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**A SCREEN FOR MUTATIONS AFFECTING PNS  
DEVELOPMENT IN *DROSOPHILA* IDENTIFIES  
THE TRIM GENE, *DAPPLED*.**

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"Äntligen!"

- Source unknown.

FOR JILL



## ABSTRACT

The peripheral nervous system of *Drosophila melanogaster* contains a variety of sense organs, ranging from the relatively simple four celled bristle organ to the more complex compound eye. The development of each organ type is well described, providing a useful backdrop for functional studies of genes acting in one or more of the many processes involved in organogenesis. We have used the bristle organ to screen for genes affecting PNS development. Two of the candidates recovered via this approach, *string* (*stg*, *Drosophila* *cdc25*, the universal regulator of the G2 to M phase mitotic transition), and *dappled* (*dpld*, a poorly described gene implicated in tumor suppression) were selected for further study. Examination of *stg* mis-expression phenotypes in the adult bristle organ revealed cell fate transformations corresponding to the generation of two pIIa structural precursor cells at the expense of a neural precursor cell. This transformation most reasonably resulted from an abnormally short G2 arrest, indicating that the time spent in the G2 phase is crucial to correct cell fate determination.

*dpld* is a member of the Tripartite Motif (TRIM) superfamily, members of which are involved in diverse biological processes *e.g.* proliferation, apoptosis and immune response. *dpld* belongs to a subgroup of NHL domain containing TRIM proteins, that are known to be involved in tumor suppression. Phylogenetic analysis placed *dpld* in the *lin-41* sub-clade of the TRIM superfamily. A combination of in-silico, genetic and cell culture assay approaches showed *dpld* to be susceptible to miRNA regulation. As homologous genes are also miRNA regulated this regulatory mechanism may be conserved throughout this sub-clade, between vertebrates and invertebrates.

Pre-existing loss of function *dpld* alleles were characterized, however, subsequent complementation studies revealed that characteristic aspects of the described *dpld* phenotype, in fact mapped outside the *dpld* locus, and were caused by mutations of nearby genes. The tumor-causing locus was mapped to the *Cytb5* gene (mutated in both pre-existing *dpld* alleles), while the embryonic lethality and PNS phenotype was mapped to the *scraps* locus. *scraps* encodes for *Drosophila* Anillin, known to be required during cytokinesis. We provide the first characterization of *scraps* null alleles and detail a biased requirement for *scraps* within neural precursor cells of the embryonic PNS.

A novel loss of function *dpld* allele was recovered. This mutation is lethal, however it does not have an associated tumor phenotype. This finding, together with our complementation study indicates that the existing classification of *dpld* as a tumor suppressor is inaccurate. Subsequent studies detail *dpld* requirements in the developing fly retina. There, *dpld* mutation resulted in excessive proliferation, while conversely, mis-expression caused a reduction. Additionally, and perhaps consequently, cell differentiation was affected. Thus, regulation of proliferation by NHL-TRIM genes seems a conserved feature. We additionally identified a novel *Drosophila* TRIM gene of the same class as *dpld*, which we have dubbed *another b-box affiliate* (*abba*), bringing the number of NHL containing TRIM genes in *Drosophila* to four.

## LIST OF PUBLICATIONS

- I. O'Farrell F. and Kylsten P. **A mis-expression study of factors affecting *Drosophila* PNS cell identity.** Biochem. Biophys. Research Comm. In Press.
- II. O'Farrell F. and Kylsten P. ***Drosophila* Anillin is unequally required during asymmetric cell divisions of the PNS.** Biochem. Biophys. Research Comm. 2008; 369: 407-413
- III. O'Farrell F., Muñoz-Alarcón A., Georgiev A. and Kylsten P. **Functional Analysis of *Drosophila* lin-41, dappled during adult eye development.** Submitted.
- IV. O'Farrell F., Esfahani S.S., Engström Y., and Kylsten P. **Regulation of the *Drosophila* lin-41 homologue dappled by let-7 reveals conservation of a regulatory mechanism within the LIN-41 subclade.** Developmental Dynamics. 2008 Jan;237(1):196-208.

## ABBREVIATIONS

|         |  |
|---------|--|
| ABBA    | Another B-box Affiliate                            |
| AC      | Achaete  |
| ASC     | achaete-scute complex                              |
| ato     | atonal   |
| bHLH    | Basic Helix loop Helix                             |
| BRAT    | Brain tumor  |
| Ch      | Chordotonal  |
| CNS     | Central nervous system                             |
| Cytb5   | Cytochrome B5                                      |
| DI      | Delta  |
| DPLD    | Dappled protein                                    |
| dpld    | dappled gene                                       |
| DPP     | Decapentaplegic                                    |
| ECM     | Extracellular matrix                               |
| EGFR    | Epidermal Growth Factor Receptor                   |
| ES      | External Sensory                                   |
| Lch5    | Lateral Chordotonal Five or pentascolopodial organ |
| lola    | longitudinals lacking                              |
| MD      | Multidendritic                                     |
| miRNA   | micro-RNA  |
| MEI-P26 | Meiotic Protein-26                                 |
| NB      | Neuroblast   |
| PAV     | Pavarotti  |
| PNS     | Peripheral Nervous System                          |
| PROS    | Prospero   |
| pIIa    | Precursor cell IIa etc                             |
| RING    | Really interesting new gene                        |
| sca     | scabrous   |
| SC      | Scute  |
| SOP     | Sensory Organ Precursor                            |
| stg     | string   |
| TRIM    | Tripartite Motif                                   |
| TRP     | Transient Receptor Potential ion channel           |



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# INTRODUCTION

## **The Peripheral Nervous System of *Drosophila*.**

The Peripheral Nervous System (PNS) of *Drosophila melanogaster* contains a wide range of sensory organ types that collectively interpret the animal's environment. External stimuli such as light, odor and movement within the immediate surroundings provide vital cues to the animal *e.g.* circadian rhythm maintenance, food source and potential danger (or potential mate) detection, respectively. The appropriate interpretation of these external signals via the various specialized sense organs can therefore be crucial to the animal. Both the larva and adult fly host a magnificent array of structurally distinct sense organs (sensilla), stereotypically positioned over the entire body. Sensilla are composed of both structural and neuronal cell types. The structural components of the various organs are morphologically specialized, likely adapted to confer optimal sensitivity to the specific stimuli they receive. In the majority of sensilla, these components are suitably located on the exterior surface of the animal, facilitating sensation of the external environment (External Sensory (ES) organs) while the neuronal components lie insulated from the exterior surface, beneath the epidermal layer (see Figure 1). At least two prominent exceptions to this exist; Chordotonal (Ch) organs, which have both structural and neuronal components beneath the epidermal layer (Matthews et al., 1990), and Multi-dendritic (MD) neurons, which are not obviously associated to other cell types, but are instead autonomously sensitive to certain stimuli (Tracey et al., 2003; Song et al., 2007) see Figure 2.

