

**Isolation and identification of molecular partners
of the proteins encoded by the *Drosophila* tumor
suppressor gene *lethal(2)tumorous imaginal discs***

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1. INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by the rapid growth and spread of abnormal cells. External factors such as chemicals or viruses as well as internal factors such as hormones or the condition of the immune system may alone, or in combination, promote cancer (NAAM, 2001). Cancerous cells differ from their normal neighbours by a host of specific phenotypic changes such as rapid division rate, invasion of new cellular territories, high metabolic rate and abnormal shape (McCormick, 1999). These changes occur by the production of multiple mutations in a single cell that cause it to proliferate out of control. Some of these mutations may be transmitted from the parents through the germ line (hereditary mutations) like those for the Retinoblastoma (Rb) protein (Weinstein *et al.*, 1997). Others arise *de novo* in the somatic cell lineage of a particular cell (somatic mutations). Two general kinds of mutations are associated with cancer: mutations in oncogenes (gain of function) and mutations in tumor suppressor genes (loss of function). Oncogenes are mutated in such a way that the proteins they encode are activated in cancerous cells carrying the dominant mutant allele. Some examples are *jun* (Weitzman, 2000), *fos* (Behrens *et al.*, 2000), *ras* (Campbell *et al.*, 1998) and *bcl2* (Hueber and Evan, 1998). Tumor suppressor genes must have both alleles mutated before cancerous growth begins (Griffiths *et al.*, 1999).

1.2 Tumor suppressor genes

Mutations in tumor suppressor genes are found in most human cancers and are believed to be a prerequisite for the accumulation of additional mutations that lead to uncontrolled growth and metastasis of cancerous cells (Bilder *et al.*, 2000). An example is the development of colon cancer displayed in Figure 1. The gene products encoded by tumor suppressor genes fall into different categories including cell surface receptors such as Patched, cell adhesion molecules such as DE-cadherin, cytoplasmic proteins such as PTEN and APC and nuclear factors such as p53 and the Rb protein (Wodarz, 2000). Most of the known tumor suppressor gene-products are involved in signal

transduction pathways that lead either to cell-cycle arrest or apoptosis. Consequently, mutations in these genes cause overproliferation and survival of cells that otherwise would have been eliminated by cell death.

COLON CANCER

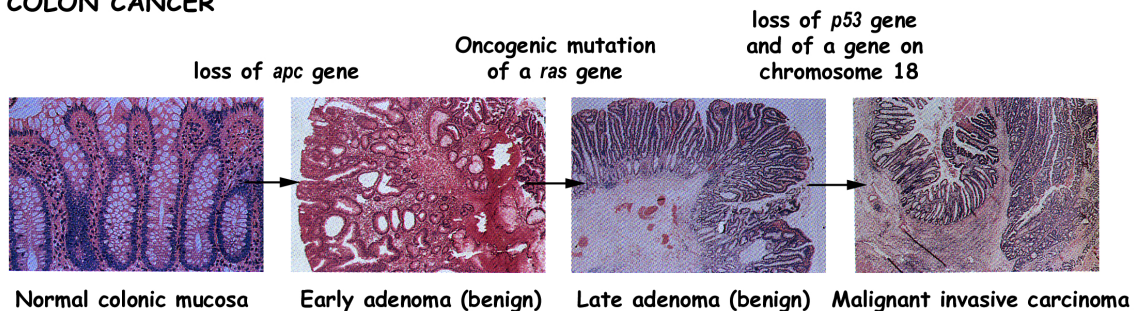


Figure 1. Multistep progression to malignancy in colon cancers. Development of colon cancer requires a series of sequential mutational events that lead to a state of uncontrolled proliferation. In this figure loss of function of the tumor suppressor gene *apc* is a prerequisite for the accumulation of additional mutations in the colonic mucosa. Several histologically distinct stages can be distinguished in the progression of the tissue from the normal state to benign tumor to a malignant cancer. Note that the tissue becomes more disorganized as the tumor progresses toward malignancy. Extracted from Cavenee and White, 1995.

1.2.1 Tumor suppressor genes in *Drosophila*

In 1916, decades before *Drosophila* became one of the most popular models for studying many aspects of modern biology, the discovery of melanotic tumor-like granules in mutant larvae by Bridges and Stark first suggested that flies could develop tumors (Stark, 1918). Later, spontaneous mutations were identified that caused animals to die at larval stages due to overproliferation of certain internal tissues (Gateff, 1978) (Mechler *et al.*, 1985). Currently, at least 29 tumor suppressor genes are known in *Drosophila*. Examples of these genes are *discs large* (Woods and Bryant, 1991), *l(2)giant larvae* (Jacob *et al.*, 1987; Gateff and Schneiderman, 1969) and *l(2)tid* (Kurzik-Dumke *et al.*, 1992). The high level of gene and pathway conservation, the similarity of cellular processes and the emerging evidence of functional conservation of tumor suppressors between *Drosophila* and mammals, argue that studies of tumorigenesis in flies can directly contribute to the understanding of human cancer (Potter *et al.*, 2000).

1.3 The Tid family of proteins

The *Drosophila* tumor suppressor gene *lethal tumorous imaginal discs* (*l(2)tid*) has been the first member of this family to be identified (Kurzik-Dumke *et al.*, 1992). Recent work describes the existence of two homologues of *l(2)tid* in mouse (*mTid-1*) (Trentin *et al.*, 2000) and human (*hTid-1*) (Syken *et al.*, 1999).

1.3.1 In *Drosophila*: the tumor suppressor gene *l(2)tid*

The *Drosophila melanogaster* tumor suppressor gene *l(2)tid* causes in homozygotes malignant growth of cells of the imaginal discs and the death of the mutant larvae at the time of puparium formation (Gateff, 1978; Gateff and Mechler, 1989; Kurzik-Dumke *et al.*, 1992). Imaginal discs are the adult integumental primordia consisting of a single-cell-thick epithelium exhibiting a pronounced basal-apical polarity (Poodry, 1980). In the early embryo the presumptive imaginal discs are present as small cell groups subdivided into an anterior and a posterior compartment (Wieschaus and Gehring, 1976) (Bate and Arias, 1991). During larval life the imaginal discs grow by cell division and acquire, in a spatial and temporal sequence, the capacity to differentiate into their respective adult integumental structures (Gateff and Schneiderman, 1974). Finally, during development in the pupa imaginal discs differentiate the adult cuticular patterns of the head, the thorax, appendages and genitalia. In *l(2)tid* mutant larvae the imaginal discs are incapable of differentiating and grow during the prolonged larval life into lethal tumors which, after transplantation into the abdomens of *wild-type* flies, grow in the same neoplastic and lethal fashion as *in situ* (Kurzik-Dumke *et al.*, 1992).

Genetic and cytogenetic analysis localized the *l(2)tid* gene to the right arm of the second chromosome at position 59F5 in a 20 kb region. cDNA-analysis of this region yielded three non-overlapping cDNAs: 10D, 10F and 11D. The germ line rescue of the tumor phenotype, the definitive proof for the cloning of a gene (Rubin and Spradling, 1982), was achieved with a 7.0 kb HindIII-fragment that just represented the genes corresponding to the cDNAs 10D and 10F completely (Kurzik-Dumke *et al.*, 1995). A transcript analysis of two mutant *l(2)tid* alleles (1 and 2) showed that the tumor phenotype of the mutant larvae is causally related to the absence of a constitutively expressed 2.0 kb transcript, one of the three transcripts recognized by the cDNA 10D,

whereas no change in the expression of the 2.2 kb fragment, recognized by the cDNA 10F, was observed. Thus the gene encoded by the cDNA 10D was designated as *l(2)tid*, whereas the gene encoded by the cDNA 10F was named neighbour of *tid* (*l(2)not*) (Kurzik-Dumke *et al.*, 1997). Sequence analysis of the cDNAs 10D and 10F and the entire genomic HindIII fragment revealed the molecular organization of the corresponding genes *l(2)tid* and *l(2)not*. The *l(2)tid* gene lies completely in the intron of the non-coding DNA-strand from *l(2)not*. Furthermore, analysis of the intronic part of *l(2)not* revealed an additional ORF representing another gene, the *lethal(2)relative of tid* (*l(2)rot*) (Kurzik-Dumke *et al.*, 1997). Similar “gene within a gene” structures have already been reported in *Drosophila* (Davis and Dauwalder, 1991), mouse (Huxley and Fried, 1990) and man (Viskochil *et al.*, 1991).

The *l(2)tid* gene spans a total of 2539 nt, including the putative promoter region. The coding region, 1554 nt, is subdivided by a 142 nt intron into exon 1, 1326 nt, and exon 2, 225 nt. The predicted amino acid sequence of the putative *l(2)tid* gene product, the Tid56 protein, consists of 518 amino acids with a putative molecular weight of 56 kDa. Search for sequence homology of the putative Tid56 precursor protein to known proteins revealed a significant amino acid identity with the DnaJ homologs from different species from bacteria to man (Kurzik-Dumke, 1996) (Figure 2).

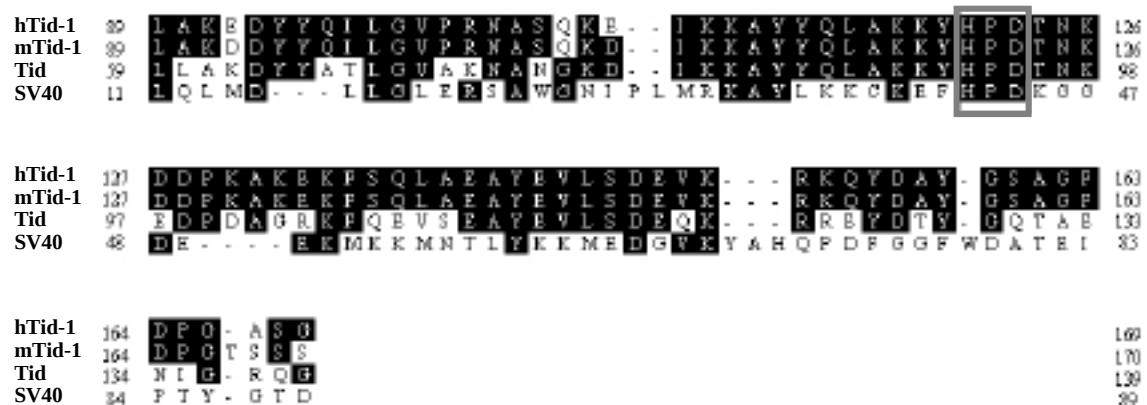


Figure 2. Sequence comparison between the J-Domains of the Tid family of proteins and SV40 T-Ag. Amino acid sequence of the human hTid-1 J-Domain (Acc. N° NP_005138) is presented in comparison with the corresponding sequences of mouse mTid-1 (Acc. N° AAG37303), *Drosophila* Tid (Acc. N° CAA54837) and SV40 T-Ag (Acc. N° AAC59346). Shaded areas mark identical amino acid residues. The box highlights the conserved HPD motif important for the binding of DnaJs to Hsp70.

To identify the protein encoded by the *l(2)tid* gene, three polyclonal rabbit antibodies, anti-Tid, anti-Tid^(J) and anti-Tid^(C), directed against different parts of the putative Tid56 protein, were generated and used for staining of developmental Western blots (Kurzik-Dumke *et al.*, 1998). Three specific bands, 50, 47 and 40 kDa in size respectively (designated in the following as Tid50, Tid47 and Tid40) were recognized. To determine the intracellular localization of these proteins, *Drosophila* embryos and Schneider cells were fractionated by differential centrifugation and sucrose gradients. Immunoblots of these fractions indicated a localization of Tid50 and Tid40 in the mitochondria, whereas Tid47 was located in the cytosol. The putative Tid56 precursor protein could be recognized only in highly enriched mitochondrial fractions. Tid56 is soluble in the mitochondrial matrix, whereas Tid50 and Tid40 are peripherally associated to the inner membrane (Kurzik-Dumke *et al.*, 1998), (Debes, 1997).

In situ hybridisation on wholemount embryos and larval tissues revealed a ubiquitous expression during the whole of the embryonic life and in larval tissues such as fat body, Malpighian tubules, the gut, the hematopoietic organ and in imaginal discs. *l(2)tid*-RNA is not expressed in salivary glands and in larval brain (Kurzik-Dumke *et al.*, 1998). Similarly, the developmental expression pattern and the tissue specificity of the *l(2)tid*-encoded protein immunostaining showed equivalent results. The mitochondrial subcellular localization of the Tid50 protein revealed a distribution of the signals in a spot-like manner, resembling the pattern obtained by staining of Rhodamine 123. Electron microscopy evaluation revealed signals on the outer mitochondrial membrane, in the mitochondria and cytosol (Debes, 1997; Kurzik-Dumke *et al.*, 1998).

1.3.2 In mouse: *mTid-1*

The mouse *Tid-1(mTid-1)* gene is a novel member of the Tid family of chaperone proteins. It has been identified as a binding partner of the GAP protein (p120 GTPase activating protein) (Trentin *et al.*, 2000). The function of GAP is to downregulate Ras by stimulating GTP hydrolysis of active Ras in one of the main signaling pathways in the organism, which is also involved in malignant transformation (Bourne *et al.*, 1990). The *mTid-1* gene encodes three alternatively spliced forms: mTid-1_L, mTid-1_I and mTid-1_S. Immunofluorescence experiments revealed that mTid-1 proteins are localized in the

cytosol, mitochondria or nucleus, depending on the particular cell type. Moreover, in response to epidermal growth factor stimulation, GAP and mTid-1 colocalize to distinct perinuclear subdomains resembling mitochondrial membranes. Furthermore, mTid-1 can associate with both cytoplasmic and mitochondrial Hsp70 chaperone (Trentin *et al.*, 2000). The current hypothesis points mTid-1 to a potential role in GAP-mediated regulation of cell growth through the control of GAP-conformation, assembly of complexes and sequestering of GAP from the cytosol to the mitochondria. In absence of functional mTid-1 proteins GAP may escape this regulation and consequently affect GAP's ability to effectively downregulate Ras, which may contribute to hyperproliferative phenotype (Trentin *et al.*, 2000).

1.3.3 In human: *hTid-1*

The human homolog of the *Drosophila* tumor suppressor protein Tid was identified and cloned 1998 in Münger's lab by virtue of its ability to form complexes with the human papillomavirus E7 oncoprotein (Schilling *et al.*, 1998). It has been mapped to the short arm of chromosome 16 and its mRNA is widely expressed in human tissues. *HTid-1* encodes two mitochondrial matrix localized splice variants of 43 and 40 kDa, named hTid-1_L and hTid-1_S, respectively. Both hTid-1_L and hTid-1_S contain the characteristic J domain of the HSP40 family of proteins and co-immunoprecipitate with mitochondrial Hsp70 (Syken *et al.*, 1999). Given the mitochondrial localization of Tid and the important role of mitochondria in regulating apoptosis (Kroemer *et al.*, 1997), (Green and Reed, 1998) it has been postulated that the tumorous imaginal discs phenotype may reflect a failure of imaginal disc cells to properly integrate stimuli of cell death and survival signals (Syken *et al.*, 1999). hTid-1_L and -1_S expression studies on cell lines showed an opposing effect on a cell's ability to respond to an exogenous apoptotic stimulus. A J domain mutant of hTid-1_L is able to suppress apoptosis to levels well below control cells. In sharp contrast, hTid-1_S increases apoptosis. Hence, hTid-1_L and hTid-1_S represent two mitochondrial matrix localized proteins that can regulate apoptotic signal transduction and may comprise a mechanism by which the mitochondria amplify or dampen apoptotic signals (Syken *et al.*, 1999).

Moreover, several studies on hTid-1 expression in different human tissues have been carried out. Work done by Kurzik-Dumke *et al.* showed a strong expression of hTid-1

in rheumatoid arthritis synovial tissue (Kurzik-Dumke *et al.*, 1999). Furthermore, preliminar studies on the expression of hTid-1 in varios tumors revealed an abnormal staining of the protein. Therefore, it has been postulated a potential use of the expression levels of hTid-1 as an indicator of the pathological state of cells (Kurzik-Dumke *et al.*, 1997).

1.4 Molecular chaperones: the Hsp40 family of proteins

The tumor suppressor Tid protein has been identified as a member of the chaperone Hsp40 family of proteins (Kurzik-Dumke *et al.*, 1995) (Section 1.3.1). The Hsp40 family consists of a diverse group of proteins that are related to the DnaJ protein of *E.coli* (Bardwell *et al.*, 1986). They typically share 35-50% overall sequence identity with DnaJ and contain a highly conserved domain (termed the J-domain) corresponding to the N-terminal 70 amino acids of this protein. As shown in Figure 2, members of this family are known to have 4 distinct domains: (i) a highly conserved J domain of approximately 70 amino acids in size which has been known to mediate interaction with Hsp70 and regulate its ATPase activity; (ii) a glycine and phenylalanine (G/F)-rich region possibly acting as a flexible linker; (iii) a cysteine-rich region (C domain) containing 4 [CXXCXGXG] motifs resembling a zinc-finger domain (Bork *et al.*, 1992) and (iv) a *C-terminal* domain which is less well conserved among different J proteins and is still poorly understood (Ohtsuka and Hata, 2000). However, not all DnaJ homologs necessarily contain all of these 4 domains. Recently, Cheetham and Caplan proposed the classification of DnaJ homologs into 3 groups: type I homologs that have all 3 main domains (J, G/F and CXXCXGXG), type II that have the J and G/F but not the CXXCXGXG domain, and type III that have the J domain alone (Cheetham and Caplan, 1998). The most prominent feature of Hsp40 family members is the ability they possess to stimulate hydrolysis of ATP by specific Hsp70 partners (Caplan *et al.*, 1993), (Cyr *et al.*, 1994), (Hartl, 1996). Other reactions facilitated by Hsp40 proteins include protein synthesis, protein folding (Georgopoulos and Welch, 1993), protein translocation [Pfanner, 1994 #18], signal transduction, assembly of macromolecular complexes (Cyr *et al.*, 1994), renaturation of misfolded proteins and proteolysis (Gething, 1997). Recently it has become clear that many human diseases result from improper protein folding. Despite the presence of abundant molecular chaperones *in vivo* proteins still

fail to attain their native stable structure (Kaufman, 1998). In fact, chaperones have gained an important role in medicine: ER- (endoplasmic reticulum) chaperones elicit host cell specific T-cell responses and induce tumor immunity in mice (Wassenberg *et al.*, 1999); Grp94 is described as a tumor rejection antigen because it binds peptides specific to the tumor cell and presents them in an immunocompetent manner to the host (Blachere *et al.*, 1997) and there exist chaperones such as p58IPK which are able to mediate malignant transformation (Melville *et al.*, 1999).



Figure 3. Schematic representation of the structural features of Tid. *M* corresponds to the mitochondrial pre-sequence. The *DnaJ* domain defines Tid as a DnaJ homologue and is responsible for the binding of DnaJ proteins to Hsp70. *G/F* corresponds to a region rich in glycine and phenylalanine which may have a spacer function. *CXXC* denotes a cysteine-rich region that contains four repeats of the sequence CXXGXGXG resembling a zinc-finger motif. The *C-terminal* part of Tid is conserved to a lesser extent in evolution and has no obvious sequence features.

1.5 Objectives of this work

The main goal of this thesis is the identification of molecular partners of the proteins encoded by the *Drosophila* tumor suppressor *l(2)tid* gene (section 1.3.1) in order to give them a functional context in the organism. To achieve this several approaches are going to be used. Immunoprecipitation of biotinylated protein extracts from all developmental stages with a specific anti-Tid antibody (Kurzik-Dumke *et al.*, 1998) should provide us with information about the existence of Tid molecular partners during development. Further, identification of these binding partners will be carried out with the screening of an expression library *via* the yeast two-hybrid system. Potential positive clones found in this study will be tested for interaction with independent biochemical methods such as co-immunoprecipitation and the GST-pulldown assay. Moreover, additional two-hybrid assays will be done to establish the protein domains responsible for the interaction.

Immunohistochemical studies on mutant wing imaginal discs will be performed to check whether changes in expression of the interacting proteins can be observed. Furthermore, double mutant stocks will be generated to test for genetic interaction. Finally, to better interpret the results of these immunohistochemical studies on abnormal *l(2)tid* tumorous imaginal discs and to determine the regions of the wing disc involved in tumor formation several enhancer-trap lines expressing in defined parts of the wing disc and specific antibody stainings will be used.

2. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Enzyme and chemicals

Enzyme

Restriction endonucleases (different types)	MBI Fermentas, Roche
T4-DNA-Ligase	MBI Fermentas, Roche
Klenow Fragment	MBI Fermentas
Calf Intestine Alakine Phosphatase	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas, Sigma
Big Dye Terminator Sequencing Mix	PE-Biosystems

Chemicals

All chemicals used had “pro analysis” quality and were from Gibco BRL, Roche, Difco, Merck, Roth, Serva and Sigma.

2.1.2 Culture media

Media used for the culture of *E.coli* cells

LB-Medium (Luria-Bertani)	Bacto-Trypton	20 g
	Yeast Extract	5 g
	NaCl	5 g
	Agar (for plates only)	15 g
	add H ₂ O _{dd} to a final volume of 1 l	
	adjust the pH to 7.4 with NaOH and autoclave	
NZY-Broth	Yeast extract	5 g
	NZ amine	10 g
	NaCl	5 g
	MgSO ₄ .7H ₂ O	2 g

Agar (for plates only) 15 g
add H₂O_{dd} to a final volume of 1 l
adjust the pH to 7.5 with NaOH and autoclave

Concentration of antibiotics	Ampicillin	100 µg/ml
	Kanamycin	25 µg/ml

Media used for the culture of yeast cells

YPD medium	Difco peptone	20 g
	Yeast extract	10 g
	Agar (for plates only)	18 g
	add H ₂ O _{dd} to a final volume of 1 l, adjust	
	pH to 5.8, autoclave and add dextrose to 2%	

10x Dropout (DO) supplements 10x DO contain all but one or more of the following components, depending on the selection medium desired.

L-Isoleucine	300 mg
L-Valine	1500 mg
L-Adenine	200 mg
L-Arginine	200 mg
L-Histidine	200 mg
L-Leucine	1000 mg
L-Lysine	300 mg
L-Methionine	200 mg
L-Phenylalanine	500 mg
L-Threonine	2000 mg
L-Tryptophan	200 mg
L-Tyrosine	300 mg
L-Uracil	200 mg

add H₂O_{dd} to a final volume of 1 l and autoclave

SD medium

Difco yeast nitrogen base
 w/o amino acids 6.7 g
 Agar (for plates only) 20 g
 add H₂O_{dd} to a final volume of 850 ml
 add 100 ml of the appropriate DO-solution,
 adjust pH to 5.8, autoclave and
 add dextrose to 2%

Drosophila breeding medium

Agar 40 g
 Dry yeast 90 g
 Soja flour 50 g
 Maize semolina 400 g
 Malta extract 400 g
 Sugar-beet sirup 200 g
 Acid propionic 25 ml
 add water to 5 l

2.1.3 Standard solutions**Tris/HCl buffer**

Tris 1 M
 add 1 l H₂O_{dd} and adjust pH with HCl

10x TBE buffer

Tris/HCl 0.89 M
 B(OH)₃ 0.89 M
 EDTA 20 mM
 add H₂O_{dd} to a final volume of 1 l

TE buffer

Tris/HCl pH 8.0 0,01 M
 EDTA 1 mM

10x loading buffer

Bromphenolblue 0.1 %
 EDTA 0.1 M
 Glycerin 50 %

Phenol/Chloroform	Phenol buffered with Tris	50%
	Chloroform	50%
	cover with a layer of 0.1 % Tris/HCl pH 8	
	and keep at 4°C	
10x TBS buffer	Tris/HCl pH 7.5	0.5 M
	NaCl	1.5 M
	add H ₂ O _{dd} to a final volume of 1 l and	
	adjust pH to 7.2	
10x PBS buffer	NaCl	1.3 M
	KCl	27 mM
	Na ₂ HPO ₄	65 mM
	KH ₂ PO ₄	15 mM
	add H ₂ O _{dd} to a final volume of 1 l and	
	adjust pH to 7.2	
TKM buffer	Tris/HCl pH 7.5	50 mM
	KCl	150 mM
	MgCl ₂	5 mM
Protease inhibitor solution	One “Complete TM ” tablet (Roche) was used for 10 ml protein extract.	

