Functional genomics of the *Drosophila melanogaster* X Chromosome and the role of *DWnt5* during development

Dissertation

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Abbreviations

AP  Alkali Phosphatase
ATP 2’-Desoxyadenosine-5’-Triphosphate
BCIP 5-Bromo-4-chloro-3-indolyl Phosphate
BDGP Berkeley Drosophila Genome Project
bp  Base pair
biot  biotinylated
Cy  Curly
CyO Curly of Oster
TEM  Stable transposase source integrated into the genome
DAB Diaminobenzidine
DIG Digoxigenine
DNA Desoxy-ribonucleic acid
dNTP Desoxynucleotide
EDGP European Drosophila Genome Project
EDTA Ethylene diamin tetraacetic acid
EGTA Ethylene glycol-bis-(b-aminoethylether)-N,N,N’,N’-tetraacetic acid
EMS Ethylmethanosulfonate
EST Expressed Sequence Tag
GXP Göttingen X-Chromosome Project
h hour
Hepes 4-(2-Hydroxyethyl)-1-piperazine-ethansulfonic acid
Mb Megabase
min minute
µg microgramm
µl microliter
NBT Nitro Blue tetrazolium salt
NCBI National Center for Biotechnology Information
ng nanogramm
O/N overnight
PCR Polymerase Chain Reaction
RNA Ribonucleic acid
RT Room Temperature
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1. Introduction

1.1. P elements

Genetics provide a powerful approach to understand the function of each gene and to decipher its role during development. Model organisms, such as the fruit fly *Drosophila melanogaster*, share many genes with humans whose genome sequences and functions have been conserved. The fruit fly provides an advanced system to study the function of conserved genes since, unlike human, any open reading frame (ORF) within the fly genome can be mutated in principle. In such experimental systems, powerful techniques have been developed towards this analysis, using transposable elements to trap genes by disruption of their function in genetic screens, in order to identify novel genes and analyze their functions. Understanding the function of these genes can lead to better understanding of gene function in higher organisms, such as mammals.

1.1.2. Transposable elements

Several classes of transposable elements have been identified in the genome of *Drosophila melanogaster*. The term transposable element usually refers to all DNA segments, which, as discrete units, are capable of changing their positions within the genome (Shapiro 1982). Transposable elements (transposons) are mobilized by DNA rearrangement reactions that do not require homology between transposon sequence and target DNA.

The best characterized transposable elements in the fruit fly are the P elements, which transpose at very high rates when certain genetic criteria are met. The P factors are responsible for the phenomenon of P-M hybrid dysgenesis in *Drosophila melanogaster* that occurs among the progeny of matings between certain strains (Kidwell *et. al.* 1977, Engels 1989; 1992). Dysgenesis occurs when males of the strain P (paternally contributing) are mated with females from strain M (maternally contributing). The reverse cross, with an M father and a P mother, produces normal offspring, as do crosses within a strain (P x P or M x M).

Further studies showed that the P factors are only present in P strains, where their transposition is repressed, and they are absent from M strains. Intact and functional P
elements are 2907bp in length, have 31bp terminal inverted repeats (TIRs) and they are autonomously functional for transposition (O’Hare and Rubin 1983; Spradling and Rubin 1982). When chromosomes carrying P factors are placed in the M cytotype, the transposable elements become “active” and transpose at high rates. Among other effects, P factors would then induce mutations by inserting into and disrupting genetic loci.

1.1.3. Gene disruption using transposable elements

P elements are very important tools for functional genetics, not only because of their usefulness as transformation vectors, but also as potential mutagenic agents as well. Early efforts focused on cloning specific genes by mobilizing large numbers of natural P elements (reviewed by Spradling et al. 1995). The resulting genetic lines were unsuitable for genetic or phenotypic studies without extensive outcrossing to remove extraneous P elements. P element mediated transformation allowed strains containing just one or few elements to be constructed. However, microinjecting DNA could generate only a limited number of strains, and the insertions could not be targeted into genes of particular interest.

In 1988, Cooley and her colleagues showed that individual, experimentally modified P elements could be used in large genetic screens to generate thousands of stable mutant strains (Cooley et al. 1988). They proposed that single-insertion lines can be generated by mobilizing a transposon already present in the genome (mutator). By this method, in order to initiate mutagenesis, the mutator strain is crossed with another strain (jumpstarter), which contains a single modified element encoding the transposase enzyme. The produced transposase will catalyze transposition of the mutator to a new site in the genome. Progenies lacking the jumpstarter are mated with normal flies carrying no element. Offsprings that carry the new insertion can be analyzed in detail because the new insertion is stable. By now, this method became a powerful technique, since transposable elements provide a potent mean of correlating genetic and molecular information. P elements generate a simple, reproductive lesion upon insertion that can be detected much more easily than the point mutations produced by other mutagens such as chemical mutagens or radiation.

During the last decade, numerous mutant strains containing single P element insertions were generated in a number of laboratories (Spradling et al. 1999; Salzberg et al. 1997;
Bier et al. 1989; Karpen and Spradling 1992; Gaul et al. 1992; Török et al. 1993; Chang et al. 1993). These insertions have been associated with recessive or sterile phenotypes and led to tremendous wealth of information about the Drosophila genome. The Berkeley Drosophila Genome Project (BDGP) has made the most systematic effort to characterize available single P element insertion lines and use them on mutagenesis screens, to generate a gene-disruption library (Spradling et al. 1995; Rubin et al. 2000a).

However, these efforts were primary focused on the isolation of essential genes on the autosomes. The current collection disrupts at least 1200 different genes, representing about 30% of the estimated 3600 genes on the autosomes (Spradling et al. 1995; 1999). However, no systematic gene disruption experiments on the X chromosome had been initiated.

1.1.4. Functional analysis of genes on the X chromosome

The cytogenetic map of the X chromosome has been subdivided into 20 divisions (Bridges 1935; 1938) and contains about 1000 chromosome bands. This chromosome contains about one fifth of the euchromatic region of the Drosophila melanogaster genome, and corresponds to roughly about 22 Mb of DNA, coding for some 2182 predicted proteins (Adams et al. 2000). Two-thirds of all Drosophila genes show no obvious loss of function phenotype (Miklos and Rubin 1996), leaving about 800 of the X chromosome genes which carry viable or phenotypically distinct functions.

A X chromosome project has been initiated to systematically identify the essential genes that are located on the X chromosome. As pioneered with autosomes (Spradling et al. 1995; 1999), this approach is based on a large-scale P element insertion screen on the X chromosome, aims on genes essential for viability (lethal lines) as well as for visible mutations or mutations sterile only for one sex (Peter et al. 2002).

The transposable element that was used in the initial X chromosome screen was a modified version of a naturally occurring P element, called the PlacW element (Bier et al. 1989). This element (Figure 1) is flanked by inverted repeats (31bp) that are essential for transposition. The transposable vector contains a bacterial origin of replication, the β-lactamase gene coding for ampicillin resistance and a lacZ gene from E.coli, which is
under the control of the weak P element promoter. Therefore, the lacZ gene can be activated by nearby regulatory elements. The element also contains a mini-white gene in the middle of the PlacW vector, serving as a visible marker (red eye pigmentation) for the presence of the PlacW element in the genome of Drosophila melanogaster.

Figure 1. Schematic structure of PlacW vector used from the X chromosome project.
The transposable portion of the PlacW vector is 10.6kb long. At the 5′ end of the transposon is the P element trasposase-lacZ fusion (pTps-lacZ), which is followed by a mini-white gene (in the same orientation for transcription). At the 3′ end of the PlacW there is the plasmid origin of replication (Origin) and the β-lactamase gene (AmpR).

The crossing scheme for generating X chromosomal PlacW insertion lines (Figure 2) is the following: Females containing the PlacW element on the second chromosome are mated to males with the transposase source P[2-3]{D2-3}(99B) on the third chromosome. In the next cross females are mated to the F1 males in whose germ cells the PlacW can be mobilized. Appropriate marker such as the absence of the dominant second chromosome marker Curly and the white+ eye color from PlacW, allow the identification of F2 females with a PlacW that has jumped on a different chromosome. Such an F2 female is individually mated to FM7c males. In the F3 generation, segregation of the white marker allows the identification of PlacW in the X chromosome or in an autosome. In the X chromosome integrations, the occurrence of only males with FM7c balancer chromosome indicates that a vital gene has been inactivated by the new PlacW insertion. The progenies are then used to establish a PX fly stock. PX is a line where a vital gene is affected by the insertion of a single PlacW element.

57105 individual F2 females were initially mated and 39900 (69.9%) of them produced progenies. From these crosses, 501 candidate PX lines were isolated (2.5%). These PX lines are either lethal or semilethal. Semilethal lines are the lines, which show less than 20% viability compared to the subling balancer males. One of the genes identified carries a P element insertion affecting the gene encoding the Drosophila homologue of the signaling molecule Wnt5. In the study described here, I have focused on the analysis of its function in some detail.
Figure 2. Crossing scheme for isolating lethal insertion lines in the X-Chromosome.
The sex chromosome (left pair in each genotype) and the two large autosomes are shown schematically with females on the left. Relevant mutations are labeled accordingly. The PlacW insertion is indicated by the triangle and the white+ marker is represented by the red chromosome. The transposase source is indicated by the double-headed arrow. Balancer chromosomes are hatched. Absence of the crossed-out males in the F3 generation indicates a potential lethal PlacW insertion (After Peter et. al. 2002, EMBO Reports, p. 36).

1.2. Signaling Molecules

1.2.1. Wnt signaling

The Wnts are a large family of secreted glycosylated ligands that bind to a class of seven-pass transmembrane receptors encoded by the frizzled genes. Wnt proteins are involved in a wide variety of biological processes. The first Wnt gene, mouse Wnt-1, was discovered in 1982 as a protooncogene activated by integration of mouse mammary tumor virus in mammary tumors (Nusse and Varmus 1982). Consequently, the potential involvement of Wnt genes in cancer was the main area of research in 1980s. With the molecular identification of the Drosophila segment polarity gene wingless (wg) as an orthologue of Wnt-1 (Cabrera et. al. 1987; Rijsewijk et. al. 1987), it became clear that Wnt genes are important regulators for many developmental decisions (reviewed by Nusse and Varmus 1992). At this moment, close to 100 Wnt genes have been isolated from the nematode C. elegans to mammals (a regularly updated list of Wnt genes can be viewed at: http://www.stanford.edu/~rnusse/wntwindow.html). The Wnt signal transduction pathway is involved in the establishment of the body axis at the very earliest stages of embryogenesis and Wnts are later required for development of many organs (e.g. kidney and brain) (Smalley and Dale, 1999).
For several years, it has been known that many Wnt proteins activate gene transcription indirectly by activating a pathway that is controlled by β-catenin. (Figure 3). This is the best-characterized signaling cascade triggered by Wnt proteins and it called “the canonical pathway” or Wnt/β-catenin pathway (Brown and Moon 1998; Wodarz and Nusse 1998). Details about this cascade first emerged from genetic analyses of *Drosophila melanogaster*, where it functions in developmental processes such as the patterning of body segments and appendages (Nüsslein-Volhard and Wieschaus 1980). Work in both invertebrates and vertebrates indicates that the canonical pathway is actually a large network. In the absence of signaling from Wnt, a large molecular complex including GSK-3, Axin and APC, ensures that β-catenin is rapidly targeted to the cellular protein-degrading apparatus. Activation of the pathway by a Wnt protein results in β-catenin being stabilized (reviewed by Niehrs 2001). Free β-catenin forms nuclear complexes with members of the TCF/LEF transcription factor family to regulate expression of numerous genes (e.g. c-myc, fibronectin, mab-5, NT-3, siamois, sloppy paired, twin, ubx) and to control cell fate (Wodarz and Nusse 1998; Patapoutian and Reichardt, 2000).

The second Wnt signaling cascade controls the planar cell polarity (PCP) as revealed by genetic analysis of *Drosophila* as well (Mlodzik 1999). Cuticle cells in adult flies secrete hairs, which are polarized by the PCP pathway so that all point in one direction. The PCP pathway is also at work in vertebrates during gastrulation- the massive rearrangement of cells that produce the three main tissue layers, endoderm, mesoderm and epidermis, in the early embryo. During gastrulation, migrating cells become polarized by the PCP pathway and extrude lamellipodia along one axis only (Wallingford *et. al.* 2000). The common theme in both processes is the polarization of the cytoskeleton. The activation of the Frizzled receptor by the Wnt protein results in the activation of D ishevelled. The PCP pathway works through small GTP-binding proteins of the Cdc42/Rho family, which activate the transcription factor Jun (reviewed by Niehrs 2001).

Hints that there might be a third Wnt-triggered pathway came from the discovery that Wnt5a, together with Frizzled-2, mobilizes Ca\(^{2+}\) ions within cells and thereby activates certain Ca\(^{2+}\)-dependent enzymes, including protein kinase C (PKC) (Slusarski 1997; Sheldahl 1999; Kühl 2000).
Figure 3. Three signaling cascades that are triggered by Wnt proteins.

Wnt proteins are outside the cell. In the canonical pathway, the stabilization of $\beta$-catenin results in the activation of specific genes in the nucleus. In the PCP pathway, instead of using $\beta$-catenin, the Cdc42 activates the transcription factor Jun. In the third pathway, the Wnt works to induce an increase in the Ca$^{2+}$ level inside the cells and thereby activate protein kinase C (After Niehrs, 2001, Nature).

In *Xenopus*, it has been reported that the ligand-activated serotonin type 1C receptor, which stimulates PI cycle activity and Ca$^{2+}$ signaling independent of Wnts, phenocopies embryonic responses to Xwnt-5A (Slusarski *et. al.* 1997). These results suggested that intracellular signaling by a subset of vertebrate Wnts involves modulation of intracellular Ca$^{2+}$ signaling pathway, which may arise from phosphatidylinositol cycle activity. In zebrafish, the rat protein Frizzled-2 causes an increase of intracellular calcium, which is enhanced by Xwnt-5a. This release of intracellular calcium is suppressed by the enzyme inositol monophosphatase and hence the phosphatidylinositol signaling pathway (reviewed by Niehrs 2001).
1.2.2. Drosophila Wnts

In Drosophila, there could be a total of 7 Wnt genes identified after the complete sequence of the Drosophila genome. So far, mutations in only three of the seven Drosophila Wnt genes, namely Wingless, DWnt2 and DWnt4 have been reported (Figure 4). Wingless, the first Wnt gene identified in Drosophila, is acting through the canonical pathway and plays an important role in almost every aspect of development. Wingless shows a pattern of 16 regularly spaced bands in the extended germ band stage (Rijsewijk et al. 1987). These bands coincide with the posterior aspect of each parasegment. Wingless is also expressed in head segments as well as being required for heart development (Schmidt-Ott and Technau 1992; Wu et al. 1995). Wingless acts non-autonomously to specify the fate of a specific neuronal precursor, NB4-2 (Bhat 1996). The Wg pathway is also required for tracheal development, since in wg- embryos a substantial amount of the dorsal trunk (DT) is not formed (Llimargases and Lawrence 2001). Later on in larvae development, wingless is a negative regulator of the morphogenetic furrow and affects tissue polarity in the developing Drosophila compound eye (Ma and Moses 1995). Wg protein has been detected in Wg-expressing cells at the dorsoventral (DV) boundary of the wing disc and in an irregular pattern of spots in nearby cells. The intensity and number of spots decreases with distance from the source of Wg, providing indirect evidence that Wg protein forms a gradient across the disc (Strigini and Cohen 2000).