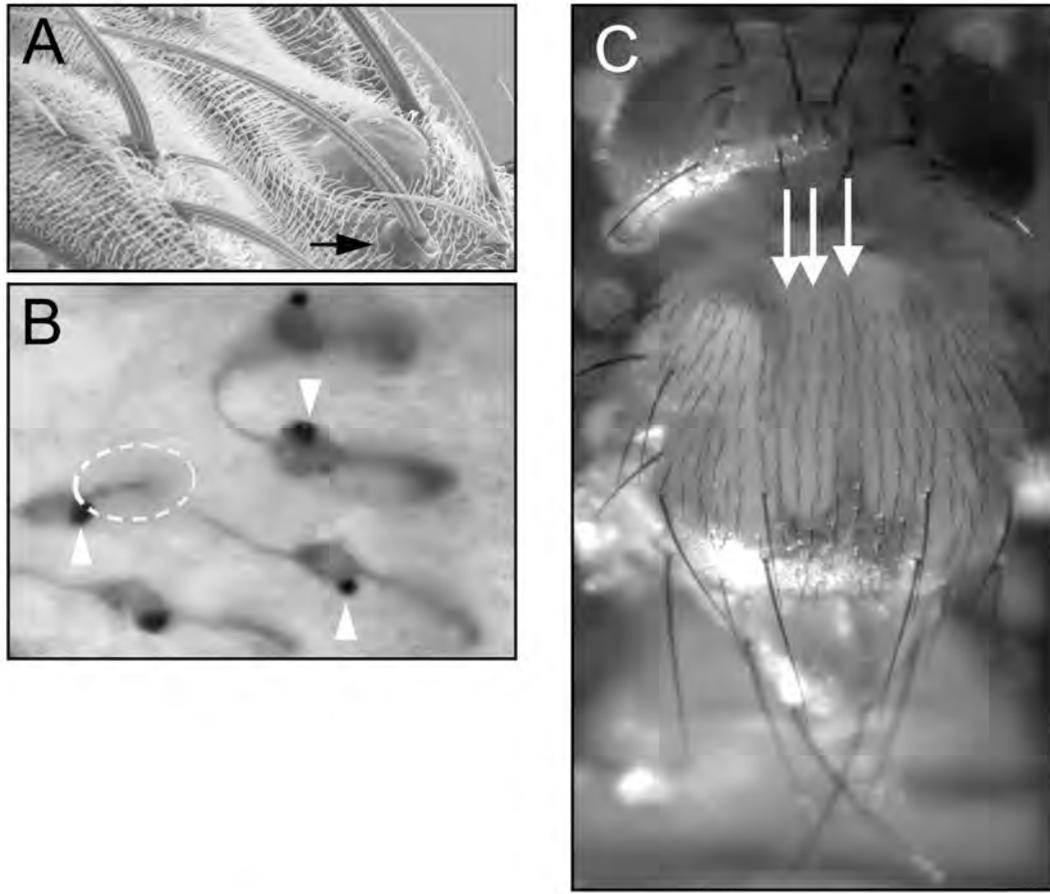
The structural cells of the PNS facilitate the transmission of stimuli to the internal neuronal cell. This (often external) stimulus must be transduced to a neural impulse, interpretable by the animal's brain. The physical interface between the structural cell and neuron must facilitate this. Here, the transmembranous protein Transient Receptor Potential (TRP) ion channels are of particular relevance. TRP's are a family of broadly conserved ion channels, with demonstrated expression in organisms ranging from yeast to vertebrates. Activation of the channel results in an influx of ions from the exterior of the cell and can occur in response to diverse stimuli, *e.g.* mechanical strain, temperature, osmotic pressure and volatile substances, for review see (Christensen and Corey, 2007; Venkatachalam and K. Montell, 2007). To facilitate activation by mechanical means, specific TRP subtypes possess intra- and/or extracellular protein domains thought to enable protein-protein associations that bridge

the interior to the exterior of the cell, *e.g.* forming stable attachments with both the neuronal cytoskeleton interiorly and specialized extracellular structures. Such physical associations permit immediate activation of the TRP channel, as determined by the (short) interval between mechanical stimulation and neuronal depolarization (Walker et al., 2000). TRP channels are not however limited to direct mechanical stimuli but can be also activated indirectly via second messenger systems, *e.g.* the *Drosophila* Rhodopsins, in the photoreceptive neurons of the eye (Dolph et al., 1994; Niemeyer et al., 1996). TRP ion channels are expressed and facilitate sensation in the PNS organ types most relevant to this work, the ES, MD, Ch and the eye.

### External Sensory Organs.

The larval PNS develops during embryogenesis, whilst the adult PNS forms during larval and pupa stages. Relatively few larval PNS cells are kept to adulthood. The physical differences between these two stages naturally require that the PNS be rebuilt during adult tissue development in a manner fitting the physical appearance of the adult fly and its many appendages. Both larval and adult PNS organs are generated in a strikingly analogous manner that I will attempt to describe.

Both the larva and adult possess numerous sensory bristle organs (thousands in the adult case), including the mechanosensory ES-organs that confer sensitivity to touch (Walker et al., 2000). Other adult bristle organs, such as those located on the antennae, while appearing morphologically similar, are in fact specialized chemosensory organs, having porous bristle structures that facilitate the animals sense of smell (odorant sensilla) and taste (gustatory sensilla) see (Vosshall and Stocker, 2007). Within the adult, the mechanosensory ES-organs (referred to simply as ES-organs from here onwards) can be sub-divided into two groups based on size, macro- (large) and microchaetae (small). Both organ types are composed of a single enervated bristle, held in place via a socket cell embedded within the epidermis, a neuron and supporting sheath cell below the epidermal layer (see Figures 1 and 2). The sheath cell envelopes and insulates the neuronal dendrite from the surrounding cellular environment and moreover, secretes a specialized extracellular matrix (ECM) structure around the tip of the ES-neuronal dendrite, known as the dendritic sheath. This dendritic sheath structure represents the interface between the structural and neuronal cells of the organs. It lays in contact with both the neuronal dendrite and the base of the bristle shaft, within a  $K^+$  rich endolymph filled pocket, formed by the socket cell, see (Jarman, 2002; Kernan, 2007) and Figure 2. Movement of the bristle exerts force upon



**Figure 1. Mechanosensory External Sensory organs.**

(A) Macro- and microchaetae bristle organs on the adult fly head. Arrow indicates a socket cell that holds the bristle in place, while allowing its movement.

