finger proteins compose the largest ZF protein family and their specific DNA binding is achieved by zinc finger domains with ββα structure, each consisting of 20-30 amino acid residues stabilised by a zinc ion that binds to a pair of cysteine and a pair of histidine residues. Each zinc finger binds to 3 nucleotides in the major groove of the DNA, with specificity provided by side chains of several amino acids on the α helices. Unlike other DNA binding proteins, zinc fingers can be linked linearly in a tandem to recognize nucleic acid sequences of different lengths, which offers a large number of combinatorial possibilities for the specific sequence recognition /Iuchi and Kuldell, 2005/.

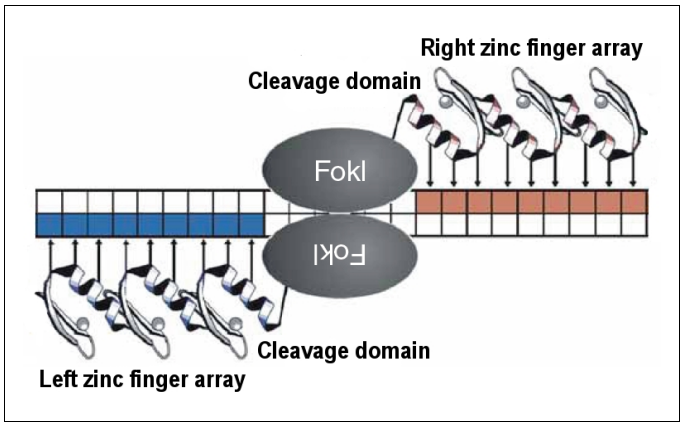

Fig. 1. 7 Sketch of a ZFN dimer bound to a nonpalindromic DNA target. Each ZFN consists of the cleavage domain of FokI fused to an array of three zinc fingers, each recognizing 3 nucleotides. Recognition sequences are separated by a spacer of either 5 or 6 bp. Simultaneous binding of both ZFNs enables dimerization of the FokI endonuclease and DNA cleavage. (Figure adapted from Miller et al., 2007.)

The first scheme of ZFNs-mediated targeted mutagenesis in *Drosophila* required P element-mediated introduction of the sequence coding for the ZFNs into the genome. Expression of ZFNs was heat-shock innducible and if introduction of specific mutation via homologous recombination was desired, a donor construct had to be involved alongside with flippase and I-SceI in order to activate it (see 1. 5. 3) /Bibikova et al., 2002; Bibikova et al., 2003/. However, with regard to the elaborate genetic constructions required, and controversy about reliability of the engineered ZFs /Ramirez et al., 2008/, this approach did not offer any significant advantage over the previously described method for directed mutagenesis (1. 5. 3).

Nevertheless, an improved protocol has been published recently, offering the possibility of yielding transgenic flies within only 2-3 generations and with such a high frequency, that new mutations can be recovered by molecular analysis, without reliance on a known mutant phenotype. In this approach, *in vitro* transcribed mRNAs coding the engineered ZFNs are injected directly into the fly embryo with the optional circular donor. The mRNAs need to be 5´-capped, 3´-polyA-tailed and have to contain the nuclear localization signal (NLS) in order to ensure proper translation and translocation of the ZFNs into the nucleus. After the assembly and DNA cleavage in wild-type flies, most of the resulting mutant flies are due to non-homologous end joining (NHEJ). However, if homologous recombination in order to introduce specific mutations is desired, the proportional yield of such mutant flies can be significantly increased to the vast majority by injecting into the *Drosophila* mutant strain lacking DNA ligase IV ($lig4^{-}$), a component of the canonical NHEJ machinery /Beumer et al., 2008/.

1. 6 AIMS OF THE THESIS

In the frame of my master thesis, I wanted to re-create mutations found within CKIε-coding sequence in breast cancer tissue samples of three patients in the genome of *D. melanogaster* and to examine the phenotype of such mutants. Since misregulation of mere wild-type *CKIε*/*dco* genes affects the cell proliferation, it would be of the greatest significance to introduce the mutation exactly to the endogenous locus via targeted mutagenesis.

2. Material and Methods

2. 1 Maintaining Fruit Fly Culture

Flies were raised on a standard cornmeal diet, consisting of 120 g cornmeal, 75 g sacharose, 60 g instant yeast, 15 g agar and 1,5 l water, supplemented with 25 ml of 10% methylparaben in ethanol. For experimental purposes, flies were raised at 25 °C, whereas in order to maintain the stocks the temperature of 18 °C was used.

Fly stocks used in this work are listed in Tab. 2. 1.

Internal stock number	Genotype	Origin	Note
121	yw; (v?); [ry+, 70FLP]4 [v+, 70I-SceI]2B Sco / S(2)CyO	Kent Golic	HS-FLP and HS-SeI endonuclease, II chromosome
123	w; [1118]; [ry+, 70FLP]10	Kent Golic	Constitutively active FLP, II chromosome
181	w; ADGF-A^{karel} / TM6B	Tomáš Doležal	III chromosome
281	yw; Xa / Cyo;MKRS	Sangbin Park	Compound II and III chromosome with Xa marker
573	yw	Sangbin Park	
1015	Df(3R)A177der22, ry[+] / TM6B	Markus Noll	*dco* deficiency, III chromosome

Tab. 2. 1 List of fly stocks used in this work. Used genetic aberrations are explained in Lindsley and Zimm, 1992.

2. 2 Constructs for P Element-Mediated Germline Transformation

I was provided with 3 pTV2 constructs bearing regions of the *dco* gene with mutations found in 3 patients (Tab. 2. 2) introduced to the coding sequence. Mutation combinations from the particular three patients were chosen from the six shown in Tab. 1. 1 because they were found most frequently. The pTV2-P3/4/6 constructs were prepared by Tomáš Doležal as follows: Fragment 1 was amplified from the genomic clone containing the *dco* gene (NotI of lambda88.9 in CasPer4#7 provided by Prof. Markus Noll, described in Zilian et al., 1999) using Phusion proof-reading polymerase (Finnzymes) and primers Kpn-dco-F2 and Xba-dco-R (Tab. 2. 3). The 2,4 kb long PCR product was cut by KpnI and XbaI restriction enzymes and cloned into the pGem-3Zf+ plasmid (Promega) cut by the same enzymes. The resulting vector was cut by SalI and XbaI restriction enzymes and I-SceI dsDNA-oligo was ligated into this linearised vector. I-SceI dsDNA-oligo was

made by annealing sal-sce-2 and spe-sce oligonucleotides (Tab. 2. 3).

Patient	Mutations found
P3	L39Q (leucine39→glutamine) and S101R (serine101→arginine)
P4	L39Q, L49Q (leucine49→glutamine) and N78T (asparagine78→threonine)
P6	L39Q

Tab 2. 2 Mutations found in breast cancer tissue of three patients /Fuja et al., 2004/.

Fragment 2 was cut out of the mentioned genomic clone containing the *dco* gene by XhoI and BamHI restriction enzymes. The 6 kb long fragment was cloned into the pBluescript II SK(+) (Stratagene) XhoI/BamHI cloning sites.

Desired point mutations were introduced into the resulting construct by QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene) using following primers: L39Q, L49Q, N78T and S101R (Tab. 2. 3).

Oligonucleotide	Sequence 5′→3′	Note
Kpn-dco-F2	GCTTGGTACCcgggcaacggagagctattc	KpnI site in nonmatching capitalized sequence
Xba-dco-R	ACTATCTAGatgcattcaaaggcccatctg	XbaI site in nonmatching capitalized sequence
sal-sce-2	TCGACtagggataacagggtaat	Capitalized is the sequence re-creating SalI site after ligation
spe-sce	CTAGattaccctgttatccctag	Capitalized is the sequence matching the XbaI overhang
L39Q	gtggccatcaagcAggagtgcatccgc	Mismatch capitalized
L49Q	ccaaacacccccagcAgcacatcgagtcaaa	Mismatch capitalized
N78T	cagcgagggcgactacaCtgtgatggtgatg	Mismatch capitalized
S101T	cttttgttcacgccgctttCGgttgaagacggttctgct	Mismatches capitalized

Tab. 2. 3 Oligonucleotides used in preparation of the pTV2 construct carrying defined regions of the *dco* gene and the point mutations found in breast cancer tissue.

Fragment 1+I-SceI was subsequently cut out from the pGem-3Zf+ vector by KpnI and SalI restriction enzymes and then ligated into into the site-mutagenized Fragment 2-pBluescript II SK(+) vector cut by KpnI and XhoI restriction enzymes.

In order to facilitate its introduction into the fly genome, the insert was subsequently cut out from the pBluescript II SK(+) using KpnI and NotI restriction enzymes and ligated into the pTV2 vector /Rong et al., 2002/ cut by the same restriction enzymes (Fig. 2. 1).

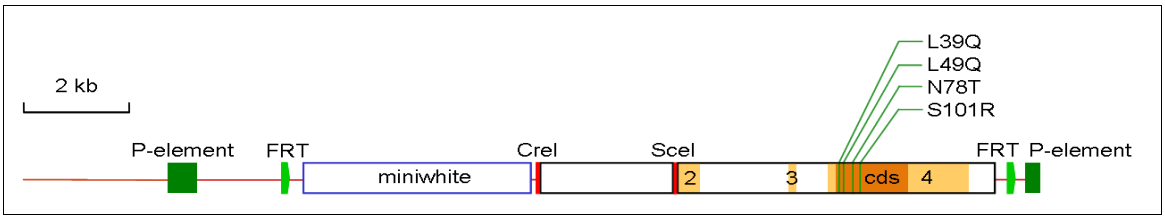

Fig 2. 1 Scheme of the pTV2 construct carrying defined regions of the *dco* gene, with the introduced mutations shown above.

2. 3 P ELEMENT-MEDIATED GERMLINE TRANSFORMATION

The pTV2 constructs were introduced into the fly genome using standard P element-mediated germline transformation via microinjection into the *Drosophila* embryos.

2. 3. 1 PREPARATION OF THE DNA FOR MICROINJECTIONS

The pTV2-P3, pTV2-P4 and pTV2-P6 vectors were transformed into the DH5α *E. coli* competent cells using standard heat-shock transformation protocol. Since the plasmids kept recombining if the cells were cultivated under standard conditions, the cultivation temperature was lowered to 30 °C, which successfully suppressed the recombination events in the host cells. The plasmids were isolated by the Qiagen Plasmid Midi Preps Kit according to the manufacturer´s protocol.

The presence of introduced mutations as well as absence of any additional mutations in the coding sequence was confirmed by sequencing using primers shown in Tab. 2. 4.

Oligonucleotide	Sequence 5´→3´
dco-seq1	GTCCGAACTCTACCACAC
dco-seq2	CCTCGATTGTGGTCGTGTGC
dco-seq3	AAGTTGCGGAAGAGTTTG
dco-seq4	TGTTTGTCTACTGTGTCGTGTCCA

Tab. 2. 4 Primers used for sequencing the *dco* gene.