The distribution of DWnt2 transcripts in embryos is predominantly segmented, with the additional presence of transcripts in the presumptive gonads. Transcripts of DWnt2 appear to be associated with limb primordia in the embryo and may therefore specify limb development. DWnt2 is expressed in embryos in all mesodermal cells of the gonad, before the mesoderm and germ cells have condensed to form a compact gonad. DWnt2 expression is limited to the posterior mesodermal cells of the gonad late in embryogenesis (Russell et al. 1992). This late pattern of expression is apparently maintained in the male, as DWnt2 is expressed at the posterior of the pupal gonad, in cells that will become the terminal epithelia. In the male pupal genital disc, DWnt2 is expressed in the epithelial cells at the apical tip of each developing seminal vesicle (Kozopas et al. 1998). Loss of DWnt2 produces a muscle migration defect in the male gonads, resulting in male sterility and a lack of the characteristic pigment cells that migrate over the male testis. Ovaries are normally not surrounded by pigment cells, but misexpression of DWnt-2 in females can
induce ectopic male-specific pigment cells. (Willert et al. 1999). In addition to these functions, DWnt2 can function together with wg in the developing trachea: when both genes are removed together, the phenotype is identical or very similar to that observed when the Wnt pathway is shut down. DWnt2 is expressed near the tracheal cells in the embryo in a pattern different from wg, but is also transduced through the canonical Wnt pathway (Llimargas and Lawrence 2001).

DWnt4 is 60kb upstream from wingless and is transcribed from the opposite strand of DNA than wingless. Further studies show that DWnt4 and wingless developmentally interact. wingless and DWnt4 are transcribed in overlapping embryonic territories under the control of the same regulatory molecules (Gieseler 1996). The ability of DWnt4 to induce additional wings, as Wg does, indicates that the two molecules can elicit similar cellular responses. Strong support for this conclusion is provided by rescue experiments of wg loss-of-function phenotypes. DWnt4 can restore normal wing development in the absence of a functional Wg protein at the second instar (Gieseler 2001). Recent studies revealed another function for DWnt4, the regulation of cell movements during Drosophila ovarian morphogenesis pathways (Cohen et al. 2002). These authors show that DWnt4 can facilitates cell movements through a signaling mechanism that results in focal adhesion kinase (FAK) accumulation. A model has been suggested in which DWnt4 promotes motility and regulates FAK through a distinct mechanism that bring together components from multiple Wnt.

The completion of the Drosophila genome sequencing revealed the presence of three more previously unrecognized Wnt genes: DWnt6, DWnt8, and DWnt10 (Adams et al. 2000). During embryogenesis, DWnt6 appears to be expressed at low levels. By late third instar larva however, this gene is expressed in a pattern that is identical for wingless. DWnt10 is expressed in the embryonic mesoderm, central nervous system and gut, whereas its expression was below detection in imaginal discs (Janson et al. 2001). For DWnt8 there is no information in the literature, with the exception of Llimargas and Lawrence (2001), in which overexpression of DWnt8, as well as DWnt6, were shown to rescue a tracheal dorsal trunk phenotype of double mutants for wg DWnt2, indicating a possible role in the tracheal development.
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**Figure 4. Drosophila Wnt genes after completion of the Drosophila Genome Project.**

Two functions of wg gene are shown here, its function as a segment polarity gene and the role of wingless in the tracheal system development. DWnt2 gene has a major role in the gonads development. DWnt4 can induce additional wings and it is important for the regulation of cell movements and focal adhesion kinase during Drosophila ovarian morphogenesis. Mutation for DWnt5 was not available up to now. For DWnt6, DWnt8 and DWnt10 there are no mutations. All Drosophila Wnt names have been given in accordance with homologies to the mouse orthologs.

Expression data for DWnt5 have existed since 1992, but there was no mutation available and therefore no functional data presented up to now for DWnt5. In the literature there is confusion concerning naming of the gene. In 1992, Russel and his colleagues isolated two Wnt genes DWnt2 and DWnt3. They named them 2 and 3 because they were the second and third genes isolated after wingless. The same year, Eisenberg and his colleagues (1992) isolated the DWnt3 gene, but named it as DWnt5 in view of the gene’s homology with the vertebrate Wnt5 gene pair. Since then, DWnt5 and DWnt3 were used as synonyms or referred to as DWnt3/5. In the following work we chose to use the name DWnt5.
1.2.3. *Drosophila Wnt5* gene

The existence of a fourth *Drosophila Wnt* gene, *DWnt5* has been discussed in the past (Eisenberg *et. al.* 1992; Russel *et. al.* 1992; Franklin *et. al.* 1995). The transcripts consist of 3862bp containing a long open reading frame sufficient to encode 1004 amino acids (Figure 5A). The amino-terminal sequence constitutes a largely hydrophobic domain consistent with the presence of a secretory signal peptide, as expected for Wnt family member.

The putative signal peptide is followed by a domain of 518 amino acids which show no significant homology with any sequence in protein databases, and only the carboxy-terminal half of the *DWnt5* product displays homology with known Wnt sequences. Within this region there is a 45% amino acid identity with Wg over a 304 amino acid overlap (excluding gaps) and 22 of the 24 cysteine residues present in this domain are found in corresponding positions in the Wg sequence. Relative to vertebrate sequences, the Wingless protein contains an insertion of 93 amino acids within the cysteine-rich region. This insert is not found in DWnt5 which contains an insertion of 145 amino acids is present in a more upstream position instead (Figure 5B). The insert constitutes a strongly hydrophilic domain and shows no relationship to other sequences in the database.

*DWnt5* gene has 49% similarity with mouse *Wnt5a* and 50% similarity with mouse *Wnt5b*. In addition to 49-50% sequence identity to both mouse gene products within the 304 amino acid conserved region; all 24 cysteine residues in both *Wnt5a* and *Wnt5b* are present in *DWnt5* in equivalent positions. Therefore, it is likely that *DWnt5* constitutes the *Drosophila* ortholog of the vertebrate *Wnt5* gene pair and perhaps the long amino terminal domain was lost during evolution (Eisenberg *et. al.* 1992).
Figure 5. Comparison of wingless, Drosophila Wnt5 and mouse Wnt5a molecules.

A. Amino acid sequence of DWnt5 protein aligned with those of the Drosophila wingless and mouse Wnt5a. Underlining indicates the putative secretory signal peptide at the aminoterminus. Conserved aminoacids are indicated by dots and cysteine residues are highlighted. Dashes represent gaps introduced to optimize the alignment.

B. Diagrammatic comparison of the proteins.
Ectopic expression of \textit{DWnt5} in transgenic \textit{Drosophila} embryos bearing a HS-\textit{DWnt5} construct leads to specific disruption of the commissural axon tracts of the central nervous system, indicating that DWnt5 protein might display a role in neuronal processes (Franklin \textit{et al.} 1995). In the same work, it was also shown that \textit{DWnt5} does not functionally replace \textit{wg} during segmentation in vivo, since the uniform \textit{DWnt5} expression in HS-\textit{DWnt5} embryos cannot alter en expression and cuticle morphology. Furthermore, experiments with a tissue culture cell line with a construct encoding the \textit{DWnt5} gene show that the DWnt5 protein is efficiently synthesized, glycosylated, proteolytically processed, and transported to the extracellular matrix and medium (Franklin \textit{et al.} 1995). \textit{DWnt5}, therefore, seems to encode indeed a secreted protein.

The lack of a mutant strain of flies missing a functional \textit{DWnt5} gene has been a limiting factor for the analysis of \textit{DWnt5}’s role during development. We have generated mutations in the \textit{DWnt5} gene that allowed a first evaluation of the role of \textit{DWnt5} activity. We show that \textit{DWnt5} gene has two distinct functions, one in the developing nervous system and a second function in regulation of cell numbers in animal development. Our results suggest therefore a novel function different from those reported for the known Wnt molecules.
2. Results

2.1. Characterization of the PX lethal lines

Analysis of the X chromosome insertions (PX) lethal lines starts with cloning of the insertion sites, mapping within genomic DNA and determination of the reporter gene expression patterns. This information will reveal the identification of genes that are responsible for the lethality, report their expression profile and finally will determine the correlation of biological and genomic information concerning the X chromosome in *Drosophila melanogaster*.

In collaboration with Peter Deak (University of Cambridge, U.K.) the time point of lethality was determined for the PX lethal lines using a balancer chromosome, which carried a GFP-transgene (coding for the green fluorescent protein from the jellyfish *Aequorea victoria*; Chalfie et. al. 1994). The balancer serves as a genetic marker in order to distinguish late embryonic and larvae stage lethal hemizygous males by the lack of fluorescent protein. The lethal phase was determined in 497 of the 501 PX lines. In 75 lines (14.9%) hemizygous males die in embryogenesis, in 14 lines (2.8%) the lethal phase is varied from embryonic to larval stages. The most frequent lethal stage is the larval stage, with 29.0% (145 lines). The rest are distributed as followed: 4 lines (0.8%) at prepupal stages, 81 lines (16.2%) at pupal stages, 53 lines (10.6%) between larval and pupal stages and 79 lines (15.7%) show a polyphasic lethality (Peter 2001).

The presence of the *lacZ* gene in the *PlacW* element was used to visualize the expression pattern of adjacent genes, using anti-[-]-gal antibody to stain mounted embryos carrying the P element. In 120 lines (20.9%) there was no expression detected. The remaining 381 lines (79.1%) showed specific expression in cells of specific organs, with the most frequent expression in the nervous system from 138 lines (37.5%).

In collaboration with Yuchun He and Hugo Bellen (Baylor College of Medicine, Houston) 400 from the 501 PX lines were analyzed by in situ hybridization in the polytene chromosomes, using a specific *PlacW* probe, in order to identify the localization of the P element on the X chromosome and to sort out lines with more than one element inserted. The majority of the insertions, 359 lines (89.5%), showed one hybridization signal, 39 lines (9.7%) showed two signals and 3 lines (0.7%) showed three (Peter et. al. 2001).
2.1.2. Molecular analysis of P element insertions

The flanking genomic sequence of the P insertions was revealed by “plasmid rescue” experiments. Annette Peter, Petra Schötter and Ulrich Schäfer initiated this work. Due to the fact that “plasmid rescue” requires many steps I used “Inverse PCR” method in order to identify, in a faster way, the P element adjacent genomic sequence. The analysis of 23 PX lines is presented in Figure 6 (see also Appendix), as an example for the characterization of the PX lines. The generated STS flanking the P element insertion sites were submitted to the EMBL nucleotide database. The integration sites were determined by BLAST search to the published Drosophila genomic sequence (Adams et. al. 2000). In cases where the gene annotation was supported by EST data, the identification of the affected genes was possible. For predicted genes whose open reading frame is only annotated, it is likely that the insertion is integrated in the vicinity of a putative transcription start codon, since there is a strong preference for integration in the 5’ end of genes (Spradling et. al. 1995).

A total of 513 STS sequences were generated from 496 strains. In five strains, repeated attempts to isolated flanking sequences failed, and in 16 the identification of the integration site was uninformative, since the P element has been inserted in repetitive DNA. In 11 of these lines, the insertion was within a yoyo retrotransposon (Whalen and Grigliatti 1998). The other 497 STSs were generated from the remaining 480 lines and are derived from unique X-chromosomal sequences. Five insertions were >7 kb away from any annotated gene and it is unclear whether the P integration is responsible for the observed lethal phenotype. Three additional P insertions occurred in the vicinity of known EP insertions but were at some distance from the next annotated gene, suggesting that the P elements have inserted into a putative promotor region of an unknown gene. Fourteen P elements, representing five composite genes, might affect two genes since a gene is localized within a large intron of another gene.

183 insertions affect 52 different genes that were previously characterized at both a molecular and genetic level. They include for example Notch, pebbled and short gastrulation. In addition, there are insertions in two phenotypically well characterized genes: troll, formerly known as zw1, with 12 insertions and stardust with one insertion. The largest group of insertions disrupts genes for which no mutation has previously been
reported. In most cases, some molecular information is available or the genes have been predicted by computer algorithms (Adams et al. 2000). Finally, 301 insertions affect 130 genes that remain to be further characterized (Peter et al. 2002).