(B) Beneath the epidermis lie the neuron and sheath cells (arrowheads) that are associated to the bristle organs, microchaetae in this case. Hatched circle denotes the location of an overlaying socket cell.

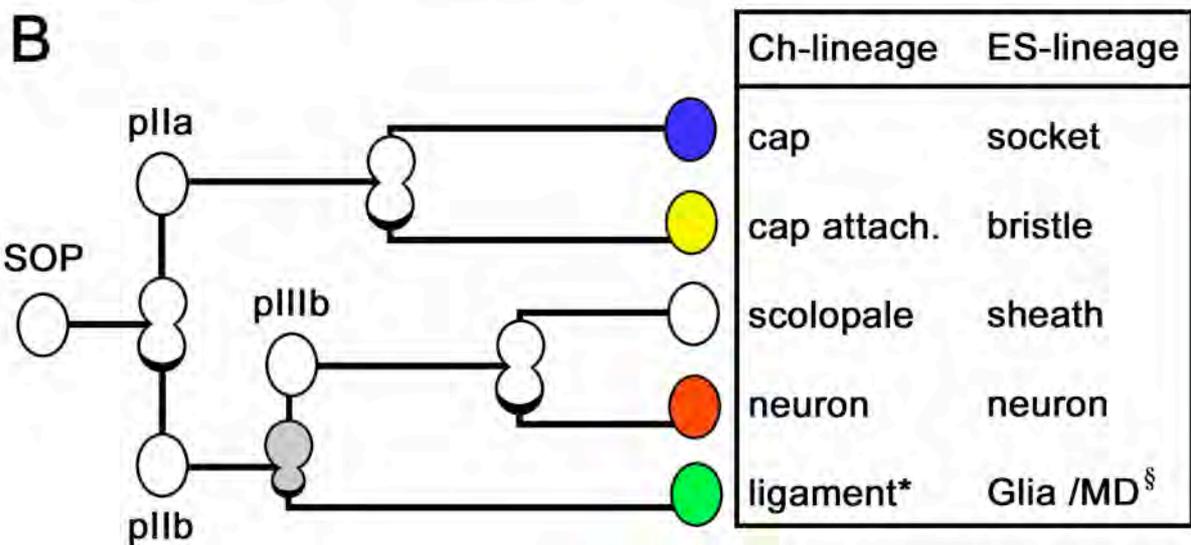
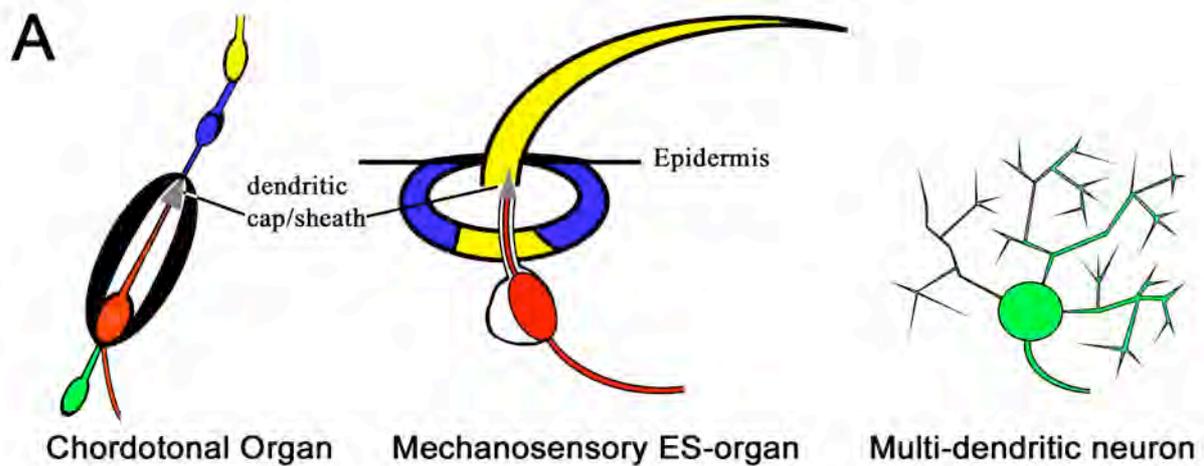
(C) The adult nota host numerous microchaetae that are evenly spaced over the thorax (arrows), and macrochaetae that are stereotypically positioned.

the dendritic sheath, potentially pulling it, and in doing so, applying stretch to the dendrite. This results in the opening of TRP ion channels (NOMPC in this case (Walker et al., 2000)) in the neuronal membrane. The consequent influx of ions (presumably  $K^+$  (Jarman, 2002; Kernan, 2007)) depolarizes the neuronal membrane and leads to an action potential. Due to the rapid response of the ES-neuron to bristle movement, direct stimulation of the TRP channels is implied (Walker et al., 2000). Movement of any of the macrochaetae is sensed by an enervating neuron which relays the stimulus to the brain, allowing the animal to react accordingly, with a grooming action being one of the most frequent responses (Vandervorst and Ghysen, 1980).

### Multi-Dendritic Neurons.

The sensory neurons of the PNS are classified into two broad groups, those that enervate a sense organ (type I, which are ciliated bipolar neurons like those of the ES-organs) and those that do not (type II). Type II sensory neurons are called multi-dendritic (MD) and as the name suggests they possess multiple, highly branched dendrites that project freely over relatively long distances under the epidermis *e.g.* spanning the larval segment in which the neuronal body resides (Bodmer and Jan, 1987). Found in both larval and adult animals, their physiological functions are not yet fully understood, although two distinct roles in nociception (detection of noxious stimuli) and locomotion have thus far been described. In a study performed by Tracey *et al.*, mutation of the *painless* gene, encoding a TRP ion channel expressed in MD-neurons (and Ch-organs), left larvae insensitive to heat and pressure stimuli (Tracey et al., 2003). Ablation of the Ch-organs (via mutation of *atonal*, required for Ch-development) had no effect on larval sensitivity to the noxious stimuli, whereas ablation of the MD-neurons rendered larvae insensitive.