1,6 µg of each pTV2 construct was separately mixed with 0,8 µg of the "helper" plasmid pUC hsPI[delta2-3} carrying the coding sequence of the transposase and the DNA was precipitated by adding 1/10 volume of 3M sodium acetate and 2 volumes of chilled 96% ethanol. After incubation at -20 °C for 15 minutes, the solution was centrifuged for 15 min at 4 °C and maximum speed, supernatant was discarded and pellet washed with chilled 70% ethanol. Dried DNA was resuspended in 25 µl of freshly prepared injection buffer (5 mM KCl; 0,1 mM NaH_2PO_4 - pH 6.8; 20x diluted green food colour – Schilling) and additionally spun at maximum speed for 5 min to remove possibly present particles that could clog the needle. A small fraction (1-2 µl) of the

injection mixture was examined by the agarose-gel electrophoresis in order to check the quantity and quality of the DNA.

2. 3. 2 PREPARATION OF MICROINJECTION NEEDLES

The microinjection needles were pulled from Capillary Glass Standard Wall Borosilicate Tubing With Filament (Sutter Instruments) using the P-97 Flaming/Brown type micropipette puller (Sutter Instruments). The program used is described in Tab. 2. 5.

Step	Heat	Pull	Velocity	Time
1	539	0	10	5
2	515	0	15	5
3	X	150	55	20

Tab. 2. 5 Program used for pulling microinjection needles. The value X ranged between 515-560, depending on the batch of the needles.

2. 3. 3 PREPARATION OF THE JUICE-GEL PLATES AND GEL-COATED SLIDES

4 g of Bacto-Agar (Scharlau) in 100 ml of black currant juice was boiled and poured into small petri dishes. Glass slides were repeatedly submerged in the same mixture until a layer of ~3 mm was built.

2. 3. 4 MICROINJECTION PROCEDURE

The *yellow white* (*yw*) flies were kept for few days on juice-gel plates with yeast paste and during the last day the plates were changed every 2 hours. These plates were supplemented with few drops of ethanol instead of yeast paste.

During the microinjection procedure, plates were changed every 30 min and within this time period, eggs laid on the plate were collected and washed with 96% ethanol. Washed embryos were aligned on the gel-coated slides and injected into the posterior end with the prepared DNA mixture using standard micromanipulator (Narishige) and the prepared needles.

It is essential to finish the microinjection within the 30 min from eggs collection, as after about 1 hour after the fertilization, the syncitial blastoderm becomes partitioned into separate cells (cellular blastoderm).

The stripes of juice-gel with the injected embryos were then transferred to the standard LB agar plates supplemented with ampicillin (50-100 μg/ml) and kept for 24 hours at 25 °C. After the first instar larvae had hatched, they were carefully transferred to the standard cornmeal diet at the density of 20 larvae per vial and cared for as usual.

2. 4 CROSSES AFTER INJECTION, MAPPING

After the injected individuals had eclosed, males and virgin females were individually crossed with the *yw* flies. The transgenic red/orange-eyed flies from their progeny were collected and crossed individually with the *yw* flies. Normally the transgenic flies are mated with *yw;Xa/CyO; MKRS* flies at this step, but for time reasons we decided to carry it out few generations later. During the next few generations, the transgenic siblings were crossed *inter se*.

In order to determine the position of the insertion, single transgenic males were mated with *yw;Xa/CyO; MKRS* virgins (Fig. 2. 2). To establish a line, siblings of the F_2 generation were mated as shown in Fig. 2. 3 and the individuals carrying both the insertion and corresponding balancer were crossed *inter se*. In case of insertion on the X chromosome, lines were established by mating flies with darker shading of the eye colour, representing homozygotes.

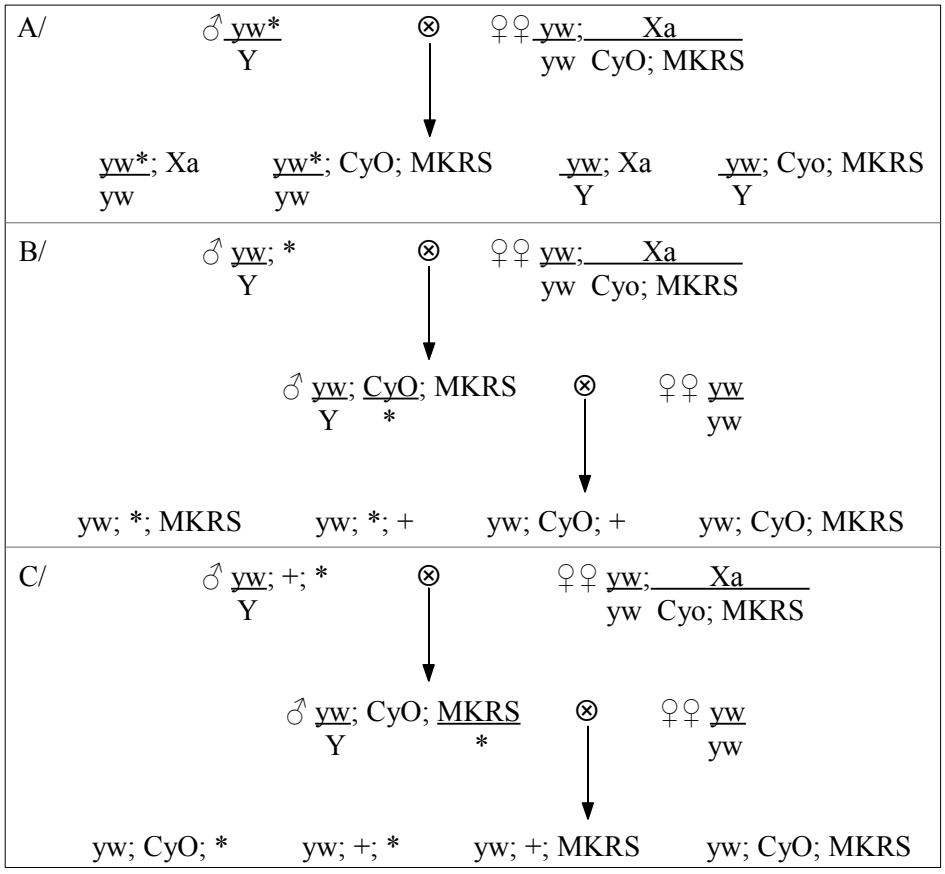

Fig. 2. 2 Mating scheme used to determine the chromosome carrying insertion. A/ In case of insertion on the X chromosome, a transgenic male will give rise only to red-eyed females, whereas all the male offspring will be white-eyed. B/ In case of insertion to the second chromosome, a red-eyed fly carrying both balancers CyO and MKRS has to be selected and mated with *yw* flies. In this case, the phenotype of red eyes will be found in no progeny carrying the CyO, whereas all flies lacking CyO will be red-eyed. C/ The case of insertion on the third

chromosome is analogous to the latter described, only the F_2 progeny will show segregation of red eyes away from the balancer MKRS.

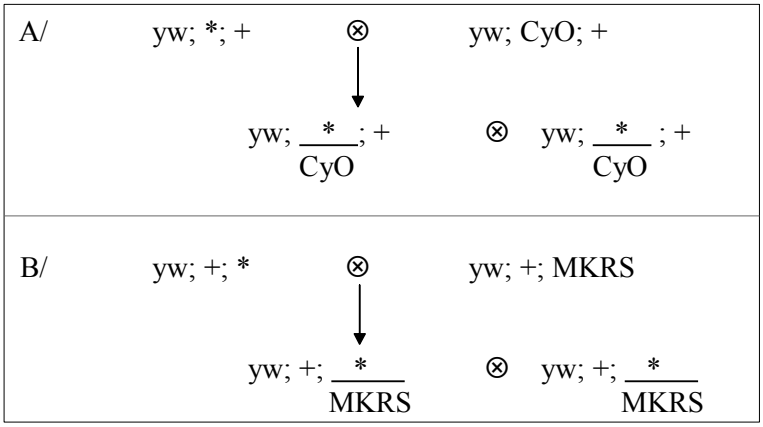

Fig. 2. 3 Establishment of a line. A/ Siblings from the cross shown in Fig. 2. 2. were crossed to obtain flies carrying both the insertion and corresponding balancer that were then crossed *inter se*. A/ Insertion on the second chromosome. B/ Insertion on the third chromosome.

2. 5 FIRST STEP OF THE HOMOLOGOUS RECOMBINATION - DUPLICATION

Transgenic flies (homozygous as well as heterozygous for the insertion) were crossed to flies carrying *FLP recombinase* and *SceI endonuclease* genes on the second chromosome under control of the heat shock-promotor (Fig. 2. 4). Mated flies were allowed to lay eggs for 24 hours and the progeny was twice heat shocked at 38 °C for 1 hour on the second and third day after oviposition.

After hatching, only virgin females were selected for the next cross, since the recombination is much more frequent in the female than in the male germline /Rong and Golic, 2000/. Females without the CyO balancer, thus carrying *FLP recombinase* and *SceI endonuclease,* were crossed to males with constitutively active FLP recombinase to mobilize every construct that had escaped mobilization after the heat shock (Fig. 2. 4). Selected females had white or mosaic eyes due to the high rate of somatic excision and loss of the donor.

The progeny was screened for re-appearance of red eyes and such individuals were crossed to *yw; Xa/ CyO; MKRS* in order to map the localization of the construct (see chapter 2. 4).