Figure 6. Analysis of the PX lethal lines by Inverse PCR

<table>
<thead>
<tr>
<th>Insertion l(1)G0#</th>
<th>Accession number in EBI</th>
<th>enhancer trap expression</th>
<th>Localization by in situ</th>
<th>Putatively affected gene</th>
<th>Lethal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>031</td>
<td>AJ299996</td>
<td>CNS, PNS</td>
<td>12C</td>
<td>CG4262 (elav)</td>
<td>embryonic</td>
</tr>
<tr>
<td>042</td>
<td>AJ300020</td>
<td>Brain specific, CNS, PNS</td>
<td>2B1-8</td>
<td>CG11491 (broad-complex)</td>
<td>L3, prepupae</td>
</tr>
<tr>
<td>058</td>
<td>AJ299998</td>
<td>-</td>
<td>1C1-2</td>
<td>CG16983 (skpA)</td>
<td>L3</td>
</tr>
<tr>
<td>179</td>
<td>AJ427040</td>
<td>-</td>
<td>19F</td>
<td>(repetitive DNA)</td>
<td>pupae</td>
</tr>
<tr>
<td>191</td>
<td>AJ427041</td>
<td>PNS, glial cells</td>
<td>-</td>
<td>Yoyo element</td>
<td>Semilethal</td>
</tr>
<tr>
<td>207</td>
<td>AJ426685</td>
<td>Epidermis, amnioserosa, muscles</td>
<td>4C11-14</td>
<td>CG6998 (cutup-dynein light chain)</td>
<td>pharate adult</td>
</tr>
<tr>
<td>222</td>
<td>AJ426971</td>
<td>-</td>
<td>16B6-11</td>
<td>CG8465 (hypothetical protein KIAA0692 with ankyrin human repeats)</td>
<td>L3</td>
</tr>
<tr>
<td>318</td>
<td>AJ300017</td>
<td>Midgut, CNS, fatbody</td>
<td>2B1-8</td>
<td>CG11491 (broad-complex)</td>
<td>pupal</td>
</tr>
<tr>
<td>319</td>
<td>AJ299994</td>
<td>CNS, muscles</td>
<td>1B7-10</td>
<td>CG4262 (elav)</td>
<td>embryonic</td>
</tr>
<tr>
<td>329</td>
<td>AJ426827</td>
<td>-</td>
<td>1B7-10 &amp; 9D</td>
<td>CG12639 (novel)</td>
<td>L3, prepupal, pupal, pharate adult</td>
</tr>
<tr>
<td>376</td>
<td>AJ426784</td>
<td>Tracheal, muscles, CNS</td>
<td>7E5-10</td>
<td>CG11195 (TATA box-binding protein)</td>
<td>pharate adult</td>
</tr>
<tr>
<td>378</td>
<td>AJ299995</td>
<td>CNS, PNS</td>
<td>1B7-10 &amp; 4E1-2</td>
<td>CG4262 (elav)</td>
<td>embryonic</td>
</tr>
<tr>
<td>379</td>
<td>AJ426768</td>
<td>CNS</td>
<td>7D20-22</td>
<td>CG2151 (thioredoxin reductase)</td>
<td>pharate adult</td>
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<tr>
<td>394</td>
<td>AJ427046</td>
<td>-</td>
<td>12C4-5</td>
<td>Yoyo element</td>
<td>L3, prepupal, pupal, pharate adult</td>
</tr>
<tr>
<td>398</td>
<td>AJ427047</td>
<td>-</td>
<td>12C</td>
<td>Yoyo element</td>
<td>viable</td>
</tr>
<tr>
<td>399</td>
<td>AJ299993</td>
<td>CNS, brain, PNS</td>
<td>1B3-4</td>
<td>CG13372 (EG:171D11.2 ATP-dependent permease)</td>
<td>L3</td>
</tr>
<tr>
<td>422</td>
<td>AJ300003</td>
<td>-</td>
<td>1C</td>
<td>CG7434 (ribosomal protein L22)</td>
<td>-</td>
</tr>
<tr>
<td>431</td>
<td>AJ300011</td>
<td>Fatbody</td>
<td>2A</td>
<td>CG14788(EG:BACN32G11.5 GTP-binding protein)</td>
<td>semilethal</td>
</tr>
<tr>
<td>448</td>
<td>AJ300016</td>
<td>-</td>
<td>2B1-4</td>
<td>CG14792 (stubarista)</td>
<td>L1, L2</td>
</tr>
<tr>
<td>467</td>
<td>AJ427050</td>
<td>Fatbody, gut</td>
<td>12D</td>
<td>Yoyo element</td>
<td>-</td>
</tr>
<tr>
<td>471</td>
<td>AJ299997</td>
<td>Brain, CNS, PNS, tracheal</td>
<td>1B11-14</td>
<td>CG7622 (ribosomal protein L36)</td>
<td>Male sterile</td>
</tr>
<tr>
<td>472</td>
<td>AJ426897</td>
<td>-</td>
<td>12C-D</td>
<td>CG10997 (chlorine channel protein P64)</td>
<td>semilethal</td>
</tr>
<tr>
<td>501</td>
<td>AJ300094</td>
<td>Amnioserosa, malpigian tubes</td>
<td>3E5-F2</td>
<td>CG2849 (ralA)</td>
<td>pharate adult</td>
</tr>
</tbody>
</table>
2.2. Functional studies of the l(1)G0265 line

For a detailed and paradigmatic characterization I chose the lethal insertion l(1)G0265 line, which is located in the *Drosophila* Wnt5 locus. First, I applied molecular techniques to determine the gene structure and the expression patterns and continued with genetics in order to reveal the role of the gene during development.

2.2.1. Genomic organization of the Wnt5 locus

Plasmid rescue as well as Inverse PCR was performed using the *PlacW* insertion line and the cloned PCR genomic fragments were sequenced. The sequence analysis showed that the l(1)G0265 insertion occurred at the AC018226 Celera clone (Mark et al. 2000). Moreover, the analysis showed similarities to seven EST’s clones (LD35976, LD28671, LD27626, LD22778, LD22614, LD32139, LD30719) of *Drosophila melanogaster*. The 5’prime ends of all the above EST clones were similar to M97450 cDNA, which represents the complete cDNA (3812bp) of the *Drosophila melanogaster* Wnt oncogene analog 5 region (DWnt5).

Alignment of all the above sequences showed that the *PlacW* element is located 21 bp (Figure 7) upstream the Wnt5 transcribed region (17B5). Comparisons of the AC018226 Celera clone (covers a genomic DNA region of 73617bp) with the M97450 complete cDNA reveals no difference in the sequence, indicating that DWnt5 gene has no introns. The transcript comprises of 3862bp, including a 5’UTR of 552bp, a coding region of 3015bp and a 3’UTR of 295bp.

The position of the l(1)G0265 as identified molecularly is in accordance with results obtained from *in situ* hybridization to squashes of polytene salivary gland chromosomes which pace the insertion into region 17A-17C of the X chromosome (Figure 8).

2.2.2. Isolation of DWnt5 alleles

In addition to the lethal *PlacW* element insertion l(1)G0265, three different DWnt5 mutations were recovered from another independent screen of *Drosophila melanogaster* by the Göttingen X Chromosome Project (U. Schäfer, personal communication). This second screen is currently in progress and its aim is the identification of new genes, whose
elevated expression levels cause lethality or other visible phenotypes (U. Schäfer, unpublished data). The lines that were isolated from the second screen are the homozygous viable lines: \( P\{y+\}G1278, P\{y+\}G5874, P\{y+\}G3069. \)

The insertion positions of these three \( P\{y+\} \) elements are 24, 30 and 546 bp upstream the 5’UTR of \( DWnt5 \) gene, respectively (Figure 7). The three \( P\{y+\} \)-lines contain 5xUAS constructs at the 5’end of the P-element and the orientation of two of them allow overexpression of the \( DWnt5 \) gene using the Gal4-UAS system (Brand and Perrimon, 1993) for directed misexpression of the gene. In order to test these lines for overexpression, homozygous females for \( P\{y+\}G1278 \) and \( P\{y+\}G5874 \) were crossed with \( enGAL4 \) males. Embryos from this cross were examined for \( DWnt5 \) RNA expression (\textit{in situ}). The detection of \textit{engrailed} stripes (Figure 9) indicate that the \( DWnt5 \) gene can be activated by ectopically expressed GAL4 and thus the two \( P\{y+\} \) lines can be used for \( DWnt5 \) overexpression studies.

**Figure 7. Genomic organization of \( DWnt5 \)**

A. Polytenic X chromosome (top) and annotated sequences of the 17B region of the X chromosome (red box in X chromosome on top)).

B. Transcription is from left to right, and the relative positions of the five P-element integration sites are shown. The putative transcript starts 21 bp from the end of \( Wnt(PlacW)\)\textit{G0265}. Comparison of the genomic and cDNA sequence revealed the \( DWnt5 \) transcript contains no intron. Filled boxes represent translated regions and open boxes indicate untranslated regions.
Figure 8. Localization of \textit{DWnt5}

A. Schematic draws of the \textit{PlacW} integration into the X chromosome. The genome sequence is in black; the 8 bp duplication upon insertion is in blue and the \textit{P} element sequence in red.

B. In situ hybridization to squashes of polytene salivary gland chromosomes shows one signal in position 17A-17C.
Figure 9. The P(y+)G1278 and P(y+)G5874 lines allow overexpression of the DWnt5 gene using the Gal4-UAS system.

A. An embryo at stage 14 is stained with DWnt5 RNA DIG probe (see Material and Methods, 4.2.1.1). The genotype of the embryo is P(y+)G1278/+;enGAL4/+ or P(y+)G1278/Y;enGAL4/+.

B. In situ hybridization (with DWnt5 RNA DIG probe) showing the engrailed stripes on an embryo at stage 16. The genotype of the embryo is P(y+)G5874/+;enGAL4/+ or P(y+)G5874/Y;enGAL4/+.

2.2.3. Characterization of the l(1)G0265 line

The time point of lethality of animals having the insertion l(1)G0265 was determined using the green fluorescence protein as a marker to select individuals of the proper genetic background. Heterozygous females l(1)G0265/FM7i,pActGFP were crossed with FM7i,pActGFP balancer males. The GFP marker allows the identification of larvae having the balancer chromosome and therefore males hemizygous for the mutation can be distinguished by the lack of the GFP marker. These males die at late pupae stage.

In order to check whether the l(1)G0265 insertion and the associated lethal phenotype can be linked, experiments for precise excision of the PlacW element was performed. Heterozygous females of the l(1)G0265 line were crossed with males having the transposase source P[ry+]/(2-3)(99B) (Zhang and Spradling 1994). In the next generation (F1) females in whose germ cells the PlacW can be mobilized, were mated to FM6 males (Figure 10A). In the subsequent F2 generation, the reversion of the eye color from w+ to w− in the fully viable males suggested that the P element was excised (Figure 10A). Sequencing analysis (See Material and Methods, 4.3.6.) of PCR fragment from genomic DWnt5 region of the viable males showed that the inserted sequences of the P element were excised and the wild type sequence was restored (Figure 10B). These white-eye flies that
are therefore “revertants” indicating that the lethality associated with the \( l(1)G0265 \) insertion could be reverted by mobilizing the P element.

The line Dp(1;Y)W39, Bloomington Stock line 1538, carries a duplication of the region 16F1-3 to 18A5-7 and 19E5-7 to 20F on the Y chromosome. Heterozygous females for the \( l(1)G0265 \) mutation were crossed to Dp(1;Y)W39 males (see Material and Methods, 4.1.2.). The resulting males (“rescued males”) carry the mutation in the X and the duplication in their Y chromosome and are viable and fertile. This is the second indication that gene disruption caused by the P element is the reason for the \( l(1)G0265 \) lethality.

**Figure 10. Reversion of the \( l(1)G0265 \) lethality by precise excision of the PlacW element.**

A. Genetic cross for the remobilization of the PlacW element (for details see text).
B. Molecular analysis of one homozygous revertant line. The mobilization of the PlacW element was precise, the wild type sequence was restored as revealed by sequencing analysis. The genome sequence is represented with black color; the 8 bp duplication upon insertion is in blue and the P element sequence in red.
Figure 11. Reversion of the l(1)G0265 lethality by rescue experiments.

The genomic rescue construct encompassing the DWnt5 transcript and its endogenous regulatory regions and on the lower picture a schematic draw of the genetic cross for the rescue experiment using the genomic rescue construct. The introduction of one copy of the rescue construct is sufficient to revert the lethality in the l(1)G0265 males.

Since the duplication covers many genes, a 5371bp genomic DWnt5 rescue construct (Figure 11) was used for the generation of transgenic flies, in order to confirm that only the activity of the DWnt5 locus can rescue the lethality of the l(1)G0265 males. Homozygous transgenic flies for the rescue construct (R) were crossed to flies heterozygous for the l(1)G0265 mutation. In the offspring, mutant males bearing one copy of the genomic rescue construct were fully viable (Figure 11). Hence, the lethality of the l(1)G0265 line is rescued by the genomic rescue construct, which encompasses the DWnt5 transcription unit and endogenous regulatory regions sufficient for the proper activity of the gene. These data,
together, are the final evidence that the lethality of the \( l(1)G0265 \) line is due to the disruption of the \( DWnt5 \) gene by the \( PlaCw \) element insertion.

2.3. \( DWnt5 \) expression

In order to examine the expression pattern of the \( DWnt5 \) gene, I performed whole mount in situ hybridization of ovaries and embryos of various stages of development (see material and Methods, 4.2.1.). High levels of \( DWnt5 \) RNA are provided maternally as the transcript is present in the nurse cells, from where it is transported to the oocytes (Figure 12). The early embryonic stages showed a ubiquitous distribution of the \( DWnt5 \) transcript. Later on, and as the germ band extends, the transcript levels increased specifically in distinct groups of cells, which, on the basis of their position, appear to correspond to the limb primordial of the antennal, maxillary and labial segments. In addition, two groups of cells were labeled in the clypeolabrum. At later embryonic stages, the transcript distribution become modulated in every segment until early stage 14, when the RNA persists in the ventral neuroectoderm and become restricted to the CNS.

![Image](image12.png)

**Figure 12. \( DWnt5 \) expression during development**

The gene shows a strong maternal contribution. The transcript is ubiquitously distributed at the early embryonic stages. Later it is strongly expressed in the limb primordial. After stage 14, the gene product accumulates in the CNS.
Genomic and cDNA fragments from DWnt5 were used to probe Northern blots of RNA from different stages of Drosophila development (Eisenberg et al, 1992, Russel et. al, 1992). The transcript is detectable within the first 3 hours of embryogenesis, reaches a peak between 3 and 9 hours and then declines. The transcript is most abundant in the larvae (with the exception of stage L2) and pupae stage, as well as in the adults. Therefore, it seems that the DWnt5 gene is necessary throughout development, from early embryogenesis till the adult fly (Eisenberg et. al. 1992).

2.4. Germ line clones using the PlacW insertion l(1)G0265 line

The DWnt5 transcripts are expressed in the nurse cells of the ovary, indicating that DWnt5 is maternally transcribed and transported into the growing oocyte and egg, where it remain at least until stage 5 of embryogenesis (Figure 12). Therefore, activity related to the maternal transcripts could fulfill essential functions for embryonic development. By using the dominant female sterile technique (Xu and Rubin 1993), it is possible to produce mutant DWnt5 eggs, which lack the maternal contribution. The crossing scheme, the genetically marked chromosomes and the processes involved are schematically shown in Figure 13.

The result of such an experiment was no egg production by mothers with germ line clones. In order to check whether the females carrying the germ-line clones show defects in their reproductive system, ovaries and ovarioles were dissected and stained for DNA and actin (see Material and Methods, 4.4.8.). The confocal pictures showed no significant abnormalities between ovaries with mutant germ-line clones and ovaries from wild-type females (data not shown). The only phenotype that could be detected was that mutant ovaries seemed to have a delayed development and contained more unmatured ovarioles, but the DNA as well as the actin cytoskeleton appeared normal (see also below for more details). Therefore it looks like the ovaries have no obvious defects other than grow very slow.
Figure 13. The dominant female sterile technique for selecting homozygous germ-line clones.

The FLP induces specific recombination at the FRT sites and after chromosome segregation, homozygous mutant germ-line clones are produced. The homozygous for the ovoD2 as well as the non recombinant embryos will be destroyed after the fixation procedure (see Material and Methods, 4.2.1.2.).
2.5. **DWnt5 function in the developing nervous system**

2.5.1. **DWnt5 mutant embryos show defects at the exit junction**

The late embryonic RNA expressions of DWnt5 in the CNS (Figure 12) as well as published overexpression data (Fradkin et. al. 1995) suggest that *DWnt5* might play a role in the development of the embryonic CNS. Therefore, the 22C10 as well as fasc II antibodies were employed to detect a possible mutant phenotype in the nervous system. 22C10 is a monoclonal antibody specific for Futsch protein, which is expressed in some CNS neurons as well as in all neurons in PNS and fascII antibody recognizes Fasciclin II protein important for the fasciculation of the axons (Hummel et. al. 2000; Interactive Fly).