During larval locomotion, MD-neurons are thought to “read” the peristaltic contractions, monitoring their rhythm. Loss of this sensation irreversibly stalls larval crawling (Song et al., 2007), a phenotype that was less evident but also commented upon in the earlier nociception study (Tracey et al., 2003). In other insect systems MD-neurons have been reported to sense stretch (proprioception) within the epidermis (Grueber et al., 2001). Potentially this proprioceptive nature is conserved to *Drosophila* MD-neurons, enabling them to sense the rhythmic movement of the animal, detecting inconsistent locomotion so that it may be corrected. Apparently, not only does the animal require this sensory input to rectify inconsistencies, but importantly, this input from the MD-neuron also seems to act as a stimulus for normal locomotion.



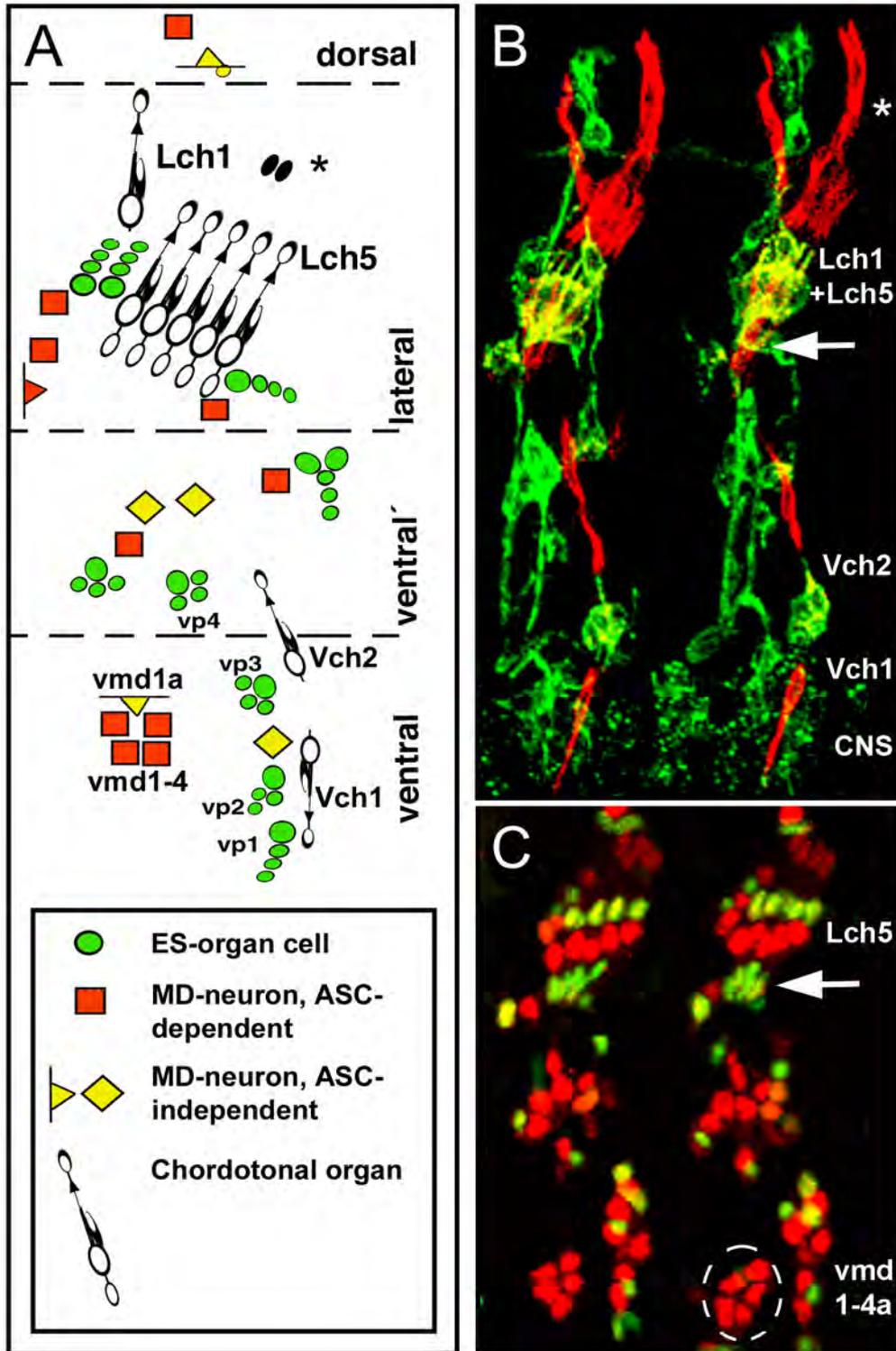
**Figure 2. Distinct PNS organs and cells are generated from similar and sometimes overlapping cell lineages.**

(A) Chordotonal (Ch) organs, Mechanosensory External Sensory (ES) organs and Multi-dendritic (MD) neurons, derived from the lineages depicted in (B). Cells are color coded accordingly. (B) The asymmetric distribution of Numb at each precursor cell division is indicated as a black crescent. The pIIb cell division generates daughter cells of unequal size (shaded) while divisions of the SOP and pIIa cells are equal and generate daughter cells of comparable size. \* Ligament cells are seemingly absent from many Ch-organs, a MD-neuron may replace this missing cell type, see text. § Within the adult ES-lineage a suicidal glial cell stems from the pIIb division, whereas in the embryonic lineage an MD-neuron is born. Adapted from Kernan MJ., *Eur. J. Physiol.* (2007).

### Chordotonal organs.

Chordotonal (Ch) organs are also proprioceptive, providing feedback to the animal regarding the positioning of the individual appendages relative to each other. This facilitates the coordination of complex body movements required during flight, walking or crawling, with Ch-organs suitably located at the base of the wing, within leg joints and throughout the larval body wall respectively. Additionally, Ch-organs are used in a form of insect auditory reception, in which sound vibrations elicit rotation of an antennal segment, loaded with Ch-organs (approx. 200) referred to as Johnston's organ, the resulting stretch of which is interpreted as sound, see (Eberl and Boekhoff-Falk, 2007) for an overview of hearing in the fly. The Ch-organ is frequently composed of five clonally related cells, each with distinct terminal cell fates. In addition to the neuron, these are the scolopale (a sheath cell which insulates the dendrite and secretes an ECM structure akin to the ES-dendritic sheath: the dendritic cap), the cap cell (which attaches to the dendritic cap), the cap attachment cell (which anchors the cap cell to the epidermal layer) and the ligament cell (which anchors the organ to the epidermis at its opposite end), see (Kernan, 2007) and Figures 2 and 3. The scolopale cell creates a luminal space surrounding the dendrite and the dendritic cap. This "scolopale space" is presumed to be filled with  $K^+$  rich endolymph. Longitudinal stretch imposed upon the organ results in mechanical strain across the dendritic cap/neuronal dendrite interface, leading to activation of TRP channels (Eberl, 1999; Jarman, 2002).