Solutions used to prepare competent *E.coli* cells

RF1 buffer	RbCl	0.1 M
	MnCl ₂	0.05 M
	Potassiumacetat	0.03 M
	CaCl ₂	0.01 M
	Glycerol	15 %
	adjust pH to 5.8 with acetic acid	

RF2 buffer	MOPS	0.01 M
	RbCl	0.01 M
	CaCl ₂	0.075 M
	Glycerol	15 %
	adjust pH to 8.0 with NaOH	

Solutions used to generate recombinant proteins

Buffers for purification of 6xHis-tagged proteins under denaturing conditions

Lysis buffer	Urea	8 M
	NaH ₂ PO ₄	0,1 M
	Tris/HCl	0,01 M
	adjust pH to 8.0	

Wash buffer	Urea	8 M
	NaH ₂ PO ₄	0,1 M
	Tris/HCl	0,01 M
	adjust pH to 6,3	

Elution buffer D	Urea	8 M
	NaH ₂ PO ₄	0,1 M
	Tris/HCl pH 8.0	0,01 M
	adjust pH to 5,9	

Elution buffer E	Urea	8 M
	NaH ₂ PO ₄	0,1 M
	Tris/HCl pH 8.0	0,01 M
	adjust pH to 4,5	

Buffers for purification of 6xHis-tagged proteins under native conditions

Lysis buffer	NaH ₂ PO ₄ pH 8,0	50 mM
	NaCl	300 mM

	Imidazole	10 mM
Wash buffer	NaH ₂ PO ₄ pH 8,0	50 mM
	NaCl	300 mM
	Imidazole	20 mM
Elution buffer	NaH ₂ PO ₄ pH 8,0	50 mM
	NaCl	300 mM
	Imidazole	250 mM

Solutions used in immunoprecipitation

Lysis buffer	Core buffer(Roche)	50mM
	NaCl	150 mM
	PMSF	0,1mg/ml
	Aprotinin	1µg/ml
	Leupeptin	1µg/ml
	Nonidet P-40	1%
	Sodium deoxycholate	0,5%
	add H ₂ O _{dd} to a final volume of 12 ml	
Dilution buffer	Tris/HCl pH 7,5	50mM
	NaCl	150 mM
	PMSF	0,1mg/ml
	Aprotinin	1µg/ml
	Leupeptin	1µg/ml
	Nonidet P-40	0,1%
	add H ₂ O _{dd} to a final volume of 12 ml	
Wash buffer 1	Tris/HCl pH 7,5	50mM
	NaCl	150 mM
	Nonidet P-40	0,1%
	add H ₂ O _{dd} to a final volume of 48 ml	

Wash buffer 2	Tris/HCl pH 7,5	50mM
	NaCl	500 mM
	Nonidet P-40	0,1%
	add H ₂ O _{dd} to a final volume of 48 ml	

Wash buffer 3	Tris/HCl pH 7,5	10 mM
	add H ₂ O _{dd} to a final volume of 24 ml	

Solutions used in the GST-pulldown assay

Lysis buffer	Tris/HCl pH 7,5	50 mM
	KCl	100 mM
	ZnCl ₂	10 µM
	Dithiothreitol (DTT)	0,1 mM
	Glycerol	10 %

GST buffer	Tris/HCl pH 7,5	50 mM
	KCl	100 mM
	MgCl ₂	10 mM
	DTT	0,3 mM
	Glycerol	5 %
	Nonidet P-40	0,1 %

Solutions used in the two-hybrid system

Cracking buffer stock solution	Urea	8M
	SDS	5% w/v
	Tris/HCl pH 6.8	40 mM
	EDTA	0,1 mM
	Bromophenol blue	0,4 mg/ml
	add H ₂ O _{dd} to a final volume of 100 ml	

Cracking buffer	Cracking buffer stock sol.	1 ml
	β-mercaptoethanol	10 µl

	PMSF	50µl of a100x stock solution
Herring testes carrier DNA		10mg/ml
10X LiAc (Lithium Acetate)	lithium acetate	1M
	adjust pH to 7.5 with acetic acid and autoclave	
PEG/LiAc solution	PEG4000	8 ml
(Polyethylene Glycol/	10X-TE buffer	1 ml
Lithium Acetate)	10X LiAc	1 ml
Yeast lysis solution	Triton X-100	2 %
	SDS	1 %
	NaCl	100 mM
	Tris/HCl pH 8.0	10 mM
	EDTA	1 mM
Z buffer	Na ₂ HPO ₄ ·7H ₂ O	16,1 g
	NaH ₂ PO ₄ ·H ₂ O	5,50 g
	KCl	0,75 g
	MgSO ₄ ·7H ₂ O	0,25 g
	add H ₂ O _{dd} to a final volume of 1 l,	
	adjust pH to 5.8 and autoclave	
X-gal stock solution	20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) diluted in 1 ml of N,N-dimethylformamide (DMF)	
Z buffer/X-gal solution	Z buffer	100 ml
	β-mercaptoethanol	0,27 ml
	X-gal stock solution	1,67 ml

Solutions used in immunostainings of embryonic and larval tissues

PBT	PBS	50 ml
	Triton X-100	150 µl
PBTs	PBT	10 ml
	Bovine Serum Albumin	0,2 %
PBTw	PBS	10 ml
	Tween 20	10 µl
DAB stock solution	3,3'-Diaminobenzidine	0,35 mg/ml

Solutions used in SDS-Polyacrylamidgelelectrophoresis (PAGE)

Acrylamid-Stock solution	Acrylamid	30 %
	N,N-Methylenbisacrylamid	0,8 %
	in H ₂ O _{dd}	
Lower Tris	Tris	18,2 g
	10% SDS	4 ml
	add H ₂ O _{dd} to a final volume of 100 ml	
	and adjust pH to 8.8	
Upper Tris	Tris	6,1 g
	10% SDS	4 ml
	add H ₂ O _{dd} to a final volume of 100 ml	
	and adjust pH to 6.8	
Sacking gel (5%)		
Acrylamid stock solution	0,83 ml	
Upper tris	1,25 ml	
10% Ammoniumpersulfate	50 ml	

TEMED 10 ml

H₂O_{dd} 2,9 ml

These quantities are sufficient to prepare two (9x8cm) minigels

Running gel	8%	10%	12,5%
Acrylamid stock solution	2,65 ml	3,3 ml	4,1 ml
Lower tris	2,5 ml	2,5 ml	2,5 ml
10% Ammoniumpersulfate	100 µl	100 µl	100 µl
TEMED	20 µl	20 µl	20 µl
H ₂ O _{dd}	4,8 ml	4,1 ml	3,3 ml

These quantities are sufficient to prepare two (9x8cm) minigels

1x SDS-Running buffer	Glycin	192 mM
	Tris/HCl pH 8.3	25 mM
	SDS	0,1%

Coomassie staining solution	Methanol	40%
	Acetic acid	10%
	Coomassie Brilliant Blue R-250	

Destaining solution	Methanol	5%
	Acetic acid	7,5%
	add H ₂ O _{dd} up to 1 l	

Solutions used in Western Blot

Anode buffer I	Tris/HCl pH 7,5	300 mM
	Methanol	20%

Anode buffer II	Tris/HCl pH 7,5	25 mM
	Methanol	20%

Catode buffer	6-Amino-hexan acid	40 mM
	Methanol	20%
Ponceau-S	Ponceau-S	0,5%
	in 5% TCA	
2xLaemmli buffer	Tris/HCl pH 6.8	125 mM
	Glycerin	20%
	SDS	4%
	β-mercaptoethanol	10%
	Bromphenol blue	0,1%
Stripping solution	2-mercaptoethanol	100 mM
	SDS	2%
	in TBS	

2.1.4 Equipment

Autoclaves	Aesculap 420 Q
	Gössler Laboratory autoclave GLA 40
Computer	Power Machintosh G3
	IBM-compatible computer
Computerprogramms	Microsoft Word 97
	Microsoft Excel 97
	Microsoft Power Point 97
	Adobe Photoshop TM 5.5
	Fotolook SA 2.07.2
Electrophoresis appliances	Hoefer SE250 Mighty Small II Electrophoresis appliance
	LKB Multiphor Nova Blot appliance

	Pharmacia Gel Electrophoresis Apparatus GNA-200 and GNA-100 Biometra Maxigel, vertical Electrophoresis appliance Vivascience Microconcentrators
Fridges an freezers	Liebherr GS 3702 Liebherr Glassline Bosch Electronic Luxus
Microscopy	Leitz binocular Leica Confocal Laser Scanning microscope Zeiss Axiophot microscope
Photometry	Kontron Spectralphotometer Uvikon 810 LKB Novaspec 4049 Spectrophotometer
Power Supplies	Pharmacia Gene Power Supply GPS 200/400 Pharmacia Electrophoresis Power Supply EPS 3500
Waterbath/incubators	Desaga-Frigostat waterbath Köttermann Shake-waterbath Heraeus Incubators
Centrifuges	Centra Coolingcentrifuge IEC 7R Heraeus Christ Biofuge A (Table centrifuge) Sorvall high-speed centrifuge RC-5B (GS3, GSA, SS34) Kontron ultracentrifuge Centrikon T-2060 (TST 41.14) Hettich Micro Rapid/K table centrifuge Hettich Universal/K25 table centrifuge Braun Sigma 3K20

Other equipment and material

Eppendorf Pipette tips
Falcon tubes (15 ml, 50 ml)
Filterpaper Schleicher&Schuell
Teflon homogenisator
Nitrocellulose membrane Schleicher&Schuell (0,2mm)
PVDF membrane Millipore (0,45mm)
Greiner plates
Lunik200 Microwave
Heraeus Sterilhood Laminair HLB 2472
Heildoph Vortex REAX 2000
IKS Combimag REO Magneticmixer
Braun Shaker Certomat R
Heidolph REAX rocking platform
Eppendorf Manual Pipettes
Film (Fuji Medical X-Ray Film RX, Polaroid 665, Kodak 5018 EPY, Agfa Agfachrom CTX, Kodak Gold 200)
Whatman Chromatography paper 3MM
Eppendorf tubes (1,5 and 2 ml)
Polaroid Land Instantcamera MP-40
Bachofer Speed Vac Concentrator
Sartorius Analysis scale
Vetter UV-Transilluminator Chroma 43
Corex Centrifuge glass tubes (15 ml, 30 ml)

2.1.5 Vectors

pBluescript I KS(+/-)	(Stratagene)
pUC18	(Fermentas)
pQE-30, 32	(Qiagen)
pACT2	(Clontech)
pAS2-1	(Clontech)
pGEX5X-3	(Pharmacia)

2.1.6 Plasmid constructs

The following plasmid constructs were obtained and used in this work:

Construct	Coding for	Reference
cDNA 10D in pBluescript I KS (+/-)	<i>l(2)tid</i> gene complete	(Kurzik-Dumke <i>et al.</i> , 1995)
pQE31- Tid ^{aa 98-495}	BamHI-fragment from the <i>l(2)tid</i> gene	(Kurzik-Dumke <i>et al.</i> , 1998)
pVA3-1	DNA-BD/murine p53 protein in pAS2-1	Clontech
pTD1-1	AD/SV40 large T-antigen protein in pACT2	Clontech
pLAM 5'-1	DNA-BD/human lamin C protein in pAS2-1	Clontech

2.1.7 Bacteria and yeast stocks

<i>DH5α</i>	(Hanahan, 1983)
<i>M15 (pREP4)</i>	(Qiagen)
<i>RR1</i>	(Bolívar, 1977)
<i>Y187</i>	(Clontech)
<i>Y190</i>	(Clontech)

2.1.8 Fly stocks

The following fly stocks of *Drosophila melanogaster* were used in this work:

Name and Genotype	Origin	Reference
Wild-type: Oregon R	Stocks U. Kurzik-Dumke	(Lindsley and Zimm, 1992)
2 nd chromosome balancers: <i>Pm/CyO</i> <i>Pm/CyO*</i>	Mainzer Stock collection	
3 rd chromosome balancer: <i>TM3/TM6B</i>	Mainzer Stock collection	
<i>l(2)tid¹ a px/CyO</i>	Stocks U. Kurzik-Dumke	(Kurzik-Dumke <i>et al.</i> , 1992)
<i>ptc^{tuf-1} ltd[1]</i>	Stocks I. Guerrero	(Phillips <i>et al.</i> , 1990) (Capdevila <i>et al.</i> , 1994)
<i>ptc^{HW109} cn[1] bw[1] sp[1]</i> <i>dpp β-gal/SM5</i>	Stocks I. Guerrero	(Tearle and Nusslein-Volhard, 1987)
<i>ptc^{IN} cn[1] bw[1] sp[1]/CyO</i>	Stocks I. Guerrero	(Tearle and Nusslein-Volhard, 1987)
<i>P{UAS-mCD8::GFP.L}</i> <i>LL4,y(1)w[*];Pin[Yt]/CyO</i>	Mainzer Stock collection	Bloomington stock center
<i>w⁺; ;p{gawB}</i>	Mainzer Stock collection Stock MZ 0426	Urban, 1990
<i>w⁺; ;p{gawB}</i>	Mainzer Stock collection Stock MZ 0853	Urban, 1990
<i>w⁺; ;p{gawB}</i>	Mainzer Stock collection Stock MZ 1229/1	Urban, 1990

(For details about genetic markers, see Lindsley and Zimm, 1992)

2.2 METHODS

2.2.1 DNA manipulation

2.2.1.1 Purification of Plasmid DNA

The protocols used in this work for large- and small-scale DNA preparations are based on the alkaline lysis procedure (Sambrook *et al.*, 1989). To obtain high quality DNA kits from Qiagen or Gibco^{BRL} were used.

2.2.1.2 Quantitation of double stranded DNA

Measurement of the DNA concentration in a sample was performed by absorption at 260 nm wavelength as described in Sambrook *et al.*, 1989.

2.2.1.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis has been used to separate, identify and purify DNA fragments. Depending on the size of these fragments the concentration of agarose in the gel was between 0.8% and 1.2%. To directly visualize DNA upon illumination with UV light 3µl Ethidium bromide solution (10mg/ml) was incorporated in each 30 ml agarose gel.

2.2.1.4 DNA extraction from agarose gels

Extraction of DNA from agarose gels was performed using the Jetsorb, gel extraction kit (Genomed). Jetsorb is the core-element consisting of a high quality chromatography material based on spherical 4µm silica gel particles. The surface of these particles has been modified to purify all types of DNA fragments.

2.2.1.5 DNA digestion with restriction endonucleases

Digestion of DNA probes with restriction endonucleases was performed using 3 to 5 U enzyme per microgram DNA. Selection of suitable conditions for digestion depended on the enzyme used, according to manufacturer's instructions (see section 2.1.1). Double digestion reactions were performed when possible, adding up to 4 fold excess of enzyme to the reaction depending on its activity in the selected buffer.

2.2.1.6 Recovery of DNA

DNA precipitation was performed by addition of 1/10 volume of 3M Sodium acetate, pH 5.2 and 2-3 volumes ethanol or 1 volume isopropanol to the DNA solution. After 15-30 minutes incubation on ice, DNA was recovered by centrifugation at 4°C, 12.000g for 20 minutes. Then, the pellet was washed with 70% ethanol solution, dried and resuspended in adequate amounts of TE-buffer or H₂O_{dd}.

2.2.1.7 DNA dephosphorylation

Dephosphorylation of linearized DNA is an important step in the cloning procedure in order to minimize recircularization of the plasmid. It was performed with 1U of Calf Intestine Alkaline Phosphatase according to the manufacturer's protocol (MBI Fermentas).

2.2.1.8 DNA ligation

Cloning of DNA fragments into linearized plasmid vectors was performed with 2 to 4 U of T4-DNA ligase following manufacturer's instructions (MBI Fermentas). The concentrations of the two types of DNA in the ligation were carefully adjusted and optimized for each reaction (Sambrook *et al.*, 1989).

2.2.1.9 DNA amplification by PCR

PCR (Polymerase Chain Reaction) allows the production of more than 10 million copies of a target DNA sequence from only a few molecules. Several parameters should be considered in order to obtain DNA amplification. Following are listed the conditions used in this work.

Components of the reaction:

20 ng of plasmid DNA / 1µg of library DNA
0.2 mM dNTPs
5 µl Buffer for the Taq Polymerase
1 µl Taq Polymerase
100 pmol primer (each)
2 mM MgCl₂
H₂O_{dd} up to 50 µl

Conditions of the reaction:

1. 2 min 94°C
2. 25 cycles:
 - 1 min 94°C
 - 1 min at annealing temperature
(-5°C compared to the melting-
temperature of the primer pair)
 - 3 min 72°C
3. 7 min 52°C

2.2.1.10 Nucleotide sequencing and sequence analysis

The nucleotide sequence of inserts of plasmids was carried out by the firm Genterprise using plasmid/gene specific primers, the Big Dye Terminator Sequencing Mix (PE-Biosystems) and an automated DNA sequencer (ABI, model 377). Analyses of nucleotide and deduced amino acid sequences were done using the Sequencher and DNAsis software packages. Gene and protein identifications were accomplished by alignments using the BLAST algorithms (Altschul *et al.*, 1997). Comparisons of related sequences were undertaken using the MegaAlign and ClustalW programmes.

2.2.1.11 Amplification of a bacteriophage library and DNA extraction

Amplification of the Human fetal brain cDNA-bacteriophage library was performed at low multiplicity (Sambrook *et al.*, 1989). Extraction of bacteriophage DNA was carried out according to Sambrook *et al* (1989).

2.2.1.12 Transformation of DNA using Rubidium Chloride

Competent cultures of *E.coli* DH5- α strains were prepared according to the method developed by Hanahan (Sambrook *et al.*, 1989). Exposure to Rubidium ions rendered the cells able to take up DNA. Transformation of plasmid DNA was performed by heat shocking a mixture of this DNA and 50 μ l of competent cells for 90 sec at 42°C. Transformed cells were allowed to recover in non-selective medium for 45 min and then plated on antibiotic containing medium to permit identification of colonies carrying the plasmid.

2.2.1.13 Transformation of DNA by electroporation

Electroporation with high voltage is currently the most efficient method for transforming *E.coli* with plasmid DNA. Preparation of *E.coli* RR1 electrocompetent cells was done according to Ausubel *et al.*, 2001. 30µl of electrocompetent *E.coli* cells were mixed with 1µl salt-free DNA and incubated 1 min on ice. DNA and cells were transferred into an electroporation cuvette that had been chilled 5 min on ice. Transformation was done with a pulse of 1.8 kV during 5 msec. After 45 min recovery in LB medium aliquotes were plated on LB plates containing antibiotics and incubated overnight at 37°C.

2.2.1.14 Assessment of competency of cells

In order to determine the transformation efficiency of the prepared competent cells several aliquots of a control transformation with 10ng of plasmid DNA were plated on LB/ampicillin plates and incubated at 37°C overnight. Transformation efficiencies of 10^7 are usually obtained with the rubidium chloride method, whereas electroporation routinely gives more than 10^9 bacterial transformants per microgram of input plasmid DNA (Ausubel *et al.*, 2001).

2.2.1.15 Isolation of positive clones by α -complementation

This method was used to easily identify recombinants constructed in pUC18 and pBluescript vectors. It exploits the phenomenon called α -complementation discovered by Ullman, Jacob and Monod in 1967. X-Gal and IPTG were added to premade LB agar plates containing the appropriate antibiotics. Bacteria carrying recombinant plasmids were plated on them and incubated 12-16 hours at 37°C. Colonies containing empty plasmids turned blue whereas those containing the recombinant construct remained white (Sambrook *et al.*, 1989).

2.2.1.16 Plasmid constructs generated

Following tables contain a description of all plasmid constructs engineered during the work of this thesis:

Table 1. Constructs designed for the two-hybrid system

Name of the construct	Insert (bp)	Insert (aa)	Cloning strategy	Comments (**)
pAS2-1-Tid ^{aa45-426}	806-1949	45-426	The SmaI fragment of the cDNA10D (*) was first cloned into pUC18. Introduction into the pAS2-1 vector was done using the EcoRI- and SalI sites of the polylinker.	It contains the DnaJ, G/F, Zn-Finger and part of the C-terminal domain.
pAS2-1-Tid ^{aa98-495}	962-2298	98-495	The BamHI fragment of the cDNA10D (*) was cloned into pAS2-1 with NcoI engineered adaptors (5'-CATGGCGAACA AGGAG-3'; 5'-GATCCTCCTTGTTTCGC-3')	It contains a partial DnaJ, complete G/F and Zn-Finger and a nearly complete C-terminal part.
pAS2-1-Tid ^{aa45-161}	806-1154	45-161	The SmaI-Xho fragment of the cDNA10D (*) was first cloned into pUC18. Recloning into the pAS2-1 vector was done using the EcoRI- and SalI sites of the polylinker.	It contains the DnaJ domain alone.
pAS2-1-Tid ^{aa45-212}	806-1309	45-212	The SmaI-HincII fragment of the cDNA10D (*) was first cloned into pUC18. Introduction into the pAS2-1 vector was done using the EcoRI- and SalI sites of the polylinker.	It contains the DnaJ and G/F domains.
pACT2-Tid ²⁰⁶⁻³¹⁸	1210-1624	206-318	The fragment corresponding to nt 1210 to 1624 of the gene was engineered to have NcoI and EcoRI sites by PCR amplification with the following primers: 5'-CATGCCATGGTGTGTAACCT CGTTCGACGAC-3'; 5'-GGAATTCTGACGAACACCTCCT TGC-3' and then cloned into pACT2.	It contains the Zn-finger domain.

pAS2-1-Tid ^{aa359-518}	1740-2539	359-518	The AccI (from the cDNA10D (*)) /PstI (from the pBluescript polylinker) fragment was cloned into pUC18. Introduction into the pAS2-1 vector was done using the EcoRI site of the polylinker and the imported PstI.	It contains the C-terminal part.
pAS2-1-Ptc ^{aa1143-1286}	4214-4559	1143-1286	The fragment corresponding to the nt 4124 to 4559 of the protein was cloned into pAS2-1 using the EcoRI sites from the pGAD10 polylinker.	It contains the C-terminal cytosolic part of the Ptc protein.
pAS2-1-hTid1 ^{aa66-480}	228-1653	66-480	The fragment corresponding to nt 228 to 1653 of the gene was amplified with the following primers: 5'-CAGGATCCGTGT CAGCCTTACAGGAA-3'(BamHI); 5'-TCCGTGGAGAGAG GTGTT-3'. The PCR product was digested with BamHI (from the primer) and EcoRV (nt1474) and cloned in the pAS2-1 vector <i>via</i> BamHI and a SalI site blunted with Klenow.	It contains all 4 protein domains.
pACT2-hPtc ^{aa1027-1296}	3551-4430	1027-1296	The fragment corresponding to nt 3551-4430 of the gene was amplified using 1µg of a Human Fetal Brain cDNA bank (see sections 2.2.1.9 and 2.1.1.11) with the following primers: 5'-GAGGATCCATGCCACGGCTTGAA-3' (BamHI); 5'-CGA GCTCTCCATAACTCCAACCA-3' (SacI). The PCR product was digested with BamHI and SacI and cloned into pAS2-1.	It contains the whole C-terminal cytosolic region.

(*) see section 2.1.6

(**) see section 1.4

Table 2. Constructs designed for the generation of recombinant proteins

Name of the construct	Insert (bp)	Insert (aa)	Cloning strategy	Observations
pGEX5X-3-GST-Tid ^{aa45-426}	806-1949	45-426	The construct was assembled by insertion of the SmaI fragment of the cDNA10D into the pGEX5X-3 vector.	It contains the DnaJ, G/F, Zn-Finger and part of the C-terminal domain
pQE30-His ₆ -Ptc ^{aa1143-1286}	4125-4559	1143-1286	The fragment corresponding to nt 4125 to 4559 of the gene was cloned into pQE30 using the BamHI and HindIII sites from the pGAD10 polylinker.	It contains the complete C-terminal cytosolic region of the protein.

2.2.2 Protein analysis

2.2.2.1 Protein extracts

Different extraction methods were performed depending on the approach and the type of organism used. Protein extracts for developmental Western blots were obtained by homogenization of the tissue in TKM buffer with a glass/teflon homogenizer. Denaturation of the proteins was performed by incubating the extracts at 95°C during 5 min in Laemmli buffer. Extraction of proteins for immunoprecipitation is described in section 2.2.6. Furthermore, protocols for protein extraction in bacteria and yeast are described later in this chapter (see sections 2.2.4 and 2.2.7.5 respectively).

2.2.2.2 Measurement of protein concentration

Protein concentration in extracts was measured using the BIO-RAD, protein assay and BSA standard curves, following the manufacturer's protocol (Bio-Rad).

2.2.2.3 SDS-PAGE (Polyacrylamide Gel Electrophoresis)

Electrophoresis of proteins was carried out in 8 to 12,5 % polyacrylamide gels under denaturing conditions using a discontinuous buffer system (Ausubel *et al.*, 2001). Protein migration through the gel was compared to an appropriate protein marker (Sigma/GibcoBRL). Electrophoresis was run at 20 mA/plate until the bromophenol blue reached the bottom of the plate.

2.2.2.4 Staining of SDS-polyacrylamide gels

Polypeptides separated by SDS-polyacrylamide gels were simultaneously fixed and stained with a concentrated methanol/acetic acid solution of Coomassie Brilliant Blue R250 for 20 minutes. Excess dye was then allowed to diffuse from the gel during a prolonged period of destaining, usually for 24 hours (Sambrook *et al.*, 1989).

2.2.2.5 Western Blotting

The transfer of proteins from SDS-polyacrylamide gels to solid supports (PVDF or Nitrocellulose membranes) was performed by electroblotting in a semidry system using

a Multiphor II Nova Blot transfer unit (Pharmacia Biotech). The gel was stacked horizontally on top of the membrane in a transfer sandwich of filter paper wetted in three different buffers (Ausubel *et al.*, 2001). To monitor the transfer efficiency reversible staining of blot transfer membranes was performed with Ponceau S. Immobilized proteins were then probed with specific antibodies. First, the membrane was immersed in blocking buffer to fill all protein binding sites. Next, it was placed in a solution containing an antibody directed against the antigen of interest (see section below for a list of all primary antibodies used and working dilutions). Then, the blot was washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (see section below). Proteins were revealed by chemiluminescence. Exact conditions are described in the Chemiluminescence protocol of Roche.

2.2.2.6 Antibodies used in Western Blot

The following table includes the antibodies used in Western blot, the dilution for a staining reaction and references. All primary antibodies were aliquoted and conserved at -20°C . Antibodies were diluted with 0.5% Blocking solution (Roche) including 0.1% sodium azide to prevent bacterial growth. Secondary antibodies were stored at 4°C and diluted in 0.5% Blocking solution.

Antibodies	Animal raised in	Dilutions used in μl	Origin
Anti-Tid ^{BIO}	Rabbit	1 in 500	I.Canamasas, see section 2.2.4.4
Anti-Ptc	Mouse	1 in 2000	P. Ingham (not published)
Anti-Ptc	Rabbit	1 in 3000 (0,3 $\mu\text{g/ml}$)	I.Canamasas, see section 2.2.4.1.2
Anti-Smo	Rat	1 in 100	(Denef <i>et al.</i> , 2000)
Anti-E-APC	Rabbit	1 in 10.000 (pre-adsorbed)	(Yu <i>et al.</i> , 1999)
Anti-DE-Cad	Mouse	1 in 4	(Oda <i>et al.</i> , 1994)
POD-conjugated anti-rabbit, mouse or rat	Goat	1 in 15.000	Jackson Immuno Research Lab.
Biotinylated anti-mouse	Goat	1 in 15.000	Vector Laboratories

2.2.2.7 Re-use of protein blots

Membrane stripping was performed incubating the used blot in a TBS/100mM 2-mercaptoethanol/2% SDS solution for 30 min at 50°C by gentle shaking. After washing the membrane twice in a copious volume of TBST the blot was blocked in 1% blocking reagent (Roche) for 1 h and probed with a new primary antibody.

2.2.2.8 Storage of protein blots

Immunoblots wetted in 1% Blocking solution were stored at –20°C wrapped in plastic. Alternatively, blots were air dried and rehydrated for 5 sec in 100% methanol before use.

2.2.3 Generation of recombinant proteins

2.2.3.1 Production and purification of 6xHis-tagged recombinant proteins

The QiaexpressTM System (Qiagen) was used to express, purify and detect 6xHis-tagged proteins. High-level expression of 6xHis-tagged biomolecules in M15[pREP4] bacteria is based on the T5 promoter and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the promoter. Expression of recombinant proteins is rapidly induced by the addition of IPTG, which binds to the *lac* repressor protein and inactivates it. Protein purification is based on the selectivity and affinity of nickel-nitrilotriacetic acid (Ni-NTA) sepharose for 6xHis-tagged biomolecules. Following are listed the procedures and conditions used for the production and purification of recombinant Ptc and Tid proteins.

2.2.3.1.1 Identification of clones expressing the His₆-Ptc^{aa 1143-1286} recombinant protein by colony blot

The colony blot procedure was used for the identification of clones expressing the His₆-Ptc^{aa 1143-1286} recombinant protein (see Table 2 for more information about the recombinant construct). This method allows the distinction between clones that express the recombinant protein from those that express the short peptide sequence encoded by pQE plasmids lacking an insert. Following the protocol of the QiaexpressTM System, a

replica of the master plate with the original transformants was made with nitrocellulose membrane. This membrane was transferred to a new plate containing IPTG in order to induce expression of the protein. Then, bacteria were alkaline lysed and the membrane used for immunodetection with the specific anti-6xHis antibody (Qiagen).