![Figure 14. DWnt5 mutants show problems in the exit junction.](image)

Preparations of stage 17 embryos. Central nervous system (CNS) stained for the presence of fasc II (A,D) and mAb22C10 (C,F) antibodies. In wild-type embryos (A,C), shortly after leaving the ventral cord, the intersegmental nerve (ISN) and the segmental nerve (SN) come together at the exit junction, in contrast to the DWnt-5 mutants (D, F), where ISN and SN nerves stay apart from each other. In B and E pictures, schematic drawings represent of the development of the axonal pathways in the wild type and the mutant respectively. The penetrance of the phenotype is 90.2% (36 hemisegments) while in wild-type embryos is only seen at a frequency of 7.4% (36 hemisegments).
In the late stage mutant embryos, the distance between intersegmental (ISN) and segmental nerve (SN) is enlarged. To examine this phenotype in detail, flat preparation of embryos (Material and Methods, 4.2.3.) stained with 22C10 and fasc II antibodies was performed, in order to verify the defects in the ventral nerve cord. In wild-type embryos (Figure 14) shortly after leaving the ventral cord, the ISN and the SN nerves come together at the exit junction, so that anastomosis can happen. In DWnt5 mutant embryos the ISN and SN nerves are leading towards each other, however, their distance appears abnormal. It is possible that anastomosis might not be complete and as a secondary effect, the innervated muscles might have defects.

During the development of the nervous system, the ISN nerve uses as a substrate the dorsal trunk of the tracheal system in order to extend. Using the A12 antibody as a marker for the tracheal system we checked the possibility that defects in the distance between ISN and SN nerve might arise from defects in the dorsal trunk. No major differences in the development of the tracheal system was observed between DWnt5 mutants and wild type embryos (data not shown), suggesting that the observed phenotype in the exit junction is indeed caused by defects in the nervous system.

2.5.2. \textit{l(1)G0265} is a hypomorph allele of DWnt5 gene

In the Bloomington stock collection the deficiency Df(1)N19 exist (BL#970, its breakpoints include 17A1; 18A2) which, in addition to other genes, lacks the DWnt5 gene. Embryos with the genotype \textit{PlacW}/Df(1)N19 are lethal and have a much more severe nervous system phenotype (Figure 15), indicated by an even larger distance between ISN and SN nerves. In addition, these embryos appear to have some defects in the anterior and posterior commissures. The axons appear as if they have not separated from each other and the longitudinal axons are sometimes missing. The fact that the P element over the deficiency impairs the nervous system phenotype suggests that \textit{l(1)G0265} is a hypomorph allele.
Figure 15. The *PlacW* element over the deficiency enlarges the nervous system phenotype.

A. The anterior and posterior commissure have not separated properly and longitudinal connectives are missing.
B. The distance between ISN and SN is larger and the connectives are abnormal.

2.5.3. **Mutants for DWnt5 have problems in fasciculation of the longitudinal axons**

Beside the defects at the exit junction, we observed pronounced increase of longitudinal connectives in *DWnt5* mutant embryos (Figure 16). Using an antibody directed against the Fasciclin II protein, we found that longitudinal connectives do not properly form and axons are occasionally thinner. In wild type embryos of the same stage (st. 16), three longitudinal axons in each side can be observed, in contrast to the *DWnt5* mutant embryos where the number is increased, individual fascicles are difficult to recognize and breaks are often observed in the connectives.
Figure 16. DWnt5 mutants have problems in fasciculation of the longitudinal axons.

Frontal views of embryonic (st.16) nerve cords stained for axons where fasciclin II protein is expressed. Anterior is up and posterior is down.
(a): In wild-type embryos three fascicles are observed in each side.
(b): In mutant embryos, individual fascicles look thinner, with small gaps in between. Pronounced increase in the number of longitudinal connectives are found.

2.5.4. **DWnt5 does not affect PNS or glial cell development.**

In addition to the CNS, the peripheral nervous system was analyzed using the 22C10 antibody (Hummel *et al.* 2000). No gross alterations were observed in the embryonic PNS and the axonal progression toward the ventral nerve cord as well as the pentascalopodia organ (Campos-Ortega and Hartenstein 1997) appears to be normal (data not shown).

The midline glial cells are required for the proper formation of the ventral nerve cord commissures, (reviewed in Goodman and Doe, 1993). Since DWNT5 protein is thought to be a secreted molecule, it could well be that it affects the development of glial cells. In order to detect if DWnt5 plays a role in neuron-glial cell interactions, i.e. in the development or maintenance of glial cells, staining with δ-repo antibodies was performed.
(Figure 17). The phenotypic analysis of DWnt5 mutant embryos showed an apparently regular pattern of the glial cells, suggesting no obvious function of the DWNT5 protein in the development midline glial cells.

Figure 17. Glial cell development using a [‑]‑repo antibody.
A. The pattern of midline glial cells after antibody staining with [‑]‑repo in a wild type.
B. The pattern seems normal in a l(1)G0265/Y mutant embryo and no obvious defects are observed in the development of glial cells.

2.5.6. Overexpression experiments in the nervous system.

In order to check if high levels of the protein can cause any effect in the nervous system, overexpression experiments were performed using the UAS/GAL4 system (Brand and Perrimon, 1993). The lines P {y+}G1278, P {y+}G5874 generated by the second screen of the Göttingen X chromosome project were used, since previous experiments (Figure 9) showed that they can drive expression of DWnt5 gene when a GAL4 driver line is introduced. Embryos, having two copies of simGAL4, which drives expression in the midline cells (Luer et. al. 1997) and one copy of either the P {y+}G1278 or P {y+}G5874 element, are stained with the BP102 antibody which recognizes an antigen on the cell surface of all neuronal cells. Flat preparations of embryos were checked under the light microscope in order to observe the ventral nerve cord in detail. Ectopic DWnt5 expression leads to abnormalities in the axon tracts of the embryonic CNS (Figure 18) and occasionally the anterior commissure is not observed.
Figure 18. Overexpression of DWnt5 in the nervous system.

A. and B. Embryos overexpressing DWnt5 show problems in the anterior commissures (ac Genotypes: (A): P (y+)G1278/+;simGal4/+;simGal4/+ and (B): P (y+)G5874/+;simGal4/+;simGal4/+ ; simGal4/+. ). Anterior is to the left. The embryos have been immunostained with BP102 (DHIB) antibody which marks the commissures (see Material and Methods, 4.2.2.1.).

2.6. DWnt5 altering body size by regulation of cell number

2.6.1. Homozygosity of l(1)G0265 produces dwarf female flies

Heterozygous female flies for the l(1)G0265 mutation were mated to males carrying the duplication on the Y chromosome (Figure 19). Homozygous female flies were among the offsprings, which indicates that the l(1)G0265 is a partial loss-of-function mutation in DWnt5 gene.

Flies that are homozygous for the l(1)G0265 mutation show a phenotype similar with that previously described for weak heteroallelic combinations of insulin receptor (Tatar et. al. 2001). The body size is proportionally reduced. These small flies show an overall delay in development and enclose 5-9 days after their heterozygous siblings (Figure 20).
Closer investigation revealed that many of these small flies are dying just before they can hatch from the pupal cages and interestingly, when the pupae cages are removed, they seem to have defects (>90%) in their abdomen (Figure 21). The small proportion of the \(DWnt5\) mutant flies that are able to hatch (~0.7%) show less severe or no obvious abdomen defects. However, although the imaginal hypoderm of the last abdominal segment, including the outer genitalia of the fly, has no obvious damages, all of the small flies fail to lay eggs within more than seven weeks after hatching.

**Figure 19. Schematic cross for the production of the homozygous \(DWnt5\) small flies.**

Heterozygous for the P element females are crossed to “rescued” males (carrying a duplication of the \(DWnt5\) locus on the Y chromosome). The progenies can be followed by the GFP balancer chromosome as well as by the Barr marker. Homozygous for the mutation females are B' and lack GFP. These flies show a reduction in body size.
2.6.2. Dwarf DWnt5 female flies enclose with extremely immature ovaries.

In order to learn whether the inability of the small DWnt5 mutant flies to lay eggs is due to defects in their reproductive system, ovaries and ovarioles were stained for DNA and actin, using fluorescent secondary antibodies and observed under fluorescent light. In wild type
ovaries prepared 24h after eclosion, germanium and stages 1 up to early 9 can be seen (Figure 22A and B). In contrast, in ovaries of homozygous DWnt5 mutant flies 24h after eclosion, all ovarioles are immature (Figure 22C and D). The latest stage that can be observed is stage 6. Although the morphology of the whole ovariole in the mutants seems normal, the total number of ovarioles in each ovary is reduced. DWnt5 homozygous ovaries from mutant flies 18 days after eclosion (Figure 22E and F) have developed further but the degree of maturation still resembles that of 24h eclosed wild type ovaries.

Figure 22. Ovaries and ovarioles of wild type and DWnt5 small flies stained with rhodamine-phalloidin for actin and Sytox for DNA.

A. and B. Wild type ovary prepared 24h after eclosion. Germanium and stage 1 up to begin stage 9 are present.
B. and D. DWnt5 homozygous ovary 24h after eclosion. All ovarioles are immature and no more than stage 6 can be seen. These two pictures represent the whole homozygous ovary.
E. and F. DWnt5 homozygous ovary 18 days after eclosion. The degree of maturation resembles that of 24h enclosed wild type flies.
2.6.3. **The developmental delay in homozygous ovaries is not caused by cell death.**

The possibility that the female infertility of the mutant flies is caused by nuclear death can be excluded by looking at the DNA in early as well as in late stage ovaries in Figure 22. There was no observation of degenerated egg chambers, fragmented or condensed nurse nuclei and the nuclear shape was regular.

To obtain further proof that lack of *DWnt5* is not leading to the destruction of the ovaries, staining with acridine orange (AO) was performed. AO is a vital dye that is known to selectively stain apoptotic cells in insects (Spreij. *et. al.*, 1971, Abrams *et. al.*, 1993) and has successfully been used to study the distribution of apoptosis in *Drosophila* ovaries (Foley and Coley *et al.*, 1998). No AO positive nuclei were detected in these experiments and the DNA seemed to be normal with no indications of degeneration (data not shown). These observations exclude the possibility that apoptotic cell death.

2.6.4. **Slow development of dwarf DWnt5 flies shortens the life span**

It has been proposed (Bartke *et. al.*, 2000) that reduced body size per se can result in life-span extension in mammals. To check if the delay in development of mutant *DWnt5* flies is connected to mechanisms underlying aging and longevity, we performed measurements of the life-span of the dwarf *DWnt5* females.

While there were no significant differences between wild type flies and heterozygous flies concerning longevity, homozygous mutant flies did not survive longer than 55 days. In fact, they reached 50% of their expected life-span 31 days after eclosion, while wild type flies reach the same levels 60 days after eclosion (Figure 23). Thus, it appears that the dwarf phenotype of *DWnt5* mutants does not contribute to extension of life-span but in contrast, they have short life-span.
Figure 23. Life-span determination of homozygous dwarfs DWnt5 mutant flies.
Note the significantly decreased life-span of females. They reached 50% of their expected life-span 31 days after eclosion, while wild type flies and heterozygous flies for the Wnt5/PlacWf^G0265 allele are living longer.

2.6.5. DWnt5 regulates body size

The genetic crosses were adjusted in a way (Figure 19) that DWnt5 mutants can be followed by the lack of the GFP protein in the balancer chromosome as well as with the different gonad size in larvae, which is used for the identification of the two sexes (Ashburner 1989). This situation serves the possibility to follow development in all stages after larval development has been completed. Comparison of the DWnt5 mutants with their siblings revealed a body size reduction of the homozygous animals in almost all the developmental stages (Figure 24A). Comparison of the embryos was not possible, since they have maternal DWnt5 contribution of the heterozygous mother (see above, 2.4.). In addition, homozygous DWnt5 female adults are sterile, as well as there are no eggs produced by adults with germ line clones. Therefore, the size of eggs with strongly reduced DWnt5 activity could not be reliable assessed.
To quantify the size differences between wildtype and mutants, I determined the weight of individual flies (Figure 24B). Flies homozygous for the P-element l(1)G0265 have a weight reduction of 45.2% compared with revertant flies of the same age. All flies were reared under the same growth conditions and were aged matched (2 days old) before weighing.

Figure 24. Proportional body size reduction of DWnt5 mutant animals
A. Homozygosity causes an overall delay in all developmental stages. Since the female adults are sterile, and adults with germ line clones produce no eggs, we cannot comment on the egg size.
B. Body weight of individual homozygous wnt5 flies (n=20) was measured. There is a weight reduction of 45.2% compared with control revertant flies of the same age. All flies were reared under the same growth conditions and were age matched (2 days old) before weighing.
2.6.6. *DWnt5* regulates body size by regulation of cell number rather than by affecting cell size

In order to verify that individual organs in the mutant *DWnt5* flies are also smaller, comparisons of eyes from wild type and homozygous mutant flies were performed. Scanning Electron Microscope (SEM) analysis revealed a severe reduction of the eye size of the dwarf flies (Figure 25A) under the same magnification. Furthermore, comparison of adult wings of flies with different genotypes showed reduction in the organ size of homozygous *DWnt5* mutants (Figure 25B and C). In both organs, the reduction in the body size of *DWnt5* dwarf mutant flies could be due to a reduction in the number of cells and/or a reduction in the size of the individual cells (Conlon and Raff 1999). To determine cell number, eyes and wings of the adult animals were analyzed.

In the wings, each epithelial cell secretes cuticle with a single hair; thus counting the number of hairs provides a direct measure of cell number. As shown in Table 1 (Figure 25D), there is a 21% reduction of the cell number of homozygous mutant wings compared with the wings of the P element revertants. There is 8.7% reduction in cell number of heterozygous wings compared with revertant wings as well. The area that has been chosen for counting is the region between vein IV and V (Figure 25B). Similar results obtained by counting the ommatidia in the adult eye. These data show a cell number reduction is contributing to the overall reduction in the animal’s size.

In order to examine the cell size in the *DWnt5* mutants during development, we generated genetically marked homozygous mutant cells using the FLP/FRT system (Xu and Rubin 1993). This allows direct comparison between homozygous mutant clones and their wild type sister clones, called twin spots, that are generated at the same developmental time due to the mitotic recombination event. Imaginal wing discs were examined for mutant clones and their corresponding twin spots. The green fluorescent protein (GFP) marker on the wild type chromosome allowed for distinguishing wild type cells from mutant cells. The experimental design of the mitotic recombination analysis is outlines in Figure 26.
Figure 25. Mutation in DWndt5 Decreases Organ Size

A. Scanning Electron Micrographs (SEM) of wild type adult eye compared with

B. An eye with mutation that disrupts the Drosophila WNT5 Homologue.

C. Comparison of adult wings reveals significant differences between the different phenotypes.

D. Counting of single hairs in each genotype gives a direct measurement for the cell number in the wing. The counting area is marked on the wild-type picture in figure C. The columns represent the cell number counted from 5 different wings (n=5) of each genotype.

E. Reduction in the cell number accounts for 21% in the homozygous and 8.7% in the heterozygous, of the total reduction in the wing size (compared with cell number in the revertant wing).
Figure 26. Using the FLP/FRT system to generate mitotic clones.