In addition to the NOMPC TRP channel, Ch-cilia express two other TRP's, Nanchung (NAN) and Inactive (IAV), which are thought to function in a heteromeric complex (Gong et al., 2004). Both possess intracellular tails and NAN possesses a long extracellular loop that may mediate external contacts. It is thought that stretch first acts on the NOMPC channel, located at the tip of the cilia. The resultant ion influx elicits an ion-stimulated movement of the cilium which activates the NAN/IAV complex, located more proximal to the base of the cilia. This leads to a second NAN/IAV dependent ion influx that both modulates the motility of the cilia and triggers the neuronal action potential (Gopfert et al., 2006).



**Figure 3. The embryonic PNS.**

(A) A schematic representation of the PNS, adapted from Hartenstein and Campos-Ortega (1986). Asterisks indicates the two cap attachment cells that derive from the Lch5 organ. Dorsal most cells are not shown. (B) Labelling neurons in green, and Ch-organ cap, cap attachment (asterisks) and ligament cells (arrow) in red. (C) General labelling of PNS cell nuclei in red and ligament (arrow), scolopale and sheath nuclei in green.

## Peripheral Nervous System Development.

Organs of the PNS are positioned throughout the larva and adult fly in strikingly stereotypical patterns (see Figures 1 and 3). Organ placement is achieved through the interpretation of positional cues by cells within the ectodermal layer. These signals control proneural gene expression, by transcriptional regulation of a series of proneural gene activators and repressors. Within a proneural gene expressing region, the relatively broad proneural expression pattern is refined to a single cell, the Sensory Organ Precursor (SOP) cell, from which ES, Ch, and MD cells derive. PNS organogenesis begins thereafter.

### Prepatterning.

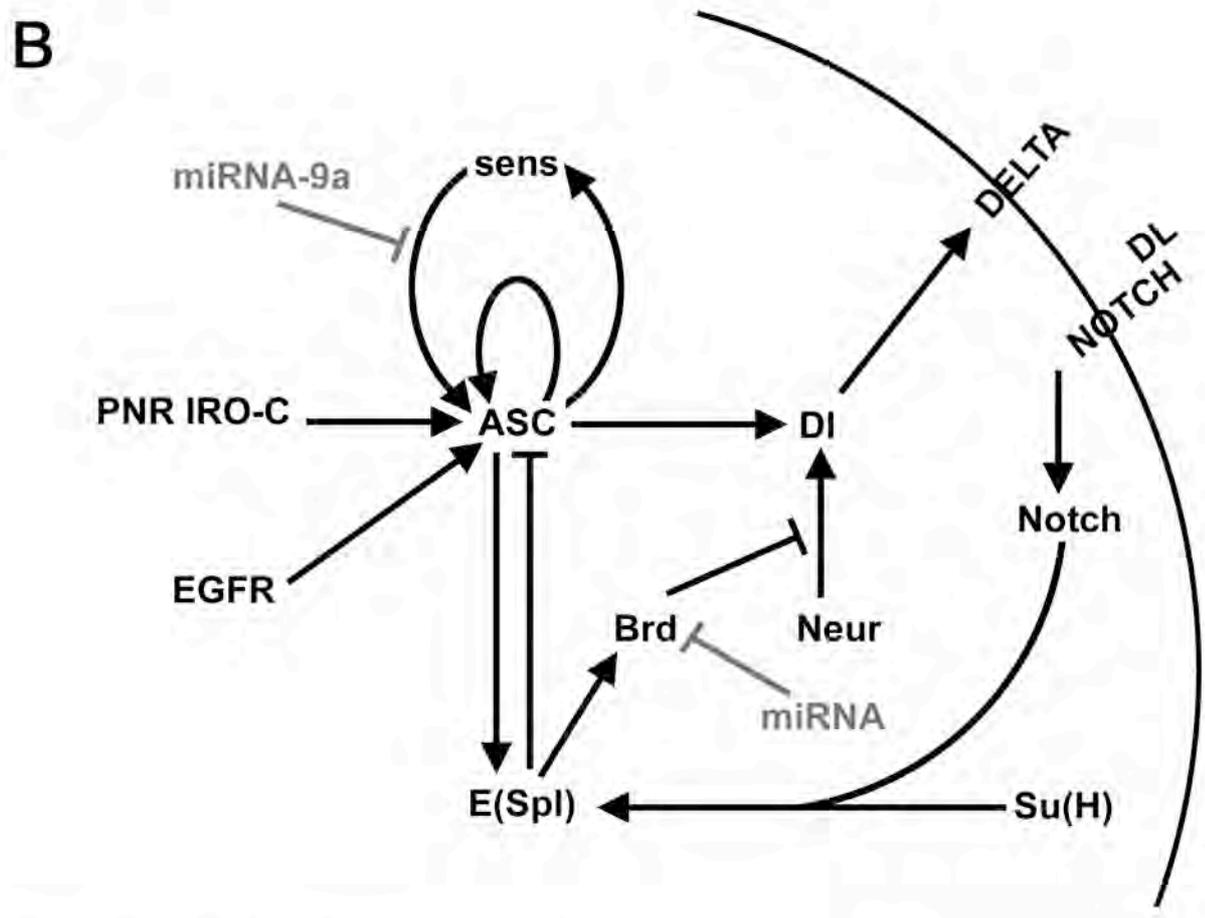
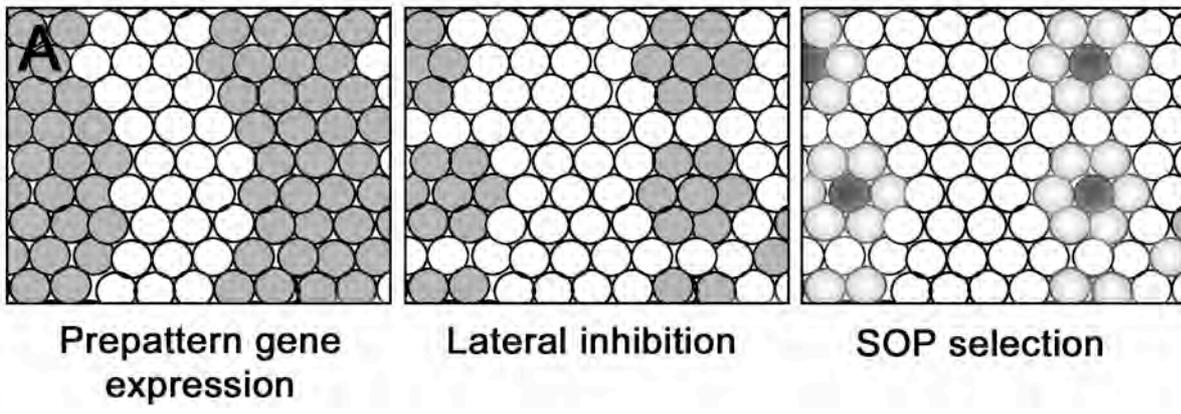
The early events leading up to SOP selection are perhaps best described during the specification of ES-organ SOP cells within the developing notum of the adult fly. In brief, the Dorsal/Ventral and Anterior/Posterior polarities of imaginal discs (the anlagen to the adult body structures) are specified during embryogenesis. These inherent tissue polarities are kept in place by the expression of genes such as *wingless* and *decapentaplegic (dpp)* within distinct regions of the imaginal disc. DPP, a transforming growth factor (TGF) family member, acts as a morphogen, *i.e.* a secreted signaling factor that elicits a graded transcriptional response (Campbell and Tomlinson, 1999). *dpp* is expressed at the Anterior-Posterior compartment boundary and provides cues to prepattern genes (*e.g.* *pannier* and *iroquois* complex (Iro-C) genes), driving their expression in a mutually exclusive fashion along the Anterior posterior axis of the notum (Letizia et al., 2007). *Pannier* (a GATA transcription factor (Ramain et al., 1993)) and *Iro-C* (encoding homeodomain transcription factors (Gomez-Skarmeta et al., 1996)) have both been shown to genetically interact with the proneural *achaete-scute* gene complex (ASC) as well as bind the ASC in-vitro (Gomez-Skarmeta et al., 1996; Ramain et al., 2000). The ASC encodes for four basic Helix Loop Helix (bHLH) transcription factors, required for PNS organogenesis (Alonso and Cabrera, 1988; Vervoort et al., 1997), *achaete (ac)*, *scute (sc)*, *lethal of scute* and *asense*, the most relevant to ES-organ formation in the adult notum being *ac* and *sc*. Each of the encoded bHLH proteins function as heterodimers, requiring partnership with another bHLH protein, Daughterless (DA) to elicit transcription of target genes (Murre et al., 1989). Specific mutation of one or other ASC genes leads to a loss of a subset of PNS organs, while mutation of *daughterless* leads to a loss of all PNS organs (Caudy et al., 1988). Once translated, ASC products are able to maintain their own transcription, forming a