2.2.3.1.2 Expression and purification of His₆-Ptc^{aa 1143-1286} and His₆-Tid^{aa 98-495} recombinant proteins

Expression and purification of the recombinant proteins was performed according to the protocol of QiaexpressTM System (Qiagen). Exact conditions were established by a time-course analysis of expression and are described in the following table:

Recombinant protein	Concentration of IPTG used	Time of induction	Temperature of induction
His ₆ -Ptc ^{aa 1143-1286}	1 mM	4 hours	37°C
His ₆ -Tid ^{aa 98-495}	2 mM	4,5 hours	37°C

Proteins were extracted under native or denaturing conditions and purification of the recombinant proteins was done on Ni-NTA columns.

2.2.3.2 Production and purification of GST-tagged recombinant proteins

In order to perform a GST-pulldown assay the Glutathione S-transferase (GST) Gene Fusion System from Pharmacia was used to express and purify Tid fusion proteins in *DH5-α* cells. The pGEX plasmid used is designed for IPTG-inducible, high-level intracellular expression of genes fused to GST. Purification was done on Glutathione Sepharose 4B beads following manufacturer's instructions (Pharmacia).

2.2.3.2.1 Expression and purification of the GST-Tid^{aa45-426} recombinant protein

Expression and purification of the recombinant protein was performed according to the protocol of the GST Gene Fusion System (Pharmacia). In order to identify clones expressing the GST-tagged Tid protein (see Table 2 for more information about the recombinant construct) several small-liquid cultures (2ml) of transformed colonies were

grown to an A_{600} of 0.6-0.8 at 37°C and fusion protein induced with 1mM IPTG for 2 hours. After protein extraction and purification under native conditions, extracts were analyzed by SDS-PAGE and Coomassie staining.

2.2.4 Immunological methods

2.2.4.1 Antibody production

2.2.4.1.1 Production of a polyclonal anti-Ptc antibody

The generation of a new antibody directed against the Ptc protein was taken into account. The C-terminal cytosolic part of the protein was used as an antigen because its sequence showed the lowest homology degree to its human counterparts (see Fig.4). This is an important factor in order to minimize the cross-reactivity of an antibody. The pQE30-His₆-Ptc^{aa1143-1286} construct (see Table 2) corresponding to the C-terminal region of Ptc was introduced into *E.coli* M15[pREP4] cells and the recombinant protein purified under denaturing conditions as it has been described before (see section 2.2.3.1.2). Several milligrams of this fusion protein were sent to the firm Biotrend for immunisation of two rabbits and the serums and pre-immuneserums obtained tested against the Ptc recombinant protein *via* Western blot and ELISA assays (data not shown).

2.2.4.1.2 Production of a polyclonal anti-Tid antibody

The *E.coli* M15 [pREP4] stock was transformed with the recombinant construct pQE31-His₆-Tid^{aa 98-495} designed by Anette Debes during her PhD thesis (Debes, 1997) (see section 2.1.6). Several milligram of His₆-Tid^{aa 98-495} protein were purified as described in section 2.2.3.1.2 and sent to the firm Biotrend for immunisation of two rabbits. Serums and pre-immuneserums obtained were tested against this recombinant protein by Western blot and ELISA assays for specificity (data not shown).

PTCH1	STLLGVLMLAGSEFDFFIVRYFFAVLAILTILGVLNGLVLLPVLLSFFG	↓	1181
PTCH2	STLLGLLMLAGSHFDFIVRYFFAALTVLTLGLLHGLVLLPVLLSILG		1117
Ptc	TSGVAVFMLSTSPFEFVIRHFCWLLLVLCVGACNSLLVFPILLSMVG		1109
PTCH1	PEVSPANGLNRLPTPSPEPPPSVVRFAMPPGHTHSGSDSSDSEYSSQTT		1231
PTCH2	PEVIQMY-----KESPE-----ILSPP-----		1134
Ptc	AELVPLEHPDRISTPSPLPVRSSKRSGKSYVVQGS-----		1144
PTCH1	SGLSEELRHYEAQQGAGGPAHQVIVEATENPVFAHSTVVHPESRHHPPSN		1281
PTCH2	-----APQGG-----		1140
Ptc	-----RSSRGSCQKSHHHHHKDLNDP-----		1165
PTCH1	RQQPHLDGSLPPGRQGQPPRRDPPREGLWPPPYRPRRDAFEISTEGHS		1331
PTCH2	-----		
Ptc	-----SLTTITEEPQS		1176
PTCH1	GPSNRARWGRGARSHNPRNPASTAMGSSVPGYCQPITTVTASASVTVA		1381
PTCH2	-----LRWG-----ASSSLP---QSFARVTT--SMTVA		1164
Ptc	WKSNSSIQMPNDWTYQPREQRPASYAAPPAYHKAAAQHHQHQPPTT		1226
PTCH1	HPPPVPGPGRNPRGGLCPGYPETDHGLFEDPHVPFHVRCERRDSKVE		1431
PTCH2	HPPPLPGAYIHP-----APD-----EPPWSPAATSSGNLSS-----		1195
Ptc	PPPPFPTAYPPELQSIVVQPEVTVETTHSDSNTTKVTATANIKVELAMP		1276
PTCH1	LQDVECEERPRGSSSN		1447
PTCH2	-----RGPGPATG		1203
Ptc	-----RAVRSYNFTS-		1286

Figure 4. Sequence comparison between the C-terminal cytosolic domains of *Drosophila* Ptc and two human homologs. Amino acid sequence of the C-terminal cytosolic region of PTCH1 (Acc.N° XP_005574) compared to analogous regions of PTCH2 (Acc.N° Q9Y6C5) and the *Drosophila* Ptc protein (Acc.N° AAA28696). The start of the C-terminal region is marked with an arrow. Sequence alignments were done using ClustalW.

2.2.4.2 Isolation of Antibodies using an activated column

Isolation of antibodies from the serums and pre-immuneserums obtained (see sections 2.2.4.1.1 and 2.2.4.1.2) was done with a Protein A Sepharose CL-4B column (Pharmacia Biotech) according to the manufacturer's protocol. In order to give the IgGs optimal chances for interaction with the Protein A Sepharose 1 ml of centrifuged Tid serum was passed slowly through a 3 ml column (1-2ml/min). The loaded sepharose was washed with 10-20 column volumes of PBS buffer and elution of antibodies performed with 0.2M Glycin-HCl buffer at pH 2.2 on eppendorf caps containing 100µl of 1M Tris-HCl buffer at pH 9.0 in order to neutralise the antibody solution. Samples obtained were analysed for protein (see section 2.2.2.2) and IgG concentration (see section below).

2.2.4.3 ELISA

Indirect ELISA (Enzyme Linked Immunosorbent Assay) was the technique used for titration and quality proof of an antiserum or antibody solution. Microtiter plates were first coated overnight at 4°C with 1µg antigen/ml PBS and blocked before incubating the plates with serial dilutions of the serum containing specific antibodies for 2 hours at 37°C. After washing the excess of antibodies, secondary alkaline phosphatase antibody diluted 1:5000 in PBS was incubated for 2 hours at 37°C, unbound conjugate washed away and substrate solution (1 mg pNPP/ ml TBS pH 9.5) added to the plates. Hydrolysis of the substrate was monitored 60 min later with a microtiter plate reader using a 405-nm filter.

2.2.4.4 Biotinylation of antibodies

Anti-Tid antibody (see section 2.2.4.1.2) was directly labeled with biotin in order to avoid the detection on the blot of the IgGs used in immunoprecipitation (see section 3.1.3.2). One millilitre of serum (see section 2.2.4.1.2) was passed through a Protein A Sepharose CL-4B column to affinity purify the antibodies (see section 2.2.4.2). Then, 500 µg of this purified antibody were concentrated with a Vivaspin 2 column (Sartorius) and sent to the firm Biotrend for biotinylation. Labeled antibodies were tested for specificity against Tid recombinant protein in Western blot and ELISA assays (data not shown).

2.2.5 GST-pulldown assay

The glutathione-S-transferase (GST) pulldown assay is a simple method to detect protein-protein interactions *in vitro*. In the following is described an overview of the methods required for this assay.

2.2.5.1 Midi-scale preparation of GST-fusion proteins

100 ml of induced bacterial culture including the pGEX5X-3-GST-Tid^{aa45-426} expression construct were lysed in 4 ml lysis buffer by sonication (4x30 sec). Debris was pelleted by centrifugation at 12.000 rpm for 60 minutes at 4°C and the crude extract obtained aliquoted and stored at -20°C.

2.2.5.2 Preparation of GST-beads

In order to ensure optimal binding activity in the assay beads loaded with GST-Tid^{1aa45-426} and GST alone were freshly prepared. 1000µl of GST- Tid^{1aa45-426} crude extract or 50µl of GST crude extract alone (volume increased up to 500µl with GST-buffer) (see section 2.2.5.1) were added to 100µl of equilibrated glutathione-S-sepharose slurry and incubated overnight at 4°C on a rocking table. Then, the beads were washed 3 times with 800µl GST-buffer and resuspended in 500µl of the same buffer. In order to determine protein concentration 50µl of this slurry were used for a Bio-Rad protein assay (see section 2.2.2.2)

2.2.5.3 *In vitro* protein-protein interaction study

For the *in vitro* GST-pulldown assay 5mg of GST- Tid^{1aa45-426} and equimolar amounts of GST-loaded beads were incubated with 250 ng of the native purified His₆-Ptc¹¹⁴³⁻¹²⁸⁶ protein (see section 2.2.4.1.2) in a final volume of 100µl of GST-buffer for 1 hour at 4°C. After 3 washes with 200µl of GST-buffer, proteins were eluted by boiling in 25µl of SDS-PAGE sample buffer and separated on a 12,5% polyacrylamide gel followed by Western blot analysis with anti-Ptc and anti-Tid antibodies (see section 2.2.2.6)

2.2.6 Immunoprecipitation

Immunoprecipitation of protein complexes was performed according to the protocol of Roche's Cellular Labeling and Immunoprecipitation Kit. Following are listed the exact conditions used in the assay. 10 mg of tissue were homogenized with a glass/teflon homogenizer in 0.5 ml of lysis buffer and cell debris pelleted by centrifugation. After a pre-clearing step of 2 hours with Protein A Sepharose, primary antibody (see table below) was added to the supernatant and incubated for 1 hour at 4°C. Immunoprecipitation was done overnight by addition of Protein A Sepharose and loaded beads washed as described in the manufacturer's protocol. Protein complexes were eluted by incubation in 50µl SDS-PAGE sample buffer at 95°C for 3 min and stored at -80°C.

Antibodies	Animal raised in	Quantities used	Origin
Anti-Tid	Rabbit	2,2µg for the developmental assay 4,5µg for co-immunoprecipitation studies	(Kurzik-Dumke <i>et al.</i> , 1998)
Anti-E-APC	Rabbit	1µl anti-serum (pre-adsorbed)	(Yu and Bienz, 1999)

Biotin-labeling of total protein extracts:

Labeling of total protein extracts was required for the developmental immunoprecipitation assay (see section 3.1.1). Biotin-7-NHS was used to label protein extracts prepared as described above, following the manufacturer's protocol (Roche).

2.2.7 The yeast two hybrid system

The two-hybrid system (Clontech) is a sensitive *in vivo* assay for detecting specific protein-protein interactions in yeast. This method was used i) to screen a library for a gene encoding a novel protein interacting with Tid, ii) to detect the domains involved in this interaction or iii) to test the interaction between two known proteins. The two-hybrid system exploits the two-domain nature of many site-specific eukaryotic transcription factors to detect interactions between two different hybrid proteins (Fields and Song, 1989). In this method two fusion proteins must be generated. One is a fusion between the DNA-binding domain (DNA-BD) of a transcription factor and a test protein "X" (the bait). The other is a fusion between the activation domain (AD) of a transcription factor and a test protein "Y" (the prey). Plasmids encoding these fusion proteins are introduced together into a yeast strain that contains two reporter genes (*HIS3* and *lacZ*) with upstream binding sites for the DNA-binding domain present in the first hybrid. The expression of either the hybrid of the DNA-binding domain with protein X or the hybrid of the activation domain with protein Y fails to activate transcription of both reporter genes. However, if protein X and protein Y interact, the transcriptional activator is anchored to the binding site and leads to the expression of the reporter genes (Bartel and Fields, 1995) (see Figure 5).

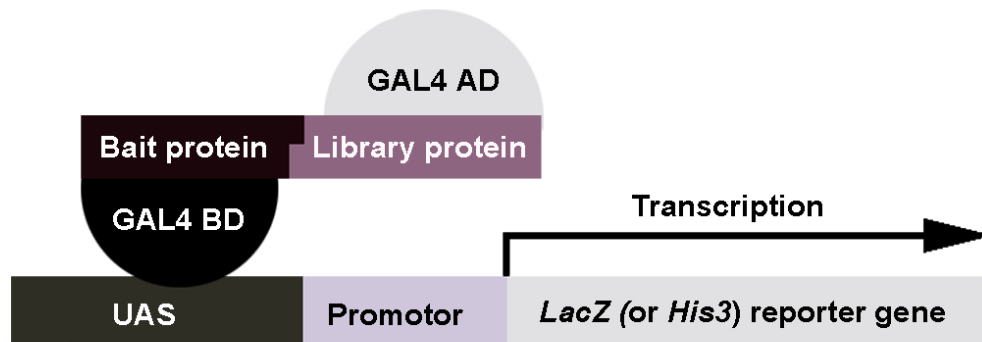


Figure 5. Schematic diagram of the GAL4-based yeast two-hybrid system. Interaction between the bait and library proteins in the yeast activates transcription of the *lacZ* and *HIS3* reporter genes. DNA-binding domain (BD) corresponds to the aa 1-147 of the yeast GAL4 protein, which binds to the GAL1-UAS upstream of the reporter gene. The activation domain (AD) corresponds to the aa 768-881 of the GAL4 protein and has transcriptional activation function.

In the following is described an overview of the methods used in order to screen a expression library for proteins that interact with Tid.

2.2.7.1 Yeast strain phenotype testing

The yeast strain *Y190* was selected for use in the library screening because it presents a high sensitive *lacZ*-reporter expression. Before using this strain in a transformation experiment it is important to verify its phenotype. This was done according to the Clontech's Matchmaker GAL4 Two-Hybrid User Manual.

2.2.7.2 Amplification of an AD-fusion expression library

A premade MATCHMAKER cDNA library from *Drosophila melanogaster* embryo fused to the GAL4-activating domain (AD) vector (Clontech) was amplified to produce plasmid DNA to screen the library in yeast. Prior to that, the titer of the plasmid library was determined according to Clontech's Matchmaker Library Protocol. To obtain at least 2-3 times the number of independent clones in the library, 200.000 colonies were plated on each of the 20 LB/amp plates (25x25cm) and incubated overnight at 37°C. The next day, colonies were scraped into 2 l of LB/amp broth and incubated for 3h at 37°C on a shaker. Highly purified plasmid DNA was extracted using the Mega-DNA Purification Kit from Qiagen.

2.2.7.3 Construction of the bait

To identify new Tid interacting proteins it was designed a bait construct containing the DnaJ, G/F and Zn Finger domains and part of the C-terminal region of the Tid protein fused to the GAL4 DNA-binding domain (pAS2-1-Tid^{aa45-426}) (see table 1 for details)

2.2.7.4 Transformation of the bait into yeast cells

The above hybrid construct was transformed into the host strain *Y190* using the small scale yeast transformation protocol (Clontech), the quick and easy transformation protocol (Gietz *et al.*, 1997) or the frozen yeast transformation protocol (Dohmen *et al.*, 1991) (Schiestl *et al.*, 1993). This last method is of particular interest because it uses frozen competent cells instead of freshly prepared cells for transformation.

2.2.7.5 Test of the bait

Before using the bait in a two-hybrid assay it should be tested for transcriptional activation and for expression of the hybrid protein. pAS2-1-Tid^{aa45-426} yeast transformants were assayed for *lacZ* reporter gene expression using the colony-lift filter assay (see section 2.2.7.7) and grown on the selective SD His⁻ Trp⁻ medium to test for a possible transcripitonal activation. Expression and stability of the hybrid protein in the reporter yeast strain was confirmed by Western blot analysis of yeast protein extracts using the anti-GAL4 DNA-BD monoclonal antibody as the primary probe (Clontech) according to the Clontech's Matchmaker Monoclonal Antibodies User Manual. Yeast protein extracts were prepared following the Urea/SDS Method described in the Clontech's Yeast Protocols Handbook.

2.2.7.6 Screening of the AD-fusion expression library

To screen the library the bait construct and the AD-library plasmids were introduced into the yeast reporter strain sequentially. At first the DNA-BD-bait plasmid was introduced using a small-scale transformation (see section 2.2.7.4). Then, 500µg of the AD-fusion library were introduced into yeast containing the bait plasmid using a large-scale transformation protocol. Two methods for large-scale transformation have been used during the work of this thesis. The method of Agatep *et al.* (1998) has been proved to be three times more effective than the method described in the Clontech's Matchmaker

GAL4 Two-Hybrid User Manual. Control transformations were run in parallel according to manufacturer's instructions. To select for positive interacting clones it was used a growth selection medium lacking His to monitor *HIS3* reporter gene expression and the colony-lift filter assay (see section 2.2.7.7) to detect β -galactosidase activity.

2.2.7.7 β -Galactosidase assay

The β -galactosidase test used to screen the cotransformants that survived the *HIS3* growth selection in the library screening was the colony-lift filter assay (Chevray and Nathans, 1992). This method uses X-gal as a substrate and requires at least one freeze/thaw cycle in liquid nitrogen to lyse the yeast cell walls (Clontech's Yeast Protocols Handbook).

2.2.7.8 Analysis and verification of positive clones

Positive clones for *HIS3* and *lacZ* activity were restreaked and retested again because some of the initial library cotransformants could have contained more than one AD-library plasmid. The next step was the isolation of plasmid DNA from positive yeast clones. Plasmid DNA isolated from yeast is often contaminated by yeast genomic DNA and is therefore not suitable for restriction analysis or sequencing. For that reason it was first isolated from yeast, transformed into *E.coli* and then isolated with any traditional method (see section 2.2.1.1). To isolate plasmid DNA from yeast the rapid procedure described in Clontech's User Manual was modified to obtain suitable plasmid for electroporation. The pellet of a 5 ml saturated yeast culture was resuspended in 0.3ml yeast lysis solution. To disrupt the cells 0.3 ml of phenol/chloroform/isoamyl alcohol and 200 μ l volume of acid-washed glass beads (Sigma) were added to the suspension and this mixture vortexed for 2 min at room temperature. After centrifugation at 12.000 rpm for 5 min the aqueous phase was again purified from particles with phenol/chloroform and chloroform steps. DNA was then precipitated (see section 2.2.1.6), washed thoroughly with 70% ethanol and resuspended in 20 μ l of H₂O_{dd}. 1 μ l of this solution was taken for electroporation (see section 2.2.1.13). The isolated library clone was then re-transformed in yeast with the original bait construct or with control plasmids in order to discard false positives for the two-hybrid assay. If the clone showed again a positive result for *HIS3* and *lacZ* activity, the cDNA inserts were sequenced and the interaction

verified using an independent biochemical method such as the GST-pulldown assay (see section 2.2.5) or co-immunoprecipitation (see section 2.2.6).

2.2.7.9 Testing for protein-protein interaction

The two-hybrid system has also been used to define the protein domains that mediate an interaction and to establish the binding between two known proteins. Basically, the methods required for this study are the same as for the library screening but the yeast strain used was *Y187*. See Table 1 for descriptions and cloning strategies of the designed constructs used in these binding assays.

2.2.8 Working with flies

2.2.8.1 Fly stock maintenance

Fly stocks were maintained in vials containing standard food media (see section 2.1.2). Stocks kept at 25°C were transferred to fresh vials every two weeks and the ones kept at 18°C were transferred every 4-5 weeks.

2.2.8.2 Embryo collection for antibody staining

To collect embryos, flies were placed in a cage with a changeable bottom. This bottom consisted of plates containing 2 % agar in apple juice. Depending on the stage required collection times were adjusted.

2.2.8.3 Larvae collection for antibody staining

Third instar wandering larvae were collected from the wall of the vials. To become a homogeneous population 3-6 hour egg-layings were performed.

2.2.8.4 Antibodies used in immunostainings

The following table includes the antibodies used in immunostainings of embryos and larval tissue, its dilution used for a staining reaction and references. All primary antibodies were aliquoted and conserved at -20°C. Dilutions were performed with 10% calf serum in PBT including 0.1% sodium azide to prevent bacterial growth. Secondary antibodies were maintained at 4°C and diluted in PBT only.

Antibodies	Animal raised in	Dilutions used in μ l	Origin
Anti-Tid	Rabbit	1 in 10 for E 1 in 50 for L3	(Kurzik-Dumke <i>et al.</i> , 1998)
Anti-Ptc	Mouse	1 in 75	(Capdevila <i>et al.</i> , 1994)
Anti-Ci	Rat	1 in 20	(Motzny and Holmgren, 1995)
Anti-Wg	Mouse	1 in 50	Hybridoma Bank
Anti-E-APC	Rabbit	1 in 10.000 (pre-adsorbed)	(Yu and Bienz, 1999)
Anti-En	Mouse	1 in 20	Hybridoma Bank
POD conjugated anti-rabbit	Goat	1 in 250	Jackson Immuno Research Lab.
Biotinylated anti-rabbit, anti-mouse or anti-rat	Goat	1 in 250	Jackson Immuno Research Lab.
Cy ³ -conjugated anti-rabbit	Goat	1 in 250	Jackson Immuno Research Lab.

2.2.8.5 Antibody staining of embryos

Fixation:

Embryos were collected in a basket with a sieve, dechorionated for 2 min in 15% bleach (Sodium Hypochloride) and rinsed thoroughly in running tap water. After fixation for 20 min in an eppendorf tube containing heptane and 10% formaldehyde in PBS in a ratio of 1:1, the lower phase was removed and 1 ml methanol was added for devitelinisation with vigorous shaking or vortexing for 30-60 sec. Then, embryos were subjected to 2-3 rinses with methanol. At this point embryos were either ready to use or could be stored at -20°C for future work.

Fluorescent antibody staining:

Embryos fixed as described above were rinsed several times with PBT and then blocked for a minimum of 1 hour in 10% calf serum in PBT. Primary antibody of an appropriate dilution (see section 2.2.8.4) was added and incubated overnight at 4°C . Excess of primary antibody was removed by washing 3 times with PBT for 20 min each. Cy3-coupled secondary antibody of an appropriate dilution was added (see section 2.2.8.4) and incubated for 2 hours at room temperature. To remove the excess of secondary

antibody embryos were rinsed 3 times with PBT for 20 min each, following 2 rinses with PBS of 15 min each. This is a critical step, as triton interferes with fluorescence. Finally, embryos were stored in dark at -20°C in 70% glycerol in PBS and mounted in Vectashield fluorescent mounting media (Vector Laboratories) for observation under the fluorescent microscope.

2.2.8.6 Antibody staining of larval tissue

Third instar wandering larvae were picked washed and dissected in cold PBS. Isolated imaginal discs were fixed in PBS containing 4% of freshly prepared paraformaldehyde for 10 minutes. The tissue was washed twice in PBT for 5 min each, following two incubations in PBT during 30 min each for blocking. Primary antibody diluted as described in section 2.2.8.4 was added to the discs and incubated overnight at 4°C . To get rid of the excess primary antibody used a short wash with PBTs following two rinses of PBTs of 30 min each at room temperature were performed. Biotin- or POD-coupled secondary antibody (see section 2.2.8.4) was added to the larval tissue and incubated for 2 hours at room temperature. At this stage, imaginal discs were treated differently depending on the type of conjugate being used:

POD-coupled secondary antibody (for Anti-Tid):

Imaginal discs incubated for two hours with POD-coupled secondary antibody (see above) were rinsed once for 20 minutes with PBTs and again 20 minutes with PBTw. DAB solution with freshly added hydrogen peroxide to a final concentration of 0.1% was added to the larval tissue. The developing reaction was allowed to take place for 1-3 minutes depending on the antibody used and stopped with 2-3 times rinsing with PBTw and 1-2 times rinsing with PBS. Stained imaginal discs were stored in 70% glycerol in PBS.

Biotin-coupled secondary antibody (for Anti-Ptc, Anti-Ci, Anti-Wg, Anti-En)

Biotin-coupled secondary antibody in combination with the ABC-Elite Kit (Vectastain) was used to amplify the signal. This kit contains Avidin DH and biotinylated horseradish peroxidase reagents, which have been specially prepared to form ideal complexes in order to increase the sensitivity of the detection assay. Imaginal discs incubated for 2 hours with biotin-coupled secondary antibody (see above) were rinsed once for 20 minutes with PBTs and again 20 minutes with PBTw. Simultaneously, the amplification

complex from the ABC-Elite kit was prepared by adding 4 µl each of solution A and B to 300µl PBTw. After both washes, this mixture was added to the larval tissue and incubated at room temperature for 1 hour. Then, imaginal discs were rinsed off twice with PBTw for 15 minutes and a final wash with PBS for 15 minutes prior to DAB-staining (section above).

2.2.8.7 Analysis of stained tissue and documentation

The analysis of embryos and wing discs stained with DAB or Alkaline Phosphatase was carried out on an Axioplan microscope using Nomarski optics. Tissue labelled with fluorescent dyes or GFP was analysed with the same microscope using a fluorescent filter or with a Leica TCS confocal microscope. Images were digitally recorded with a CCD video camera or conventional photography at 10 x 1,6 x 10 magnification.