The recombinants PlacW-FRT18A flies can be used to generate mitotic clones in the imaginal discs. Upon heat-shock FLP recombinase mediates site-specific recombination between the FRT18A (indicated with boxes) sites during replication. If recombination occurs and the chromatids segregate appropriately, two type of cells can be produces, one homozygous for the PlacW and one wild type. The use of appropriate markers, such as UbiGFP, gives the opportunity to distinguish between the two cell types by following the GFP activity.
To visualize the cell surfaces, wing imaginal discs were also immunostained with $\alpha$-spectrin antibody, which is used as a marker for the plasma membrane (Pesacreta et. al. 1989). Three differences between mutant and wild type twin clones were observed. First, $DWnt5$ mutant clones are rare. This is most likely due to the fact that small mutant clones comprise only a few cells, suggesting that either the homozygous $DWnt5$ cells proliferate more slowly than wildtype cells or undergo cell death. Secondly, the clones are significantly smaller than the wild type sister clones (Figure 27) and comprise of fewer cells than the wildtype twin clones. Both results are in accordance with previous results in the adult wing about a cell number reduction in $DWnt5$ mutants. Thirdly, there was no significant difference concerning the cell sizes of mutant and wild type clones. These results suggest that the $DWnt5$ gene is affecting cell number but not cell size.

![Figure 27. DWnt5 is affecting cell number but not cell size.](image)

A. The mutant clone (white line) is significantly smaller and comprises only a few cells than the wild type sister clone (green line).
B. A closer observation allows comparison between mutant cells (GFP) and wild type cells (GFP'). The size between cells is similar (for example a,b,c). Differences in c,f,g, are due to different confocal levels. The antibody $\alpha$-spectrin has been used to mark the cells (see Material and Methods, 4.4.6.).

The reduction in cell number caused by the absence of proper $DWnt5$ function may be the result of a prolonged cell cycle time or of impaired cell survival during development. To determine whether the reduced number of $DWnt5$ mutant clones is due to nuclear death, examination of mutant clones in the imaginal discs was performed using DAPI, a dye which stains DNA and allows estimation of the DNA content in confocal images. An increase in morphological signs of programmed cell death was not observed; such as
enlarged nuclei or cells with picnotic nuclei in *DWnt5* mutant clones, in the imaginal discs. These results are also consistent with the analysis of the ovaries of the *DWnt5* small flies, showing that the delayed growth is not a matter of cell death.

Taken together these results support the idea that *DWnt5* function is not necessary for cell survival but instead required for cell proliferation. Homozygous *DWnt5* mutant cells have a selective growth disadvantage: they grow more slowly than wild type cells, as indicated by their representation in the ovaries and in the adult eye and wing, and cannot reach the normal number seen in the wild type flies.

### 2.6.7. Elevated levels of DWNT5 protein increase cell proliferation

Since partial loss of *DWnt5* function results in a decrease in cell number, we examined whether the overexpression of *DWnt5* would have the opposite effect; i.e. increases the speed of cell proliferation. For this purpose, the *P {y+}G1278* and *P {y+}G5874* lines were used. Overexpression of a unique copy of *DWnt5* under the control of GMR-GAL4, which drive GAL4 in the eye imaginal disc, provides strong expression in all cells behind the morphogenetic furrow (Flybase), showed no detectable effect in the eye.

*DWnt5*, like all wnts, is thought to be a signaling molecule that binds to a cell surface receptor in order to activate the corresponding pathway. It has been reported (Conlon and Raff 1999) that the concentration of signaling molecules is crucial and can influence the rate of cell cycle progression. Moreover, the amount of these molecules has to reach a threshold value for the cell to pass through the checkpoint (Polymenis and Schmidt 1997). Therefore, it is possible the level of *DWnt5* activity matters to the cell and that higher levels of the protein are required in order to cause a gain-of-function phenotype in the overexpression organ. I therefore generated UAS-*DWnt5* as well as UAS-*DWnt5*GFP constructs (Figure 28) to examine whether the level of DWNT5 protein matters to the overexpressing cell.

![UAS-DWnt5 and UAS-DWnt5GFP constructs](Figure 28)

*Figure 28. Schematic representation of UAS-DWnt5 as well as UAS-DWnt5GFP constructs.*

(For the construction, see Materials and Methods, 4.3.2.).
Trangenic animals were generated (as described in Rubin and Sprandling 1982), containing the UAS-DWnt5 or UAS-DWnt5GFP construct on either the second or third chromosome. These transgenic flies were used for the generation of double homozygous flies (see Material and Methods, 4.3.2.). The latter flies allow introduction of two copies of UAS-DWnt5 or UAS-DWnt5GFP in differentiating cells of the eye imaginal disc that can be driven by GMR-GAL4. This genotype results in a rough eye phenotype in a frequency of 82%. All the flies with rough eye phenotype were used for SEM analysis. The SEM picture reveals a significant increase in the overall eye size, which is due to an increased number of ommatidia (Figure 29).

Figure 29. Overexpression of DWnt5 in the eye leads to increased number of ommatidia.

A. GMRGal4/+;+ flies have normal eyes
B. Flies in which DWnt5 is overexpressed have bigger eyes with more ommatidia (Genotype: GMRGal4/UASDWnt5GFP;+/UASDWnt5GFP)
Anterior is on the bottom and posterior on the top.

Similar results were obtained when we introduced two copies of UAS-DWnt5 in the dorsal compartment of the wing. For this purpose we used the ap-GAL4 driver. Overexpressing wings have a “bubbled” shape, probably because the ventral and dorsal epithelial surfaces have a different size and do not fit together. In some of the wings, it was possible to observe the ventral and dorsal surfaces next to each other. This view revealed a difference in the hair-density, possibly due to an increase in cell number on the dorsal side of the wing margin (Figure 30). Unfortunately, the “bubbles” shape of the adult wing does not allow precise calculation of hair number or another detailed analysis. It is remarkable that one copy of the UAS-DWnt5 or the UAS-DWnt5GFP is functional and can be used to visualize the size of DWnt5 expression.
The fact that two copies of UAS-DWnt5 is the minimum requirement in order to observe a visible phenotype indicates that DWnt5 is working in a dose dependent manner in the cells. The same results were obtained using the flies that have the UAS-DWnt5GFP construct, indicating that the fusion protein DWNT5GFP behaves the same way as the endogenous DWNT5.

Figure 30. Overexpression of DWnt5 in the dorsal compartment of the wing leads to increased cell number.

A. Wing that overexpress DWnt5 only on the dorsal epithelial surface (genotype: apGal4/UASDWnt5GFP;+/UASDWnt5GFP) has “bubble” shape.

B. Closer observation reveal differences in the hair density between the two surfaces.

2.6.8. DWnt5 overexpression causes extra rounds of cell division in eye imaginal disc

Since overexpression of DWnt5 in the adult eye leads to an increase in the number of ommatidia, we examined whether DWnt5 regulates cell proliferation. To determine if additional cell division occurred during eye-disc development, we used phosphorylated histone H3 (phospho-H3) antibody directed against rabbit (see Material and Methods, 4.4.6.), which marks M phase nuclei of mitotic cells. For these experiments, I used UAS-DWnt5GFP flies, which allows visualizing the DWnt5 overexpression cells due to the GFP marker activity. The pattern of division in the eye imaginal disc has been described in detail (Wolff and Ready 1991; Figure 31A). In the wild type disc, cells divide asynchronously anterior to the morphogenetic furrow, as the cells entering the furrow are synchronously arrested in a G0 or G1 state and differentiation is initiated. Cells that are recruited into five-cell preclusters do not divide again, but all remaining cells divide once to generate a pool of cells that serves as a reservoir from which all other cell types are recruited. This last round of division occurs relatively synchronously and appears as a
stripe (second mitotic wave) on discs stained with phospho-H3 antibody. After this position, only a few divisions will normally take place and phospho-H3 staining is rarely found in the region posterior to the second mitotic wave (Figure 31A). In discs overexpressing DWnt5, the pattern of division is not regular and it is not distinguishable where is the first or the second mitotic wave. In addition, phospho-H3 expression was significantly increased in the region posterior to the second mitotic wave (Figure 31C and E). These data suggest that in cells with increased levels of DWNT5 protein, normal cell cycle control is disrupted and have driven cells that are post-mitotic into the cell cycle, so that these cells exhibit additional cell divisions in an area which is normally mitotically silent.

To determine whether overexpression of DWnt5 is able to cause the M phase in differentiating cells, I checked if posterior to the morphogenetic furrow of the eye discs, elav and phospho-H3 staining are overlapping. In normal discs, elav-positive cells have differentiated into neurons and do not divide. In eye discs, where DWnt5 is overexpressed, the majority of the ectopic M phases are in cells that do not express elav. However, there are few cases (Figure 32D) that the elav and the phospho-H3 staining are merging, indicating that these cells re-entered the cell cycle.

2.6.9. Overexpression of DWnt5 interferes with Cyclin B levels

Progression through the various cell cycle stages occurs largely through the activity of different isoforms of cyclin-dependent kinases (Cdks) and their associated positive regulatory subunits, the cyclins (Norbury and Nurse 1992; Morgan 1995). To confirm that overexpression of DWnt5 advances post-mitotic cells to enter cell cycle we examined the expression pattern of Cyclin B in the eye disc. In a wild type eye disc of Drosophila, cells from the posterior edge of the morphogenetic furrow exit the mitotic cycle and start to differentiate. The remaining undifferentiated cells enter synchronously S phase and then G2, where they accumulate Cyclin B protein in their cytoplasm. When G2 cells enter M phase, Cyclin B enter the nucleus and become degraded (Figure 33G; Thomas 1997). In contrast, in eye disc, which overexpresses DWnt5GFP in the posterior part, cytoplasmic Cyclin B remains at significant levels (Figure 33C). In addition, the band of Cyclin B positive cells posterior to the MF is expanded. The cells surrounding the photoreceptors seem to receive a signal that forces them to upregulate the levels of Cyclin B. Despite those
alteration in the Cyclin B levels, the cells within the clusters are able to differentiate into photoreceptors as indicated by the elav staining in Figure 31.

Figure 31. DWnt5 undergoes extra rounds of cell division in the eye disc.
A. Control eye disc (CyO/UAS-DWnt5GFP;+/UAS-DWnt5GFP) can be distinguished by the lack of GFP expression and shows a normal distribution of anti-phospho-histone H3 staining. White arrowheads indicate the morphogenetic furrow.
B. The morphology of adult eyes of the genotype CyO/UAS-DWnt5GFP;+/UAS-DWnt5GFP appears normal.
C. Overexpression of DWnt5 in differentiating cells shows an increase in the number of mitotic cells, as indicated by the anti-phospho-histone H3 staining.
D. Overexpression causes an increase in ommatidia number at 25°C. Genotype in (C) and (D) is GMRGal4/UAS-DWnt5GFP;+/UAS-DWnt5GFP.
E. Shows anti-phospho-histone H3 staining together with DWnt5GFP expression in the differentiating cells.
F. DWnt5GFP expression in live eye disc.
Figure 32. Dividing cells produced after the ectopic expression of DWnt5 are included in the final number of ommatidia.

A. Eye disc, which expressed DWnt5GFP
B. In the overexpressed eye disc, M phase nuclei stained with phospho-H3.
C. $\alpha$-elav antibody marks the differentiating cells. These cells do not divide in a wild type eye disc.
D. Merge between B. and C. shows that some of the cells in M phase nuclei stained with phospho-H3 are overlapping with the cells that express elav marker, indicating that these cells have re-entered the cell cycle. Arrowheads indicate cells stained both with phospho-H3 and elav antibodies. (pink) and arrows indicate cells in M phase outside of the differentiated cells.

2.7. DWnt5 gene encodes for a secreted protein

It has been shown (Franklin 1995) that in cell culture experiments DWNT5 is transported to the extracellular matrix and medium, suggesting a secreted role for DWNT5. The overexpression of DWNT5GFP protein under the control of the GMRGal4 driver line was used to detect the localization of the protein in the eye disc, by following the GFP marker. In addition, discs were stained with $\alpha$-spectrin antibody to mark the cell membranes. Although the confocal pictures were not very clear due to diffusion of the GFP after fixation treatment of the eye discs, (Figure 34) it seems that the DWNT5GFP protein does not go into the nucleus but rather stays in the membrane. This observation leads to the conclusion that DWnt5, like the other wnts, encodes for a secreted molecule.
Figure 33. Accumulation of cyclin B in DWnt5 overexpressed region in the eye disc.

Confocal images of control wild-type eye disc (A-D), with the genotype CyO/UASDWnt5GFP;UASDWnt5GFP;+ and eye disc which overexpress DWnt5GFP (E-H), with the genotype GMRGal4/UASDWnt5GFP;UASDWnt5GFP;+.

A. The presence of GFP (green) shows that in these discs GMRGal4 is drive expression of the DWnt5GFP on the posterior part of the eye disc.

B. The pattern of neuronal differentiation is not affected by this overexpression, as shown with α-elav antibody.

C. The cytoplasmic Cyclin B in overexpressed discs is increased significantly. The band of CycB positive cells posterior to the MF is expanded and there are more Cyclin B positive cells in the very posterior region.

D. The controls eye discs have no GFP expression

E. The differentiation is normal in the control eye disc stained with α-elav antibody

F. The cyclin B is expressed in a small band of cells at the position of the second mitotic wave, but no cyclin B positive cells are observed posterior to that position.

The (D) and (H) pictures are the merge of A.,B.,C., and E.,F.,G., respectively.

Figure 34. Localization of DWnt5 in the eye disc.

DWnt5 seems not to go into the nucleus but rather staying in the membrane as shown by merge of DWnt5GFP and α-spectrin staining that marks the cell membrane (see Material and Methods, 4.4.6.).
3. Discussion

The recent annotation of the *Drosophila* genome (Adams *et al.* 2000) is a landmark achievement of *Drosophila* genetics that marks the end of a century of gene hunting and heralds a new era of exploration and analysis. The *Drosophila* research community, through the efforts of individual investigators, has accumulated a vast amount of data on the genetic and molecular organization of the structure, expression and function of individual genes. As part of the effort to develop and apply tools for functional analysis, the two major projects, the Berkeley *Drosophila* Genome Project (BDGP) and the European *Drosophila* Genome Project (EDGP) have undertaken gene-disruption projects on the autosomes and on the X chromosome respectively, which are based on mutagenesis by transposable P elements insertions. In particular, the Göttingen X chromosome project, a member of the European mapping consortium, analyzed gene mutagenesis on the X chromosome (Peter *et al.* 2002) and managed to establish a large collection of strains that each contains only one single insertion that mutates single genes.

3.1. A collection of P elements on the X chromosome

In order to be useful in assigning functions to human sequences, genome projects of model organisms must be accompanied by genetic studies so not only the sequence of the genes, but also their biological functions, are determined. To facilitate this, the Göttingen X chromosome project has adopted a broad approach that combines the determination of genomic sequence with a large-scale functional analysis.