positive auto-regulatory loop (Van Doren et al., 1992), an aspect of their regulation significant to the lateral inhibition process described below.

Together, prepattern gene expression covers the entire notum and could as such drive ASC expression within each cell therein. However, the formation of only PNS organs, without surrounding epithelial cells, would be detrimental to the animal. The majority of the notal ectodermal cells must therefore be protected from a neural fate. A wide range of additional transcription factors are expressed in longitudinal stripes on the developing notum, including transcriptional repressors such as *hairy* (encoding a bHLH transcription factor that represses ASC (Van Doren et al., 1994) and *extra macrochaetae* (encoding a HLH transcription factor, lacking the basic DNA binding domain but capable of partnership with, and hence sequestering, bHLH transcription factors of the ASC (Van Doren et al., 1992). Mutation of these transcriptional repressors causes the formation of excess PNS organs at the expense of epidermal cells (Orenic et al., 1993). Hence, they serve to limit the effects of broadly expressed ASC transcriptional activators. Thus the expression of a group of pre patterning transcription factors, expressed in either overlapping or mutually exclusive domains contribute to the even spacing of PNS organs by specifying the regions in which there is ASC activity. These regions require further refinement before PNS organogenesis can commence.

### **Lateral inhibition.**

Within the zones of proneural gene expression, which appear as longitudinal stripes (reflecting the future positioning of microchaetae, Figure 4,) or circular patches of cells (proneural clusters, reflecting the future sites of macrochaetae or photoreceptors, Figure 5, detailed later), PNS organs are permitted to develop. Each cell within such a proneural cluster is considered to possess equivalent potential for neural differentiation. Prior to the neuralizing action of proneural genes within these clusters, proneural gene expressing cells are further refined via lateral inhibition; the process in which a single cell within a field of equivalent cells assumes a distinct cell fate, and in doing so, inhibits its lateral neighboring cells from following suite. The theory was reasoned in an effort to explain the even spacing of microchaetae upon the adult fly notum. Since then, the ligand receptor pair mediating this cell-cell communication have been identified, namely Delta (Dl) and Notch respectively (Vassin et al., 1987; Shepard et al., 1989). The Notch signaling pathway is well described, although unlikely in its entirety, having multiple regulatory steps and tissue specific



**Figure 4. Lateral Inhibition.**  
 (A) The refinement of the prepattern to single evenly spaced SOP cells from which the microchaetae organs derive. (B) An overview of lateral inhibition. Notch receptor and Delta ligand are at the cell surface. Cellular compartments are not indicated. Activation of Notch via extracellular DI leads to Notch cleavage and activation of the Notch pathway. Prepattern genes drive ASC gene expression in opposition. miRNA are in grey, details in the text.

regulators. This complexity likely reflects its broad, reiterative use throughout the development of the animal, for recent review see (Fiuza and Arias, 2007).

In the proneural cluster, activation of the Notch transmembrane receptor via the membrane bound Delta ligand, situated on a neighboring cell, leads to a series of Notch receptor processing/cleavage events that result in the internalization and passage of the intracellular domain of Notch to the nucleus. There it binds/activates the Suppressor of Hairless (Su(H)) transcription factor (Fortini et al., 1993; Fortini and Artavanis-Tsakonas, 1994). Transcriptional activation of target genes of Notch located within the Enhancer of Split E(Spl) complex, which encodes for several genes including bHLH transcription factors and four members of the Bearded (Brd) family (Knust et al., 1992; Lai et al., 2000), leads to a block in neural cell fate determination, (see Figure 4). This by either direct repression of ASC genes (Oellers et al., 1994), or post-translationally antagonizing ASC target effectors *e.g.* Dl trafficking to the cell surface is modulated by several Brd proteins that compete with Neuralized (NEUR) for Dl ligand binding. NEUR is a RING finger E3 ligase that positively regulates the passage of Dl ligand to the cell surface via ubiquitination (Bardin and Schweisguth, 2006).