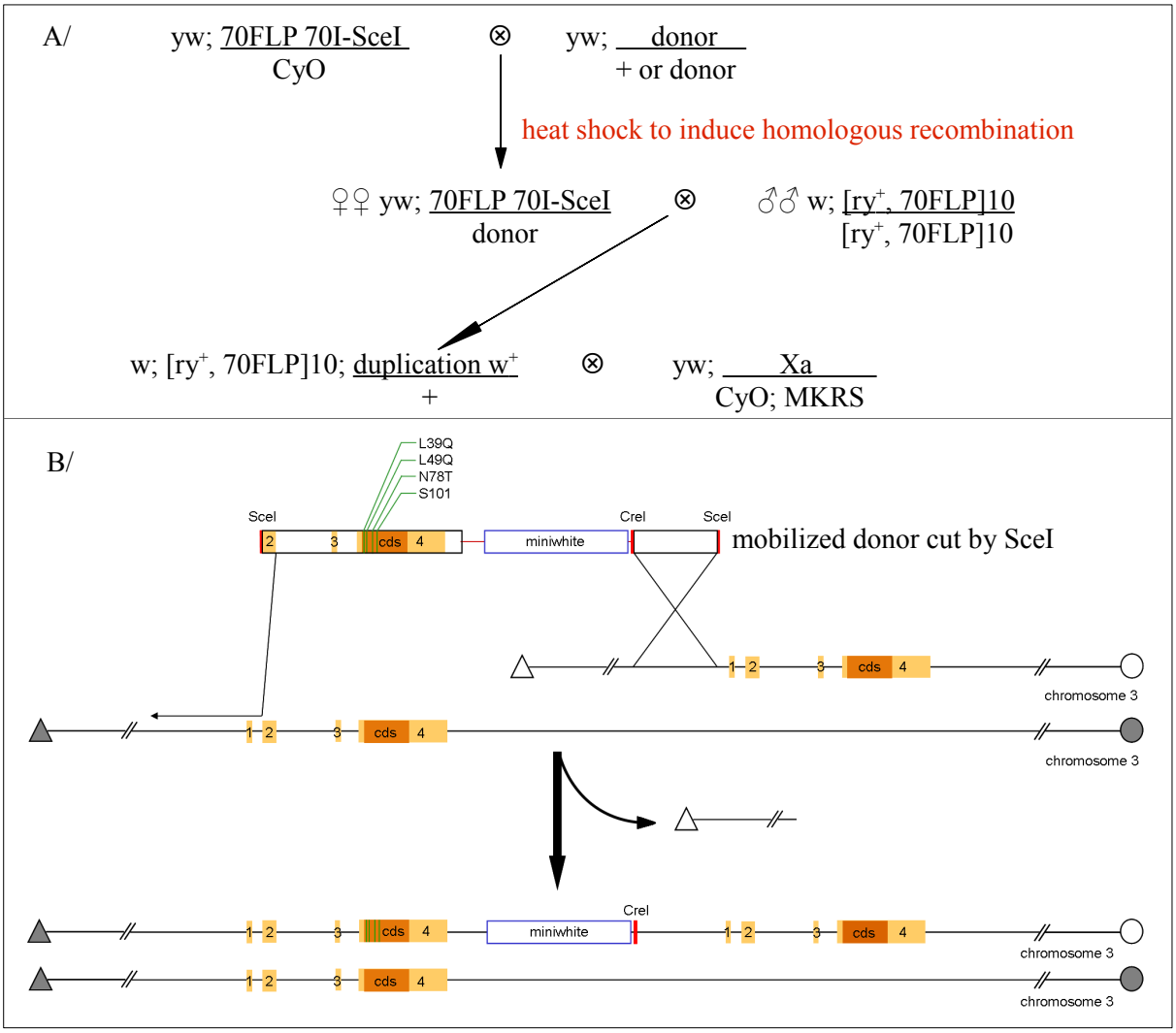

Figure 2. 4 A/ Crossing scheme used for induction of homologous recombination with resulting duplication of the target gene (for details, see chapter 2. 5). B/ Scheme of the events happening during the induction of homologous recombination with *dco* gene on chromosomal level. The ds break of the extrachromosomal donor (mobilized by FLP recombinase and cut by SceI) induces recombination with the homologous sequence, resulting in a truncated chromosome missing all DNA distal *dco*. The broken end of the centric chromosome III invades the homolog at *dco*, initiating DNA replication toward the telomere.

2. 6 CONFIRMATION OF THE DUPLICATION

2. 6. 1 PCR SCREENING

In order to screen the obtained red-eyed flies instantly, primers specific for the duplication were designed (dupl-FW and dupl-RV, see Tab. 2. 6). Since there was no possibility to test them before a duplication was created, their annealing temperature (57 °C) and specificity were confirmed in combination with another pair of primers (dupl-ctrl-FW and dupl-ctrl-RV, see Tab.

2. 6) that give rise to a product in absence of the duplication.

Since the introduced mutations could be possibly lost during the homologous recombination, allele-specific primers for the introduced mutations were designed (Tab. 2. 6) and tested using the lines bearing the P element-introduced donors with good results at annealing temperature of 58 °C. These primers should give rise to a PCR product only if the corresponding mutation is present and were designed with regard to observations of Kwok et al. In their experiments, A:G, G:A and C:C mismatches at the 3´end reduced overall PCR product yield about 100-fold, A:A mismatches about 20-fold and all other 3´-terminal mismatches were efficiently amplified /Kwok et al., 1990/.

Oligonucleotide	Sequence 5´→3´
dupl-FW	ATGGCAAACTGTCTCACGACG
dupl-RV	GGAAAGCTGTGAAATAGCCAAG
dupl-ctrl-FW	GCCAGGCAGACAAATCGAAG
dupl-ctrl-RV	CGAGTCGGTTATGCTGTTCTTC
L39Q-FW	AGGAGGTGGCCATCAAGCA
L49Q-FW	GCACCAAACACCCCCAGCA
N78T-RV	TAGCTCCATCACCATCACAG
S101-RV	AGCAGAACCGTCTTCAACCG
L/L-RV	GTGCGGTTTGTTGCAGTTTC
N/S-FW	ACAGCCGAATGCAATGAC

Tab. 2. 6 Primers used for screening for duplication of the gene *dco* and for the presence of introduced mutations.

For both types of PCR screening described, flies of interest were individually squeezed using a pipette tip in 50 µl squishing buffer (10 mM Tris-HCl pH 8,2; 1mM EDTA; 25 mM NaCl) freshly supplemented with Proteinase K (final concentration 200 µg/ml, New England BioLabs) and incubated at 37 °C for 30 min. Proteinase K was then inactivated by incubation at 95 °C for 2 min and 1-2 µl of this solution was used as a template in 15 µl PCR reaction. For screening PCR, Taq polymerase with ThermoPol Buffer (New England BioLabs) was used according to manufacturer´s instructions.

2. 6. 2 SOUTHERN BLOT

Since there was no possibility of including a positive control in the screen for duplication of the *dco* gene using PCR, it was not clear, whether the absence of a product indicated clear absence of the duplication or simple inability of the dupl-FW and dupl-RV primers to cooperate.

To confirm unambiguously whether the *dco* gene is duplicated in candidate lines or not, Southern blot was carried out.

2. 6. 2. 1 Isolation of Genomic DNA

To isolate genomic DNA, flies from each line (about 0,2 g) were grinned with pestle in liquid nitrogen-filled mortar and the powder was transferred into 10 ml of freshly prepared extraction buffer (100 mM NaCl; 10 mM Tris-HCl pH 8,0; 50 mM EDTA pH 8,0; 100 µg/ml Proteinase K; 0,5% sarkosyl). The suspension was then incubated with moderate shaking (40 rpm) at 37 °C over night.

Next day RNase A (Quiagen) at final concentration of 10 µg/ml was added and suspension was incubated under the same conditions for an additional hour and the DNA was subsequently isolated using standard phenol-chloroform extraction. Precipitation was done using 0,1 volume of 3M Na-Ac and 0,7 volume of isopropanol. The DNA in form of fibres was collected by a sterile glass hook, carefully washed in 70% ethanol and let dissolve in 100 µl of TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8,0).

The remaining solution with precipitated DNA fragments was centrifuged, the pellet was washed with 70% ethanol and then dissolved in 100 µl of TE buffer.

2. 6. 2. 2 Preparation of the DNA Probe

The template for random-primed synthesis of DIG-11-dUTP labelled DNA probe was prepared using PCR with primers dcoF and S101-RV (Tab. 2. 7) and diluted plasmid pTV2-P3 as a template. ExTaq polymerase (Takara) was used and the reaction mixture of 50 µl was prepared according to manufacturer´s instructions, with annealing temperature of 58 °C. The PCR product was then purified using Invisorb Fragment Cleanup kit (Invitek).

1 µg of the PCR product was mixed with miliQ water up to 16 µl, boiled for 10 min to completely denature the DNA, then chilled in ice-water bath and 4 µl of DIG-High Prime labelling solution (Roche) was added. The mixture was incubated for 20 hours at 37 °C and the reaction was stopped by adding 2 µl of 0,2 M EDTA.

Oligonucleotide	Sequence 5´→3´
S101-RV	AGCAGAACCGTCTTCAACCG
dcoF	GTGGCGCCTGCTGGAACAAC

Tab. 2. 7 Primers used for amplification of DNA template for synthesis of the labelled DNA probe.

2. 6. 2. 3 DNA Electrophoresis and Transfer to Membrane

6 µg of each genomic DNA was cut by EcoRV restriction enzyme using 75 units of the

enzyme in total volume of 110 µl, out of which 8 µl was subsequently checked for proper restriction using agarose-gel electrophoresis. DNA from the remaining solution was precipitated by adding 1/10 volume of 3M Na-Ac and 2,5 volume of 96% ethanol. After the incubation at room temperature for 30 min, the DNA was centrifuged for 30 min at maximum speed at 4 °C, washed with 70% ethanol and re-dissolved in 15 µl of TE buffer.

The whole amount of cut DNA was loaded on 0,7% agarose gel using 6x loading dye (10 mM Tris-HCl pH 7,6; 0,03% bromphenol blue; 0,03% xylene cyanol FF; 60% glycerol; 60 mM EDTA). The DNA Molecular Weight Marker II, DIG-labelled (Roche) was incubated at 65 °C for 10 min, chilled on ice and then loaded on the gel simultaneously with the other samples. The gel was run in TAE buffer (40 mM Tris base; 20 mM acetic acid; 1 mM EDTA pH 8,0) at 35V for the first 30 min and then at 70 V.

After separation, the gel was submerged in 0,25 M HCl for 10 min (until the bromphenol blue turned yellow) and then washed twice in distilled water. The gel was subsequently incubated twice in denaturation solution (0,5 M NaOH; 1,5 M NaCl) for 15 min, washed twice in distilled water and incubated twice in neutralization solution (0,5 M Tris-HCl; 3M NaCl; pH 7,5) for 15 min.

The DNA was then transferred over night from the gel to nylon membrane Hybond N+ (Amersham) due to capillary effect using 20x SSC buffer (3 M NaCl; 0,3 M sodium citrate; pH 7,0) as shown in Fig. 2. 5.

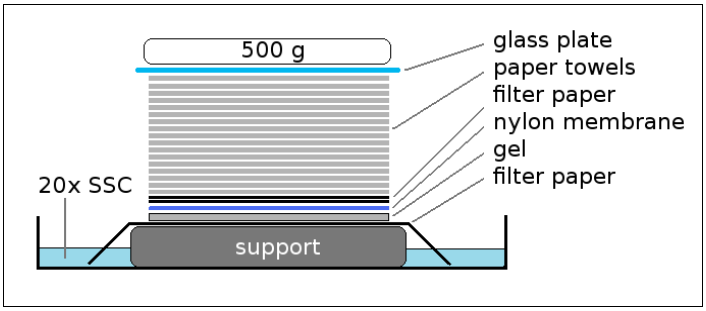

Fig. 2. 5 Layout of arrangement used for capillary transfer of DNA from agarose gel to a nylon membrane.

2. 6. 2. 4 HYBRIDIZATION AND SIGNAL EXPOSURE

After transfer, the nylon membrane was washed for 5 min in 6x SSC buffer and the DNA was fixed to membrane by UV-crosslinking (120 mJ, Stratagene). Membrane was washed in 2x SSC and prehybridized in 18 ml of pre-warmed DIG Easy Hyb solution (Roche) at 42 °C with moderate shaking for 30 min. About 200 ng of the labelled probe was mixed with 50 µl of miliQ water and denatured at 95 °C for 5 min, chilled on ice and mixed with 6,5 ml of pre-warmed DIG

Easy Hyb solution. The prehybridization solution was replaced by the hybridization solution containing the probe and incubated over night at 42 °C with moderate shaking.

Next day, the hybridization solution was removed, membrane washed twice in Stringent wash buffer I (2x SSC; 0,1% SDS) for 5 min at room temperature and then washed twice in Stringent wash buffer II (0,5x SSC; 0,1% SDS) for 15 min at 68 °C. Membrane was subsequently washed in Washing buffer (0,3% Tween; 25 mM Tris-HCl; 0,2 M NaCl; pH 7,5) and incubated in Blocking solution (5% fat free dry milk; 25 mM Tris-HCl; 0,2 M NaCl; pH 7,5) for 30 min with moderate shaking.