2.2.8.8 Phenotype of the *tid*¹ allele

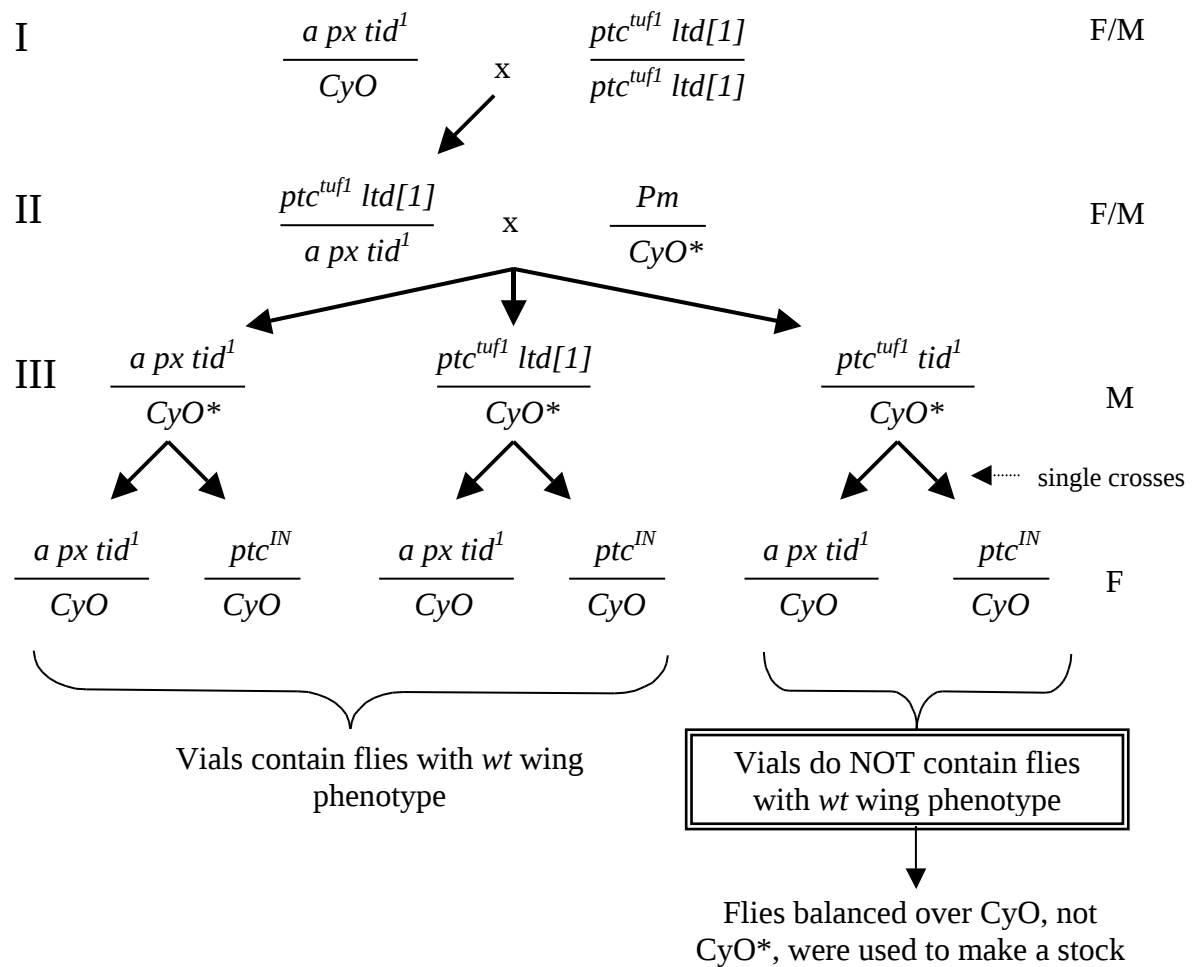
The *tid*¹ allele cause in homozygotes malignant growth of cells of the imaginal discs and the death of the mutant larvae at the time of puparium formation (Gateff, 1978; Gateff and Mechler, 1989; Kurzik-Dumke *et al.*, 1992). Third instar, homozygous *l(2)tid* larvae of *tid*¹ can readily be recognized by their larger size and transparent bodies (Kurzik-Dumke *et al.*, 1992). They show a delay in puparium formation of up to 10 days when compared to heterozygous or *wild-type* larvae. Anatomical investigations of these larvae revealed enlarged and morphologically abnormal imaginal discs (Kurzik-Dumke *et al.*, 1992).

2.2.8.9 Phenotype of the *ptc*^{*tuf-1*} allele

Homozygotes for *ptc*^{*tuf-1*} display a variable wing phenotype, ranging from *wild-type* to loss of costal bristles and moderate duplication of the base of vein 1 and associated triple row bristles (Phillips *et al.*, 1990). Flies have small tufts of hair between between the eye and antenna and show basal twinning of the anterior half of the wing (Sturtevant, 1948). Heterozygotes for *ptc*^{*tuf-1*}/*ptc*⁹ and *ptc*^{*tuf-1*}/*ptc*¹⁶ display severe outgrowth of the anterior compartment of the wing disc, loss of costal structures, duplications or plexations of veins 1 and 2, increase of the distance between veins 3 and 4 and increase in the number of scutellar bristles (Capdevila *et al.*, 1994).

2.2.8.10 Generation of double mutant stocks carrying the ptc^{tuf-1} and tid^1 alleles

Following is a description of the strategy used for the design of stocks carrying mutations for the viable ptc^{tuf-1} (Capdevila *et al.*, 1994) (Phillips *et al.*, 1990) and the tid^1 (Kurzik-Dumke *et al.*, 1992) alleles (see above).

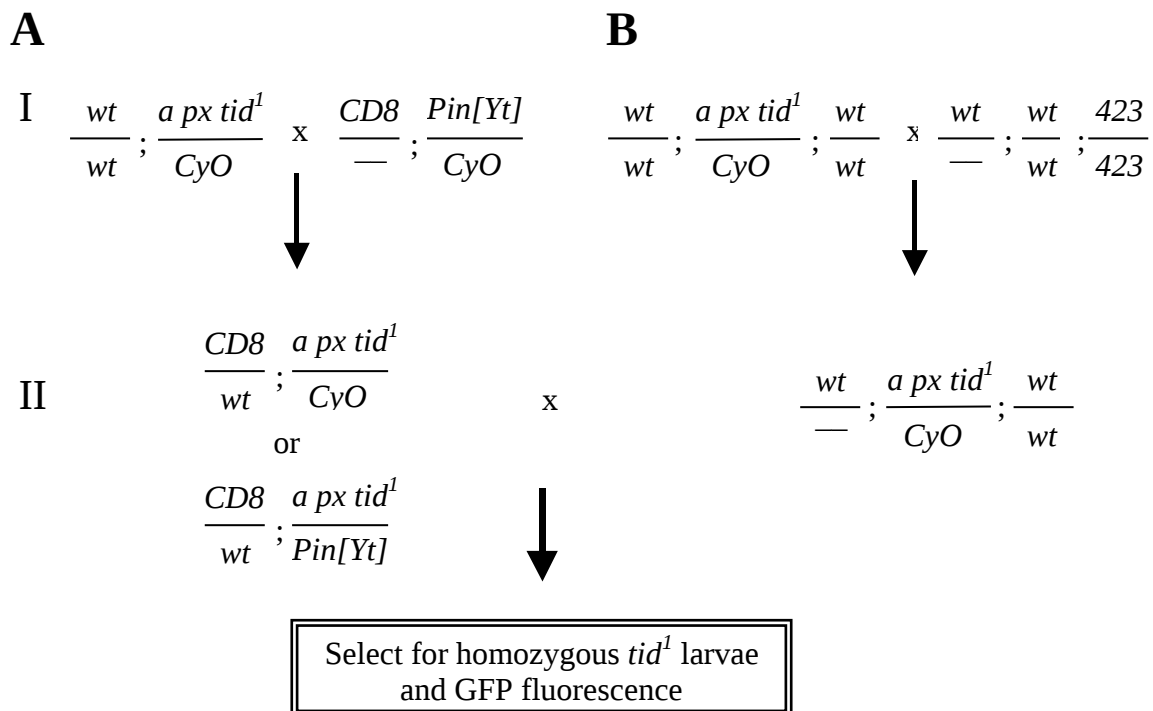


To generate fly stocks carrying mutations for the $l(2)tid$ and ptc genes, both located on the second chromosome (see above), female flies $a \text{ px } tid^1 / CyO$ were crossed to males homozygous for $ptc^{tuf1} ltd[1]$ (I). Since recombination occurs just in female flies the offspring of this cross was selected for female animals lacking a curly wing phenotype ($ptc^{tuf1} ltd[1] / a \text{ px } tid^1$). Then, these flies were crossed to a balancer for the second chromosome (Pm / CyO^*) in order to conserve the originated recombinant chromosomes (II). Descendants of this cross (III) were selected for the presence of white eyes (this phenotype is due to a genetic marker of the CyO^* balancer). To identify flies carrying

the recombined chromosome, each selected descendant male was first crossed with two females mutant for the *l(2)tid* (*a px tid¹/CyO*) allele and then with two females carrying another *ptc* (*ptc^{IN} cn bw sp/CyO*) allele to give another genetic background to the animals. This is an important step in the generation of isogenic stocks in order to avoid the influence of other mutations. Heterozygous *ptc^{IN}/ptc^{tuf-1}* flies also present the spread wings phenotype from *ptc^{tuf1}* homozygotes (Capdevila *et al.*, 1994) (see section 2.2.8.9). The identification of vials with flies containing the *ptc^{tuf1}tid¹* recombined chromosome was done selecting for the absence of animals with a wild-type wing phenotype (all flies had curly or spread wings). The recombinant stock *ptc^{tuf1}tid¹* was generated with flies from these vials balanced over *CyO* (flies over *CyO** carried the single mutant allele introduced in the last cross). (M: males; F: females).

2.2.8.11 Generation of fly stocks used for the characterisation of the tumorous wing imaginal disc

Following is a description of the strategy used for the construction of fly stocks carrying the *tid¹* allele (Kurzik-Dumke *et al.*, 1992) (see section 2.2.8.8), a UAS-mCD8::GFP construct and one of the three different GAL4 enhancer trap lines (see section 2.1.8) as an example.



To generate fly stocks homozygous for the *tid*¹ allele (located on the second chromosome) (Kurzik-Dumke *et al.*, 1992) carrying a UAS-mCD8::GFP construct (located on the first chromosome) (see section 2.1.8) and the GAL4 enhancer trap line MZ 0423 (located on the third chromosome) (see section 2.1.8) two different crosses were set up. First, *a px tid*¹/CyO female flies were crossed with *UAS-mCD8::GFP; Pin[Yt]/CyO* male flies to generate stocks carrying a combination of the first and second chromosome containing the above construct and the mutant *l(2)tid* allele (A,I). Since animals homozygous for *CyO/CyO* die at the end of embryonic life or shortly after hatching from the egg (Kidwell, 1972) all female flies generated in this second (II) generation were suitable for the next cross. Second, female flies carrying the *a px tid*¹ chromosome balanced over *CyO* were crossed with male flies homozygous for the GAL4-MZ 0423 enhancer trap line (B,I) and male descendants selected according to the absence of curly wing phenotype (B,II). Finally, *UAS-mCD8::GFP; a px tid*¹/*Pin[Yt]* or *UAS-mCD8::GFP; a px tid*¹/*CyO* female flies were crossed with *a px tid*¹; *GAL4-0423* male animals. Larvae carrying a combination of the first, second and third chromosome were selected for the presence of the homozygous *tid*¹ larval phenotype (see section 2.2.8.8) and GFP-fluorescence. Generation of the same stocks carrying the GAL4-MZ 0853 or GAL4-MZ 1229/1 enhancer trap lines instead of the GAL4-0423 line was done analogous to the procedure described above. *CD8: UAS-mCD8::GFP; 423: GAL4-MZ 0423*.

3. RESULTS

3.1 Looking for molecular partners of Tid

The main aim of this work has been the identification of molecular partners of the proteins encoded by the *l(2)tid* gene in order to functionally characterize them. Inside cells protein-protein interactions are instrumental in enzymatic actions upon protein substrates and in the fleeting and lasting protein assemblies that govern signal transduction, cell division, DNA replication and transcription initiation (Blackwood and Eisenman, 1995). The *l(2)tid* gene has been identified as a tumor suppressor in *Drosophila melanogaster*. It causes in homozygotes malignant growth of cells of the imaginal discs and the death of the mutant larvae at the time of puparium formation (Kurzik-Dumke *et al.*, 1995). Search for sequence homology of the putative Tid56 precursor protein revealed a significant degree of conservation to members of the chaperone DnaJ family of proteins (Kurzik-Dumke *et al.*, 1995). Members of this family are shown to be involved in protein folding, protein translocation, signal transduction, renaturation of misfolded proteins and proteolysis (Georgopoulos and Welch, 1993; Pfanner *et al.*, 1994; Cyr *et al.*, 1994; Gething, 1997). To identify the proteins encoded by the *l(2)tid* gene three polyclonal rabbit antibodies directed against different parts of the putative Tid56 protein were generated and used for staining of developmental Western blots (Debes, 1997; Kurzik-Dumke *et al.*, 1998). This analysis resulted in the identification of three specific proteins: Tid50, Tid47 and Tid40 (50, 47 and 40 kDa in size respectively) (see Fig.6, panel A) (Debes, 1997; Kurzik-Dumke *et al.*, 1998). Interestingly, differential centrifugation and sucrose gradients studies located Tid50 and Tid40 in the mitochondria and Tid47 in the cytosol (see Fig.6, panel B) (Debes, 1997; Kurzik-Dumke *et al.*, 1998). Further immunostainings on embryos revealed ubiquitous expression of Tid during the whole of the embryonic life. In larvae, strong expression was visible in all imaginal discs and other tissues such as the ring gland and the fat body, whereas no staining was detected in the salivary glands and in larval brain (Kurzik-Dumke *et al.*, 1998).

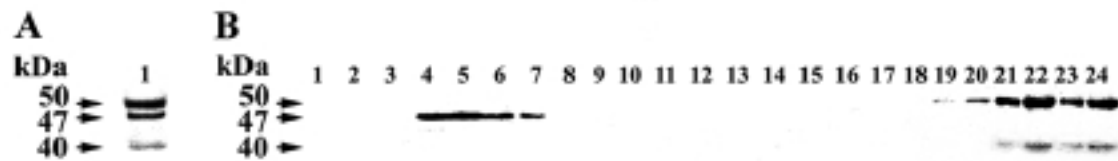


Figure 6. Subcellular localization of the Tid proteins as determined by immunoblot analysis. Detection and subcellular localization of the Tid50, Tid47 and Tid40 proteins as revealed by immunoblot analysis of Schneider cells crude homogenates (A) or subcellular fractions separated on continuous sucrose gradient (12-44%) (B). Tid50 and Tid40 are detected in mitochondrial fractions (B, column 19-24) and Tid47 in cytosolic fractions (B, column 4-7). Blots were stained with the anti-Tid antibody (see section 2.2.6). Extracted from Debes, 1997.

3.1.1 Developmental immunoprecipitation assay

The first goal of this study was to identify if Tid interacts with other proteins during development and if so, the characterization of these interacting partners in terms of their molecular weight and distribution pattern. To achieve this, immunoprecipitation techniques were applied. Immunoprecipitation of native proteins has proven to be a powerful tool and widely used approach in addressing questions about the nature of a protein and/or protein-protein interactions under various biological conditions (Lukas and Bartek, 1998). Samples from all fly-developmental stages were taken, lysed, biotinylated and the resulting extract immunoprecipitated with anti-Tid antibody (see section 2.2.6). In Figure 7 can be seen a developmental Western blot of the biotinylated Tid-immunoprecipitates. This analysis shows many Tid specific binding partners during development and reveals different sets of proteins for each developmental stage. Interestingly, the number of binding partners during development is higher at embryonic, third larval and pupal stages (lanes E,a; L3,a and P,a respectively). Furthermore, the observation that some of the protein bands detected on the blot (e.g. 150, 119, 95 kDa) are present in several developmental stages may implicate a ubiquitous function for Tid. In this figure potential bands corresponding to the Tid50, Tid47 and Tid40 proteins are labeled in color. Further studies to specifically identify Tid in immunoprecipitates will be described later in this work (see section 3.1.3.2).

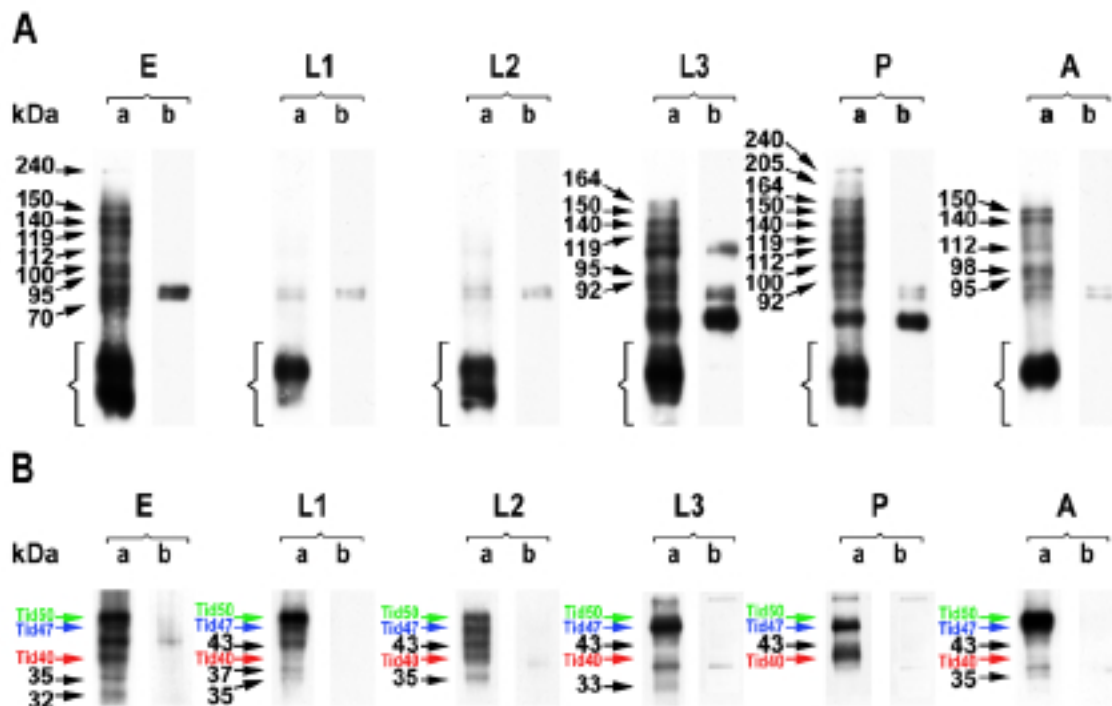


Figure 7. *In vivo* interaction study of the Tid proteins during development. *In vivo* interactions of the Tid proteins have been studied by co-immunoprecipitation from biotinylated protein extracts of all developmental stages of *Drosophila melanogaster*. Proteins immunoprecipitated with anti-Tid antibody (see section 2.2.6) were separated by SDS-PAGE and visualized by chemiluminescence after Western blot analysis. For controls, extracts were incubated with pre-immuneserum. (A) Western blot of anti-Tid and pre-immuneserum immunoprecipitates of all developmental stages probed with Streptavidin/POD. Lanes were loaded with 12% of anti-Tid immunoprecipitated material (see section 2.2.2.10) and proteins resolved on an 8% SDS-PAGE. (B) Western blot of anti-Tid and pre-immuneserum immunoprecipitates of all developmental stages probed with Streptavidin/POD. Lanes were loaded with 10% of anti-Tid immunoprecipitated material (see section 2.2.2.10) and proteins resolved on a 10% SDS-PAGE. Lanes *a* and *b* show anti-Tid and pre-immuneserum immunoprecipitates respectively. E: embryo; L1: first larval instar; L2: second larval instar; L3: third larval instar; P: pupae; A: adult. Potential bands corresponding to Tid50, Tid47 and Tid40 proteins are labeled in color. This blot reveals specific sets of Tid binding proteins during development.

3.1.2 Identification of new Tid molecular partners via the yeast two-hybrid system

Immunoprecipitation studies have shown the presence of specific binding partners for the Tid proteins in the fly (see above). This result has formed the basis for further investigations in order to characterize these interactors. The two-hybrid system, a yeast-based genetic assay for detecting protein-protein interactions *in vivo* (Bartel and Fields, 1995), has been used for this purpose.

3.1.2.1 Identification of Patched as a Tid interacting protein in a two-hybrid library screening

To identify new Tid interacting proteins in a yeast two-hybrid library screening Tid was used as a “bait” – a fusion of the gene and the sequences encoding the GAL4 DNA-binding domain in pAS2-1. An expression vector encoding the amino acids 45 to 426 of Tid (pAS2-1-Tid^{aa45-426}, see Table 1) including the DnaJ, G/F and Zn Finger domains and part of the C-terminal region fused to the GAL4-binding domain was designed in *E.coli*, sequenced and introduced into the yeast strain *Y190* (see section 2.2.7.4). Expression and stability of the hybrid protein in the reporter strain was assayed by Western blot analysis using the anti-GAL4 DNA binding domain monoclonal antibody (Clontech) (Figure 8) (see section 2.2.7.5). This assay is recommended before using a recombinant construct in a two-hybrid test. The left lane in Figure 8 shows a strong band of ~22 kDa corresponding to the GAL4-DNA binding domain protein alone. The expression of this protein was used as a control of the system. Furthermore, a band of ~71 kDa corresponding to the GAL4-DNA binding domain/Tid^{aa45-426} hybrid protein to be used in the screening can be detected on the right lane. Yeast *Y190* cells transformed with this pAS2-1-Tid^{aa45-426} construct were used to screen 500mg of an oligo(dT)-primed MATCHMAKER cDNA Library from *Drosophila melanogaster* embryo constructed in the GAL4-activating domain vector (see section 2.2.7.6). Transformants containing both plasmids were plated on Leu⁻ Trp⁻ His⁻ medium and screened by the filter X-Gal assay (Chevray and Nathans, 1992) to monitor the activity of the *HIS3* and *lacZ* reporter genes (see section 2.2.7.6). From a total of approximately 15 million transformants carrying

both the bait and the prey, 30 clones were identified as potential positives for interaction with Tid. Plasmid DNA from these clones was extracted and re-transformed back in yeast with the original bait construct or with a control plasmid in order to discard false positives for the two-hybrid assay (see section 2.2.7.8). Figure 9 shows the only clone that had again a positive result for *HIS3* and *lacZ* activity. The cDNA insert of this clone was sequenced and aligned using the BLAST algorithm (Altschul *et al.*, 1997). This analysis showed homology of this fragment with the C-terminal cytosolic part of the membrane receptor Patched (aa 1143-1286) (see Fig. 10).

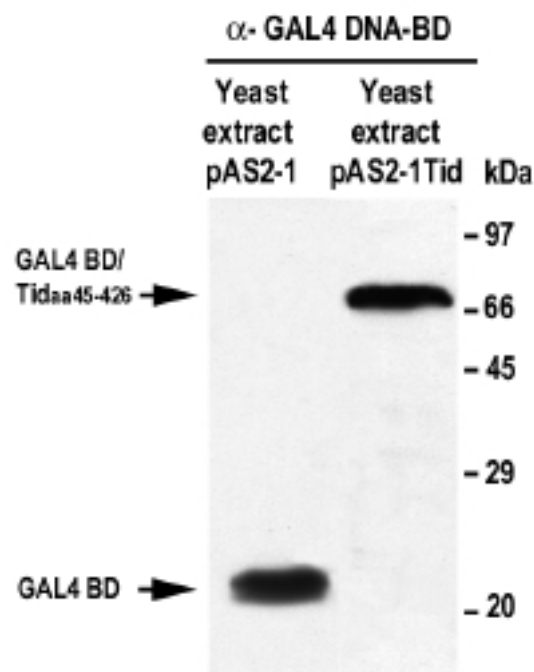


Figure 8. Expression test of the pAS2-1-Tid^{aa45-426} construct used in the library screening. Expression of the GAL4-binding domain (BD)/Tid^{aa45-426} hybrid protein was confirmed by Western blot analysis of protein extracts using the anti-GAL4-DNA BD antibody (Clontech). The yeast strain Y190 was transformed with the pAS2-1 Tid^{aa45-426} construct or the pAS2-1 vector encoding the GAL4-BD protein as a control. Transformants were selected on the appropriate SD medium and soluble protein extracts prepared using the Urea/SDS Method (see section 2.2.5.5). Protein samples equivalent to ~1.5 OD₆₀₀ units of cells were separated on a 12% polyacrylamide/SDS gel, immunoblotted with 0.5 µg/ml GAL4-DNA BD monoclonal antibody and revealed by chemiluminescence. As shown on the right lane, one protein band of ~71 kDa is detectable in extracts of yeast clones carrying the pAS2-1-Tid^{aa45-426} construct.

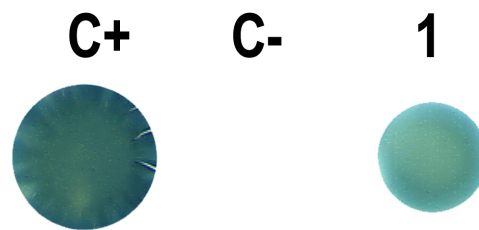


Figure 9. Interaction among the GAL4-DNA binding domain/Tid^{aa45-426} hybrid protein and the isolated library clone. Plasmids that carried chimeric gene fusions to express the GAL4-DNA binding domain fused to Tid^{aa45-426} and the activation domain of GAL4 fused to an unknown library clone were used as bait and prey respectively. Colonies of each doubly transformed clone were streaked onto agar plates deficient for Histidine and β -galactosidase activity monitored by the filter assay to assess for transcriptional activation (see section 2.2.7.7). Reporter gene activation indicates an interaction between both proteins. (1) Yeast clones containing the pAS2-1-Tid^{aa45-426} construct and the library vector coding for the potential interactor. (C+) Yeast clones including the pVA3 (DNA-binding domain and the murine p53) and pTD1 (activation domain and the SV40 large T-antigen) vectors as a positive control. (C-) Yeast clones containing the pLAM5'-1 (DNA-binding domain and human lamin C protein) and library vectors as a negative control test.