Positive transcriptional regulation of E(Spl) by AC/SC forms an ASC negative regulatory loop (Kramatschek and Campos-Ortega, 1994) see Figure 4. Mentioned previously, an ASC positive auto-regulatory loop is activated within the proneural clusters in response to prepattern genes, this opposes the Notch mediated forms of ASC repression. This aspect of ASC regulation is perhaps central to lateral inhibition. As Notch signaling converges upon E(Spl), levels of intracellular Notch can tip the balance of the ASC “switch” towards a repressed or “off” state, blocking neural differentiation. Other signaling pathways such the Epidermal Growth Factor Receptor (EGFR) also feed into this system and act (positively) upon ASC transcription (zur Lage and Jarman, 1999).

Within the proneural clusters both Dl and Notch are initially rather uniformly expressed. It is thought that stochastic differences in the starting levels of one or other of the proteins described (*e.g.* Dl, Notch, AC or SC) will be amplified over time, via some of the mechanisms described above, with neighboring cells battling to “switch off” each others proneural auto-regulatory loop, until such a time when one cell, having low levels of Notch activity, high levels of Dl at the cell surface and sustained AC/SC expression, will emerge, namely the Sensory Organ Precursor (SOP) cell. Its high levels of Dl expression will “laterally inhibit” neural differentiation in the

neighboring cells. Cell fate decisions mediated by Notch during PNS development have been demonstrated to act not only during the lateral inhibition process but also during the subsequent cell fate decisions between sibling cells of the PNS lineages.

### **Lineages of the PNS.**

Likely the positioning of the SOP cells in the embryo follows similar sets of positional cues to those provided to the adult ES-organs. The subsequent requirement of Notch mediated refinement of the pattern (lateral inhibition) has been shown (Alton et al., 1989; Kunisch et al., 1994). A fundamental difference between Ch-ES- and MD-lineages is the proneural genes they express. Ch-SOP cells (and a small subset of MD-neurons) require the action of the *atonal (ato)* proneural gene, whilst ES-MD-lineages require the action of the ASC (although a small subset of MD-lineages instead require the *absent md neurons and olfactory sensilla (amos)* proneural gene) (Bodmer et al., 1987; Jarman et al., 1993; Huang et al., 2000) see Figure 3. In general, mutation of *ato* results in the loss of Ch-organs (and a minor population of MD-neurons) while mutations of ASC leads to the loss of ES-organs and the vast majority of MD-neurons (Jarman et al., 1993). Minor exceptions are found, for example, in ASC mutations, the loss of one Ch-organ is frequently observed, while mutation of *ato* frequently leads to the loss of all but one Ch-organ. Presumably the same Ch-organ in each case as removal of both proneural gene cassettes ablates all Ch-organs (Huang et al., 2000). Hence one Ch-organ has a weaker requirement for *ato*, the significance of which is not understood.

Loss of an ASC effector, the homeodomain transcription factor Cut (an ES-organ selector gene) leads to the transformation of ES- to Ch-organs (Bodmer et al., 1987), as does mis-expression of *ato* (Jarman and Ahmed, 1998). Conversely, mis-expression of *cut* leads to a Ch- to ES-fate transformation (Blochlinger et al., 1991). These observations have collectively lead to the hypothesis that the target genes of ASC and *ato* overlap, demonstrated by (Powell et al., 2004), and moreover that a default PNS organ type is the Chordotonal (Jarman and Ahmed, 1998). Indirectly in line with this, a downstream effector of *ato*, a Ch-organ selector gene, has not been described. The specific proneural gene expressed in each SOP cell is postulated to trigger transcription of both the genes relevant to the development of each specific organ type, in addition to those in common with PNS organs in general.

Following selection of the SOP, its subsequent division marks the first in a series of asymmetric cell divisions that generate the distinct sibling cells of each PNS

organ. These lineages look remarkably alike (see Figure 2B), regardless of whether the organ being generated is Ch or ES, in the adult or embryo. Generally, the lineages consist of four precursor cells divisions, the SOP, pIIb, pIIa and pIIIb, which divide in this specific order. The asymmetric cell division machinery that is responsible for this is described later.

#### Ch-lineages.

The *ato* dependent pentascolopodial organ, or the Lateral Chordotonal 5 (Lch5), lies within the lateral region of the abdominal segments of the embryo (Figure 3). The organ is composed of five non-clonally related Ch-organs, *i.e.* each derived from a separate SOP cell (Brewster and Bodmer, 1996; Okabe and Okano, 1997). The Lch5 SOP cells are selected in a manner partially similar to that described for ES-organ SOP cells, but with notable differences. During early embryogenesis (stage 10), SOP cells of the Lch5 emerge sequentially, with the semi-ASC dependent Ch-SOP mentioned previously, specified first (zur Lage et al., 1997). This is followed rapidly by the specification of two more Ch-SOPs, each of which expresses *ato* (as does the first SOP). Subsequently, the remaining two SOP cells that will contribute organs to the Lch5, are recruited from surrounding the ectodermal cells via an EGFR dependent signaling mechanism (Okabe and Okano, 1997; zur Lage et al., 1997). This is through “active” secretion of the EGFR ligand, Spitz, from the predetermined SOP precursors. These express Rhomboid, an intramembranous protease capable of cleaving membrane tethered Spitz and releasing it from the signaling cell (Urban et al., 2001). Low levels of *ato*, in combination with EGFR signaling in the Spitz signal receiving cell, further activates *ato* (perhaps stimulating its auto-regulatory loop to a sustainable level (Sun et al., 1998; zur Lage et al., 2004) akin to the ASC “switch”) and commitment to a Ch-SOP fate follows. That this process invariably recruits exactly two SOP cells is remarkable. The predetermined SOP cells in addition to the secretion of Spitz, also secrete the negative EFGR ligand Argos, which appears to have a longer range of action than Spitz. While high levels of Spitz locally will activate EGFR signaling in proximal cells, the inhibitory effect of Argos will dampen the effect of lower Spitz levels more distant from the secreting cells. The embryonic abdominal hemisegments contain a total of eight Ch-organs, two occupying the ventral region (Vch1 and 2) and one in the lateral region (the Lch1) overlying the Lch5 (Figure 3). One of the Vch SOP cells also relies on this EGFR mediated recruitment mechanism for its specification (Okabe and Okano, 1997).