Afterwards, membrane was placed on an even surface, covered with 5 ml of Blocking solution supplemented with 5 µl of Anti Digoxigenin – POD Fab Fragments (Roche) and incubated for 30 min. The solution was then washed away by Washing buffer (3 times for 10 minutes, moderate shaking) and membrane was covered with 3 ml of compound solution SuperSignal West Dura Extended Duration Substrate (Pierce). After 5 min incubation, the solution was drained away and the wet membrane wrapped into a transparent plastic sheet was exposed in MF-ChemiBIS device (DNR Bio-Imaging Systems Ltd.).

2. 7. DESIGN OF ZINC FINGER NUCLEASES

/Carroll et al., 2006/

In order to target the *dco* gene with ZFNs, its sequence was searched for a suitable site using two on-line search engines (http://zincfingertools.org and http://www.zincfingers.org). Based on the personal communication with Kelly Beumer (Prof. Carroll lab), a sequence GTC CAC CTC gattgt GGT GCT GTG (capitalized are triplets that interact with zinc fingers in ZFNs) was selected. The amino acid sequence of zinc fingers corresponding to these triplets was designed following instructions in Carroll et al, 2006 and using the same on-line engines mentioned above (Tab. 2. 8).

Zinc finger	Triplet	Amino acid sequence
Left 1	GAC	DRSNLTR
Left 2	GTG	RSDALSR
Left 3	GAG	RSDNLTR
Right 1	GTG	RSDALTR
Right 2	GTC	QSSDLTR
Right 3	GGT	TSGHLVR

Tab. 2. 8 Amino acid sequence of zinc fingers binding specific triplets /Liu et al., 2002/.

Sequence of the specificity-determining residues was then pasted into the backbone taken from the transcription factor Zif268 (Tab. 2. 9) and reverse translated with regard to the *Drosophila* codon usage.

	Amino acid sequence
Finger L1	MEPYACPVESCDRRFS **DRSNLTR** HIRIH
Finger L2	TGQKPFQCRICMRNFS **RSDALSR** HIRTH
Finger L3	TGEKPFACDICGRKFS **RSDNLTR** HTKIHLRQK
Finger R1	MEPYACPVESCDRRFS **RSDALTR** HIRIH
Finger R2	TGQKPFQCRICMRNFS **QSSDLTR** HIRTH
Finger R3	TGEKPFACDICGRKFS **TSGHLVR** HTKIHLRQK

Tab. 2. 9 Amino acid sequence of left and right zinc finger array. The specificity determining residues (bold) are pasted into the Zif268 framework. The cysteines and histidines that coordinate the zinc atom are in red /Carroll et al., 2006/.

The engineered DNA sequence was then created using the seven primer strategy (Fig. 2. 6, Tab. 2. 10). The PCR reaction was mixed following instructions in Carrol et al., 2006 and since proof-reading polymerases were reported to have a lower yield in this approach, Taq polymerase with ThermoPol Buffer was used. Touchdown PCR was run (Tab. 2. 11) and the reaction products were fractionated by electrophoresis on a 1,5% agarose gel. The 285 bp long product was cut from the gel and purified using Invisorb Fragment Cleanup kit.

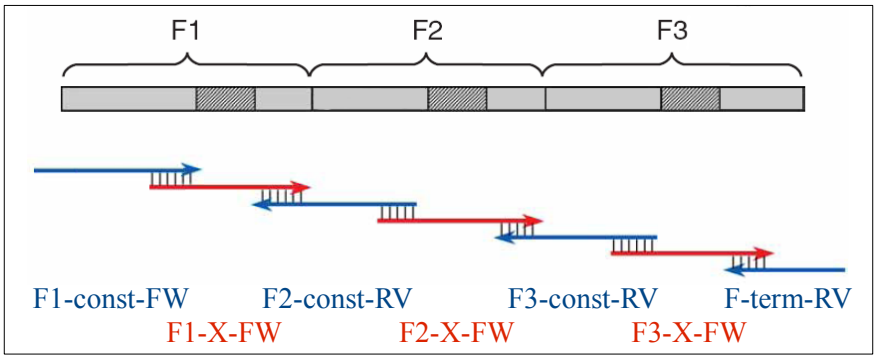

Fig. 2. 6 Scheme of seven-primer strategy for synthesizing zinc finger coding sequences. The structure of the desired three-finger product is shown above, with the specificity-determining residues textured. Each arrow represents a separate oligonucleotide, with the arrowheads on 3′ ends and the name (used in later design where X is replaced with the specific oligonucleotide name, Tab. 2. 10) shown below. The blue oligonucleotides are constant, as they encode the Zif268 framework and in red are oligonucleotides coding the specificity-determining regions. (Figure adapted from Carroll et al., 2006.)

Oligonucleotide	Sequence 5′→3′
F1-const-FW	cggcagccatATGGAGCCCTACGCCTGCCCCGTGGAGAGCTGCGATCGCCGCTTCAGC
F2-const-RV	GCTGAAGTTGCGCATGCAGATGCGGCACTGGAAGGGCTTCTGGCCGGTGTGGATGCGG
F3-const-RV	GCTGAACTTGCGTCCGCAGATATCGCAGGCGAAGGGCTTCTCGCCGGTGTGGGTGCGG
F-term-RV	ttgactagttgCTTCTGGCGCAGGTGGATCTTGGTGTG
F1-dcoLEFT-FW	CTGCGATCGCCGCTTCAGCGATCGCAGCAACCTGACCCGCCACATCCGCATCCACACCGGCC
F2-dcoLEFT-FW	CTGCATGCGCAACTTCAGCCGCAGCGATGCCCTGAGCCGCCACATCCGCACCCACACCGGC
F3-dcoLEFT-FW	CTGCGGACGCAAGTTCAGCCGCAGCGATAACCTGACCCGCCACACCAAGATCCACCTGCGC
F1-dcoRIGHT-FW	CTGCGATCGCCGCTTCAGCCGCAGCGATGCCCTGACCCGCCACATCCGCATCCACACCGGCC
F2-dcoRIGHT-FW	CTGCATGCGCAACTTCAGCCAGAGCAGCGATCTGACCCGCCACATCCGCACCCACACCGGC
F3-dcoRIGHT-FW	CTGCGGACGCAAGTTCAGCACCAGCGGCCACCTGGTGCGCCACACCAAGATCCACCTGCGC

Tab. 2. 10 Oligonucleotides used for creation of zinc finger-coding fragments. The oligonucleotides F1-const-FW, F2-const-RV, F3-const-RV and F-term-RV correspond to the sequence of the Zif268 framework and can be therefore used for creation of a variety of zinc finger arrays. The non-capitalized sequence contains the SpeI and NdeI sites used for cloning the fragments into the pENT-NLS-G-FN vector.

Cycle number	Denature	Anneal	Extend
1	95 °C for 5 min		
2 - 11	94 °C for 30 sec	62° C for 30 sec	72 °C for 30 sec
		decreasing 1 °C per cycle	
12-30	94 °C for 30 sec	52° C for 30 sec	72 °C for 30 sec
31	72 °C for 5 min		

Tab. 2. 11 Program of the touchdown PCR used for creation of the zinc finger array-coding sequences, TGradient Thermocycler (Biometra).

The fragment was then cut by SpeI and NdeI restriction enzymes, purified by Invisorb Fragment Cleanup kit and ligated into the vector pENT-NLS-G-FN (Fig. 2. 7) cut by the same restriction enzymes. This vector contains in frame sequence coding the FokI cleavage domain and the nuclear localization sequence that is essential for the proper function of the ZFNs in fly embryo.

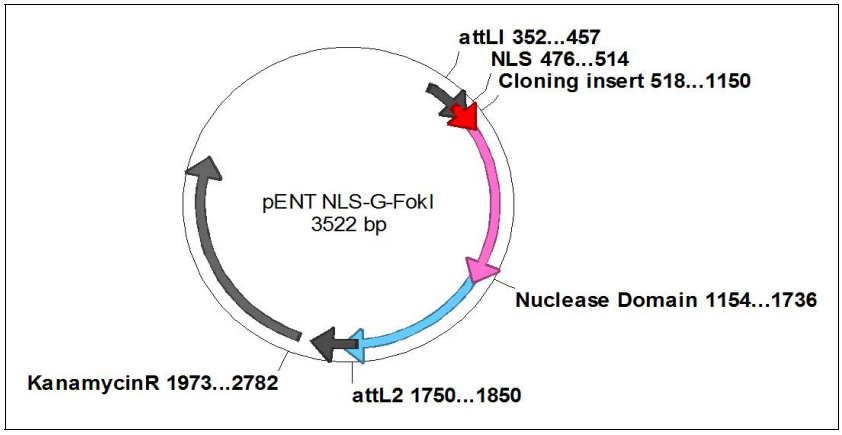

Fig. 2. 7 Scheme of the pENT-NLS-G-FN vector. The blue arrow shows the FokI endonuclease domain, red arrow shows the nuclear localization signal and the pink arrow corresponds to a spacer sequence to be replaced by ZFN-coding insert. The sequences attL1 and attL2 are important for subsequent *in vitro* recombination with a destination vector.

As the oligonucleotide synthesis as well as the usage of non-proof reading polymerase may introduce mutations into the sequence, the candidate clones were sequenced using the primers pENTseqFW and pENTseqRV (Tab. 2. 12).

Oligonucleotide	Sequence 5´→3´
pENTseqFW	CGCCGGAACCAATTCAGTC
pENTseqRV	TTCTATCCTGAGTGGAATTTCTGG

Tab. 2. 12 Primers used for sequencing the insert in pENT-NLS-G-FN and pCS2-DEST vectors.

The complete coding sequences containing the left/right zinc finger array, FokI cleavage domain and the NLS were then shuttled into the *in vitro* transcription vector pCS2-DEST using the Gateway LR Clonase II enzyme mix for *in vitro* recombination (Invitrogen) according to manufacturer´s instructions.

The whole strategy described above is summarized in Fig. 2. 8.