A

1	MDRDSLPRVP	DTHGDVVDEK	LFSDLYIRTS	WVDAQVALDQ	IDKGKARGSR	TAIYLRSVFQ
61	SHLETLGSSV	QKHAGKVLFF	AILVLSTFCV	GLKSAQIHSK	VHQLWIEGG	GLEAELAYTQ
121	KTIGEDESAT	HQLLIQTTHD	PNASVLHPQA	LLAHLEVLVK	ATAVKVHLYD	TEWGLRDMCN
181	MPSTPSFEGI	YYIEQILRHL	IPCSIITPLD	CFWEGSQLLG	PESAVVIPGL	NQRLLWTTLN
241	PASVMQYMKQ	KMSEEKISFD	FETVEQYMKR	AAIASGYMEK	PCLNPLNPNC	PDTAPNKNST
301	QPPDVGAILS	GGCYGYAAKH	MHWPEELIVG	GAKRNRSGHL	RKAQALQSVV	QLMTEKEMYD
361	QWQDNYKVHH	LGWTQEKAEE	VLNAWQRNFS	REVEQLLRKQ	SRIATNYDIY	VFSSAALDDI
421	LAKFSHPALS	SIVIGVAVTV	LYAFCTLLRW	RDPVRGQSSV	GVAGVLLMCF	STAAGLGLSA
481	LLGIVFNAAS	TQVVPFLALG	LGVDHIFMLT	AAYAESNRRE	QTKLILKKVG	PSILFSACST
541	AGSFFAAAFI	PVPALKVFCL	QAAIVMCSNL	AAALLVFPAM	ISLDLRRRTA	GRADIFCCCF
601	PVWKEQPKVA	PPVLPLNNNN	GRGARHPKSC	NNNRVALPAQ	NPLLEQRADI	PGSSHSLASF
661	SLATFAFQHY	TPFLMRSWVK	FLTVMGFLAA	LISSLYASTR	LQDGLDIIDL	VPKDSNEHKF
721	LDAQTRLFGF	YSMYAVTQGN	FEYPTQQQLL	RDYHDSFVRV	PHVIKNDNGG	LPDFWLLIFS
781	EWLGNLQKIF	DEEYRDGRLT	KECWFPNASS	DAILYKLIV	QTGHVDNPVD	KELVLTNRLV
841	NSDGIINQRA	FYNYLSAWAT	NASSPTELLR	ANCIRNRANG	ASQGLYPEP	RQYFHPQNEY
901	DLKIPKSLPL	VYAQMPPFYLH	GLTDSQIKT	LIGHIRDLSV	KYEGFGLPNY	PSGIPFIFWE
961	QYMTLRSSLA	MILACVLLAA	LVLVSLLLLS	VWAAVLVILS	VLASLAQIFG	AMTLLGIKLS
1021	AIPAVILILS	VGMLLCFNVL	ISLGFMTSVG	NRQRRVQLSM	QMSLGPLVHG	MLTSGVAVFM
1081	LSTSPFEFVI	RHFCWLLLIV	LCVGACNSLL	VFPILLSMVG	PEAELVPLEH	PDRISTPSPL
1141	PVRSSKRSGK	SYVVQGSRSS	RGSCQKSHHH	HHKDLNDPSL	TTITEEPQSW	KSSNSSIQMP
1201	NDWTYQPREQ	RPASYAAPP	AYHKAAAQQH	HQHQPPTTP	PPPFPTAYPP	ELQSIVVQPE
1261	VTVETTHSDS	NTTKVTATAN	IKVELAMPGR	AVRSYNFTS		

B

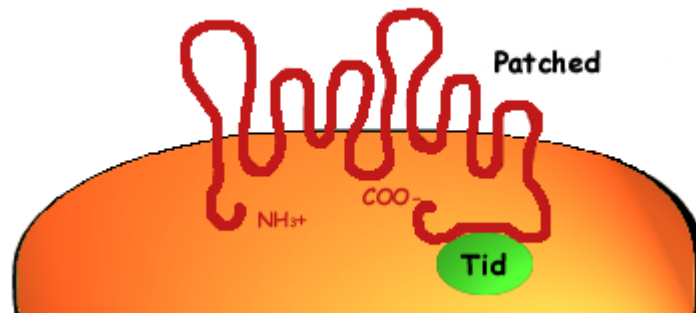


Figure 10. Sequence of the positive clone isolated in the yeast two-hybrid assay and scheme showing the putative binding of Tid to Patched in the cell. The only positive clone identified as a true interactor of Tid in the yeast two-hybrid system (see section 3.1.2.1) coded for the C-terminal cytosolic part of the Patched protein, a transmembrane receptor important in the transduction of Hedgehog signaling (Johnson *et al.*, 2000). (A) Amino acid sequence of the Patched protein (Acc. N° AAA28696). The C-terminal part of the protein deduced from the cDNA insert of the isolated library clone is outlined in yellow. (B) Scheme representing the putative binding between the cytosolic Tid protein and the C-terminal region of Patched.

3.1.2.2 Patched, an important player in development

The gene *patched* (*ptc*) encodes a large protein with multiple putative membrane spanning domains (Nakano *et al.*, 1989) (see Fig.10). This protein is a key regulator in the Hedgehog (Hh) signal transduction pathway (Johnson *et al.*, 2000). The Hh signaling pathway has an instructive role in the development of many vertebrate and insect organs. In *Drosophila* wing imaginal discs Hh produced by posterior compartment cells induces anterior cells to form a developmental organizer (Tabata and Kornberg, 1994). This effect is limited to a strip of cells adjacent to the anterior/posterior (a/p) compartment border, where Hh signals anterior cells to upregulate expression of target genes such as *decapentaplegic* (*dpp*) (a member of the TGF- β family of transcription factors) and *ptc* itself (Ingham, 1998). Ptc together with Smoothened (Smo), another transmembrane protein, is critical in receiving and regulating Hh signal (Johnson *et al.*, 2000). A model for Hh signaling has been proposed whereby Ptc, in the absence of Hh, inhibits Smo signal transduction to prevent target gene activation (see Fig. 11A). In the presence of Hh, Ptc inhibition of Smo is relieved presumably by binding of Hh to Ptc and the expression of target genes is activated (Aza-Blanc and Kornberg, 1999) (see Fig.11B). The response triggered by the Hh receptor involves in whole or in part the

transformation of a microtubule-bound cytoplasmic complex that includes Cubitus interruptus (Ci), a homologue of the vertebrate Gli transcription factors (Hui *et al.*, 1994), Fused (Fu), a putative serine/threonine protein kinase (Alves *et al.*, 1998) and Costal-2, a kinesin-related microtubule binding protein (Sisson *et al.*, 1997). As shown in Figure 11B, in presence of the Hh signal Ci is converted to transcriptional activator, the phosphorylation of Ci is altered and the limited proteolysis that converts Ci to a transcriptional repressor is inhibited. In addition, the association between the complex and microtubules is weakened and Fu is converted to a hyperphosphorylated state (Ramirez-Weber *et al.*, 2000). Interestingly, misregulation of Hh signaling has been implicated in human disease. Inactivating mutations in a human homologue of *ptc*, *PTCH1*, occur in sporadic and inherited forms of the common skin tumor, basal cell carcinoma (BCC) and the brain tumor, medulloblastoma (Johnson *et al.*, 2000), (Ingham, 1998).

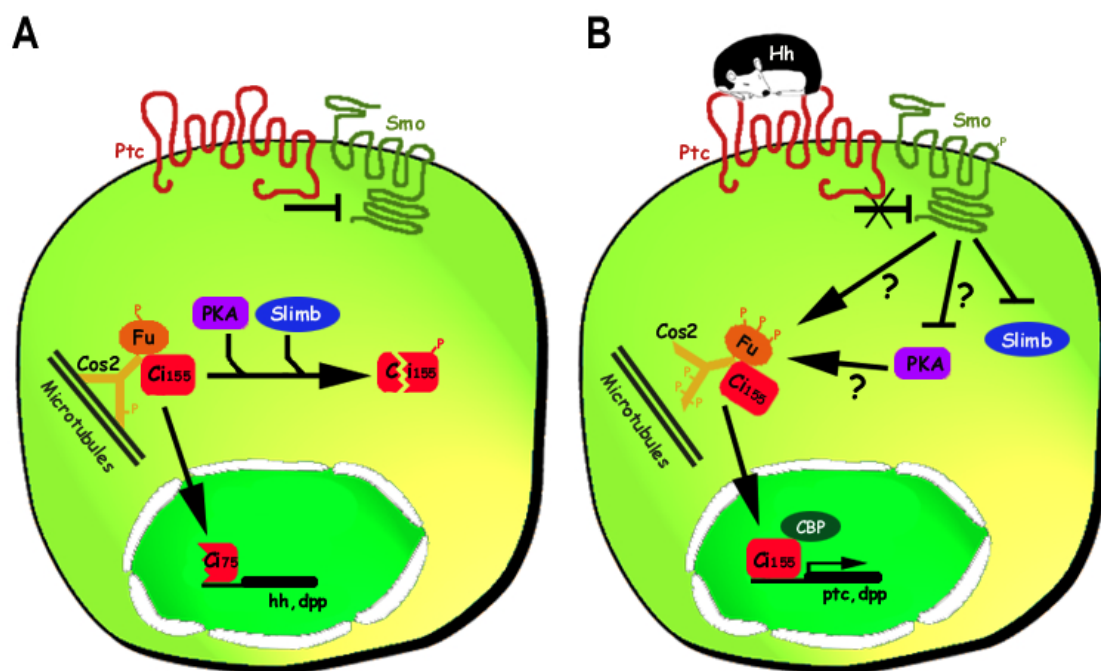


Figure 11. The Hedgehog signaling pathway. In *Drosophila*, the Hedgehog signaling pathway plays an important role in regulating cellular identity and proliferation (Ingham, 1998). Two transmembrane receptors, Patched (Ptc) and Smoothened (Smo), are important for the reception and regulation of Hedgehog signals (Johnson *et al.*, 2000). (A) In the absence of Hh, Ptc inhibits Smo activity to keep the pathway deactivated. Intracellular components of the pathway: Ci, Fu and Cos2 form a complex that

associates with microtubules. Negative regulators Cos2 and PKA inhibit Fu and Ci. The full-length activating form of the transcription factor encoded by Ci, Ci¹⁵⁵, is cleaved to yield a repressing form, Ci⁷⁵, presumably through PKA direct phosphorylation and Slimb activity. (B) In the presence of Hh, Ptc is inhibited allowing Smo activation. Smo causes – by an unknown mechanism – Cos2 and Fu hyperphosphorylation and dissociation of the complex from microtubules. Cleavage of Ci is blocked and the full-length form of the protein associates with CBP to activate transcription of target genes. Modified from Ingham, 1998.

3.1.3 Other proofs for the binding of Tid to Ptc

The binding of Tid to Ptc has been demonstrated *via* the yeast two-hybrid system (see above). However, this interaction should be further verified by an independent methodology in order to ensure the validity of the result. Therefore, GST-pulldown and co-immunoprecipitation studies have been performed.

3.1.3.1 *In vitro* binding study using the GST-pulldown assay

The glutathione-S-transferase(GST)-pulldown assay is a simple method to present a native bait protein, as a GST-fusion protein, to a test protein or a complex of proteins to test for interaction (Ausubel *et al.*, 2001). Prior to test whether Tid and Ptc interact in this assay two different expression systems were required to produce differently tagged recombinant proteins: GST-Tid^{aa45-426} and His₆-Ptc^{aa1143-1286}.

Expression and purification of GST-Tid^{aa45-426}

The GST Gene Fusion System (Pharmacia Biotech) was used to produce the recombinant Tid fragment corresponding to aa 45 to 426 fused to the C-terminus of GST, a common 26-kDa cytoplasmic protein of eukaryotes (see Table 2). The generated construct (pGEX5X-3-GST-Tid^{aa45-426}) was tested for expression by induction of an *E.coli* culture carrying this recombinant vector with 1 mM IPTG for 2 hours at 37°C (see Section 2.2.3.2.1). Figure 12 shows the Coomassie staining of a gel containing protein extracts from this induced culture (left lane) and of GST-tagged-Tid protein purified under native conditions on glutathione agarose beads (right lane). This fusion protein can be seen as a strong band of ~70 kDa in the gel. Furthermore, a second band of ~26 kDa corresponding to the GST protein alone can also be observed.

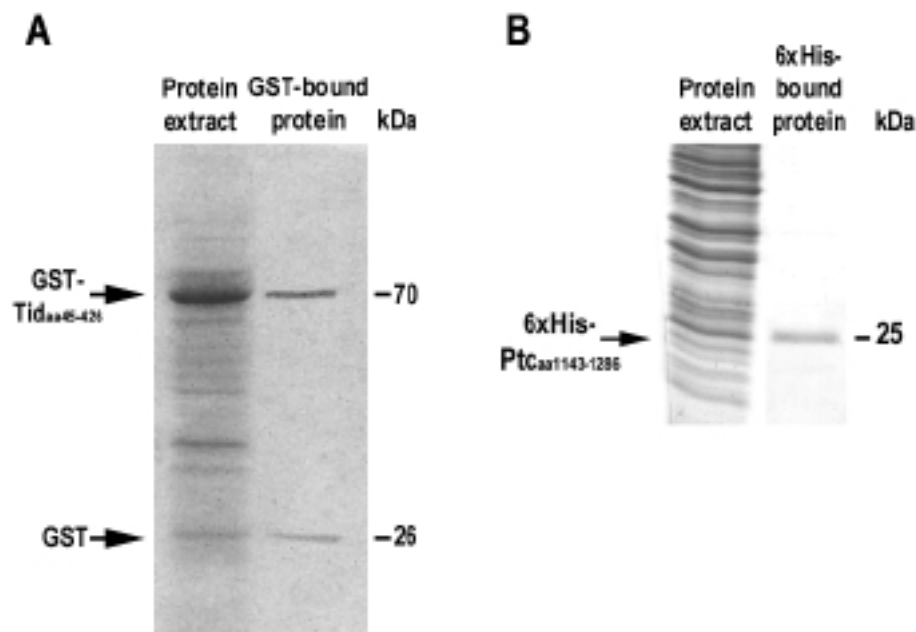


Figure 12. Expression and purification of the GST- Tid^{aa 45-426} and the His₆-Ptc^{aa1143-1286} recombinant proteins. Two differently tagged Tid and Ptc recombinant proteins were required for the GST-pulldown assay (see section 2.2.5). Exact conditions for expression and purification of the proteins have been already described in sections 2.2.3.1.2 and 2.2.3.2. In this figure are displayed coomassie stainings of protein extracts from induced *E.coli* cultures containing the pGEX5X-3-GST-Tid^{aa45-426} and the pQE30-His₆-Ptc^{aa1143-1286} vectors (left lanes in both panels) and of the corresponding purified recombinant proteins (right lanes in both panels). The GST- Tid^{aa 45-426} fusion protein can be seen as a strong band of ~ 70 kDa in the gel (A). In this same lane, a second band of ~ 26 kDa corresponding to the GST protein alone can also be observed. (B) The His₆-Ptc^{aa1143-1286} fusion product is seen as a band of ~ 25 kDa.

Expression and purification of His₆-Ptc^{aa1143-1286}

The QiaexpressTM System (Qiagen) was used to produce the recombinant C-terminal part of Ptc (aa 1143 to 1286) fused to a 6xHistidine tag (see table 2). After transformation of the construct (pQE30-His₆-Ptc^{aa1143-1286}) in a suitable bacterial host (*E. coli* strain M15[pREP4]), the culture was induced for 4 hours with 1mM IPTG at 37°C and the recombinant protein isolated under native conditions on Nickel-nitriloacetic acid (Ni-NTA) sepharose (see section 2.2.3.1.2). Figure 12 shows the Coomassie staining of a gel containing protein extracts from this induced culture (left lane) and of the purified recombinant protein (right lane). The His₆-Ptc^{aa1143-1286} fusion product can be seen as a band of ~25 kDa on the gel. Although the expected molecular weight of the recombinant protein was 17 kDa, this difference lays within the margin of error of SDS-PAGE.

GST-Pulldown assay

The *in vitro* binding study was carried out as described in material and methods (see section 2.2.4.1.2). Tid^{aa 45-426} and Ptc^{aa 1143-1286} recombinant proteins in the binding assays were detected by specific antibody staining with the polyclonal anti-Tid and anti-Ptc antibodies (see section 2.2.2.6). The output of this analysis can be followed on the blot displayed in Figure 13. Recombinant His₆-Ptc^{aa1143-1286} protein bound efficiently to GST- Tid^{aa 45-426}, whereas no binding of Ptc to the GST protein alone could be recorded (compare lanes 4 and 5). This result further implies Tid as a molecular partner of Ptc.

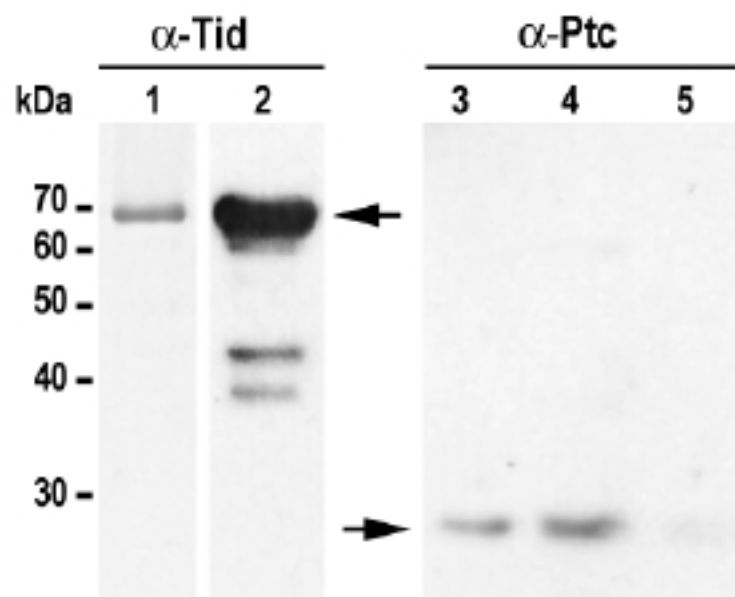


Figure 13. *In vitro* binding study of Tid to Ptc. Interaction between Tid and Ptc was tested *in vitro* by the GST-pulldown assay. This binding study was carried out by incubation of 5μg GST- Tid^{aa 45-426} loaded beads with 250 ng of purified recombinant His₆-Ptc^{aa 1143-1286} protein. After processing the beads as described in section 2.2.5.2, the presence of Ptc protein retained on them was analyzed by Western blot using a polyclonal antibody directed against Ptc (lane 4) (see section 2.2.2.6). The same procedure was done with sepharose beads coupled to equivalent amounts of GST alone as a control for non-specific binding (lane 5). Furthermore, recombinant purified Ptc and Tid proteins (100ng each) were loaded as a control in lanes 3 and 1 respectively. Moreover, lane 4 was restripped and probed with anti-Tid antibody (see section 2.2.2.6) to check for the presence of recombinant GST- Tid^{aa 45-426} fusion protein in the binding study (lane 2). Recombinant Ptc protein bound efficiently to GST- Tid^{aa 45-426}, whereas no binding of Ptc to the GST protein alone could be observed (compare lanes 4 and 5).

3.1.3.2 *In vivo* binding study by immunoprecipitation

Demonstration of a binding *in vitro* does not necessarily indicate that the binding has *in vivo* biological relevance (Clontech, 1996). For that reason, immunoprecipitation studies were performed to ensure the binding of Tid to Ptc in a cellular environment. Immunoprecipitation of native proteins is a procedure by which peptides or proteins that react specifically with an antibody are removed from a protein extract by addition of Protein A Sepharose and examined for quantity or physical characteristics. To test whether Ptc and Tid associate *in vivo*, protein extracts prepared from embryo and third instar larvae were immunoprecipitated either with anti-Tid antibody (see section 2.2.6) or pre-immuneserum as a control. These two stages were chosen since the two-hybrid screening was performed on an embryonic library and further immunohistochemical tests were done in larval wing imaginal discs (section 3.1.2 and 3.1.5 respectively). The resulting immunoprecipitates were analyzed on immunoblots probed with biotinylated anti-Tid antibody (Fig. 14A) (see section 2.2.2.6) and a mouse monoclonal anti-Ptc antibody (Fig. 14B) (see section 2.2.2.6). Biotin-labeled anti-Tid antibody was used in order to avoid the detection of the IgG antibody band in the immunoprecipitates which in part colocalizes with two Tid forms: Tid50 and Tid47. As expected, Tid50, Tid47 and Tid40 were detected in the immunoprecipitates (Fig 14A). Interestingly, Tid47 appears to be in higher amounts in immunoprecipitates as in normal protein extracts prepared under denatured conditions, whereas Tid40 concentration in this immunoprecipitates is reduced. Figure 14B shows an immunoblot of embryonic extracts and Tid immunoprecipitates from embryo and third instar larvae with anti-Ptc antibody. A protein band of ~140 kDa can be recognized in both immunoprecipitates. The mobility of this protein corresponds to the predicted molecular weight for Ptc. Furthermore, a second signal of ~ 82 kDa can also be detected. However, no explanation for such a band can be found in literature. The presence of Ptc in Tid immunoprecipitates is a further indication of the binding of Tid to Ptc in the cell.

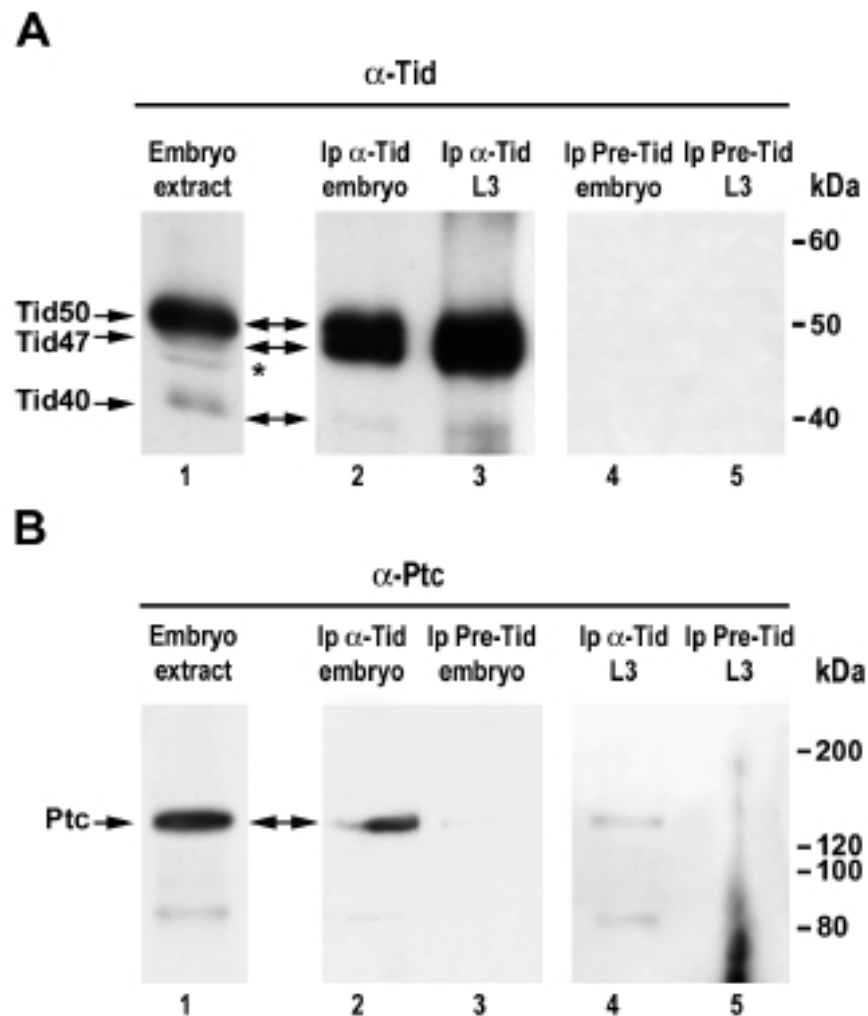


Figure 14. Interaction of Tid with Ptc *in vivo*. *In vivo* interaction between Tid and Ptc was demonstrated *via* co-immunoprecipitation. Proteins immunoprecipitated from embryonic and third instar larval extracts with anti-Tid antibody (see section 2.2.6) were separated by SDS-PAGE gels (9%) and visualized by chemiluminescence after Western blot analysis. For controls, protein extracts were incubated with pre-immuneserum. Embryo extract lanes contain 50 μ g of protein. Lanes 2-5 include 6% (upper panel) or 16% (lower panel) of the total immunoprecipitated material (see section 2.2.6). (A) Western blot of anti-Tid and pre-immuneserum immunoprecipitates probed with biotinylated anti-Tid antibody. Protein bands corresponding to Tid50, Tid47 and Tid40 can be specifically detected in the immunoprecipitates. The asterisk marks a 45 kDa protein band shown to be unspecific in previous studies (Kurzik-Dumke *et al.*, 1998). (B) Western blot of anti-Tid and pre-immuneserum immunoprecipitates probed with the monoclonal anti-Ptc antibody (see section 2.2.2.6). This antibody specifically recognizes a ~140kDa protein band. Furthermore, a second protein of ~82 kDa is also detected, but no explanation for it can be found in literature. L3: third instar larvae.

3.1.4 Characterization of Tid binding to Ptc

3.1.4.1 Identification of binding domains responsible for the interaction of Tid to Ptc via the two-hybrid system

The two-hybrid system has been used to screen a library for a gene encoding a novel protein that interacts with Tid (see section 3.1.2) but it can be also used to define the protein domains that mediate an interaction. Previous results have shown that Ptc binding to Tid involves the cytosolic C-terminal part of the protein (see Fig.10). In order to identify the Tid domains responsible for this interaction five new yeast fusion plasmids including different regions of the *l(2)tid* gene were designed (see Table 1 and Fig 16A). The first construct, pAS2-1- Tid^{aa 98-495}, contains part of the DnaJ domain and the complete G/F, Zn Finger and C-terminal regions. A second construct, pAS2-1-Tid^{aa 45-212}, contains the DnaJ- and G/F-domains. The third one, pAS2-1-Tid^{aa 45-161}, includes just the DnaJ domain. The fourth one, pACT2-Tid^{aa 206-318}, contains only the Zn Finger domain and the fifth construct, pAS2-1-Tid^{aa 359-518}, has the complete C-terminal region alone. The orientation and reading frame of the constructs was checked by sequencing and expression of the recombinant proteins verified *via* Western blot of protein extracts from yeast cultures containing the different constructs with the anti-GAL4 DNA-binding domain monoclonal antibody (Clontech). As shown in Figure 15 all clones did express the fused protein. However, differences in expression between the stocks were observed (compare Tid^{aa 98-495} with Tid^{aa 359-518} for example). For the binding study each fusion protein was tested for interaction against the C-terminal cytosolic part of the Ptc protein (aa 1143-1286) (see Fig. 16B). After co-transformation of the plasmids, positive clones could grow in medium lacking Histidine and turned blue after the b-galactosidase assay was performed. Control reactions included the vectors pVA3 (DNA-binding domain and the murine p53) and pTD1 (activation domain and the SV40 large T-antigen) for the positive test, and pLAM5'-1 (DNA-binding domain and human lamin C protein) for the negative interaction test with Ptc. This binding study shows an interaction between the regions of Ptc^{aa1143-1286} and Tid^{aa45-426} or Tid^{aa 98-495}. Both Tid constructs contained the complete G/F and Zn Finger regions. The first one has a complete J-Domain and a partial C-terminal region, whereas the second one has a partial J-Domain and a nearly complete C-terminal region. Further, none of both domains alone is capable of interact

with Ptc. However, the last part of the Tid protein is necessary for the binding since a construct without the C-terminal region also fails for interaction. Interestingly, the J-Domain increases the efficiency of the interaction as the intensity of the blue color is greater when the J-Domain is complete (Fig. 16B, compare clones 1 and 4). This experiment shows that no domain of Tid alone is capable of binding to Ptc. The nearly complete structure of the protein is required for the interaction.