The GXP collection represents mutations in slightly 25% of the X chromosomal essential genes and is similar to the data obtained for the autosomal collection (Spradling *et al.* 1999). The P element screen for X chromosomal genes required for adult viability, represent a valuable tool for the *Drosophila* research community. The collection provides the first opportunity to link ~130 newly identified X chromosomal genes with a phenotype. The majority of the lines are available from the Bloomington stock center and their *in situ* data together with the enhancer trap expression patterns have been deposited in the FlyView database (http://flyview.uni-muenster.de:8010/html/SearchPage.html).
Drosophila now has a wealth of mutants on the X chromosome that have been endowed with visible and molecular markers and other properties that facilitate genetic manipulation. Simply knowing the sites of transposon insertion makes many types of genetic studies possible. Mutations are frequently desired in an ORF that was identified by sequence similarity. The PX lines whose insertions are located nearby a gene of interest can novo be used to disrupt this gene by remobilization experiments (Zhang and Spradling 1994). These transposons contain an easily scored eye-color marker and can be remobilized efficiently. Consequently, the inherent tendency of P elements to transpose “locally” (within about 100kb) can be utilized to preferentially mutate the region containing the gene of interest. Moreover, by selecting for loss of the marker gene following remobilization, small deletions in the surrounding region can be generated, some of which are likely to remove sequences in the desired locus only (Tower et al. 1993).

Unlike mutations generated by chemical mutagens or radiation, single P element insertions allow new alleles of a gene to be generated rapidly by imprecisely excising the original element. Studying a range of mutant alleles that includes true lack-of-function mutations is frequently important for understanding gene function. Imprecise excisions can be selected that delete both the gene’s promoter and coding sequences, revealing its true “null” phenotype.

The molecular and functional analysis of Drosophila X chromosome genes, by the means of PX lines, can be used for subsequent large scale studies with an attempt to assemble a molecular outline of many cellular and developmental processes. Such an example is the identification of the DWnt5 function in Drosophila, the study of which has initiated here. Moreover, these advantages can provide entries into studies of the corresponding processes in mammals, since the majority of genes appear to be evolutionary conserved and many of the Drosophila cognates have closely related functions in mammals (Kornberg and Krasnow 2000).

3.2. DWnt5 plays an important role in the developing nervous system

As many WNT proteins are expressed in both the developing and mature nervous system, much work has been done to examine wnts’ role in neural development and function. Within the embryonic nervous system Wnt proteins are involved in almost all important
patterning events. Wnt proteins appear to be equally important at later stages of development and in the mature brain (reviewed in Patapoutian 2000).

Especially for Wnt5, in mice it has been shown that Wnt5a marks the medial margin of the developing telencephalon just dorsal to the choroid plexus. In particular it is expressed in the cortical hem, which forms the boundary between the hippocampus and the choroid plexus in the embryonic cerebral cortex (Grove et. al. 1998). Although the expression of the gene indicates an important role for the neural patterning decisions, very little is known about the function of the gene in the developing nervous system. The Wnt5 gene serves some interesting parallel features since it is expressed at late embryonic stages in the CNS, suggesting that the gene is important for the developing nervous system both in vertebrates and invertebrates. In fact, *Drosophila* mutant embryos for *DWnt5* are defective in the organization of CNS and two primary observations suggest that *DWnt5* activity affects the axon projection pattern. Firstly, misrouting and aberrant transverse of ISN and SN nerves and, secondly, there are abnormalities in the axon projection of the longitudinal commissures of the mutants. The relationship of these phenotypes to the complex pattern of nervous system is not clear. The strong disorganization of the mutant embryos at the longitudinal axons could be either due to a defect in the process of fasciculation of the longitudinal axons or could be an indirect effect due to a delay in development of the *DWnt5* embryos. However, the potential importance of cell-cell contact is apparent in proneural clusters, where signaling between cells controls the extent of neuronal determination. Therefore, future studies have to address whether *DWnt5* fails to achieve or to maintain contact between neuronal precursors, or whether positional information in the neuroblasts have not been scrambled. In both cases, lack of *DWnt5* activity would result in misleading the axons from following the proper tracks.

The *DWnt5* gene is transcribed in cells of the ventral neurogenic region at a stage where the newborn neuroblasts delaminate from the neuroectodermal sheet (reviewed in Goodman and Doe 1993). Although we do not know the downstream targets of the *DWnt5* signal, it is possible that they may be accessory cells in the CNS in the immediate environment of the axon bundles. Midline glial cells, for example, are required in the ventral nerve cord for the proper formation of the commissures of the CNS (Campos-Ortega and Hartenstein 1997). The possibility that a disrupted organization of glial cells is responsible for the *DWnt5*-dependent defects in axonal projection seems unlikely since no obvious defect in the
pattern of glial cell formation was observed in the mutant embryos. On the other hand however, overexpression of the DWNT5 protein in the midline cells causes defects in the proper patterning of the anterior commissures. This effect could be the result of the formation of the specific commissure and/or the result of the failure of the anterior and posterior commissure to separate from each other. Nevertheless, elevating levels of DWNT5 in the CNS midline cells causes improper establishment and maintenance of the neuroectodermal cell lineage. This may be attributed to the inability of the midline cells to perform the proper cell-cell interactions and/or to send or receive signals that are needed to guide axons along the commissures.

Taken together, our analysis of the expression and mutant phenotype of DWnt5 indicates that it function in a process needed for the proper organization of the nervous system. Additional studies on how DWnt5 is regulated and how it affects the cellular basis of neuronal development should yield new insights into how positional information direct neurogenesis and might reveal that DWNT5 serves as a ligand which activates a pathway of possible neuron specific signals.

3.3. Regulation of growth and proliferation and the function of DWnt5 in determining total body size

The mechanisms of how body and organ sizes are regulated in multicellular organisms are fundamental aspects of biology that remain largely unknown. In theory, the size of an organism is determined by cell size, cell number, and intercellular space. Although growth can occur by cell enlargement and accumulation of extracellular matrix, the size of an animal generally reflects cell numbers. For example, an elephant heart is larger than a mouse heart because of an increase in cell number (Raff 1996). Thus, it is likely that total size control does exert its effect through cell proliferation. In thus process, extracellular mitogens and inhibitory molecules regulate cell division to ensure that animal cells only divide when more cells are needed. In the absence of mitogen, cell cycle progression arrests, usually in G1, and the cells enter a modified G1 state (G0) in which much of the cell cycle control system is dismantled. At the heart of the control system are the cyclin-dependent protein kinases, which are cyclically activated to trigger the different phases of the cycle at the right time and space (Morgan 1995).
Cell number control however, depends on more than just cell proliferation. It is regulated by cell death, and in some organs by cell migration, emigration, or both. Division, death, and migration all depend on intracellular mechanisms that are regulated by extracellular signaling molecules produced by other cells (Raff 1996). Programmed cell death (PCD) or apoptosis occurs during the development of all animals. For example, PCD is involved in the formation of digits in some higher vertebrates in which cell death eliminates the cells between developing digits. If the cell death is inhibited by treatment with a peptide caspase inhibitor, digit formation is blocked (Jacobson et. al. 1996). In many organs, cells are overproduced and then culled by PCD to adjust their numbers. In the vertebrate nervous system, for instance, both neurons and oligodendrocytes are generated in excess, and up to half or more are eliminated by PCD, apparently to match their numbers to the number of target cells they innervate to the number of target axons they have to myelinate (reviewed by Jacobson et. al. 1997).

In higher vertebrates, an important role in the influence of the final body size is the function of hormones and growth factors (Stewart and Rotwein 1996). Growth factors activate intracellular signaling pathways that stimulate protein synthesis and other biosynthetic processes in the cell, so that the rate of macromolecular synthesis exceeds the rate of macromolecule degradation. Studies in mouse fibroblasts have demonstrated roles for several growth factors in progression of the cell cycle from G1 to the S phase (Pardee 1989). For example children that lack the growth hormone (GH) become dwarfs, while children with excessive GH become giants. GH stimulates growth by inducing the liver and other organs to produce insulin-like growth factor 1 (Conlon and Raff 1999).

However, transplantation experiments, as well as tissue culture experiments, indicate that most organs grow up to a characteristic size. In rats, for example, it was found that when a baby kidney was transplanted into an adult rat, the tissue grows at the correct size (Silbet 1976). When immature imaginal discs from young Drosophila larvae were transplanted into adult hosts, they attained a size and shape characteristic for the mature discs (Simpson 1980). These experiments indicate that organisms have specific intrinsic mechanisms to regulate the size of organs.

In some cases, the temperature could also be an important selective agent, since it has been reported that smaller body size is in some way adaptive at higher temperatures. In
Drosophila melanogaster, development at lower temperature increased both wing area and thorax length (Partridge et. al. 1994). Laboratory fruit flies are bigger when reared at low temperature owing to an increase in cell size (Robertson 1959). Another environmental factor that affects overall growth during development is the available nutrition supplements. Under low levels of nutrients, some organisms such as yeast cells have smaller size (Thomas and Hall 1997). In addition, small Drosophila flies with smaller and reduced number of cells are produced under insufficient feeding conditions (Bryant and Simpson 1984).

Very little is known about the elements that contribute to size differences between species, but recent studies suggest that intrinsic mechanisms might be conserved in regulating size. Genetic studies in Drosophila have identified a number of genes affecting the mechanisms on growth regulation. The main signaling pathway known to regulate both cellular growth and cell proliferation is the insulin-signaling pathway. Heteroallelic combinations of Inr alleles result in small flies with fewer and smaller cells (Chen et. al. 1996). Additionally, dTOR might be a major sensor for nutrient conditions in flies and effects on cell growth and organ size by converging on the insulin-signaling pathway. Mutations for dTOR in Drosophila are responsible for reduction in cell size, with a predominant role on cell growth during G1 (Oldham et. al. 2000). Furthermore, the myc gene has an important role in growth control (Johnston et. al. 1999). Ras-MARK signaling can affect cell growth as well, since cells that are homozygous for partial loss of function mutations in various components of the Ras-MARK signal transduction cascade grow poorly and stay small (Prober and Edgar 2000). Interestingly, Ras appears to upregulate dMyc at a posttranscriptional level (Sears et. al. 1999). The Drosophila gene gigas, a homologue of the human tumor suppressor gene TSC2 (tuberous sclerosis complex gene 2), functions together with Tsc1 to antagonize insulin signaling in regulating cell growth, cell proliferation and organ size (Potter and Xu 2001).

It is possible that size control is connected with the regulation of cell proliferation through a unique mechanism. If such a mechanism is disrupted, it is predicted that changes in cell numbers should lead to changes in organ size (Potter and Xu 2001). Mutations in the DWnt5 gene result in dramatic reduction of the body size, as has been observed by other mutations, for example, in several hypomorph alleles of the InR gene, in chico, and S6K gene. However, the disruption in the DWnt5 function causes a reduction in body and organ
growth by reducing cell number in the organs of the mutant fly rather than by reducing the overall size of the cells.

The observation that DWnt5 mutant larvae, pupae as well as adults are smaller suggests that the decrease in the proliferating cells occur during all developmental stages. Since DWnt5 mutant pupae are not as small as larvae compared with their heterozygous siblings, it is possible that these differences reflects that cells committed to terminal differentiation divide slower and have more time to grow. Despite the alterations in the total size, the differentiation of adult structures in the small animals is largely unaffected. Moreover, the phenotype of DWnt5 mutants is very similar to the one observed in knock-out mice (Yamaguchi et. al. 1999). Wnt5a knock-out mice show a reduced proliferation of progress zone and paraxial mesoderm progenitors and no changes in apoptosis. The similarity of the phenotype suggests that the two homologues have the same general biological function and it is likely that the Wnt5 pathway is the same between Drosophila and higher vertebrates. This is an indication that the basic mechanisms of tissue and body size regulation between invertebrates and mammals are similar. Therefore, Drosophila might be an invaluable model for dissecting the function of cell number determination and hence the mechanisms by which Wnt5 controls the growth of an organism.

3.4. DWnt5 as a cell number regulator

In DWnt5 mutant clones that were revealed by mitotic recombination, the number of cells is significantly decreased as compared to the cell number of wild type clones which was generated by the same recombination event. In addition, the adult wings of the mutant animals are smaller, with fewer hairs corresponding to a smaller number of cells. In contrast, size differences between individual mutant and wild type cells were not found, indicating that DWnt5 has a major role in division (determination of cells number) and is not an essential feature in cell growth (cell mass) which is mainly caused by an increase rate of biosynthesis and metabolism and selectively interferes with local growth rates (Conlon and Raff 1999).

Cell number depends on cell division as well as on cell death (reviewed in Conlon and Raff, 1999). Although studies combining DWnt5 with apoptotic genes have not been included in this work, the lack of apoptotic cell death after staining with AO in mutant
ovaries and the normal nuclei shape seen with DAPI staining in discs with mitotic clones, support the idea that the primary function of DWnt5 is not the regulation of programmed cell death but is instead required for cell proliferation throughout the Drosophila life cycle. Homozygous DWnt5 mutant cells appear to have a selective disadvantage: they grow more slowly than wild type cells, as indicated by their representation in the ovaries and in the adult eye and wing, and they cannot reach the normal number as established by comparison of wild type and mutant flies. However, all animal cells need signals from other cells to divide as well as signals to survive and there is actually a competition between survival and dividing signals (Raff 1996). Therefore, we cannot exclude the possibility that some cell death might occur later than the time points we examined, which results in shortening the cell cycle and limiting the time for development.

The pharate adult homozygous DWnt5 mutant females are similar to the ones that have been observed in double rbf and truncated de2f1 mutants (Du 2000), two molecules that are known to regulate the cyclin E activity (Du et. al. 1996; Datar et. al. 2000). In all cases, the flies develop defects in abdominal segmetation. This developmental defect might arise from defects in histoblast proliferation during pupal stage development, or might reflect regional cell death during the time that the adult abdomen grows. Our results do not distinguish between these possibilities.

3.5. Increased DWnt5 levels show abnormal cell cycle progression via changes in cyclin B levels

The overexpression experimental system in the eye disc allows the effects of elevating DWnt5 activity to be studied in vivo in a variety of contexts: in cells that are proliferating, in cells that have exited the cell cycle but have not committed a cell fate yet, and in cells that are post-mitotic and have started to express markers of differentiation. Ectopic expression of the DWNT5 protein in differentiating cells of the eye imaginal disc led to an increase in the number of cells which are in M phase of the mitotic cycle. Differences in the cell number observed in the adult tissue arise as a direct consequence of the effects on cell division in the eye disc posterior to the second mitotic wave.

In addition, in the eye discs, in which DWnt5 is overexpressed, there are differences in the region containing cells that normally proliferate. The Cyclin B staining in the second
mitotic wave normally includes 3-4 cell diameters, but in the overexpressed disc the Cyclin B staining is expanded and includes 7-8 cell diameters. Moreover, the levels of cyclin B are elevated in post-mitotic cells, which are clearly not arrested. Cyclins and cyclin-dependent protein kinases (Cdks) directly control the cell-division cycle. They are cyclically activated to trigger the different phases of the cell cycle at the right time and help to ensure that a cell divides only when another cell is needed (reviewed by Raff 1996).

The increased number of cells and the effects in the cell cycle after exposure to high levels of DWnt5 could be explained by two possible mechanisms. First, the relapse to G0 phase might be either blocked or slowed down in a way that the proliferating cells must exit the cell cycle. Alternatively, the post-mitotic cells posterior to the morphogenetic furrow might be re-entering the cell cycle. In either case, failure of down-regulation of Cyclin B expression results in unsuccessful establishment of G1 arrest posterior to the second mitotic wave. Thus, it seems that DWnt5 is an important factor which functions in a pathway that regulates Cyclin B levels.