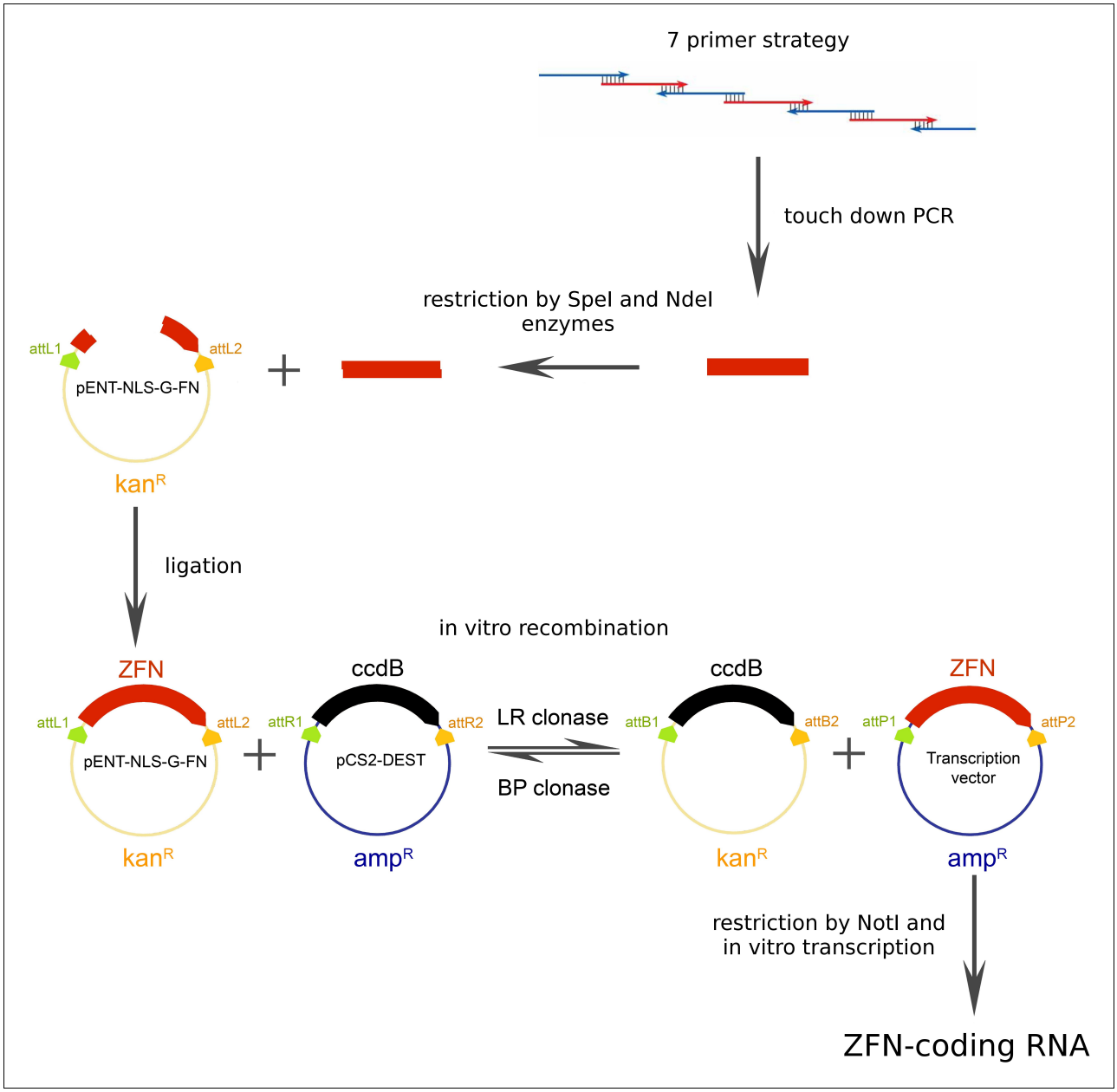

Fig. 2. 8 Summary of the strategy for creation of ZFNs-coding mRNAs. The touch-down PCR with 7 designed oligonucleotides was followed by restriction of the fragment by SpeI and NdeI restriction enzymes and ligation into the pENT-NLS-G-FN vector containing in-frame sequence coding NLS and FokI endonuclease domain. The whole ZFN-coding fragment was then shuttled into the pCS2-DEST vector using *in vitro* recombination, occurring between attL1/attR1 and attL2/attR2 sequences. The ccdB gene that encodes poison of topoisomerase II serves as a means of negative selection, causing death of majority of the laboratory strains of *E. coli*. The pCS2-DEST vector containing ZFN-coding sequence was then cut by NotI restriction enzyme and used as a template for *in vitro* transcription.

The resulting vectors were once more sequenced using the primers pENTseqFW and pENTseqRV. The confirmed and by Qiagen Plasmid Midi Preps Kit isolated plasmids were then cut by NotI restriction enzyme in order to create template DNA for *in vitro* transcription. The cut DNA was then treated by proteinase K (100 µg/ml) and 0,5% SDS (30 min at 50 °C) to remove any possibly present RNases. Proteinase K was then inactivated by incubation at 95 °C for 5 min. DNA was extracted by adding 0,5 volume of phenol and 0,5 volume chloroform, thoroughly mixed and centrifuged for 2 min. DNA in the taken aqueous phase was precipitated by adding 2 volumes of 96% ethanol and 0,1 volume of NH_4-acetate. After incubation at -20 °C for 1 hour, the solution was centrifuged for 15 min at maximum speed and the pellet was resuspended in 10 µl of DEPC-treated water.

The capped RNA was then transcribed from 1 µg of the template DNA using mMessage mMachine SP6 transcription kit (Ambion) according to manufacturer´s instructions.

The quality of transcribed RNA was examined by agarose gel electrophoresis. 1,2 µl from the total volume of 20 µl was filled up to 4 µl with water, mixed with 4 µl of the Gel Loading Buffer II (95% formamide, 0,025% xylene cyanol, 0,025% bromphenol blue, 18 mM EDTA, 0,025% SDS; Ambion) and the solution was heated to 85 °C for 4 min. After chilling on ice, the solution was loaded to 1,2% agarose gel alongside with 0,5 – 10 kb RNA Ladder (Invitrogen) that was treated in the same manner.

The remaining RNA was then precipitated by adding 0,1 volume of Na-Ac and 2,5 volumes of 96% ethanol. After incubation at -20 °C for 1 hour, the solution was centrifuged at maximum speed for 15 minutes at 4 °C and the pellet was then resuspended in 10 µl of RNase free water (Sigma Aldrich).

2. 8 CONSTRUCTION OF THE DONOR FOR ZFNS-MEDIATED MUTAGENESIS

In order to estimate the effectiveness of the approach, we decided to introduce first a marker that would be easy to score instead of the point mutations. For this reason, the *miniwhite* gene was cut out from the pTV2-P6 construct using the PstI restriction enzyme and ligated into the pBluescript II SK(+) plasmid containing the fragment 2 (and therefore the *dco* coding sequence, see chapter 2. 2) cut by the EcoT22I restriction enzyme (Fig 2. 9). This was possible, because PstI and EcoT22I produce compatible ends.

2. 9 MICROINJECTION OF ZFNS-CODING MRNA

The microinjection buffer was mixed 5x concentrated (25 mM KCl; 0,5 mM NaH_2PO_4 - pH 6.8; 4x diluted food colour) and since it was not possible to prepare the food colours under RNase-free conditions, the potential RNase activity of the injection buffer was tested. About 5 µg of total fly RNA was mixed with water and microinjection buffer up to 8 µl and incubated over night at -20 °C, then kept on ice for 2 hours and finally let stand at room temperature for 1,25 hour in order to simulate conditions of the microinjection procedure. As a control, the same amount of total fly RNA was diluted in RNase free water up to 8 µl and treated in the same manner.

The mixtures were then examined by agarose gel electrophoresis as described above. Since none of the colours showed signs of RNase activity, the usual green colour was used for preparation of the microinjection mixture.

2. 9. 1 MICROINJECTION OF MRNA WITH DNA DONOR

Various concentrations of both mRNAs and the donor were tried in order to optimize the yield of transgenic flies. Between about 0,7 µg/µl and 1,7 µg/µl final concentration of each ZFN mRNA were injected with 1,1-1,5 µg/µl final concentration of the DNA donor into the *yw* embryos as described in chapter 2. 3, only that the working place and equipment was kept as RNase-free as possible.

The eclosed injected individuals were then crossed with *yw* flies and their progeny were screened for appearance of red red eyes due to incorporation of the *miniwhite* gene (Fig. 2. 9).

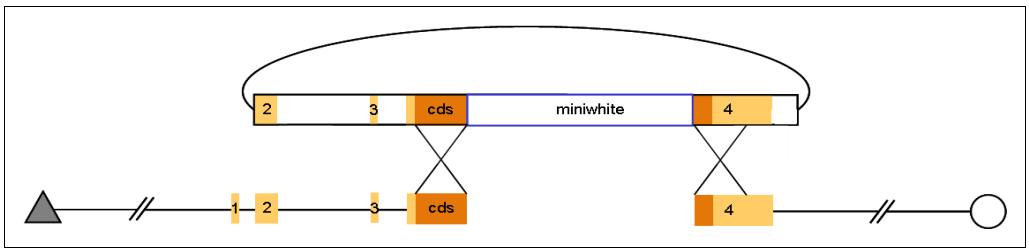

Fig. 2. 9 Scheme of mutagenesis by homologous recombination triggered by introduction of ds break due to activity of ZFNs. The *miniwhite* gene should be incorporated, thus enabling an easy screen for transgenic flies.

2. 9. 2 MICROINJECTION OF MRNAS

In order to test whether the ZFNs are able to cleave the target site at all, mRNAs were injected at concentrations about 0,9 µg/µl and 0,6 µg/µl into *yw* embryos. Since the target site of the engineered pair of ZFNs resides within the *dco* coding sequence, the following NHEJ reparation process should produce a null allele. The flies homozygous for *dco* deficiency are not viable and

the mutation is therefore possible to score genetically (Fig. 2. 10).

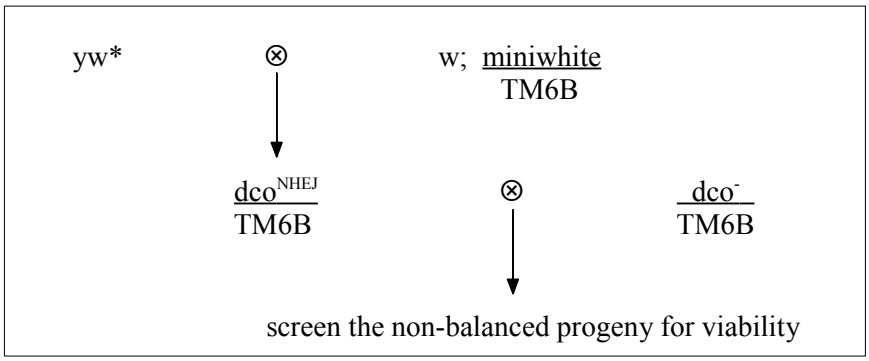

Fig. 2. 10 Scheme of the crosses after microinjection of ZFNs targeting the coding sequence of the *dco* gene. In the first step, injected flies were individually crossed with any stock carrying the balancer TM6B (here the stock number 181). From their progeny, balanced white-eyed flies are individually crossed with a stock carrying the *dco* deficiency over the TM6B balancer and their progeny is screened for absence of viable non-balanced individuals.

3. RESULTS

3.1 P ELEMENT-MEDIATEED GERMNLINE TRANSFORMATION

In order to introduce the mutation combinations found in three patients (Tab. 2. 2), three constructs for targeted mutagenesis were prepared by Tomáš Doležal.

The provided constructs pTV2-P3, pTV2-P4 and pTV2-P6 were isolated and sequenced. About a thousand *yw* embryos were injected with the injection mixture containing the pTV2 construct and a "helper" plasmid (Fig. 3. 1), out of which 381 survived the manipulation. Within their progeny, 3 transgenic individuals were found in case of pTV2-P3 construct, 31 individuals in case of the construct pTV2-P4 and 6 in case of pTV2-P6.

Crosses in order to find the chromosome bearing the insertion and subsequent experiments were carried out with all lines injected with the constructs pTV2-P3 and pTV2-P6 and with 12 lines bearing the construct pTV2-P4 (Tab. 3. 1).