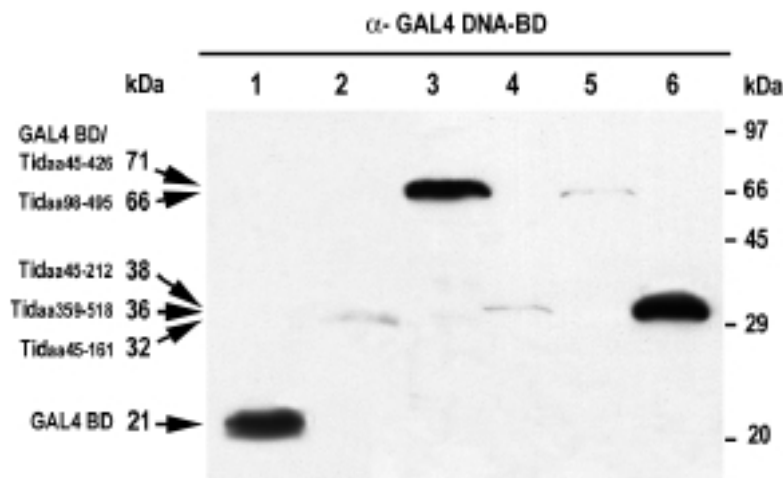


Figure 15. Expression test of the constructs used in the binding study. Expression and stability of the hybrid proteins used in the binding assay (see Fig.16) was assayed via Western blot. Soluble protein extracts from *Y190* yeast cells containing the recombinant vectors (see below) were prepared using the Urea/SDS method (see section 2.2.7.5). Samples equivalent to ~ 1.5 OD₆₀₀ units of cells were separated on 12% polyacrylamide/SDS gels, immunoblotted with anti-GAL4-DNA BD monoclonal antibody (0,5µg/ml)(Clontech) and visualized by chemiluminescence. Lane 1 contains protein extract from yeast cells carrying the pAS2-1 construct as a control. Lanes 2-6 contain protein extracts from yeast cells carrying the pAS2-1-Tid^{aa45-161}, pAS2-1-Tid^{aa45-426}, pAS2-1-Tid^{aa45-212}, pAS2-1-Tid^{aa98-495} and pAS2-1-Tid^{aa359-518} recombinant constructs respectively. In this test all clones do express the hybrid protein.

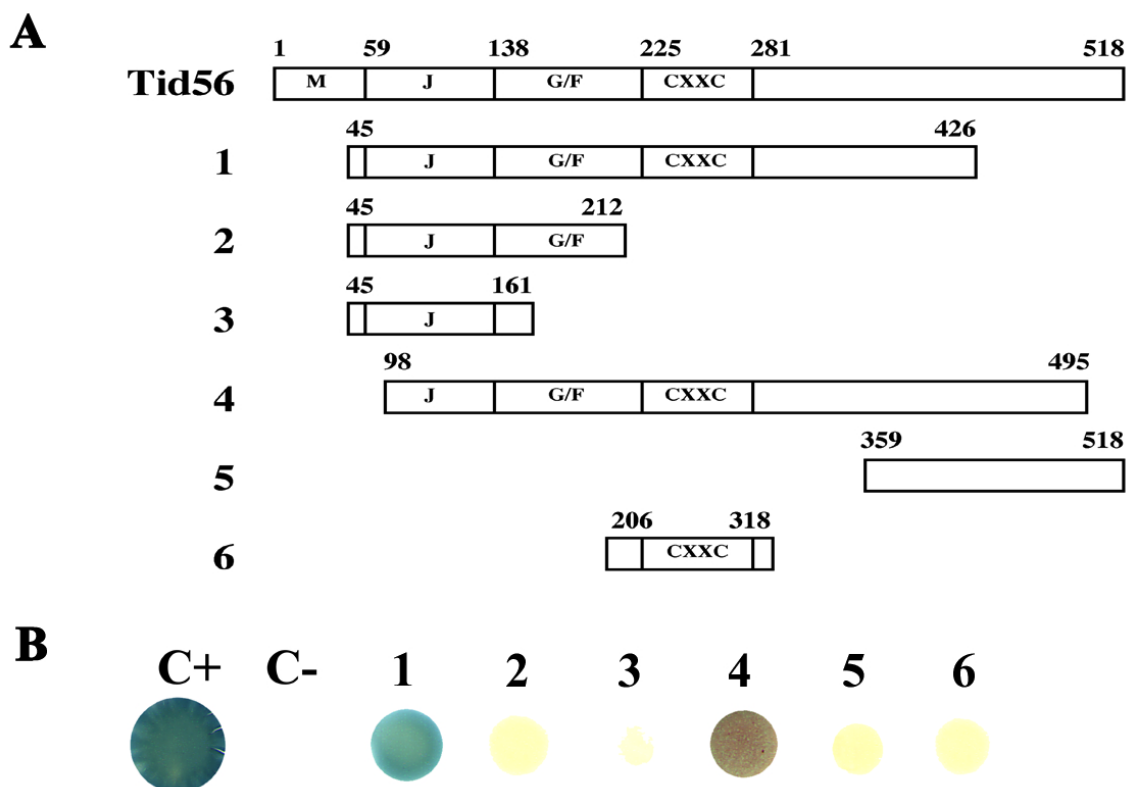


Figure 16. Identification of the Tid domains responsible for the interaction with Ptc. To determine the domains of Tid involved in the interaction with Ptc several two-hybrid assays were performed. (A) Diagram showing the different Tid fusion constructs used in this study. 1: pAS2-1-Tid^{aa 45-426}, 2: pAS2-1-Tid^{aa 45-212}, 3: pAS2-1-Tid^{aa 45-161}, 4: pAS2-1-Tid^{aa 98-495}, 5: pAS2-1-Tid^{aa 359-518}, 6: pACT2-Tid^{aa 206-318} (see Table 1). (B) Yeast clones containing the bait and prey (see below) grown on medium lacking histidine and assayed by the β -galactosidase test to select for positive interaction (see section 2.2.727). Blue colonies indicate a binding between the hybrid proteins. All binding studies were performed with the C-terminal cytosolic part of Ptc (aa 1143-1286) except for the positive control reaction. Numbers above the yeast colonies indicate the deletion construct used in each case (compare numbers in panel A and B). (+): pVA3 (DNA-BD/murine p53)-pTD1 (AD/SV40 large T-antigen); (-): pLAM5'-1 (DNA-BD/human lamin C protein)- pGAD10-Ptc^{aa 1143-1286}. This experiment shows that no domain of Tid alone is capable of binding to Ptc. The nearly complete structure of the protein is required for the interaction.

3.1.4.2 Is Tid binding to Ptc conserved in evolution? - First insights

To determine if the ability of Tid to interact with Ptc is conserved in evolution additional two-hybrid interaction assays were carried out. Experiments were done using *Drosophila* Tid and Ptc and human hTid-1 and PTCH1 proteins in all pairwise combinations. Two new yeast fusion plasmids were constructed for this study (see Table 1 for more information). The *hTid-1* gene was cloned from amino acid 66 to 280 into the pAS2-1 vector (pAS2-1-hTid-1^{aa 66-480}). This construct includes the complete protein except for the mitochondrial pre-sequence, which would interfere with the nuclear localization of the recombinant product. The second construct, pACT2-PTCH1^{aa 1027-1296}, contains the whole C-terminal part of the protein in analogy to the *Drosophila* one. The orientation and reading frame of the recombinant vectors was checked by sequencing. For the binding study each fusion construct was tested pairwise (see Fig. 17). After co-transformation in yeast *Y187* cells, interaction was assessed by the β -galactosidase assay and growth on medium lacking histidine (see section 2.2.7.7). Unfortunately, human fusion proteins were not able to substitute its *Drosophila* counterparts in this assay. Possible reasons for this result will be discussed in section 4.1.1.2.

GAL4 BD- Tid ^{aa45-426}	/ GAL4 AD- Ptc ^{aa1143-1286}	+
GAL4 BD- Tid ^{aa45-426}	/ GAL4 AD- PTCH1 ^{aa 1027-1296}	-
GAL4 BD- hTid-1 ^{aa 66-280}	/ GAL4 AD- Ptc ^{aa1143-1286}	-
GAL4 BD- hTid-1 ^{aa 66-280}	/ GAL4 AD- PTCH1 ^{aa 1027-1296}	-

Figure 17. Yeast two-hybrid assays to analyze if the binding of Tid to Ptc is conserved in evolution. For the binding study each fusion protein was tested pairwise (*Drosophila* proteins: GAL4 BD- Tid^{aa45-426} and GAL4 AD- Ptc^{aa1143-1286}; human proteins: GAL4 BD- hTid-1^{aa66-280} and GAL4 AD- PTCH1^{aa1027-1296}) (see table 1). Interaction between the hybrid proteins was assayed using the *HIS3* and *lacZ* reporter genes (see section 2.2.7). (+) indicates a positive interaction. (-) indicates a negative interaction.

3.1.5 Tid/Ptc interaction in the fly

Despite the fact that the binding of Tid to Ptc has been proved using biochemical studies it remains to be seen which function this binding could have in the *in vivo* environment of the fly. Therefore, several stainings on *l(2)tid*-tumorous wing discs and on those from a viable *ptc* mutant stock were done to monitor a possible change in expression of both proteins. However, to better interpret these results on the highly abnormal tumorous structure and to analyze the parts of the disc involved in tumor formation a rough characterization of boundaries and overgrown regions in the *l(2)tid*-wing disc was first carried out. To achieve this, three different enhancer-trap lines and specific antibody stainings against the Wingless and Engrailed proteins were used.

3.1.5.1 Characterization of the tumorous wing imaginal disc

The wing discs are simple invaginated epithelial sacs composed of a convoluted columnar epithelium that is continuous with the squamous epithelium of the peripodial membrane (Bate and Martinez-Arias, 1993). These discs can be viewed as a series of circumferentially arranged regions (Fig.18). The outermost region will make the dorsal and ventral body wall structures, the notum and pleura. The next “ring” will make the dorsal and ventral hinge region and the more centrally located wing pouch region will make the wing blade (Bate and Martinez-Arias, 1993) (Fig.18). The wing disc is structured into three axes: i) the a/p axis, structured by the segment polarity genes *engrailed*, *hh* and *dishevelled* on either side of a stripe expressing *decapentaplegic* (*dpp*); ii) the proximal/distal axis, structured by the genes *distal-less* and *aristaless* and iii) the dorso-ventral (d/v) axis, structured by *vestigial* (FlyBase, 1999).

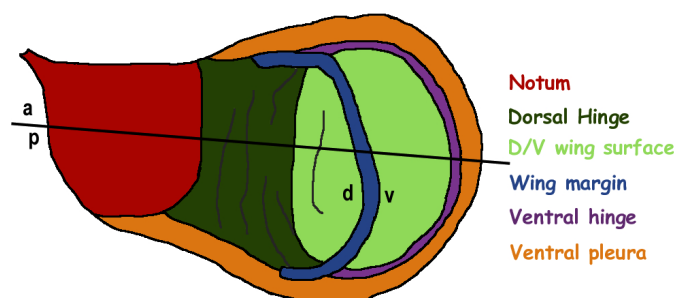


Figure 18. Fate map of the wing imaginal disc. Top view of the fate map of the wing. Regions of the wing disc that correspond to adult structures are color-coded and labeled (modified from Campuzano and Modolell, 1992). a:anterior; p:posterior; d:dorsal; v:ventral

Anatomical investigations of homozygous *l(2)tid* larvae of the *tid*¹ allele revealed enlarged and morphologically abnormal imaginal discs (Kurzik-Dumke *et al.*, 1992). To analyze this abnormal structure three different enhancer-trap GAL4 lines were used: MZ 1299/1, MZ 0423 and MZ 0853 (see section 2.1.8). These lines have an inserted P element into the third chromosome of the *Drosophila* genome close to an endogenous enhancer of gene expression. This P element contains as a reporter a yeast transcription factor (GAL4) that is functional in *Drosophila*. Rather than visualize GAL4 directly, this protein is used to drive a secondary reporter (the GFP gene) linked to a GAL4 dependent promoter (UASG). The *UAS-mCD8::GFP* stock (see section 2.1.8) with insertion in the first chromosome was the one used as a secondary reporter to allow the *in vivo* observation of the GAL-expressing cells. After preparation of fly stocks homozygous for *tid*¹ and containing the GAL4 and UAS insertions (see section 2.2.8.11) GFP-fluorescent *tid*¹-homozygous larvae were identified under a fluorescent binocular and its tumorous discs prepared. Figure 19 shows the changes in GFP expression of the GAL4-lines in tumorous imaginal discs compared to its expression in the *wild-type* counterparts.

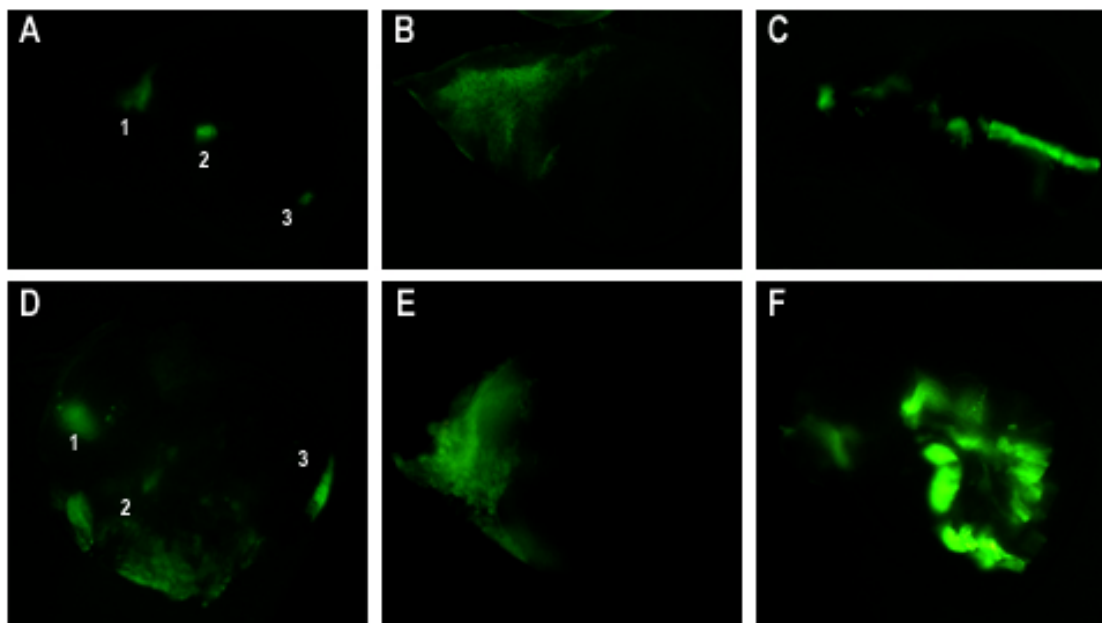


Figure 19. Characterization of the tumorous wing imaginal disc (1). In a first step to determine the regions of the wing disc which undergo neoplastic transformation in *l(2)tid* mutants three different GAL4 enhancer trap lines were used. Stocks homozygous for *tid*¹ carrying the GAL4 and UAS insertions were generated as described in section 2.2.8.11. The upper row shows GFP expression driven by the MZ 1299/1(A), MZ

0423(B) and MZ 0853(C) GAL4 enhancer trap lines in *wild-type* wing imaginal discs. The lower row shows the same lines expressing GFP in *l(2)tid*-tumors. Central regions of the wing disc corresponding to the future dorsal hinge and dorsal wing surface are broadened in the tumorous tissue (compare A,2 and D,2) whereas the notum seems not to play a crucial role in tumor development (compare B and E). Furthermore, the anterior/posterior border can be still recognized in this abnormal structure (compare C and F).

The stock MZ 1229/1 normally expresses in three defined regions located in the notum (Fig. 19A,1), in the ventral pleura (Fig. 19A,3) and at the border of the dorsal hinge and the dorsal wing surface (Fig.19A,2). GFP expression of this GAL4 line in *l(2)tid*-tumorous imaginal discs (Fig. 19D) identified the region of the dorsal hinge and dorsal wing surfaces as a potential proliferation focus. Further, analysis of the GFP expression induced by the GAL4 line MZ 0423 in *wild-type* discs and tumorous tissue showed no involvement of the notum in the development of this abnormal structure (compare Fig. 19B and E). To establish the importance of a/p border in tumor formation it was used the GAL4 line MZ 0853. This line expresses along the a/p axis in wing imaginal discs (Fig. 19C). Figure 19D shows a partial conservation of this border in the tumorous structure.

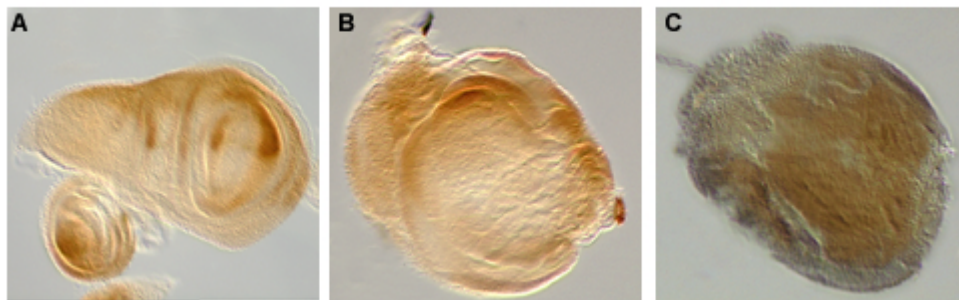


Figure 20. Characterization of the tumorous wing imaginal disc (2). To identify the anterior and posterior regions in the tumorous wing disc, staining with antibodies against the active form of *Cubitus interruptus* (Ci) (see section 2.2.8.4), a marker protein for the anterior part (Motzny and Holmgren, 1995) and against *Engrailed* (En) (see section 2.2.8.4), a marker for the posterior region (Tabata and Kornberg, 1994), were performed. (A) Staining of the posterior region of a *wild-type* wing imaginal disc with anti-En. (B) Staining of a *l(2)tid*-tumorous wing imaginal disc with the same antibody. (C) Double staining of a *l(2)tid*-tumorous wing imaginal disc with anti-En (black colour) and anti-Ci (brown color). As shown in C the major part of the tumor arises from the anterior part.

Further, to identify the anterior and posterior regions in *l(2)tid*-tumorous wing discs it was monitored the expression of En as a marker for the posterior part (Tabata and Kornberg, 1994) and of the active form of Ci as a marker for the anterior part (Motzny and Holmgren, 1995) (see section 3.1.5.2 for more information about Ci). The segment polarity gene *en* plays an important role in patterning of embryonic and adult structures. The product of this gene is a homeoprotein that controls cell segregation at the a/p boundary and is sufficient to specify the identity of posterior cells in the wing disc in the absence of Ci (Dahmann and Basler, 2000). *Wild-type* and *l(2)tid*-tumorous wing discs were stained with anti-En antibody or with a combination of anti-En and anti-Ci (see section 2.2.8.4). In Figure 20 is shown En expression in the posterior part of the *wild-type* wing disc (A) and in the external regions of the tumorous disc (B). Double staining with both antibodies (C) helped identifying the anterior region as the major part of the wing disc involved in tumor formation.

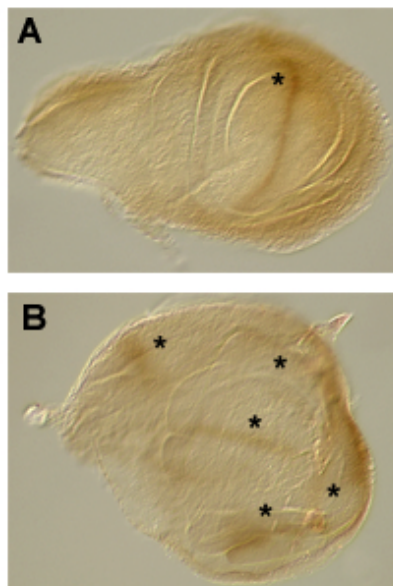


Figure 21. Characterization of the tumorous wing imaginal disc (3). To identify the dorso/ventral (d/v) boundary in the tumorous wing disc staining with anti-Wingless (Wg) antibody (see section 2.1.8.4) was performed. (A) Wg expression in *wild-type* wing imaginal discs from third instar larvae defines the d/v boundary. (B) In *l(2)tid* tumorous tissue Wg expression (marked with asterisks) is disrupted.

Moreover, studies to identify the d/v border in the tumorous structure have been done. This axis has been associated with cell proliferation and is required for the normal development of the wing (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1997). The d/v boundary in *wild-type* wing imaginal discs from third instar larvae is defined by Wg expression (Fig.21A) (Neumann and Cohen, 1997). Wg is a signaling molecule that functions in combination with Vestigial to regulate in part the proliferation of the wing disc by using the d/v boundary as an organizing center (Irvine and Vogt,

1997). Interestingly, the expression of this protein in *l(2)tid*-tumorous imaginal discs is disrupted. Several broadened stripes of Wg protein can be observed in this abnormal tissue (Fig.21B, marked with asterisks).

3.1.5.2 Genetic interactions of Tid and Ptc in the fly

To examine a possible effect of Tid on Ptc expression and *viceversa* several immunostainings on mutant wing imaginal discs have been performed. The lethal *tid¹/CyO* and the viable *ptc^{tuf-1} ltd* stocks (see section 2.1.8 for more information) were used in this analysis. Tid expression in *wild-type* wing imaginal discs shows an ubiquitous pattern but it is stronger at certain regions such as the notum and the parts of the disc that will become the wing surface (Fig.22A) (Kurzik-Dumke *et al.*, 1998). Furthermore, wholemounts of tumorous imaginal discs with anti-Tid antibody exhibit an abnormal staining in comparison to its *wild-type* counterparts (compare Fig.22A and C). Previous studies revealed that the rest of staining in the tumorous tissue only concerns the surface of the tumorous imaginal discs, while no expression was shown in the tumorous tissue (Kurzik-Dumke *et al.*, 1998). The expression of Tid in wing discs from the mutant *ptc^{tuf-1}* allele was also tested. As it can be seen in Figure 22E there are no perceptible changes in the expression pattern of Tid in this tissue. There is still a clear expression around the wing pouch and the characteristic staining in the notum. In addition to these immunostainings Ptc expression has also been checked in the same tissues. Ptc protein in *wild-type* wing imaginal discs is expressed throughout the anterior compartment with a stripe of maximal intensity near the a/p compartment boundary due to the action of Hh signal (Fig.22B)(Forbes *et al.*, 1993; Goodrich *et al.*, 1996; Hidalgo and Ingham, 1990). Further, the expression of Ptc in third instar wing discs of *ptc^{tuf-1}* larvae is changed. The stripe of maximal Ptc staining expands anteriorly several cell diameters whereas the level of Ptc protein expressed is reduced (Fig.22F)(Capdevila *et al.*, 1994). Interestingly, in *l(2)tid*-tumorous tissue there is also a change in Ptc expression. The number of cells showing a high level of Ptc protein has clearly increased (Fig.22D). These cells are not only present at the a/p boundary but throughout the anterior compartment. This result could indicate a possible activation of the Hh signaling pathway in the tumorous structure.

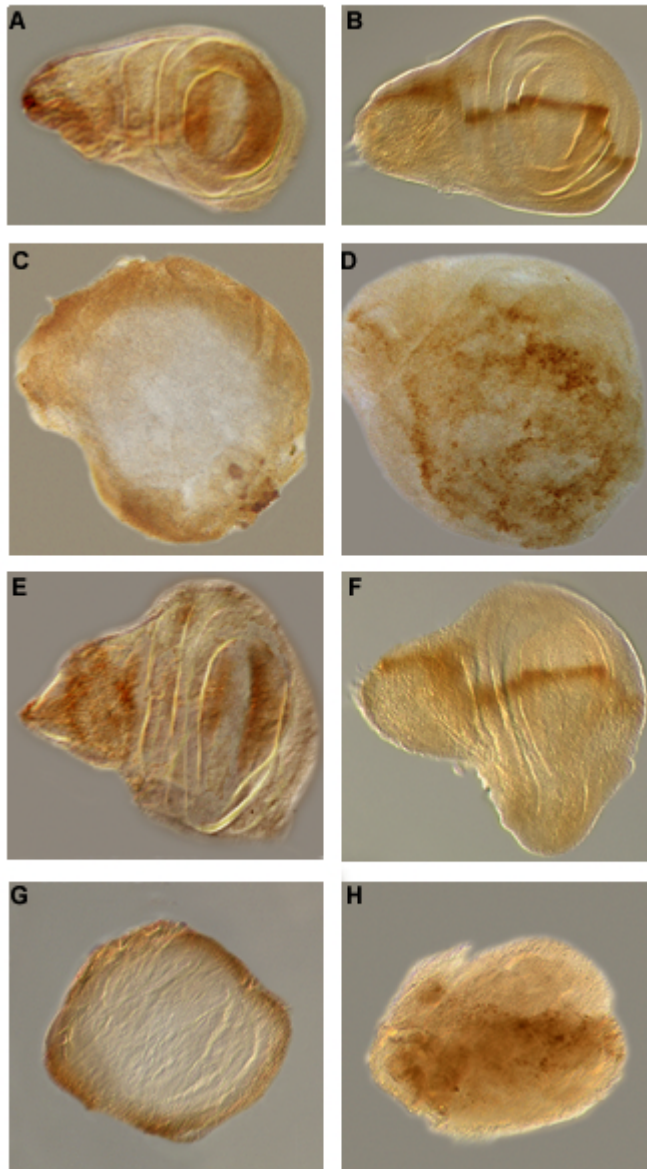


Figure 22. Analysis of Tid and Ptc expression in *tid*¹, *ptc*^{*tu**f*-1} and *tid*¹/*ptc*^{*tu**f*-1} wing imaginal discs. To examine a genetic interaction between *l(2)tid* and *ptc* in the fly stainings of *wild-type* (A,B), *tid*¹ (C,D), *ptc*^{*tu**f*-1} (E,F) and *tid*¹/*ptc*^{*tu**f*-1} (G,H) double mutant wing imaginal discs were performed with anti-Tid (panels on the left) and anti-Ptc (panels on the right) antibodies (see section 2.2.8.4). (A) Tid expression has an ubiquitous pattern in *wild-type* imaginal discs whereas staining in *tid*¹ and *tid*¹/*ptc*^{*tu**f*-1} tumorous wing discs is visible just in the partially preserved monolayer epithelium (C,G)(Kurzik-Dumke *et al.*, 1998). (E) Tid staining in *ptc*^{*tu**f*-1} mutant discs revealed no perceptible changes in the expression pattern when compared to *wild-type*. (B) In *wild-type* wing discs Ptc protein is expressed throughout the anterior compartment with a stripe of maximal intensity near the a/p boundary. In *ptc*^{*tu**f*-1} imaginal discs this stripe expands anteriorly several cell diameters showing a lower level of Ptc protein

(compare B and F) (Capdevila *et al.*, 1994). Interestingly, high amounts of Ptc protein in *tid*¹ and *tid*¹/*ptc*^{*tu**f*-1} tumorous wing discs are found not only at the a/p boundary but in many cells of the anterior compartment of the tumorous wing disc (D,H).