Moreover, the increased number of cells that stay in M phase seems not to perturb the onset of neuronal differentiation as revealed by the elav expression in the precluster cells. The pattern of differentiation appears normal suggesting that pattern formation can proceed normally despite the alterations in cell cycle. Double staining of cells in M phase (phospho-H3 antibody) with the elav antibody showed that most M phases occur in undifferentiated cells but a few M phases were in cells that expressed elav and started to differentiate, indicating that the dividing cells are included in the final number of ommatidia.

The extra ommatidia in the adult eye seem to have the same size as their neighboring cells. Thus, a dependent or independent growth factor must have also stimulated the cells to grow in order to encompass the physiological total mass. But despite the alterations in cell number, differentiation is mainly unaffected, although the overexpression has been performed in differentiating cells. Our results indicate that DWnt5 is likely to increase, through an induced signaling cascade, the ultimate number of differentiating cells.

The fact that at least two copies of DWnt5 gene are required in order to observe the overexpression phenotype implies a dose-dependent activity of the DWNT5 protein. This is in accordance with the previous observations that the concentration of a mitogen can
influence the rate of cell cycle progression and leads to either bigger or smaller individuals depending on whether the concentration is either increased or lowered (Brooks and Riddle 1988; Gao and Raff 1997). A possible function of Wnt5 in proliferation events has been discussed previously (Yamaguchi et. al. 1999). The results shown here provide the first experimental evidence that is consistent with Wnt5 acting as a mitotic regulator, which affects the cell cycle progression.
4. Materials and Methods

4.1. Genetics

4.1.1. Fly stocks

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<td>Doris Brentrup</td>
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4.1.2. Genetic crosses and *Drosophila* strains

Flies were kept under standard conditions as previously described (Forjanic *et. al.* 1997) The stock Dp (1:Y)W39 (Bloomington stock 1538) carries a duplication on the Y chromosome and was used to rescue the lethal males (*Wnt5{PlacW}\textsuperscript{G0265} /Dp (1:Y)W39). The cross was:

\[
P: \text{Wnt5}[^{\text{PlacW}}]\textsuperscript{G0265} \text{/FM7i-pAct-GFP} \times \text{Df(1)os-UE19} / \text{Dp (1:Y)W39,y+}\]

\[
\text{F1: Wnt5}[^{\text{PlacW}}]\textsuperscript{G0265} / \text{Dp(1:Y)W39 y+ fcl'Y} \quad \text{(rescued males)}
\]

Heterozygous females for the lethal mutation *l(1)G0265* with the genotype: *Wnt5[^{\text{PlacW}}]\textsuperscript{G0265} /\text{FM7i-pAct-GFP}* were crossed with the rescued males *Wnt5[^{\text{PlacW}}]\textsuperscript{G0265} /\text{Dp (1:Y)W39}*. The homozygous small flies with the genotype *Wnt5[^{\text{PlacW}}]\textsuperscript{G0265} / Wnt5[^{\text{PlacW}}]\textsuperscript{G0265}* were the progenies of the above cross. See also Figure 19.

The revertant stock consists of flies in which only the *PlacW*-insertion *l(1)G0265* was precisely excised as revealed by the sequences analysis. The genetic crosses that were performed in order to get the precise excisions have been described in Figure 10A.

The lethality as well as the small size phenotype was complemented by insertion on the third chromosome of a *DWnt5* genomic rescue construct. The “rescued” male as described in Figure 11 was crossed to heterozygous for the *DWnt5* mutation:

\[
P: \text{Wnt5}[^{\text{PlacW}}]\textsuperscript{G0265} /\text{FM7i-pAct-GFP};+/+;+/+ \times \text{Wnt5}[^{\text{PlacW}}]\textsuperscript{G0265} /\text{Y}; +/+; \text{R/+}
\]

\[
\text{F1: Wnt5}[^{\text{PlacW}}]\textsuperscript{G0265} /\text{Wnt5}[^{\text{PlacW}}]\textsuperscript{G0265};+/+;\text{R/+} \quad \text{(Females with normal body size)}
\]
4.2. Embryology

4.2.1. Drosophila RNA *in situ* hybridization in embryos

4.2.1.1. Antisense RNA probe preparation

5-10µg template DNA was linearized with a specific restriction enzyme (5’ overhang). After checking for the completeness of the digest on an agarose gel, linearized template DNA was purified using the Qiaquick Nucleotide removal kit (Qiagen) and the following 20µl *in vitro* transcription reaction was performed:

\[
\begin{align*}
1\mu g & \text{ linearized template DNA} \\
2\mu l & \text{ dithiotreitol (DTT 100mM)} \\
2\mu l & \text{ digoxigenin (DIG) RNA labeling mix} \\
& \text{ (Boehringer Mannheim)} \\
0.5\mu l & \text{ Rnasin} \\
2\mu l & \text{ 10x Transcription-buffer} \\
1\mu l & \text{ RNA polymerase} \\
\text{ add H}_2\text{O up to 20}\mu l \text{ volume}
\end{align*}
\]

The reaction was incubated at 37°C for 2 hours. Then 1µl DNaseI (RNase free) was added and the incubation was continued for 15 mins. The probe was purified using the RNeasy mini kit (Qiagen) and eluted in 50µl H2O. The purified DNA was checked on an agarose gel and the gel was blotted to test DIG incorporation and the incorporation was tittered by dot blotting of probe dilutions. Afterwards 50µl Hybe-solution was added to the purified probe and stored at -20°C.

4.2.1.2. Fixation of embryos

Collections of appropriate staged embryos from apple juice agar plates were briefly washed and dechorionated with 50% Klorix (bleach). The embryos were extensively washed with H2O. Afterwards they were fixed for 20 min with 1ml Fix-solution (10% paraformaldehyde in PBS /50mM EGTA, pH 7) + 6ml heptan on a rocking platform and devitellineazed by adding 10ml methanol and vortexing for 15 sec. The embryos were
allowed to sink to the bottom, the solution was removed and the embryos were washed 3 x with methanol. Finally, embryos were stored in methanol at -20°C

4.2.1.3. *In situ* hybridization and signal detection

The embryos were transferred to an eppendorf tube and proceeded with the following steps in 1ml volume (all steps on a rotating wheel): rinsed 1x with methanol, 1x with Methanol/PBT (1:1) and fixated with Fix-solution/PBT (1:1) for 20 min. Afterwards, the embryos were rinsed 3x and washed 1x for 5min with PBT (1x PBS, pH7 /0,1% Tween 20). Digestion with proteinaseK (5mg/ml) was performed for 3 min and again a series of rinsing 1x with 4mg/ml Glycin/PBT and 2x with PBT was done. Then, another fixation step with Fix-solution/PBT (1:1) for 20 min and a series of rinsing was followed: 3x with PBT and wash 1x for 5min with PBT, 1x with 500µl HybeB-solution/PBT (1:1), 1x with 250µl Hybe-solution (50% Formamide-Fluka /5xSSC), 1x with 250µl Hybe-solution (50% Formamide/5xSSC/5ug/ml Heparin (Fluka)/ 5mg/ml Torula yeast RNA (Sigma)/ 0,1% Tween 20 (Sigma)/ pH 6,7 (25ºC). After this step, the embryos were prehybridized with 250µl Hybe-solution at 65ºC for 30-60 min. The hybridization was performed with 1µl preheated DIG-labeled probe in 30µl Hybe-solution at 65ºC o/n. The following day, 500µl Hybe-solution added to the embryos at 65ºC and then replaced by 500µl HybeB-solution for 15min at 65ºC. 500µl PBT was added, which then was washed for 5, 10 and 20min with PBT. The incubation with the preabsorbed Anti-DIG-AP Fab secondary antibody (1:2000 in PBT, Boehringer Mannheim) was followed for 1h. The embryos were rinsed 2x with PBT, washed for 5, 10 and 15min with PBT, rinsed 1x and washed 1x for 5min with AP-buffer (20mM Tris/HCl pH9,5/100mM NaCl/50mM MgCl2). The staining was developed with 4,5µl NBT (Boehringer Mannheim) and 3,5µl BCIP (Boehringer Mannheim) solution in 1ml AP-buffer. The staining reaction was stopped by rinsing 2 x and washing at least 5 x with PBT. The embryos were mounted in Canada balsam (Sigma).
4.2.2. Drosophila embryos antibody staining

4.2.2.1. Antibody staining

Transfer the embryos to an eppendorf tube and proceed the following steps in 1ml volume (all steps on a rotating wheel): The embryos were washed 3x with PBT for 5 min and were incubated with the first antibody o/n at 4°C. The next day, were washed 4x with PBT for 5 min, 2x with 500 µl PBT+2% goat-serum (10 µl) for 20 min and the second antibody was added: 380 µl PBT+10 µl second antibody biotynylated or AP conjucated (preabsorbed)+10 µl normal goat-serum. The incubation lasted for 2h. The antibodies used for immunostaining in the embryos were: 8D12C3-[]-repo (1:10, Corey Goodman), 22C10 (1:100, Hybridoma Bank), BP102 anti CNS axons (1:100, Hybridoma Bank) and ID4G11-[]- fascII (1:10, Corey Goodman) for the nervous system; the A12 antibody (1:100, Hybridoma Bank) for the analysis of the tracheal system.

4.2.2.2. Signal detection with peroxidase staining

When the secondary antibody was biotinylated, the embryos were washed 4x 10min with PBT and incubated for 30-60 min at RT with the ABC- Colour-solution (10µl A (HRP) + 10µl B (Streptavidin) + 500µl PBT, freshly prepared 30min before use). Afterwards, the embryos were washed 4xwith PBT for 10 min and the development of the staining performed.

The embryos were transferred to a glass tube and 20µl DAB + 450µl PBT+0.3% H₂O₂ added. After 15min the reaction was stopped with PBT. The embryos were checked under the binocular and after 5-10min the reaction was completed by rinsing 2x and wash 3x with PBT for 5min. The embryos were mounted in glycerol.

4.2.2.3. Signal detection goat []-rabbit AK Alkal-Phosphatase(AP)

When the secondary antibody was AP conjucated, the embryos were washed 10x with PBT for 20min, rinsed 1x and washed 3x with AP-buffer for 5min and the staining developed with 4.5µl NBT and 3.5µl BCIP solution in 1ml AP-buffer. The staining was checked
under the binocular and the reaction stopped by rinsing 2x and washing at least 5x with PBT. The embryos were mounted in glycerol.

4.3. Molecular Biology

4.3.1. Preparation of plasmid DNA

The plasmid DNA was isolated according to Plasmid Mini Kit (Qiagen) for small amount of DNA or Plasmid Midi Kit (Qiagen) for larger amounts of plasmid DNA.

4.3.2. Transgene Construction and Overexpression Experiments

The genomic rescue construct was isolated by PCR amplification in genomic DNA from wild type flies. The primers used for the amplification were: GCG GCC GCC TAC GCG TGC GAT TCC TCA ACT GT (R5-NotI) and GCG GCC GCC TAC GCG TGC GAT TCC TCA ACT GT (R3-NotI). The Advantage 2 PCR kit (Clontech) which includes a proofreading polymerase, was used to amplify the rescue fragment. The PCR program that was used is:

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The resulting 5371bp fragment (Figure 11) was isolated from an agarose gel using the Gel Extraction Kit (Qiagen) and cloned (as described in Sambrook and Russel 2001) in a pCaspeR4 vector using the NotI sites that were introduced by the amplification primers and the full construct was used for transformation of white flies (Rubin and Sprandling 1982).

The Advantage 2 PCR kit was also used to amplify the ORF of the DWnt5 gene that was used for the rescue construct (R). The primers for the amplification were: C C C A C A T C T T A C A C A T A C T C C A A A G C G A (B g 1 1 1 - 5 ) and CCCGGTACCAGTTTACATGTGTGCTCCTC (KpnI-3)
and the conditions of the PCR program:

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<tr>
<th>PCR Program</th>
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<th>Time</th>
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The PCR fragment was isolated from an agarose gel using the Gel Extraction Kit (Qiagen) and cloned in a UASpCaspeR4 vector as well in a UASpCaspeR4-3’GFP vector (GFP is after the DWnt5 gene, kindly provided by Britta Linder-Stuart). The full constructs used for the transformation of flies was described previously. We obtained several transformant lines homozygous on the second chromosome and several transformant lines homozygous on the third chromosome for each construct. These lines used for the production of strains that had the UASDWnt5 homozygous on both the second and third chromosomes (+/++;UASDWnt5/ UASDWnt5; UASDWnt5/ UASDWnt5) The same was done for transformants lines with the UASDWnt5GFP construct (+/++;UASDWnt5GFP/UASDWnt5GFP; UASDWnt5GFP/ UASDWnt5GFP).

The UAS-DWnt5 and UAS-DWnt5GFP behaved similarly when they were overexpressed with different GAL4 lines.

4.3.3. DNA preparation for embryo injections

Midi- or maxiprep of the constructs was prepared Quiagen and their DNA concentration determined spectrophotometrically.

Phenol extraction

We added H2O to 20 µg DNA-construct to a final volume of 300 µl and then 300 µl Phenol and vortex for 2 sec. The mixture was centrifuged for 2min at 12000 rpm and carefully we transferred the upper phase to new Eppendorf cup. Afterwards, 300 µl Phenol–Chloroform were added ,vortexed and centrifuged as before. 300 µl Chloroform (or Chloroform/Isoamylalcohol) were added to the upper phase, vortexed and centrifuged again. 30 µl 3M NaAc (pH 5.2) + 750 µl 100% EtOH were added to the upper phase, vortexed and centrifuged for 15 min at 12000 rpm. We carefully removed the supernatant
and washed the pellet with 500 µl 70% EtOH. After 5 min centrifugation at 12000 rpm the supernatant was carefully removed and the pellet was dried in a SpeedVac for 5 min. Afterwards, the pellet was resuspended in 20 µl H2O (or TE) and was incubated on rocking Thermomixer at 60°C for 5 min.

Preparation of injection mixture
12 µg DNA-construct, 4 µg Helper and H2O to a final volume of 200 µl was mixed with 20 µl 3M NaAc (pH 5.2) and 500 µl 100% EtOH. The mixture was centrifuged for 15 min at 12000 rpm and the pellet was washed with 500 µl 70% EtOH. Afterwards we centrifuged for 10 min at 12000 rpm and dried the pellet pellet in the SpeedVac for 5 min. The pellet was resuspended in 20 µl and was incubated on rocking Thermomixer at 60°C for 5 min. Then 15 µl were injected to the posterior part of Drosophila embryos.

4.3.3. Preparation of genomic DNA

Around 30 anesthetized flies were collected and genomic DNA was prepared according to Dneasy Tissue Kit-Isolation of genomic DNA from insects (Qiagen). After a phenol-chloroform extraction and ethanol precipitation, the DNA was digested for 1.5 h at 37°C with RNAse A (10 mg/µl, Sigma,).