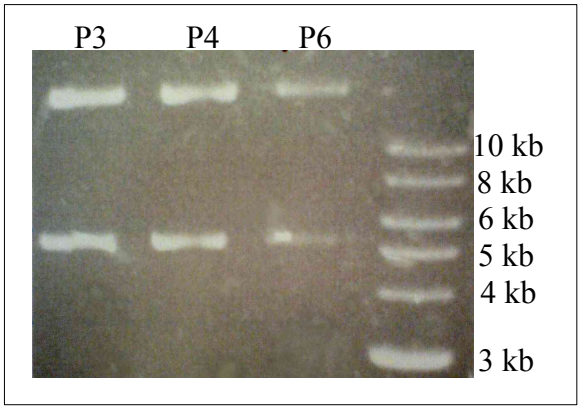

Fig. 3. 1 Agarose gel electrophoresis of microinjection mixtures containing pTV2-P3, pTV2-P4 or pTV2-P6 construct (19,2 kb) respectively and the "helper" plasmid pUC hsPI[delta2-3} (7,2 kb). 2 µl of the mixture were loaded to the gel in case of P3 and P4 constructs and 1 µl was loaded in case of the construct P6.

Site of insertion	Stock code
X chromosome	P3-7SV, P3-10, P4-36, P4-47, P4-51, P4-111, P6-54, P6-66, P6-32, P4-72, P4-88
II chromosome	P3-7TM, P4-69, P4-93, P4-98, P4-172, P6-48
III chromosome	P4-70, P4-124, P6-15, P6-22

Tab. 3. 1 List of the transgenic lines prepared by P element-mediated germline transformation and used in further experiments.

3. 2 FIRST STEP OF RECOMBINATION – DUPLICATION OF THE *DCO* GENE

To induce the first step of recombination mutagenesis, all above mentioned transgenic flies were crossed to flies bearing the FLP recombinase and I-SceI endonuclease. Since we had decided to perform the mapping simultaneously with the first step of recombination in order to speed up the process, we employed the unbalanced lines, using therefore not only homozygotes, but also flies hemizygous for the insertion. This approach compromises somewhat the effectiveness of this step since not all individuals carry the targeting construct, but saves about 2 months.

In order to induce expression of FLP recombinase and I-SceI endonuclease, total amount of 270 vials with larval progeny from the above described cross was twice heat shocked (second and third day after oviposition). After hatching, 3000 heat-shocked virgin females (a thousand for each construct) were individually crossed to flies constitutively expressing the FLP recombinase and their progeny was searched for stable, i.e. non-mosaic red eyes indicating construct mobilized to new location. As a result, 2 individuals from the line P3, 10 individuals from the line P4 and 4 individuals from the line P6 were picked and individually crossed to *Xa/CyO; MKRS* flies to determine the localization of the *miniwhite* marker (Tab. 3. 2).

Code of picked individual	Chromosome
S1-P3	sterile
S2-P4	III
S3-P6	III
S4-P4	III
S5-P4	II
S6-P6	III
S7-P4	sterile
S8-P4	X
S9-P4	III
S10-P3	sterile
S11-P6	X
S12-P6	III
S13-P4	II
S14-P4	sterile
S15-P4	II
S16-P4	II

Tab. 3. 2 List of flies obtained after the first step of homologous recombination. Four of them were not able to reproduce and the *miniwhite* gene mapped to the third chromosome in case of only 6 lines.

Since the *dco* gene resides on the third chromosome, only lines with *miniwhite* mapping to that chromosome were taken for further analysis.

As there were no fertile individuals with potential *dco* duplication in case of the pTV2-P3 construct, the first step of homologous recombination was repeated simultaneously with the subsequent experiments that were carried out with the lines potentially bearing the duplication. Since the S1-P3 and S10-P3 were both progeny of the P3-7TM line, only flies from this line were crossed as already described, their progeny in 52 vials heat shocked and 1000 virgin females were crossed as already described. Their progeny was searched for re-appearance of red eyes and 7 individuals were found (Tab. 3. 3) and mated individually to *Xa/CyO; MKRS* flies in order to find localization of the *miniwhite* gene.

The proportion of recombination events is summarized in Tab. 3. 4.

Code of picked individual	Chromosome
H1-P3	III
H2-P3	III
H3-P3	III
H4-P3	II
H5-P3	X
H6-P3	II
H7-P3	X

Tab. 3. 3 List of flies obtained after repeated first step of homologous recombination. The miniwhite gene mapped to the third chromosome in case of the first three lines.

Mobilized construct	Number of insertions to the chromosome III	Number of insertions to other chromosomes
pTV2-P3	3	4
pTV2-P4	3	5
pTV2-P6	3	1

Tab. 3. 4 Summary of re-insertion sites with respect to the mobilized construct.

3. 3 MOLECULAR CHARACTERIZATION OF THE DUPLICATION

3. 3. 1 PCR ANALYSIS

As a means of instant screening, primers specific for the duplication were designed (dupl-FW and dupl-RV; Fig. 3. 2). The primer dupl-FW recognizes part of the construct containing CreI restriction site, whereas the primer dupl-RV binds to the region of *dco* gene that was not involved in creation of the construct and can be therefore found only in the endogenous locus of *dco* gene. With

respect to the impossibility of testing the primers before a duplication was created, their properties were tested using another pair of primers (dupl-ctrl-FW and dupl-ctrl-R; Fig. 3. 3).

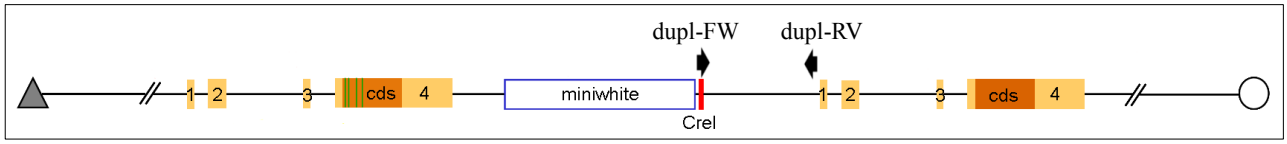

Fig. 3. 2 Primers specifically recognizing duplicated *dco* gene. The forward primer binds to the sequence of introduced vector containing CreI recognition site, whereas the reverse primer recognizes region of *dco* gene that is not involved in the construct. Size of the product ought to be 2,7 kb.

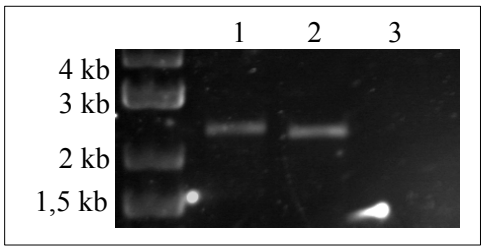

Fig. 3. 3 Products of PCR reaction using duplication-specific primers and genomic DNA (squishing protocol) of a transgenic line bearing the pTV2-P3 construct. 1/ dupl-FW with dupl-ctrl-RV, expected size 2,5 kb. 2/ dupl-RV with dupl-ctrl-FW, expected size 2,4 kb. 3/ dupl-FW with dupl-RV.

In order to screen the lines for presence of introduced point mutations, allele-specific primers were designed and tested using stocks with P element-introduced constructs (Fig. 3. 4).

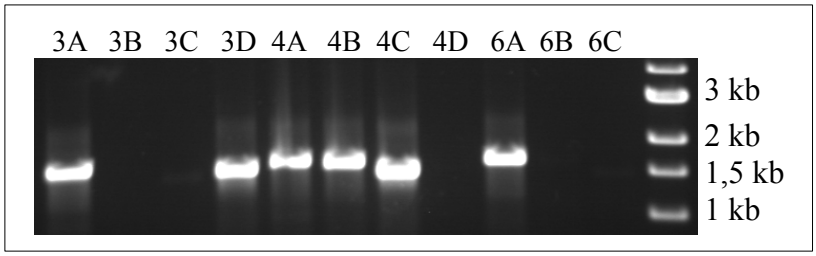

Fig. 3. 4 Allele-specific primers tested on the genomic DNA (squishing protocol) of transgenic lines bearing the construct pTV2-P3 (marked with 3; mutations L39Q and S101R), pTV2-P4 (marked with 4; mutations L39Q, L49Q and N78T) or pTV2-P6 (marked with 6; mutation L39Q). A stands for primers L39Q-FW with L/L-RV (expected size 1,64 kb), B stands for primers L49Q-FW with L/L-RV (expected size 1,6 kb), C stands for primers N78T-RV with N/S-FW (expected size 1,47 kb) and D stands for primers S101-RV with N/S-FW (expected size 1,54 kb). Strong bands occur only in expected lines, although some faint bands are visible also in the lines 3C and 6C, where the mutation N78T is not present.

Lines potentially bearing the duplication of *dco* gene were then tested by PCR from squished template using the primers dupl-FW/dupl-RV with no line giving rise to the expected band of 2,7 kb (Fig. 3. 5).

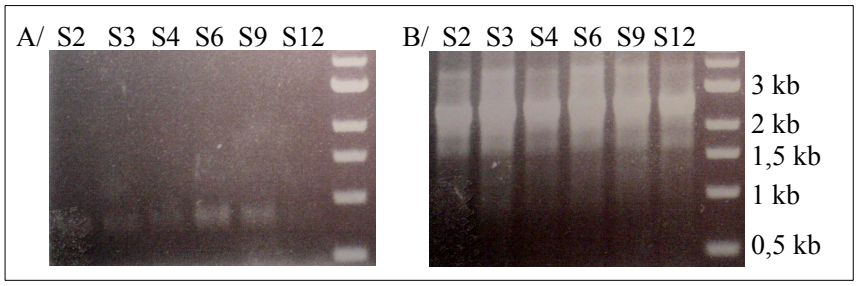

Fig. 3. 5 PCR screening of the lines potentially bearing the duplication. A/ Reaction using primers dupl-FW and dupl-RV, size of the expected product is 2,7 kb. B/ Positive control using primers dupl-ctrl-FW and dupl-RV, expected size 2,4 kb.

The somewhat later produced lines with potential duplication H1-H3 were also screened by PCR using the primers dupl-FW and dupl-RV (Fig. 3. 6) with the same negative result.

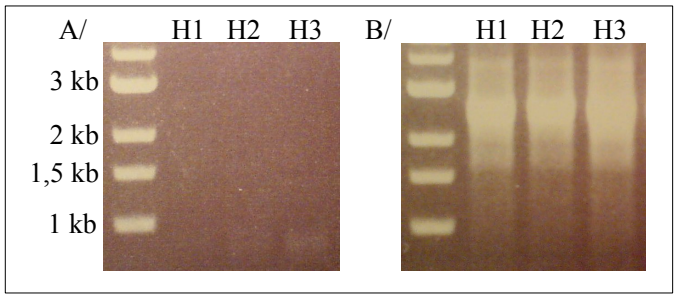

Fig. 3. 6 PCR screening of the later produced lines potentially bearing the duplication. A/ Reaction using primers dupl-FW and dupl-RV, size of the expected product is 2,7 kb. B/ Positive control using primers dupl-ctrl-FW and dupl-RV, expected size 2,4 kb.

3. 3. 2 SOUTHERN BLOT ANALYSIS

Since it was not possible to show, whether the primer pairs used above do not work together or actually no line bears the duplication, Southern blot was carried out to analyse the lines further. The genomic DNA was isolated from the balanced lines (from both flies homozygous in the observed locus and flies also bearing the balancer chromosome MKRS) potentially carrying the duplication on the third chromosome and from *yw* and P3-7TM as controls.