In order to further prove this idea expression analysis of Ci, a transcription factor that mediates all outputs of the Hh signal transduction pathway (Methot and Basler, 2001; Ramirez-Weber *et al.*, 2000), was performed. Ci is the *Drosophila* homologue of the vertebrate Gli proteins (Alcedo and Noll, 1997; Ingham, 1998) and in wing imaginal discs is present in an active full-length form of 155 kDa or in a repressive proteolysed form of 75 kDa (see Fig.11). Hh signaling is believed to inhibit proteolysis of Ci primarily by controlling its cytoplasmic association with multiprotein complexes,

leading to Ci activation of target genes such as *dpp* and *ptc* (Methot and Basler, 1999) (Fig.11). Therefore, to monitor an activation of the Hh signaling cascade it was used an anti-Ci antibody that recognized only the active form of the protein. In Figure 23A can be seen the normal staining pattern of Ci in *wild-type* wing imaginal discs, where it is expressed throughout the anterior compartment with a stripe of maximal intensity at the a/p boundary due to Hh signaling (Motzny and Holmgren, 1995). In *ptc^{tuf-1}* wing imaginal discs the stripe of maximal expression expands anteriorly and the low levels of Ptc protein give rise to ectopic Ci expression in the anterior edge of the presumptive wing pouch. This area roughly corresponds to the presumptive costal region (Bryant, 1975) (Fig.23B). As expected, Ci staining in *l(2)tid*-tumorous imaginal discs correlates well with the Ptc staining described above. The number of cells expressing Ci at high amounts has increased when compared to *wild-type* wing discs (Fig 23C compared to A). This expression expands throughout the anterior compartment of the tumorous structure.

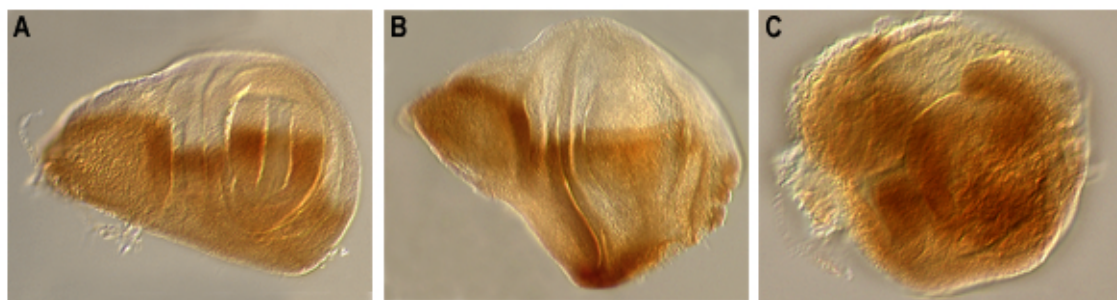


Figure 23. Analysis of Ci expression in *tid¹* and *ptc^{tuf-1}* wing imaginal discs. Staining of the active form of Ci has been used as a reference for positive activation of the Hh signaling cascade. Expression of Ci in the *wild-type* (A), *ptc^{tuf-1}* (B) and *tid¹* (C) wing imaginal discs as revealed by wholemount staining with the appropriate antibody (see section X). This analysis shows an up-regulation of Ci expression in overgrown *ptc^{tuf-1}* wing discs and *l(2)tid*-tumorous tissue.

To further analyze the functional relationship between *ptc* and *l(2)tid* in the fly a mutant stock carrying both the *tid¹* and *ptc^{tuf-1}* alleles was generated (see section 2.2.8.10). Animals carrying both mutations were characterized by larval lethality and tumorous imaginal discs resembling those of the *tid¹* mutant. Furthermore, Tid and Ptc expression in these discs was comparable to that observed in the single mutant tumorous tissue (Fig.22D compared to H).

3.1.6 *In vivo* study to detect a possible binding of Tid to Smo

Previous studies have shown an interaction of Ptc to Tid *in vivo* (see section 3.1.3.2). Ptc together with Smo, another transmembrane protein, is critical in receiving and regulating Hh signal (Johnson *et al.*, 2000). Genetic analysis have shown that Ptc activity antagonizes Hh signaling by blocking the intrinsic signaling activity of Smo (Alcedo *et al.*, 1996; van den Heuvel and Ingham, 1996). The mechanism by which Ptc regulates Smo is not understood but recent evidence supports the model that Ptc acts indirectly to regulate Smo (Denef *et al.*, 2000). Interestingly, Tid has been postulated to be a negative regulator of Smo signal transduction because the Hh signaling pathway seems to be activated in *l(2)tid*-tumorous tissue (see section 3.1.5.2). To test if Tid could be the connecting link between Ptc and Smo, Tid immunoprecipitates were probed with anti-Smo antibody (see section 2.2.2.6). This antibody recognizes a faster (inactive) and a slower (active) migrating forms of Smo in embryonic protein extracts (see Fig.24 lane 1) (Denef *et al.*, 2000). However, none of both forms is present in Tid immunoprecipitates (see Fig.24 lane 2).

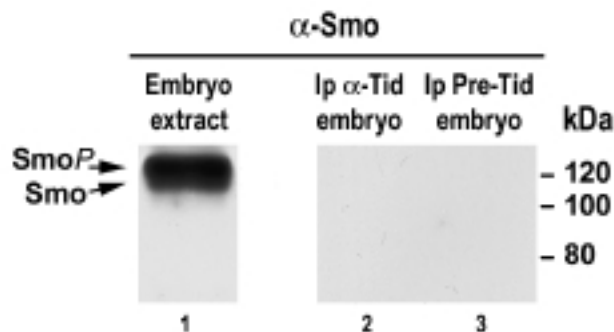


Figure 24. Test for interaction of Tid with Smo *in vivo*. *In vivo* interaction between Tid and Smo was checked via co-immunoprecipitation. Proteins immunoprecipitated from embryonic extracts with anti-Tid antibody (see section 2.2.6) or with pre-immuneserum as a control were separated on SDS-PAGE (9%) and visualized by chemiluminescence after Western blot analysis. Lanes

loaded with 50 μ g of embryonic protein extract (lane 1) or with 16% of the total immunoprecipitated material (lanes 2-3) (see section 2.2.6) were probed with anti-Smo antibody (see section 2.2.2.6). Protein bands corresponding to the active (SmoP) and inactive (Smo) forms of the protein were detected on embryonic extracts but not in Tid immunoprecipitates.

3.2 Tid influence on other developmental pathways – first insights

Several immunohistochemical studies with anti-hTid-1 antibody on colon cancer tissue preparations have shown a correlation of the tumor progression with the lost of hTid-1 expression (Kurzik-Dumke, pers.com.). Furthermore, hTid-1 has been found to associate *in vitro* (Polakis, 1997), in the two-hybrid system (Polakis, 1997) and *in vivo* (Kurzik-Dumke, pers.com.) with the Adenomatous polyposis coli (APC) protein, an important tumor suppressor in the human colon and a component of the Wg/Wnt signal transduction pathway (Bienz, 1999). To check if this binding is conserved in evolution co-localization and immunoprecipitation studies on the *Drosophila* counterparts, Tid and E-APC, have been performed.

3.2.1 APC is the clue of many colon cancers

Defects in the APC gene are undoubtedly linked to the progression of colon cancers (Polakis, 1997). APC is a component of the Wnt signaling pathway and functions to destabilize β -catenin during normal development. This ability of APC to downregulate β -catenin maps to a central domain within APC that is commonly deleted in cancer cells (Polakis, 1997). β -catenin was initially identified as a constituent of the adherens junctions in *Drosophila* (Tepass, 1997). However, in addition to this adhesion function, β -catenin has a second function in transducing the Wnt signal (Waltzer and Bienz, 1999). In the presence of Wnt protein, free cytoplasmic β -catenin accumulates to high levels and associates with transcription factors of the T-cell factor (TCF) family in the nucleus to activate the transcription of Wnt target genes (Korinek *et al.*, 1997; Morin *et al.*, 1997) (see Fig.25). How APC destabilizes β -catenin is unknown. This process is mediated by a multi-protein complex containing APC as well as Axin and GSK-3 (Hart *et al.*, 1998). In this complex Axin functions as a scaffold protein to facilitate phosphorylation of β -catenin by GSK-3 (Ikeda *et al.*, 1998). Phosphorylation causes β -catenin to be recognized by the SCF ubiquitin ligase complex which targets it for subsequent degradation by the proteasome pathway (Maniatis, 1999) (Fig.25A). Overexpression of Axin is sufficient to downregulate soluble β -catenin in APC mutant cancer cells, thus bypassing the function of APC (Hart *et al.*, 1998). This result indicates

a regulatory role of APC in the process of β -catenin destabilization. Furthermore, it has been suggested that APC may somehow derepress Axin, stimulating its activity in the β -catenin-destabilizing complex (Hart *et al.*, 1998). Therefore, Wnt signaling may inhibit the activity of this complex by causing dissociation of APC from it (Yu *et al.*, 1999) (see Fig.25B).

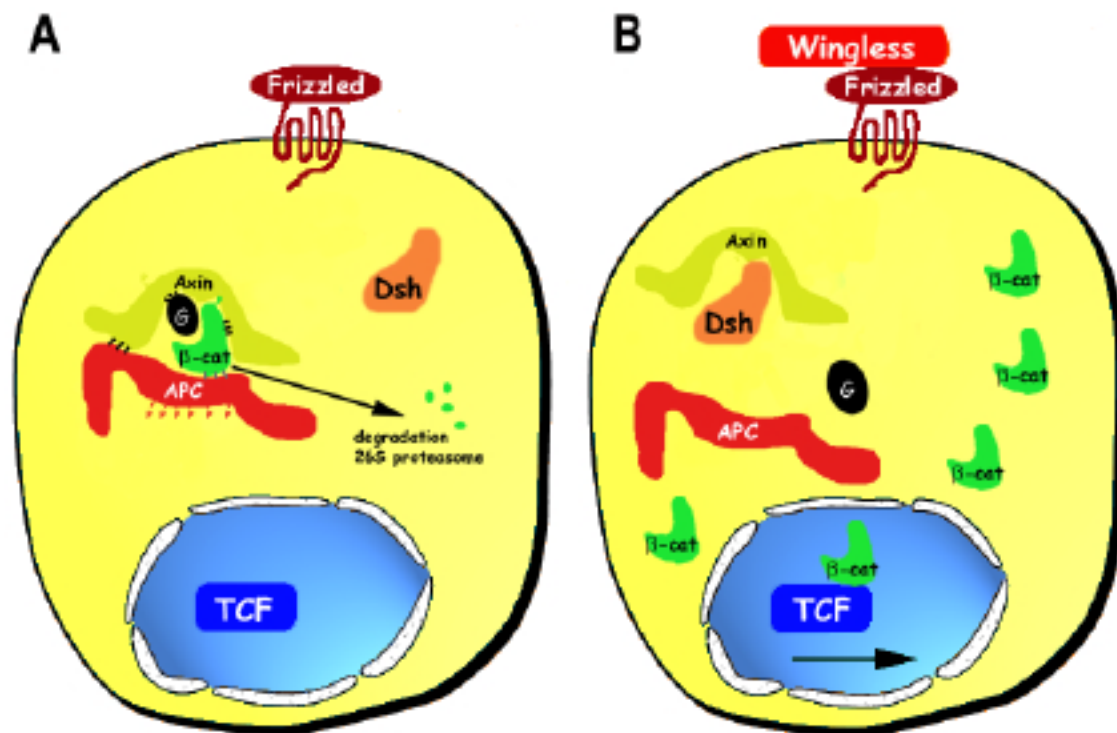


Figure 25. Wnt/Wingless signal transduction. The Wnt signaling pathway controls many developmental decisions during animal development (Waltzer and Bienz, 1999). Following is a description of the current model for transduction of the Wnt signal. (A) In the absence of Wnt, Axin binds simultaneously to APC, GSK3 and β -catenin (β -cat), thus stabilizing the interaction of the different components of the complex with each other and facilitating phosphorylation of β -cat and APC by GSK-3. β -cat phosphorylation earmarks it for degradation by the proteasome. (B) Binding of secreted Wnt protein to its receptor Frizzled activates Dishevelled (Dsh). This leads to inhibition of the Axin-APC-GSK3 complex, which in turn causes free β -cat to accumulate. Stabilized β -cat translocates to the nucleus and is recruited by TCF to activate transcription of Wnt target genes. APC: Adenomatous polyposis coli. TCF: T-cell factor. G: glycogen synthase kinase 3 (GSK3). Figure modified from Waltzer, 1999.

3.2.2 *Drosophila* E-APC and DAPC

The fruit fly *Drosophila* has two APC genes, one encodes the ubiquitous E-APC protein (also known as DAPC2) (McCartney *et al.*, 1999; Yu and Bienz, 1999) and the other, DAPC, is mainly expressed in neuronal cells (Hayashi *et al.*, 1997). Both *Drosophila* APCs can complement the function of human APC and will destabilize β -catenin when introduced into APC mutant cancer cells (Hamada *et al.*, 1999; Hayashi *et al.*, 1997). Moreover, *Drosophila* APCs have been shown to function in various embryonic and larval tissues to antagonize Armadillo (Arm), the *Drosophila* homologue of β -catenin (Ahmed *et al.*, 1998; McCartney *et al.*, 1999; Yu *et al.*, 1999).

3.2.3 Studies on the subcellular localization of E-APC and Tid in the blastoderm embryo

Previous studies on the subcellular localization of E-APC have revealed that this protein is in the cytoplasm, the nucleus and apical-membrane-associated (Yu and Bienz, 1999). Furthermore, it coincides spatially and temporally with the sites at which the adherens junctions are first assembled (Yu *et al.*, 1999). Intact adhesive junctions are important for the polarity of epithelial cells and for the development of epithelia as a whole (Yu and Bienz, 1999). The first discrete subcellular localization of E-APC is seen at the late syncytial blastoderm stage during which E-APC is concentrated in the cortical actin caps that lie above each nucleus. As cellularization begins these caps dissolve and E-APC staining diminish in the cortical caps and starts to associate with the incipient membranes at their apical parts (Yu *et al.*, 1999). From the cellular blastoderm stage onwards it exists E-APC staining in apicolateral regions of all epithelial cells that have been looked at. If these epithelia are viewed from the top E-APC shows a web-like pattern (Yu *et al.*, 1999)(Fig. 26D). Furthermore, studies on Tid expression pattern in development have shown an ubiquitous staining during the whole of the embryonic life with an intense immunoreaction in the larval presumptive sensory organs, the Gopplet cells and in the presumptive gonads (Kurzik-Dumke *et al.*, 1998). In the blastoderm, Tid staining is stronger at the cytoplasmic border on the periphery of the embryos (Kurzik-Dumke *et al.*, 1998). Figure 26 shows anti-Tid (A-C) and anti-E-APC (D-E) stainings of blastoderm embryos in confocal sections through the cortex of the newly formed epithelium shortly after cellularization (at ~2.5 h) (C) and through epidermal

epithelia at ~ 3.5 h of embryonic development (A,B,D,E). The staining of Tid is mainly concentrated in the apical part of the cells (A and D) revealing co-localization of Tid with E-APC at this stage (compare A and D).

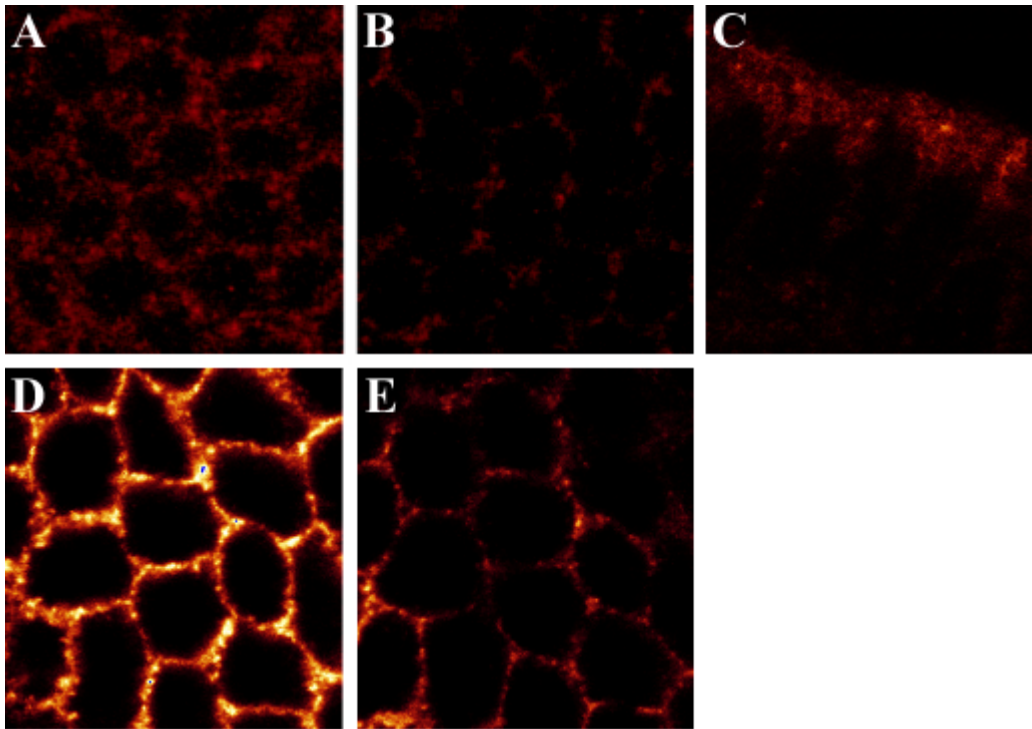


Figure 26. Subcellular distribution of E-APC and Tid in cells of the blastoderm embryo. Confocal sections through the cortex of newly formed embryonic epithelium shortly after cellularization (at ~ 2.5 h) (C) and of epidermal epithelia at ~ 3.5 h of embryonic development (A,B,D,E) stained with anti-Tid antibody (A-C) or anti-E-APC antibody (D,E) (see section 2.2.8.4). (A) and (D) show the apical regions of epithelial cells. (B) and (E) show the mid/basal zones of the same cells. Tid staining is mainly concentrated in the apical regions (A,C) co-localizing with E-APC staining at this embryonic stage (compare A and D).

3.2.4 *In vivo* study to detect a possible binding of Tid to E-APC

In order to test a direct interaction between E-APC and Tid *in vivo* embryonic protein extracts were immunoprecipitated either with anti-Tid or anti-E-APC antibodies (see section 2.2.6). These immunoprecipitates were analyzed by Western blot using anti-E-APC or a biotinylated anti-Tid antibody (see section 2.2.2.6). Immunoblots of embryonic extracts tested with anti-E-APC antibody showed a protein band of ~ 143 kDa (Figure 27 lane 1). Furthermore, another protein band of ~ 100 kDa was also seen in the

same lane. Although E-APC has an expected molecular weight of ~120 kDa there are many other proteins that have another molecular weight as expected in SDS-PAGE. Two examples for that are the GAL4 DNA-BD and AD proteins used in the two-hybrid system (see section 2.2.7). Interestingly, the ~143 kDa protein band could be detected in immunoblots of Tid immunoprecipitates (see Fig. 27 lane 4). To check the validity of this result reverse studies were performed. Immunoblots of E-APC immunoprecipitates were tested with anti-Tid antibody. As it has been shown before, all three Tid bands (50, 47 and 40 kDa in size) could be detected in Tid immunoprecipitates (see Fig.27B, lane 2). However, just the cytosolic Tid47 form was identified in E-APC probes. Together, these results correlate with the co-localization study described above (see section 3.3.3) and classify Tid as a molecular partner of E-APC in the embryo.

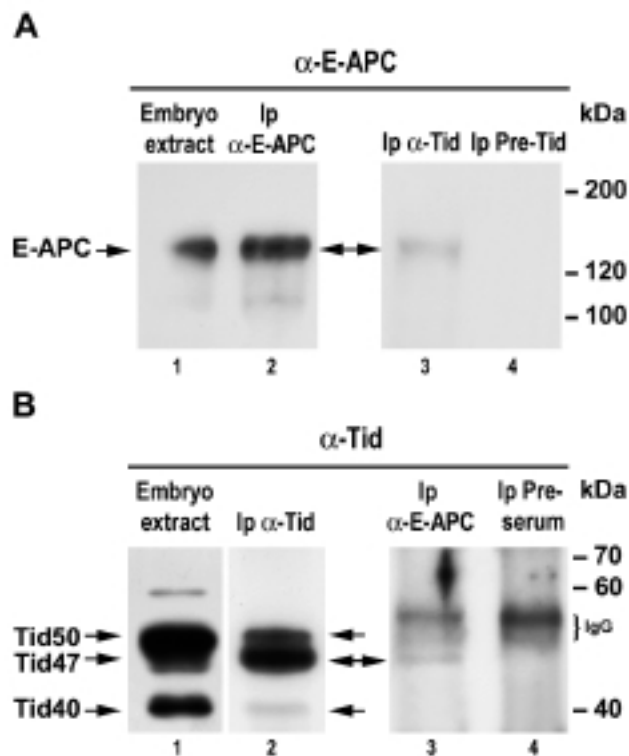


Figure 27. Tid and E-APC do bind *in vivo*. *In vivo* interaction between Tid and E-APC was tested by co-immunoprecipitation. Proteins immunoprecipitated from embryonic and third instar larval extracts with anti-Tid or anti-E-APC antibodies (see section 2.2.6) were separated on a 9% SDS-PAGE and visualized by chemiluminescence after Western blot analysis. For controls, protein extracts were incubated with pre-immuneserum. Embryo extract lanes contain 50 μ g of protein. (A) Western blot of anti-E-APC (lane 2), anti-Tid (lane 3) and pre-immuneserum immunoprecipitates probed with anti-E-APC antibody. A major

band of ~ 143 kDa corresponding to the E-APC protein can be detected in Tid immunoprecipitates (compare lanes 2 and 3). (B) Western blot of anti-Tid (lane 2) anti-E-APC (lane 3) and pre-immuneserum (lane 4) immunoprecipitates probed with biotinylated anti-Tid antibody (see section 2.2.2.6). This blot shows that anti-E-APC specifically co-immunoprecipitated Tid47. Lane 2 and lanes 3-4 (upper and lower panels) include 6% and 16% respectively of the total immunoprecipitated material (see section 2.2.6).

3.2.5 *In vivo* study to detect a possible binding of Tid to DE-Cadherin

It has been described an apical-membrane-associated staining of E-APC which indicates a recruitment of the protein to adherens junctions (Townesley and Bienz, 2000; Yu and Bienz, 1999) (section 3.2.3). These junctions are composed of the transmembrane protein DE-Cadherin which, on the cytoplasmic side of the membrane, links up with actin filaments through binding to Arm and α -catenin (this junctional Arm functions in cellular adhesion, in contrast to the free Arm which transduces the Wg signal). Yu *et al.* (1999) have postulated that E-APC is anchored in zonula adherens not by Arm directly but by an unknown protein that is recruited to adherens junctions from the beginning of zonula adherens formation. To test if Tid could play such a role *in vivo* binding studies to detect a possible interaction of Tid to DE-Cadherin were performed. DE-Cadherin can be seen as a ~150 kDa protein band in immunoblots of normal embryonic extracts probed with the specific antibody (see section 2.2.2.6) (Fig.28 lane 1) (Oda *et al.*, 1994). However, immunoblots of Tid immunoprecipitates tested with anti-DE-Cadherin antibody did not show the expected protein band (Fig.28, lane 2).

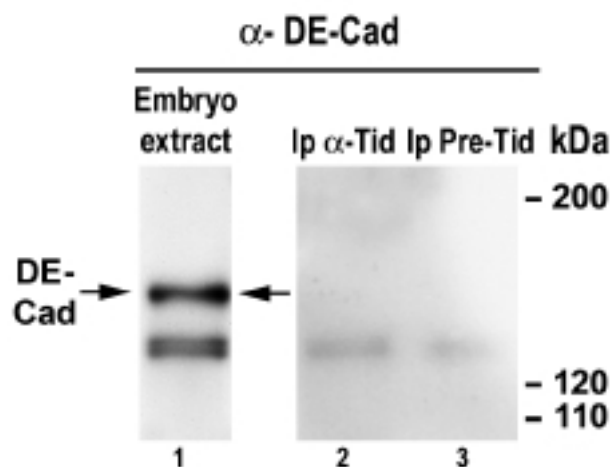


Figure 28. Tid does not bind to DE-Cadherin *in vivo*. To test a possible role of Tid in anchoring E-APC to adherens junctions *via* DE-Cadherin co-immunoprecipitation studies were performed. Proteins immunoprecipitated from embryonic extracts with anti-Tid antibody (see section 2.2.6) or with pre-immuneserum as a control were separated on 9% SDS-PAGE gels and visualized by chemiluminescence after Western blot analysis. Lanes loaded with 50 μ g of embryonic protein extract (lane 1) or with 16% of the total immunoprecipitated material (lanes 2-3) (see section 2.2.6) were probed with anti-DE-Cadherin antibody (see section 2.2.2.6). DE-Cadherin can be detected as a protein band of ~150 kDa in protein extracts but not in Tid immunoprecipitates.

4. DISCUSSION

The *l(2)tid* gene has been identified as a tumor suppressor in *Drosophila melanogaster* (Kurzik-Dumke *et al.*, 1995). Its sequence shows a high homology with all DnaJ chaperones known to date (Kurzik-Dumke *et al.*, 1995). The DnaJ family of proteins serve as regulatory factors to the evolutionary conserved heat shock 70 (Hsp70) superfamily of molecular chaperones (Cyr *et al.*, 1992; Laufen *et al.*, 1999). This protein family is defined by a highly conserved J-domain which functions as the binding region for Hsp70 chaperones and orchestrates their interaction with specific substrates (Kelley, 1999). Furthermore, DnaJ chaperones are shown to be involved in protein folding, protein translocation, signal transduction, renaturation of misfolded proteins and proteolysis (Georgopoulos, 1993; Pfanner, 1994; Cyr, 1994; Gething, 1997). The *l(2)tid* gene encodes four proteins which have been designated as Tid56, Tid50, Tid47 and Tid40 (56, 50, 47 and 40 kDa in size respectively) (Debes, 1997). Using differential centrifugation and sucrose gradient studies Tid56, Tid50 and Tid40 were located in the mitochondria and Tid47 in the cytosol (Debes, 1997). To analyse the function of these proteins in the cell, studies on identification of its molecular partners have been carried out. Protein interaction studies are a useful tool to give proteins a functional context in the organism. They have been used for example in the analysis of regulatory complexes controlling the mitotic checkpoint (Chan *et al.*, 2001), the molecular interactions of the p53 family of proteins (Kojima *et al.*, 2001) or the establishment of protein interaction maps (Stanyon and Finley, 2000).