4.3.5. Inverse PCR

4.3.5.1. Digestions (Sau3A I, HinP1 I, or Msp I)

The reaction was:
10.0 µg genomic DNA
10X buffer (NEB Sau3A or NEB 2)
10 units enzyme Sau3A I, HinP1 I, or Msp I
up to 10 µl ddH2O

The digestion was incubated 2.5 hrs at 37°, then heated for 20 min to 70°C to stop the reaction and the DNA was ethanol precipitated.
4.3.5.2. Ligation

All the above digested genomic DNA
10X ligation buffer (+ATP)
2 Weiss units Ligase-T4
up to 200µl ddH2O
The reaction was incubated O/N at 16°C. Afterwards, followed the ethanol precipitation on ice, for 10min, and the resuspension of the pellet in 150µl TE.

4.3.5.3. PCR

10.0µl ligated genomic DNA
2µl 2mM each dNTP 25mM
1µl forward primer (10µM)
1µl reverse primer (10µM)
10X Qiagen Taq buffer
2 units Taq polymerase
up to 50µl ddH2O

PCR-Programm:

1x 95°C 5min
1x 75°C 2min
36x 95°C 30sec
55°C/60°C 1min
68°C 2min
1x 72°C 10min
1x 4°C

Primers for PCR:

For the 5' end:  
Pr4:  5’ CAA TCA TAT CGC TGT CTC ACT CA 3’
Plw3-1: 5’ TGT CGG CGT CAT CAA CTC C 3’

For the 3' end:  
Plac4: 5’ ACT CTG GGT TAG GTC CTG TTC ATT GTT 3’
Plac1: 5’ CAC CCA AGG CTC TGC TCC CAC AAT 3’

The PCR products were isolated from an agarose gel by Gel extraction kit (Qiagen).
4.3.6. Sequencing

The sequencing of DNA fragments was done with the ABI Prism 377/96-Sequencer (Applied Biosystems) and the DNA Sequencing kit (Applied Biosystem) was used. The analyses of the sequences were done with the help of Sequencer Software (Gene Codes Corporation) and Blast-Algorithms (Altschul et. al. 1990) from NCBI and BDGP.

4.4. Phenotypic Analysis

4.4.1. Adult cell number analysis

Wings from adult flies of the following genotypes: DWnt51/DWnt51, DWnt51/FM7c, as well as wings from oreR and revertant flies, were removed and washed with SH buffer (1:2 Glycerol: Ethanol) and mounted in 6:5 Lactic:Ethanol. The columns in Figure ? represent the cell number which has been counted in 5 different wings (n=5) from each phenotype. Genotype was taken and images analyzed using the Adobe PhotoShop Software. Adult eyes of the same genotypes were analyzed as described in Rein et. al.

4.4.2. Weight Analysis

Body weight of individual female flies (n=20) was measured precisely (range 0.001-10mg) using “Sartorius Micro Elektronische Analysen-Semimmikro- und Mikrowaagen”. Flies were age matched –2 days old- and reared under the same growth conditions before weighing. The genotypes analyzed were Wnt5{PlacW}G0265/ Wnt5{PlacW}G02651, Wnt5{PlacW}G0265/FM7c, wild type as well as revertant flies.

4.4.3. Life-span Measurements

Up to 10 enclosed females (0-24h old) of the genotype described in the text were kept in small food vials and transferred into vials with freshly prepared food every 2 days. Survival of flies was monitored in 48h intervals. For each genotype 300 females were monitored.
4.4.4. Clonal Analysis

The l(1)G0265 was recombined onto the neoFRT18A chromosome (Xu and Rubin, 1993) and FRT9-2 chromosome. The recombinants were balanced with FM7i-pAct-GFP, which allowed identification of hemizygous female mutants, by the absence of green fluorescent protein (GFP) expression. Germline clones of the l(1)G0265 allele were generated using the autosomal dominant female-sterile technique in combination with the FLP recombinase system (Chou and Perrimon, 1996).

For the generation of clones in the imaginal discs, females with the genotype wUbiGFPnslneoFRT18A were crossed to hsFLP;+/+; dppGAL4/TM6B males which express GFP in the nucleus of all cells. Female offsprings with the genotype l(1)G0265,neoFRT18A/FM7i-pAct-GFP were crossed to hsFLP;+/+;TM6/+ males. All larvae were subjected to a heat shock, 1h at 38°C, to induce mitotic recombination. Selections of female larvae lacking the FM7i-pAct-GFP were dissected and imaginal discs were fixed, permeabilized, stained with appropriate antibodies, mounted and examined under a confocal microscope. The identification of the clones was done as following: wild type clones were GFP+ and mutant clones were lacking GFP.

4.4.5. Scanning Electron Microscope

The dehydration of the adult flies was done through a series of ethanol concentrations. Critical point drying was performed using a Balzers Union dryer and sputter coat at 0.1 mbar Argon, 220V/40mA for 2 min and at 5cm distance (800A blickness). Pictures of the adult eyes were taken using Electron Scanning microscope.

4.4.6. Immunostaining in imaginal discs and Microscopy

Discs were dissected on ice (within 30min) and fixed in 4% paraformaldehyde in PBS for 30min at RT. The discs were permeabilized in 1% Triton X-100 in PBS for 15 min at RT and then blocked in PBS+5% normal goat serum (Vectorlabs) for 1h at RT. The incubation with the first antibody was done in PBT+5% normal goat serum o/n at 4°C. Afterwards the discs were washed 4x with PBT for 20min and then followed the incubation with the
secondary antibody in PBT+5% normal goat serum for 2h at RT. Then the discs were washed 4x with PBT for 20min and mounted in Vectashield mounting medium (Molecular probes).

The primary antibody α-spectrin (mouse, 1:200, DSHB) and FITC-conjugated secondary antibodies (1:20000, Jackson) were used to label cell outlines. To identify the nucleus morphology discs were mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Molecular probes).

For the overexpressed discs, we used phospo-Histone 3 antibody (1:100, Upstate) to visualize mitotic nuclei. The differentiating cells in eye discs were marked with α-elav antibody (mouse, 1:100, DSHB). The α-cyclin B (rabbit, 1:200) and α-cyclin E (rabbit, 1:200) antibodies were kindly provided by Dr. Christian Lehner. Alexa Fluor 633 goat anti-mouse IgG (1:1000, Molecular probes) and Cy3 anti-rabbit (1:2000, Jackson / dianova) were used as secondary fluorescent antibodies.

4.4.7. Ovaries analysis

Ovaries from 2 days old adult flies were dissected in PBS and fixed with Fix-solution for 20min, washed 4x with PBT and Sytox (1:100, Molecular Probes) and Rhodamine-phalloidin Alexa (1:100, Molecular Probes) added to stain DNA and actin respectively. The incubation was O/N at 4°C. Afterwards the samples were washed 4x with PBT and high magnification fluorescent confocal images were collected using the Carl Zeiss LSM microscopy.

4.5. In situ hybridization to polytene chromosomes of Drosophila

4.5.1 Polytene chromosome squashes

Animals were grown at 18°C using food with extra yeast up to 3rd instar larval stage. With the help of the GFP marker the larvae, which carried the balancer chromosome were sorted out and the remaining larvae washed in PBS to remove the food. Dissection of the salivary glands was performed in 45% acetic acid. The sample was covered with a clean slide, inverted and squashed with the help of the eraser of a pencil. The spreading of the
chromosomes checked under a phase contrast microscope. The dish was placed on blotting paper, coverslip side down, and firm thumb pressure applied to the back of the slide over the preparation, without allowing the coverslip to move. Freezing of the preparation was done on liquid N₂ for 5-10 min. The coverslip was flipped off with a razor blade and immersed immediately into freshly made ethanol:acetic acid (3:1) for 10 min. The slide was dehydrated in a series of 70% up to 100% ethanol for 10 min, air dried and stored in a dry place. The slide and the borders of the coverslip were marked with a black permanent marker in order to identify the position of the chromosomes for the rest of the procedure.

4.5.2. Slide treatment

The chromosomes were heat-treated in 2xSSC at 68°C for 30 min. After transferring to RT in 2xSSC for 2 min, the chromosomes were denatured in freshly prepared 70mM NaOH at RT for 2 min. The slides were washed with 2xSSC, dehydrated in the ethanol series as before and dried.

4.5.3. Probe preparation and hybridization

The digoxigenin labeled probe was denatured in boiled H₂O for 10 min. For every slide 20µl of hybridization solution was used, containing:

- 5µl digoxigenin labeled probe
- 7µl H₂O
- 2µl DNA from salmon’s sperm
- 4µl 20xSSC
- 2µl 50x Denhardts solution

After adding the 20µl of the hybridization solution, the samples were covered with a 22 x 22 mm coverslip, sealed and incubated O/N at 37°C.

4.5.4. Immunological detection

The slides were placed 3x for 3 min in 2xSSC at 37°C and washed for 2x 5 min in 2x SSC at RT. Afterwards followed washing with 2x 3 min in PBS, 1x 3 min in PBS + 0.1% Triton-X 100 and again 2x 3 min in PBS. The biot-[¶]-DIG antibody diluted 1:20 with 0.1 M
Tris/0.15 M NaCl pH 7.5. Incubation with the antibody solution was performed for 1.5 h at RT and afterwards the samples were washed with PBS and PBS + 0.1% Triton-X 100. Then, the enzymatic color reaction of the biot-[]-DIG antibody was performed using 25ml staining solution containing PBS, 12.5 mg Diaminobenzidine (DAB) and 12.5 ml 30% H₂O₂. After 10 min, the progress of the reaction started to be monitored using a microscope with phase contrast optics. When the reaction was complete, the coverslip washed off and the slide was placed in water for 5 min. The chromosomes were mounted in Euparal (Roth), covered with a new coverslip and viewed under the microscope for signals.
5. Appendix

A. Sequence of cDNA M97450

1  CAGTGTGTAA CAATTGGTC TGAGGATTGG ATTAATCTGTT CCGCGATTTC GTTGCGCAT
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**V. Sequence of AJ427050**

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**X. Sequence of AJ426897**

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Y. Sequence of AJ300094

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240  TTATCTGTTA ATGCTTTTGC TTCTTTTGC TCTTCTTTCC CGACTTTTC GACATTTTTG TCCTTTTTCT
300  TTATTTATG TATTTTTGGC GTCCGCTTTG GCCGGCGCT TGTTTTTGTG TTGTTTTGCT
350  GTTGTTCTG GCACTTGATG CACTAAACTA AACAAAAAAG TTACAGGCGC
6. Summary

The collection of X chromosome insertions (PX) lethal lines, which was isolated from a screen for essential genes on the X chromosome, was characterized by means of cloning the insertion sites, mapping the sites within genomic DNA and determination of the associated reporter gene expression patterns. The established STS flanking the P element insertion sites were submitted to EMBL nucleotide databases and their *in situ* data together with the enhancer trap expression patterns have been deposited in the FlyView database. The characterized lines are now available to be used by the scientific community for a detailed analysis of the newly established lethal gene functions.

One of the isolated genes on the X chromosome was the *Drosophila* gene *Wnt5* (*DWnt5*). From two independent screens, one lethal and three homozygous viable alleles were recovered, allowing the identification of two distinct functions for *DWnt5* in the fly.

Observations on the developing nervous system of mutant embryos suggest that *DWnt5* activity affects axon projection pattern. Elevated levels of DWNT5 activity in the midline cells of the central nervous system causes improper establishment and maintenance of the axonal pathways. Our analysis of the expression and mutant phenotype indicates that DWnt5 function in a process needed for proper organization of the nervous system.

A second and novel function of Wnt is the control of the body size by regulation of the cell number rather than affecting the size of cells. Moreover, experimentally increased *DWnt5* levels in a post-mitotic region of the eye imaginal disc causes abnormal cell cycle progression, resulting in additional ommatidia in the adult eye when compared to wild type. The increased cell number and the effects on the cell cycle after exposure to high DWNT5 levels is the result of a failure to downregulate cyclin B and therefore the unsuccessful establishment of a G1 arrest.
7. Zusammenfassung

Eine Kollektion letaler P-Insertionslinien, die bei der systematischen Suche zur Identifikation von essentiellen, X-chromosomalen lokalisierten Genen isoliert worden sind, wurden mittels Klonierung genomischer DNA-Sequenzen um die Insertionsstelle des P-Elements kartiert. Es wurde die Lage der Insertionsstelle innerhalb der genomischen DNA bestimmt und das Expressionsmuster des Reportergen-Konstrukts charakterisiert. Die flankierenden “STSs” wurden der EMBL Nukleotid-Datenbank zu Verfügung gestellt und die in-situ-Daten zusammen mit den „enhancer trap“-Expressionsmustern über die FlyView-Datenbank zugänglich gemacht. Die so charakterisierten Linien sind für die wissenschaftliche Gemeinschaft im Internet abrufbar und ermöglichen eine detaillierte Analyse der X-chromosomal-kodierten Genfunktionen.

Eines der Gene auf dem X-Chromosom ist das *Drosophila* Wnt5-Gen (*DWnt5*). Es wurden zwei unabhängige letale und drei homozygot lebensfähige Allele isoliert, welche die Identifikation von zwei unterschiedlichen Funktionen von *DWnt5* in der Fliege erlaubten.


8. References


EDGP. European *Drosophila* Genome Project. [http://edgp.ebi.ac.uk/](http://edgp.ebi.ac.uk/)


GXP. Göttingen X-Chromosome Project
http://www.mpibpc.gwdg.de/abteilungen/170/proj.html


Janson, K., cohen, E.D., Wilder, E.L. (2001). Expression of DWnt6, DWnt8, and DWnt10,


Ma, C. and Moses, K. (1995). Wingless and ptc are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing Drosophila compound eye. Development 121: 2279-2289


Curriculum vitae

Foteini Mourkioti

1979-1986: Primary School, Nea Manolada, Ilias, Greece
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1986-1989: 19th Gymnasium, Patras, Greece

1989-1993: 3rd General Lyceum, Patras, Greece

1994-1998: Bachelor in Biology (Note: very good)
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“Molecular analysis of the subclones of the neural specific gene chiP12-13”
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1998-2002: Dissertation:
"Functional genomics of the Drosophila X-Chromosome and the role of DWnt5 during development".
Max-Planck Institute for Biophysical Chemistry, Department Molecular Developmental Biology,
Karl-Friedrich Bonhoeffer Institute,
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Supervisor: Prof. Dr. Herbert Jäckle
**Publications**


**Mapping and identification of essential gene functions on the X chromosome of Drosophila.**


**From first base: the sequence of the tip of the X chromosome of Drosophila melanogaster, a comparison of two sequencing strategies.**

Genome Res. 2001 May; 11(5): 710-30.


**From sequence to chromosome: the tip of the X chromosome of D. melanogaster.**


**Announcements**

Gronke, S., **Mourkioti, F.**, Hader, T., Steuernagel, A., Eulenberg, K., Bronner, G., Jackle, H., Kuhnlein, R.

Regulation of energy homeostasis in Drosophila.


**Mourkioti, F.**, Peter, A., Schafer, U., Jackle, H.

**DWnt-5 function in the developing nervous system.**


**Mourkioti F.**, Peter A., and Jäckle H.

**Analysis of DWnt-5 function during CNS development**

**3rd GfE, 2000**, Günzburg, Ulm, Germany (Poster).

Foteini Mourkioti