The proper restriction by EcoRV enzyme was checked using 1/14 volume of the reaction mixture (Fig. 3. 7).

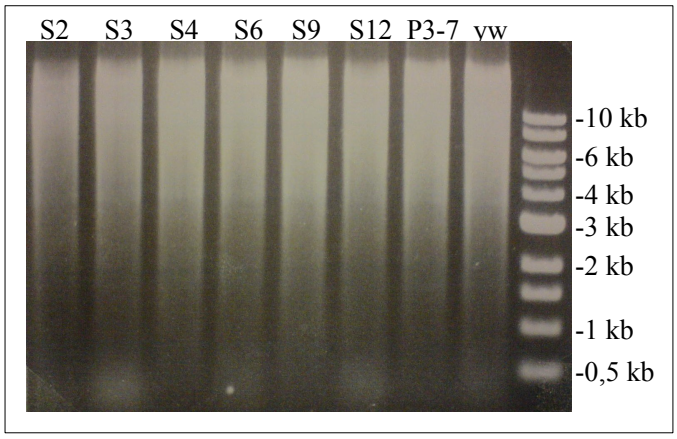

Fig. 3. 7 Control of proper restriction of genomic DNA by EcoRV restriction enzyme for Southern blot (1/14 of total volume loaded to 0,7% agarose gel).

The wild type allele of the *dco* gene is cleaved by the EcoRV restriction enzyme into 2 fragments of 5,9 and 7 kb that can be both visualized by an overlapping probe (Fig. 3. 8). If a P element-mediated insertion of the pTV2-P3/4/6 construct is present in the genome, an additional fragment of 7,45 kb and another of unpredictable size (min. 5 kb, up to the particular site of insertion) are created. In case of successful mobilization of the construct by FLP recombinase and induction of the duplication at the *dco* gene (Fig. 2. 4, chapter 2. 5), the locus would be cut by EcoRV into 4 fragments of 5,7 and 7 kb (identical to the wt allele) and of 6,1 and 8,3 kb. However, if the mobilized construct cut by SceI endonuclease is not able to induce the duplication and the construct is re-inserted into the genome non-specifically, only fragments of 6,1 kb and another one of unpredictable size occur additionally to the 5,9 and 7 kb long fragments.

The Southern blot analysis of the genomic DNA of the 6 lines potentially bearing the duplication (Fig. 3. 8) revealed, that no line observed carried the 8,3 kb long fragment characteristic for the duplication, although all of them lacked the 7,45 kb long fragment corresponding to P element-mediated insertion. Every line carried the 6,1 kb long fragment characteristic for either non-specific re-insertion or duplication and the fragment of unpredictable size characteristic for non-specific re-insertion was visible in the line S3. This result shows unambiguously, that the mobilization of the construct was successful, although not sufficient to induce the duplication of the *dco* gene and all tested lines therefore lacked the desired gene alterations.

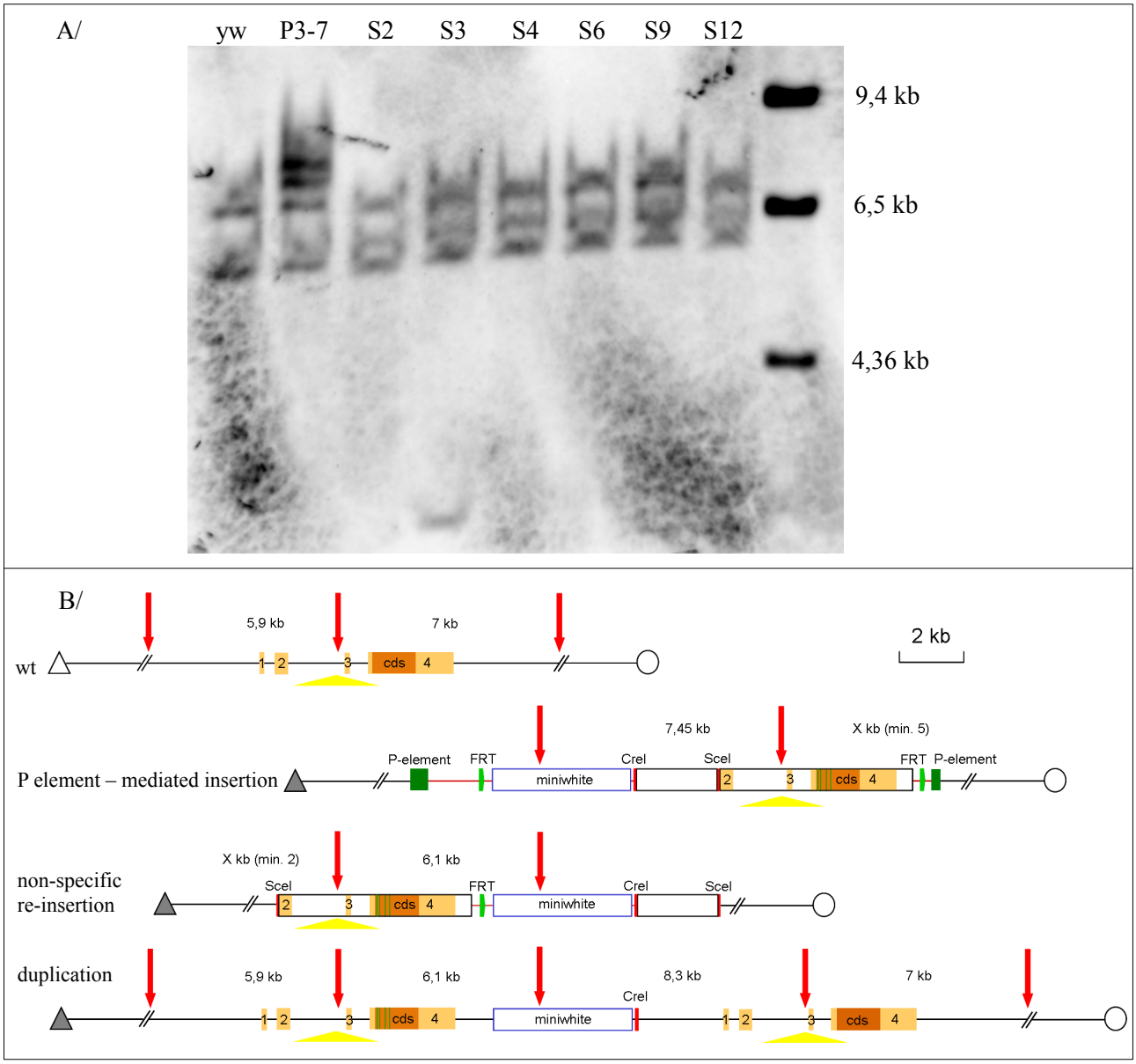

Fig. 3. 8 A/ Southern blot analysis of EcoRV-cut genomic DNA of lines potentially bearing duplicated *dco* gene (S2-S12) and the lineP3-7 with P element-introduced construct pTV2-P3 as well as a wild type control (*yw*). B/ Scheme of possible configurations of the construct and *dco* gene on chromosomal level. Red arrows show the EcoRV recognition sites, yellow triangles show the binding region of the DNA probe. Sizes of visualized fragments are shown above each region. All the lines potentially bearing the duplication show pattern of unaltered wt *dco* allele with additional 6,1 kb band corresponding to the non-specific re-insertion (the second band of unpredictable size is visible only in the S3 line). Mobilization of the construct was therefore successful, nevertheless insufficient to induce the duplication of the *dco* gene.

3. 4 CREATION OF ZFNS-CODING MRNAS

Since the previous approach did not lead to desired targeted events, we decided to use a new method that has been described only recently /Beumer et al., 2008/. This novel procedure is much more rapid than earlier approaches and it makes possible to generate and recover targeted gene alterations within as few as 2 fly generations.

The ZFNs-coding sequences were designed following instructions in Carroll et al., 2006; Beumer et al., 2008 and advices from personal communication with Kelly Beumer. The seven-primer strategy was applied to create the ZFs-coding fragments without any complication (Fig. 3. 9).

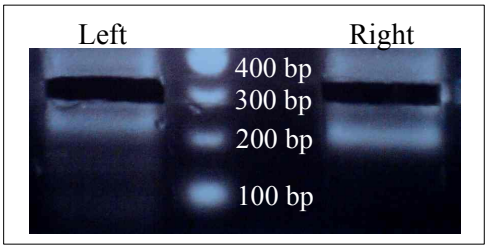

Fig. 3. 9 Products of touchdown PCR reaction used for creation of left and right zinc finger array-coding fragments. The fragments of desired size (285 bp) were cut out from the gel. Smaller products as well as smearing of bands are quite common and do not compromise the protocol.

Sequencing of several clones bearing each fragment ligated into the pENTR-NLS-G-FN plasmid revealed a large number of introduced mutations arising from the character of oligonucleotide synthesis. Due to mistake in design of 2 oligonucleotides that unintendedly contained recognition sequence for NotI restriction enzyme (needed later for linearisation of pCS2-DEST vector prior to *in vitro* RNA transcription), correct oligonucleotides were ordered, this time HPLC purified. Already these 2 HPLC purified oligonucleotides out of the 7 improved the quality of the PCR product to that extent, that a mutation-free clone for both of the ZFNs was obtained immediately.

The coding sequence was then shuttled to the pCS2-DEST *in vitro* transcription vector, mRNAs were transcribed *in vitro* from the cut pCS2-DEST plasmids bearing the coding sequences and examined using agarose gel electrophoresis (Fig. 3. 10).

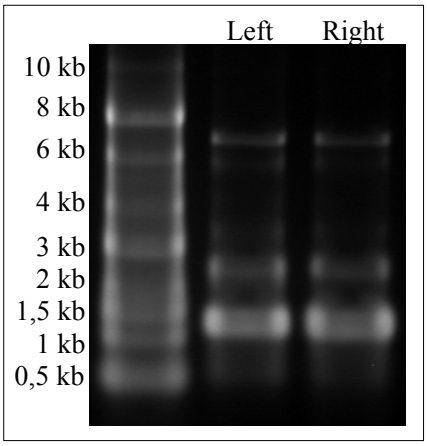

Fig. 3. 10 Agarose gel electrophoresis of ZFNs-coding RNAs. Majority of products migrate at expected size of 1,33 kb and since the electrophoresis was not run under denaturing conditions, remaining bands occurred most probably due to intermolecular interactions.

3. 5 RNA MICROINJECTION

Since we did not have any guaranteed RNase-free food colour for preparation of microinjection buffer, RNase activity of several colours was examined using total fly RNA treated similarly to the microinjection procedure (Fig. 3. 11). As none of the tested colours showed signs of RNase activity, green colour was used for microinjection procedure as usual.

About 1000 embryos were injected with ZFNs-coding RNAs mixed with the DNA donor in form of circular plasmid that should introduce *miniwhite* into the *dco* locus. 130 larvae had hatched, nevertheless only 48 individuals survived to the adulthood. Their progeny was searched for red eyes, but not a single one was observed.

However, it was not clear, whether the failure was due to the subsequent recombination with the donor or already due to the ZFNs-induced cleavage of the target sequence. In case of the former, several strategies to improve the outcome are available. First, the flies deficient for ligase IV can be used to increase the ratio of homologous events. The proportion of DNA donor in the microinjection mixture should be also optimized and the fact that the DNA donor contains the target sequence should be taken into consideration as well.