4.1 Tid has specific molecular partners during *Drosophila* development

To examine the presence of binding partners of the Tid proteins during *Drosophila* development immunoprecipitation techniques were applied (section 2.2.6). Western blot analysis of biotinylated Tid immunoprecipitates (see Fig.7) resulted in the identification of specific sets of binding proteins for each developmental stage. Furthermore, Tid was shown to have a higher amount of molecular partners in embryonic, third larval and pupal stages. Since some of the protein bands detected on the blot are present in several

developmental stages Tid may have a ubiquitous function during development. Confirming the results obtained in plain developmental Western blots (Debes, 1997; Kurzik-Dumke *et al.*, 1998), bands corresponding to the Tid50, Tid47 and Tid40 proteins can be detected in immunoprecipitates of embryonic and larval extracts (Fig14). Interestingly, Tid47 appears to be in higher amounts in immunoprecipitates compared to normal protein extracts prepared under denatured conditions. Furthermore, the concentration of Tid40 in this immunoprecipitates is reduced. This effect could be due to incomplete disruption of mitochondria with the mild lysis buffer used. Moreover, the fact that Tid50 can be extracted faster than Tid40 from mitochondrial inner membranes with 1M NaCl solution may have also an influence (Debes, 1997).

4.2 Ptc has been identified as a novel binding partner of Tid

The presence of specific binding partners of the Tid proteins *in vivo* demonstrated via immunoprecipitation formed the basis to further investigate the identity of these interactors. The two-hybrid system was chosen for this purpose because it presents an elegant way to detect protein-protein interactions *in vivo* (Bartel and Fields, 1995). This yeast-based genetic assay was used to screen an expression library of *Drosophila melanogaster* with a “bait” containing most of the Tid protein in order to reach many of the detected cellular interactors in immunoprecipitation (see section 3.1.2). Several potential positive clones were identified as a result of this screening. These clones coded for proteins such as Cytochrome B or ribosomal RNA, two of the most common false positives in the two-hybrid assay (Hengen, 1997). Furthermore, a homologue of the L35 ribosomal protein in *Drosophila melanogaster* could be also identified and an interesting clone coding for Shaggy, a protein kinase involved in the Wingless (Wg) pathway, was also isolated. However, the only potential positive clone identified as a true interactor of Tid in this assay was that coding for the membrane receptor Patched (Ptc). The Ptc protein is a key regulator of Hedgehog (Hh) signaling (Johnson *et al.*, 2000). In *Drosophila*, *hh* plays an important role in regulating cellular identity and proliferation, in most cases by mediating the local cell-cell interactions that control the transcription of *wg* and of a second signal-encoding gene, *decapentaplegic (dpp)* (Ingham, 1998) (Fig 11). Ptc, together with the transmembrane protein Smoothed (Smo), plays an important role in receiving and regulating Hh signals. Ptc functions in

Hh binding and Smo functions in transducing the signal (Johnson *et al.*, 2000). In the absence of Hh, Ptc is proposed to inhibit Smo to prevent target gene activation. In the presence of Hh, Ptc inhibition of Smo is relieved, presumably by binding of Hh to Ptc (Aza-Blanc and Kornberg, 1999) (Fig.11). The binding of Tid to Ptc described in the two-hybrid system was confirmed using independent biochemical methods such as the GST-pulldown assay and co-immunoprecipitation (see section 3.1.3). The GST-pulldown assay is a widely used system to detect protein-protein interactions *in vitro* (Cosson *et al.*, 1998; Uhrig *et al.*, 1999). In this assay differently tagged Tid and Ptc proteins bound efficiently to each other in the absence of any cellular cofactors (Fig.13). However, the two-hybrid system and the pulldown assay do not address the biological significance of the interactions observed. A good step to show biological significance is to verify the interaction by co-immunoprecipitation from a tissue in which both proteins are expressed (Brent and Finley, 1997). Interestingly, Ptc could be identified in Tid-immunoprecipitates from embryonic and larval extracts (Fig. 14) confirming the results of the GST-pulldown assay and the two-hybrid system. This may be of particular importance because it classifies Tid as a novel component of the Hh signaling pathway. Other cases of chaperones involved in signaling pathways have been described. The case of P58IPK in a protein kinase signaling cascade (Gale *et al.*, 1998; Melville *et al.*, 2000) or the involvement of hsp70/hsp40 complexes with the Retinoblastoma and p53 proteins, two tumor suppressors involved in important developmental pathways, are examples of these (Hansen *et al.*, 1996; Nihei *et al.*, 1993).

4.3 Tid47 may be the form involved in the interaction with Ptc

Several Tid forms are present in the cell (see Fig.6). The precursor, Tid56, has been only identified in the matrix of enriched mitochondrial fractions (Kurzik-Dumke *et al.*, 1998). Tid50 and Tid40 are also present in mitochondrial fractions associated to the inner membrane of the organelles, whereas Tid47 has been localized in the cytosol (Kurzik-Dumke *et al.*, 1998). Ptc is a transmembrane receptor localized to the apical regions of the lateral cell membranes in imaginal discs (Denef *et al.*, 2000). Because of its distribution pattern the only Tid protein able of binding to Ptc would be Tid47. Interestingly, Tid immunoprecipitates including Ptc contain high amounts of this protein (see Fig.14). But little is known about Tid47 location in the cytosol. Analogous to mTid-

1, Tid47 cytoplasmic location could be due to post-translational modifications that would retain this protein in the cytoplasm or signalize Tid exit from the mitochondria (Trentin *et al.*, 2000). Specific export mechanisms by which certain proteins exit mitochondria have been already described (Soltys and Gupta, 2000).

4.4 Tid and Ptc domains involved in the interaction

Studies to identify the protein domains involved in the binding between Tid and Ptc have been carried out. The isolated Ptc clone coded for the C-terminal cytosolic region of the protein (see Fig.10). Ptc has two other cytosolic sections but the two-hybrid binding assay implies an interaction involving just the last 155 amino acids of the protein. Therefore, further studies to identify the Tid binding domain in Ptc have not been required. On the other hand, characterization of the Tid domains involved in this interaction was taken into account. As it has been described before Tid has four different domains (Fig.3): i) the DnaJ domain, which defines a protein as a member of the DnaJ family and interacts with Hsp70 stimulating its ATPase activity; ii) the G/F domain that appears to be a flexible linker; iii) the Zn finger peptide binding domain and iv) the C-terminal domain, which has yet to undergo thorough characterization but appears to be a more specialized region for binding some DnaJ substrates (Ohtsuka and Hata, 2000) (Cheetham and Caplan, 1998). Several Tid domain combinations were tested for binding to Ptc in a two-hybrid assay (see section 3.1.4.1). Surprisingly, it was not possible to define a specific domain for interaction. None of the domains alone was capable of binding to Ptc. Furthermore, a complete DnaJ domain is not necessary for the interaction. Although the pAS2-1-Tid^{aa 98-495} construct included half of this region and did not contain the characteristic HPD domain it retained its binding capacity. Moreover, the presence of the less conserved C-terminal region was partially required as constructs missing the complete domain failed for interaction. There are other examples in literature describing the requirement of multiple domains for protein interaction such as the binding between HIV TAT and TAF(II)250 (Weissman *et al.*, 2001) or the binding of pRB to PP1delta (Bianchi and Villa-Moruzzi, 2001). Mutagenesis studies are the future approach to be used in order to identify important functional regions involved in the binding of Tid to Ptc. Furthermore, bioinformatic tools such as the Consurf tool (Armon *et al.*, 2001) or other multiple-sequence alignment programs should provide

information about hot spots and surface patches that are important for Tid binding to other proteins.

4.5 Structural characterization of the *l(2)tid*-tumorous wing imaginal disc

The binding of Tid to Ptc has been proved using biochemical studies (see above). To examine the function of this interaction in the fly several stainings on *l(2)tid*-tumorous wing discs and on those from a viable *ptc* mutant stock were done to monitor a possible change in expression of the proteins. However, the *l(2)tid*-tumorous wing disc does not present a normal phenotype when compared to its *wild-type* counterpart. Anatomical investigations of homozygous mutant larvae revealed enlarged and morphologically abnormal imaginal discs having a highly distorted epithelial folding pattern (Kurzik-Dumke *et al.*, 1992). Therefore, to better interpret the results from these immunohistochemical studies on this abnormal structure and to identify the regions of the disc involved in tumor formation a rough characterisation of boundaries and overgrown regions in the tumorous disc has been carried out. To achieve this, three different enhancer-trap lines driving GFP expression in defined parts of the disc and antibody stainings against Engrailed, Cubitus interruptus (Ci) and Wingless proteins were used (see section 3.1.5.1). The outcome of this study revealed that most of the tumor corresponds to anterior parts and regions of the dorsal hinge and the dorso/ventral (d/v) wing surfaces (see Fig.19 and 20). On the other hand, the notum and the anterior/posterior (a/p) boundary seem not to play a crucial role in the development of the tumorous tissue (see Fig.19). Together, these results may involve Tid in developmental controls concerning the regulation of gene expression in the anterior region of the wing disc. Moreover, the d/v boundary defined by Wg expression was disarranged in the tumorous tissue (Fig.21). Nevertheless, it is not known if Tid has a direct effect on the establishment of this boundary or if this disrupted pattern of expression is a result of the autonomous growth of the neoplastic tissue. Further studies should help clarifying the involvement of Tid in these processes.

4.6 Tid influence on the Hh signaling pathway

To analyze the function of Tid in the Hh signaling pathway, expression of Ci and Ptc has been used as a reference to monitor a positive activation of the cascade in *l(2)tid*-tumorous tissue. Ptc is a reliable indicator of Hh signaling in wing imaginal discs (Capdevila *et al.*, 1994). Furthermore, recent studies have demonstrated an absolute requirement of Ci for all examined aspects of Hh outputs (Methot and Basler, 2001). Ci is the *Drosophila* homolog of the vertebrate Gli proteins (Alcedo and Noll, 1997; Ingham, 1998) and acts as a transcription factor to regulate Hh target genes. In presence of the Hh signal, Ci is converted to transcriptional activator, the phosphorylation of Ci is altered and the limited proteolysis that converts Ci to a transcriptional repressor is inhibited (Ramirez-Weber *et al.*, 2000) (see Fig.11). Immunostainings with anti-Ptc antibody on *l(2)tid*-tumorous tissue showed high levels of Ptc expression not only at the a/p boundary as in *wild-type* discs but also in most of the anterior compartment of the tumorous wing disc (Fig.22). Moreover, the expression of Ci is also upregulated in the anterior part of the tumorous structure (Fig.23). It has been shown in previous studies that the a/p boundary is conserved in *l(2)tid* tumorous imaginal discs (see section 3.1.5.1). Therefore, increase in Ptc and Ci expression may be due to loss of Tid control over the Hh signal transduction pathway in anterior regions of the wing disc. Moreover, this result discards a possible role of Tid in controlling Ptc binding to Hh, otherwise Ptc and Ci expression should have been suppressed or at least reduced in the tumorous tissue (Capdevila *et al.*, 1994).

4.7 Tid and the Hh receptor complex

Tid has been identified as a possible inhibitor of Smo constitutive signal transduction in the Hh signaling pathway (see above). In the cell, Smo intrinsic activity is inhibited by Ptc to prevent target gene activation (Ingham *et al.*, 1998). The mechanism by which Ptc regulates Smo is not understood but the observation that the two proteins were co-immunoprecipitated from transfected cells first led to the idea that Ptc and Smo are present in a preformed complex (Stone *et al.*, 1996). Hh binding to Ptc would induce a conformational change in the complex alleviating Ptc-mediated repression on Smo (Alcedo and Noll, 1997; Chen and Struhl, 1998; Stone *et al.*, 1996). Interestingly, recent

studies have identified the C-terminal domain of Ptc as an important regulatory region (Ingham *et al.*, 2000; Johnson *et al.*, 2000). Deletion of the final 156 residues of Ptc permits Hh sequestration but abolishes inhibition of Smo and so of Hh targets (Johnson *et al.*, 2000). Since Tid binds to Ptc on its C-terminal region (see section 3.1.2.1) studies to clarify if Tid influence on this pathway may occur through a direct interaction with Smo were performed. Tid immunoprecipitates of embryonic extracts were tested with anti-Smo antibody but no presence of this protein could be observed on the blots (Fig. 24). Recently, Denef *et al.* have postulated an indirect regulation of Smo by the C-terminal part of the Ptc protein in the Hh pathway. They favor the model that the constitutive activity of Ptc stimulates activity of a phosphatase (probably a type 2A protein phosphatase) that leads to reduced phosphorylation of Smo (Denef *et al.*, 2000). It has been already described an implication of a DnaJ homolog (Hsp40) in controlling the phosphorylation state of a protein (interferon) (Melville *et al.*, 1997). Hsp40 forms a complex with p58IPK through a DnaJ domain at the C-terminal part of the protein (Melville *et al.*, 2000). Under cellular stress (for example viral infection) hsp40-p58IPK inhibitory complexes are dissociated (Gale *et al.*, 1998). Once released, p58IPK functions to repress PKR-mediated phosphorylation of the eukaryotic initiation factor 2alpha subunit (eIF-2alpha) through direct interaction, thereby relieving the PKR-imposed block on mRNA translation and cell growth (Gale *et al.*, 1998). A similar role for Tid could also be possible. The absence of Tid in the Hh receptor complex would relieve the control over the phosphatase leading to activation of the Hh pathway in the cell. Further studies should help identifying new players in this context.

4.8 Influence of other downstream components of the Hh pathway on tumor formation

In wing imaginal discs Dpp is one important outcome of the Hh signaling pathway (Capdevila *et al.*, 1994) (see Fig.11 and 29). Dpp has been proposed to function as a morphogen to pattern cell fields in a number of developmental contexts (Podos and Ferguson, 1999). In the developing wing disc Dpp is expressed in a narrow stripe of cells that are adjacent to the a/p compartment boundary (Ramirez-Weber *et al.*, 2000). Interestingly, by abolishing Ptc function it has been observed a misexpression of Dpp leading to overgrowth of the anterior part of the wing disc (Capdevila *et al.*, 1994). It has

been already mentioned that Tid seems to mimic the effect of Ptc function in the control of Hh signaling. Therefore, it should be expected a misexpression of Dpp in *l(2)tid*-tumorous tissue. Further studies such as immunostaining or clonal analysis should be directed in order to analyze the importance of Dpp in tumor development.

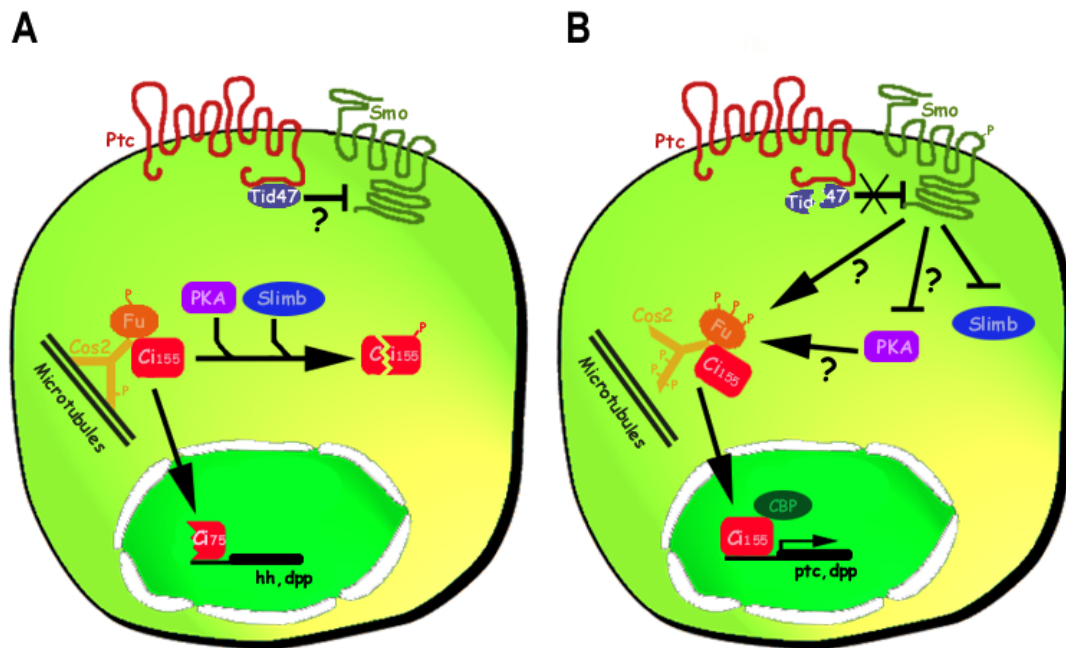


Figure 29. Regulatory function of Tid in the Hh signaling cascade. During the work of this thesis Tid has been identified as a novel binding partner of Ptc, an important component of the Hh signaling pathway (see section 3.1.2). Furthermore, Tid has been shown to have a negative effect on Smo constitutive signal transduction (see section 3.1.5). However, this effect does not occur through a direct interaction of Tid with Smo (panel A, question-mark)(see section 3.1.6). Therefore, Figure 11 has been modified to introduce Tid action in the Hh signaling pathway. For more details on this cascade see section 3.1.2.2 and Figure 11. (A) In absence of Hh signaling Tid47 inhibits Smo signal transduction – by an unknown mechanism – to keep the pathway deactivated. (B) Resembling the situation in *l(2)tid*-tumorous discs, in absence of Tid47 Smo signal transduction and the expression of downstream components such as Ci¹⁵⁵ and Ptc is activated.

4.9 Ptc control of Tid expression

Immunohistochemical analysis with anti-Tid antibody on wing discs from a *ptc* viable allele (*ptc^{tuf-1}*) did not show any perceptible changes in the expression pattern of the Tid proteins (see Fig.22). Further, wing discs of larvae carrying mutations for the *tid¹* and

ptc^{tuf-1} alleles showed the characteristic Tid phenotype. What is more, Tid and Ptc expression in discs lacking both alleles was comparable to that in the single mutant tumorous tissue (see Fig.22). These results let postulate that Ptc genetically acts downstream of Tid in cells that receive the Hh signal in wing imaginal discs. However, for further insights about a possible effect of the Hh signaling pathway on Tid expression clonal analysis should be carried out. This site-specific turn-off of gene expression in different regions of the wing disc would help to precisely analyze the existence of a possible effect of the Hh pathway on Tid expression.

4.10 hTid-1 and basal cell carcinomas

Basal cell carcinoma (BCC) is the most frequent skin cancer in the white population (Miller, 1991). BCCs mostly occur sporadically in relation to sun exposure although their incidence has increased significantly in some rare genetic disorders. For instance, patients with the nevoid BCC (NBCC) syndrome, also known as Gorlin's syndrome, have numerous BCCs together with characteristic developmental abnormalities (Gorlin, 1987). Evidently, the gene responsible for BCCs must be crucial in both normal development and regulation of cell division (Booth, 1999). Recent work has implicated the human gene homologous to the *Drosophila* segment polarity gene *ptc* in this type of cancers (*PTCH1*) (Hahn *et al.*, 1996; Johnson *et al.*, 1996). Moreover, *PTCH1* has been involved in medulloblastoma and rhabdomyosarcoma formation (Taipale *et al.*, 2000). Since studies on the human Hh signaling pathway have revealed a high degree of conservation with the *Drosophila* signaling cascade (Hunter, 1997) it would be interesting to study the role of hTid-1 in this pathway and in BCCs. A first approach has been done using the two-hybrid system to test the binding capacity between *PTCH1* and hTid-1 with analogous constructs to the *Drosophila* vectors (see section 3.1.4.2). Unfortunately, no interaction between the hybrid proteins was detected in this study. This could have several reasons. Post-translational modifications may not be always completed in a yeast expression system (Bartel and Fields, 1995). Moreover, hTid-1 and mTid-1 proteins have been reported to be toxic in many cell systems (Kurzik-Dumke, pers.com.). Therefore hTid-1 and *PTCH1* recombinant constructs should be tested for expression in yeast. Furthermore, there are three Ptc homologues in humans. *PTCH1* has been chosen for this study as it shows the highest homology degree to the *Drosophila*

protein (Fig.4). However, the recently described PTCH2 should be also tested for interaction (Zaphiropoulos *et al.*, 1999). The best way to detect an implication of hTid-1 on BCC formation *in vivo* would be to molecularly characterize possible mutations of the gene in tumorous tissue as it has been done before for PTCH1 (Bodak *et al.*, 1999). Similarly to the *Drosophila* counterpart, hTid-1 could play an important role in regulating the signaling cascade because about three quarters of PTCH1 mutations are predicted to prematurely truncate the protein, eliminating its C-terminal part (Chidambaram *et al.*, 1996; Wicking *et al.*, 1997). Other possibilities would be to immunoprecipitate both proteins from extracts or to use the newly derived mammalian two-hybrid system.

4.11 Tid binding to E-APC is conserved in evolution

The *Drosophila* E-APC (epithelial adenomatous polyposis coli) protein is associated with adherens junctions in epithelial cells and functions to antagonise Armadillo (Arm) (Bienz, 1999). Its human homolog, APC, has been linked to the progression of colon cancers and is a component of the Wnt/Wg signaling pathway (Polakis, 1999). Similarly, loss of hTid-1 expression has been shown to correlate in most of the investigated cases with colon tumor progression (Kurzik-Dumke, pers.com.). Since hTid-1 has been found to associate *in vitro* (Polakis, 1997) and *in vivo* (Kurzik-Dumke, pers.com.) with APC, co-localisation and immunoprecipitation studies on the *Drosophila* counterparts, Tid and E-APC, have been carried out to test for a conserved function in evolution. In the blastoderm embryo E-APC and Tid co-localize in the apical part of the cells indicating a possible membrane association of Tid during cellularization (Fig.26). Furthermore, *in vivo* binding was confirmed by immunoprecipitation with Tid and E-APC antibodies (Fig.27). Again, cytosolic Tid47 is the protein implicated in the interaction. Anchoring of E-APC to adherens junctions has been postulated to occur indirectly through an unknown protein (Yu *et al.*, 1999). To test if Tid could play such an anchor role in the cell, Tid immunoprecipitates were checked for the presence of DE-Cad, an important component of these junctions (Townesley and Bienz, 2000) (Fig.28). However, this was not confirmed in this experiment. The function of Tid in the cell in relation to E-APC still remains to be discovered. Interestingly, Polakis *et al.* (1997) have postulated an auxiliary role for hTid-1 in the turnover of β -catenin (the

counterpart of Arm in human) similar to the requirement for the rapid turnover of the cyclin Cln3 of another DnaJ homolog in yeast (Ydji) (Yaglom *et al.*, 1996). Further, one putative positive isolated in the two-hybrid screening corresponded to the *Drosophila* Shaggy protein (see section 4.1.2). Its human counterpart, GSK3, phosphorylates β -catenin, targeting it for subsequent degradation by the proteasome pathway (Maniatis, 1999) (Fig.25). Once more Tid may be involved in the control of the phosphorylation-state of a known oncogene.

5. RESUME

The subject of this work has been the identification of molecular partners of the proteins encoded by the *Drosophila melanogaster* *dnaJ*-like tumor suppressor gene *lethal(2)tumorous imaginal discs (l(2)tid)* in order to give them a functional context in the organism. Mutations in this gene cause, in the homozygous state, tumorous imaginal discs (Kurzik-Dumke *et al.*, 1992). The first part of this study describes different approaches used to identify the molecular partners of the Tid proteins in the cell. Immunoprecipitation of protein extracts from all developmental stages revealed specific binding partners during development. To characterize them, the screening of an expression library via the yeast two-hybrid system was performed. This assay resulted in the identification of Patched (Ptc) as a novel Tid binding protein. Ptc is a key regulator of the Hedgehog (Hh) signaling pathway, which is conserved in evolution and implicated in some types of human cancer (Johnson *et al.*, 2000). Tid/Ptc interaction was confirmed using independent biochemical methods such as the GST-pulldown assay and co-immunoprecipitation. The next part of this work examines whether changes in expression of the interacting proteins can be observed in mutant wing discs. First of all, studies to map the regions of the disc involved in tumor formation were performed. Three different enhancer trap lines driving the expression of UAS-GFP in defined parts of the wing disc helped identifying the dorsal hinge and dorsal wing surfaces as potential proliferation focus. Further delimitation of the neoplastic tissue was performed by double staining with antibodies directed against the proteins Engrailed and the active form of Cubitus interruptus (Ci) which are specifically expressed either in the posterior region or in the anterior part of *wild-type* wing discs (Motzny and Holmgren, 1995; Tabata and Kornberg, 1994). This study revealed that most of the tumor arises from the anterior part of the structure. Moreover, the dorso/ventral boundary defined by Wg expression was disarranged in the tumorous tissue. To understand the functional relationship between Tid and Ptc in the cell, antibody stainings on *tid*¹ and *ptc*^{tu^f-1} mutant discs were carried out. Ptc is a reliable indicator of Hh signaling in wing imaginal discs. Interestingly, there is an upregulation of Ptc expression throughout the anterior compartment in the tumorous tissue. On the other hand, no perceptible changes in Tid expression are observed in discs mutant for Ptc. Furthermore, wing discs extracted from

larvae carrying mutations for both genes showed the characteristic Tid phenotype and pattern of expression. These results let postulate that Ptc acts genetically downstream of Tid in wing imaginal discs. To further prove a positive activation of the Hh signaling cascade in the tumorous tissue, expression of Ci has been used as a reference. Ci is a downstream component of the pathway shown to be required for all examined aspects of Hh outputs (Methot and Basler, 2001). As expected, Ci staining in tumorous discs correlates with Ptc staining in this tissue (see above). These results may be an indication of an inhibitory function of Tid on Hh signal transduction. Finally, since Adenomatous polyposis coli (APC) has been shown to interact with the human homologue of Tid *in vitro* and in the two-hybrid system (Polakis, 1997), co-immunoprecipitation studies on the *Drosophila* counterparts, E-APC and Tid, were performed. These assays revealed a direct interaction of the proteins *in vivo*.

6. BIBLIOGRAPHY

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Abbreviations and Measuring-units

AD	Activation Domain
Acc.N°	Accession Number
BD	Binding Domain
bp	Basepairs
BSA	Bovin Serum Albumin
cDNA	copy-DNA
°C	Grad Celsius
DAB	3,3'-Diaminobenzidine
DNA	Desoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylendiamintetraacetat
<i>et al.</i>	<i>et alii</i> or <i>et aliorum</i>
Fig.	Figure
g	Gram
h	Hour
kb	Kilobase
kDa	Kilodalton
l	Liter
min	Minute
M	Mol; molar
mA	Milliampere
mg	Milligram
µg	Microgram
mM	Millimol; millimolar
µM	Micromol; micromolar
pers.com.	Personal communication
POD	Peroxidase
RNA	Ribonucleic acid
SDS	Sodiumdodecylsulfate
TBE	Tris-Borat-EDTA buffer
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) aminomethan
U	Unit
wt	<i>wild-type</i>

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