With respect to the mentioned actualities, we decided to test whether the designed ZFNs cleave the target locus at all. For this purpose, approximately 1300 embryos were injected with the ZFNs-coding RNAs alone in order to induce NHEJ reparation of the double strand break within the *dco* coding sequence. 310 larvae had hatched from the injected embryos, out of which 79 survived to adulthood and were fertile. They were individually crossed to a line bearing the TM6B balancer

and 1176 males from their progeny with the balancer were mated individually to flies bearing *dco* deficiency over the balancer TM6B. Their progeny was screened for absence of non-balanced individuals, however offspring bearing *dco* deficiency and the potentially mutated chromosome was found in every vial.

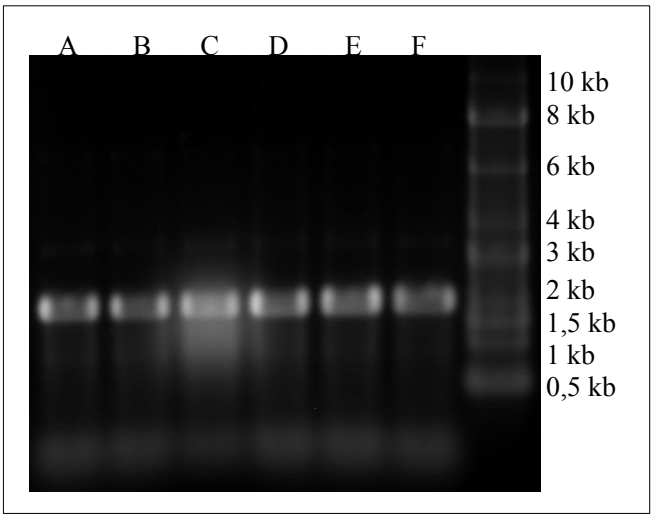

Fig. 3. 11 Agarose gel electrophoresis of total fly RNA diluted in microinjection buffer after incubation under conditions similar to microinjection procedure. A/ Buffer with yellow food colour. B/ Buffer with orange food colour. C/ Buffer with red food colour. D/ Buffer with green food colour. E/ Buffer with blue food colour. F/ Pure RNA diluted in RNase-free water.

4. DISCUSSION

Using the microinjection technique and P element-mediated germline transformation, I produced transgenic flies bearing three constructs designed for targeted mutagenesis via homologous recombination. These constructs carry regions of *dco* gene with introduced point mutations corresponding to those found in the coding sequence of CKIε (mammalian homologue of *dco*) in breast cancer tissue samples of three patients /Fuja et al., 2004/. It has not been shown yet whether the observed mutations play an important role in the development of breast cancer or are mere passenger mutations. Since a known allele of the *dco* gene shows phenotype of overgrowing imaginal discs in fly /Zilian et al. 1999/, eventual appearance of the same phenotype after introduction of the observed point mutations would be a strong indication that the gene is involved in carcinogenesis.

After the first step of targeted mutagenesis using homologous recombination, the *dco* gene ought to be duplicated /Rong et al, 2002/. Already high rate of obviously non-targeted re-insertions to other chromosomes rather than the third suggested very low effectiveness of the homologous recombination. Moreover, the flies potentially bearing the duplication showed different shadings of the eye colour, suggesting different sites of re-insertion that would affect the intensity of *miniwhite* transcription due to position effects. When using PCR analysis, duplication was not revealed in any of the examined flies.

The absence of the duplication in the *dco* gene was ultimately confirmed by Southern blot analysis. The desired duplication would be cut by EcoRV restriction enzyme giving rise to four fragments of different sizes and all would be visualized by an overlapping DNA probe. Restriction digestion of the wt locus, the P element-mediated insertion and a non-specific re-insertion by the same enzyme appear on Southern blot as distinct patterns of bands that can be unambiguously distinguished from the targeted event (Fig. 3. 8). The fragment of 8,3 kb that would confirm the duplication was not observed in any of the six examined lines. However, all lines showed pattern of re-insertion of the construct without creating the desired duplication, although the fragment of unpredictable size was visible only in the line S3. With respect to the small size differences between the expected bands, the gel was run for a long time period, so that the fragments of unpredictable size had already left the gel in cases of the remaining five lines.

Southern blot analysis has thus confirmed that the targeting construct was actually mobilized from the original insertion, however it did not induce the recombination resulting in proper duplication of the *dco* region (or at least not efficiently enough to be captured in our screen).

With regard to the scale of the experiment that was carried out to induce the homologous recombination and to the fact that even in the case of successful duplication the introduced mutations could be possibly lost due to the repair machinery, we decided to abandon this method as inapplicable for our purpose.

The reason for failure of the used approach remains unclear, however from personal communication with other groups it seems not exceptional that some particular genes resist the targeted mutagenesis via the described method. One of the potential explanations could be a physical unavailability or a difficult accessibility of the targeted sequence due to the higher organisation of chromatin structures. Since *dco* resides near to the telomeric region of the third chromosome and thus near to the heterochromatin, this explanation seems plausible.

For reasons described above, we decided to employ another method to introduce targeted gene alterations, namely a novel method described only recently that takes advantage of so called zinc finger nucleases /Beumer et al., 2008/. In this approach, the targeted sequence is cleaved itself instead of inducing cleavage of extrachromosomal construct bearing homologous sequence. Since the extrachromosomal double strand break proved to be insufficient to induce homologous recombination with the *dco* gene, we considered the new method to be a possible solution to our problem.

The new method would allow us to introduce the desired alterations at such rates, that recovery should be possible using only molecular analysis, which would be in our case PCR screening using allele-specific primers (Fig. 3. 4). Nevertheless, we did not know what effectiveness we could expect and we have decided first to introduce the marker gene *miniwhite* instead of the point mutations (Fig. 2. 9).

The sequence of *dco* was searched for combination of nucleotide triplets for which good zinc fingers would be available. Since the ZFs for GNN triplets are the longest known and best characterized /Liu et al., 2002/, sequence where all 6 ZF-interacting triplets were GNN was chosen. The seven primer strategy combined with touchdown PCR for creation of the ZFs-coding fragments proved to be easy and efficient in our case (Fig. 3. 9), although I would highly recommend to use HPLC-purified oligonucleotides.

In order to shuttle the whole ZFNs-coding sequences to a vector designed for the *in vitro* RNA transcription, *in vitro* recombination using the Gateway cloning technology by Invitrogen was carried out. After transformation of competent *E. coli* cells by the reaction mixture, only clones bearing the desired vector should survive upon ampicillin selection. Although some undesired

colonies apparently bearing the construct with the ccdB gene (topoisomerase II toxin) appeared on the background, they were characteristically small in size and easily distinguishable from the desired clones. The method was therefore efficient enough to offer readily the construct of interest.

After the ZFNs-coding mRNAs were transcribed *in vitro* (Fig. 3. 10), *yw* embryos were injected by mixture of mRNAs and DNA donor containing the *miniwhite* gene inserted into the coding sequence of *dco*. Successful mutagenesis would therefore be easily recognizable as appearance of the red-eyed flies within progeny of the injected individuals. With respect to the percentage of the red-eyed flies we would have been able to plan the scale of the following experiment in order to introduce the point mutations.

The microinjection procedure was somewhat more difficult than the standard DNA microinjection, with more frequent clogging of the needle probably due to high concentrations of RNA and DNA. The mortality among injected embryos was also higher in comparison to standard DNA microinjection and dependent on the concentration of RNA injected, with higher survival-rate when injected with less RNA. Regardless to the RNA concentration, only less than a half of the hatched larvae survived to adulthood. Among their progeny, no red-eyed fly was found in the second generation after microinjection, which could have been either due to unsuccessful recombination step or due to the failure of ZFNs to cleave the target sequence.

If the recombination step was the one that did not occur properly, there would be several factors that can be altered for the purpose of optimizing the process such as optimization of the DNA proportion in the microinjection mixture or using flies lacking functional ligase IV in order to suppress the non-homologous end joining events. Before we would proceed to the optimizing the conditions for the recombination, we decided to find out, whether the designed ZFNs are able to recognize and cleave the target locus at all. Therefore, *yw* embryos were injected only with the ZFNs-coding mRNAs without any DNA donor. The target sequence resides within the coding sequence of the *dco* gene, therefore a null allele should be created due to NHEJ reparation of the double strand break. Flies lacking functional *dco* gene are not viable, which was used in the screening for targeted events.

The microinjection procedure was this time somewhat easier than the previous one, apparently due to lower concentration of nucleic acids in the mixture, since the donor was omitted. However, the survivors-rate remained low, with maximum of 50% hatched larvae when injected with smaller amount of injection mixture. Similarly, only about one third of hatched larvae survived to adulthood.

Unfortunately, no NHEJ events were observed among the progeny, indicating that the mutagenesis-scheme failed already at the first step.

Although we do not know the exact reason for the failure of the ZFNs-mediated mutagenesis of the *dco* gene, we may speculate that the zinc finger arrays did not recognize the target sequence specifically enough. Although all the ZFNs were designed to recognize GNN triplets maximizing the chance for specific recognition based on the current knowledge /Ramirez et al., 2008/ there is still a risk that the particularly used ZFNs would not work for the particular sequence. High mortality of hatched larvae in our case may indicate low specificity of the ZFNs cleavage, when the ZFNs could assemble as homodimers or heterodimers at another sites than expected, disrupting also genes important for the proper development. This low specificity might be again influenced by the physical unavailability or difficult accessibility of the *dco* sequence discussed above therefore pushing the ZFNs rather to unspecific cleavage.

As already shown, neither the homologous recombination at the *dco* locus induced by extrachromosomal double strand break nor direct cleavage of the gene by ZFNs was successful, both suggesting difficulties when recombination within this region is considered. For a time reason there was no opportunity for further trouble-shooting, for example trying different ZFNs, and the strategy to introduce mutations into the *dco* gene by homologous recombination was abandoned. At present time, a different strategy has been designed and is now being carried out.

The point mutations are being re-cloned into the pCasPeR 4 vector carrying the *dco* gene that has already been shown to completely rescue the *dco* deficient mutants /Zilian et al., 1999/. This construct is being introduced into the *yw* embryos by the P element-mediated germline transformation. Resulting transgenic flies carrying modified genomic fragments will be crossed into the *dco* null background and their phenotype will be analysed. With respect to the random insertion of the construct, regulation of the *dco* copy containing the introduced mutations may be subjected to position effects. Nevertheless, if sufficient number of transgenic lines bearing the construct at distinct sites is compared, the information on the effect of introduced mutations will be valuable.

5. Conclusions

Two distinct strategies to introduce point mutations into the *dco* gene were applied. The directed mutagenesis via homologous recombination failed at the level of inducing homologous recombination with the *dco* locus by introducing extrachromosomal double strand break within homologous sequence, as shown unambiguously by this thesis.

The second approach, ZFNs-mediated mutagenesis, failed at the level of introduction of double strand break into the *dco* coding sequence.

Since both methods for directed mutagenesis failed in producing the desired genome alterations, an alternative approach for introduction of the altered *dco* gene into the fly genome is being applied.

5. References

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