SOP cells of the Lch5 are born and divide in the dorsal region of each abdominal hemi-segment. However, upon completion of the series of asymmetric cell divisions, the nascent Lch5 rotates approximately 180° (during stages 12-13) and migrates ventrally to the more lateral region shown in Figure 3, its final position by embryonic stage 16 (Inbal et al., 2003). The cap attachment cells of these organs mediate contact with the epidermal layer forming an anchoring point at one end (see Figure 2). Potentially, this stable contact to the epidermis, in combination with fasciculation of the Lch5 axons with the intersegmental nerve, gives two fixed points during dorsal closure, that are sufficient for organ rotation (Inbal et al., 2003). The ligament cells are not required for organ rotation, rather, after rotation they guide/drag the organ ventrally to its final resting place prior to the end of embryogenesis (Inbal et al., 2003). There, the secretion of the EGFR ligand Vein, from the ligament cells, stimulates EGFR signaling within cells of the epidermis, leading to the recruitment of 1-2 ligament attachment cells to which the ligament cells adhere (Inbal et al., 2004). In this manner the Lch5 becomes sturdily attached at either end, facilitating proprioception across the epidermis.

Only two cap attachment cells are associated to the Lch5 organ (Matthews et al., 1990; Brewster and Bodmer, 1995) rather than the five one would expect. One of these cells stems from the three predetermined SOP cells, implying that the other cell stems from one of the two recruited SOP cells (Inbal et al., 2004). This implies that some inherent difference between predetermined and recruited Ch-organs could account for the lack of cap attachment cells. Their fate is unknown. Development of the other embryonic Ch-organs is less well documented, however it seems that all three additional abdominal Ch-organs (Vch1 and 2 and Lch1) lack ligament cells. Based upon the observations made in *ato* mutant backgrounds where both Ch- and MD-neurons were lost (Jarman et al., 1993), taken together with the fact that the MD-neuron of the ES/MD-lineage (described next) stems from the pIIb cell division (where a ligament would normally be born in the case of a Ch-organ, see Figure 2), one can postulate that the Ch-organ associated MD-neuron stems from the Ch-pIIb division at the expense of the ligament cell. How these organs sense stretch without an apparent ligament attachment is currently unknown.

#### ES- and MD-lineages.

MD-neurons emerge from at least two distinct lineages, although in both cases they stem from the division of the pIIb precursor cell. In the MD-solo lineage the

MD-neuron is the only terminal cell generated from the SOP cell, while in the MD/ES-lineage an ES-organ (or Ch presumably, see above) is clonally related (Orgogozo et al., 2001; Orgogozo et al., 2002). MD-neuron sibling cells within the MD-solo lineages are selectively removed via Notch induced apoptosis (Orgogozo et al., 2002). Genetically blocking apoptosis restores these lost cell types, leading to the formation of ectopic ES-organs. Similarly, mis-expression of Numb, an intracellular inhibitor of Notch signaling, can restore the latent cells of the lineage. The MD/ES-lineage generates the four ES-organs situated in the ventral and ventral' region (vp1-4) together with four of the MD neurons in the vmd cluster of five (Orgogozo et al., 2001) see Figure 3. The remaining vmd MD-neuron derives from a solo MD-lineage (Orgogozo et al., 2002). The adult microchaetal lineage is also subjected to controlled apoptosis (Fichelson and Gho, 2003). In this lineage however, it is the pIIb daughter glial cell that dies (which emerges in place of the MD-neuron in this lineage, see Figure 2B). There are slightly conflicting views on whether it migrates away from the organ (Reddy and Rodrigues, 1999a) and subsequently undergoes controlled cell death, or just initiates the apoptotic program (Fichelson and Gho, 2003). The role of Notch in this cell death was not investigated, however, as the glial cell normally inherits Numb, this apoptosis may be a Notch independent event.

### **Asymmetric cell divisions.**

The mechanisms by which asymmetry is generated within a cell prior to division, such that distinct daughter cell fates are established, are well conserved between different lineages of the PNS and the Neuroblast (NB) divisions of the *Drosophila* CNS (Lai and Orgogozo, 2004). These can be broadly categorized into three major mechanistic events; the asymmetric distribution of cellular determinants prior to division (Rhyu et al., 1994; Spana and Doe, 1995); the positioning of the mitotic spindle fitting to this asymmetric distribution and so that daughter cell size is controlled, being deliberately equal or unequal (Bellaiche et al., 2001; Albertson and Doe, 2003; Cai et al., 2003; Izumi et al., 2004); and Notch signaling between sibling cells, reinforcing the uptake of distinct cell fates after division has occurred, see (Roegiers and Jan, 2004; Suzuki and Ohno, 2006; Yu et al., 2006). These processes are tightly linked, to the extent that in some cases the complexes controlling one aspect of the division also directly influences another, *e.g.* the machinery in part responsible for positioning of asymmetric determinants also functions in control of daughter cell size (the polar complexes described below (Cai et al., 2003)) and *e.g.* some

determinants directly affect the Notch pathway of the receiving daughter cell, biasing Notch signaling between daughter cells after cell division (Guo et al., 1996).

While divisions of the CNS NB's and those of PNS precursors have many similarities, with the mechanisms underlying the establishment of asymmetry conserved to both lineages, there is an obvious difference. NB cells divide in a self-replenishing stem-cell-like fashion, preserving the NB cell identity whilst generating a distinct daughter cell, the ganglion mother cell (GMC). The NB divides repeatedly in this manner, always in an apical basal orientation. These divisions are moreover distinct in that two daughter cells of unequal size are generated, the smaller being the GMC (Cai et al., 2003). This size disparity between mother daughter cells is thought to preserve the size of the NB, enabling its numerous divisions. The GMC subsequently divides to generate terminally differentiated neurons or glia. There is no self-replenishing division within PNS lineages, rather here each division generates progressively more differentiated daughter cells. Despite this, the pIIb cell division represents the PNS precursor cell division most like the NB, dividing in the same apical-basal orientation and giving rise to two disproportionate daughter cells (Roegiers et al., 2001). This aspect of asymmetric cell division is discussed further in the following section.

During asymmetric cell divisions, both the PNS and NB lineages make use of the Bazooka/PAR-6/atypical PKC (BAZ/PAR-6/aPKC) and Partner of Inscuteable/Discs Large/G-protein coupled receptor subunit Gai (PINS/DLG/Gai) cortical complexes to direct protein determinants asymmetrically (Wodarz et al., 1999; Yu et al., 2000; Schaefer et al., 2001). Well known determinants include Numb, an intracellular inhibitor of Notch, that upon inheritance to a daughter cell, blocks Notch signaling intracellularly. This strong biasing factor reduces the need for lateral inhibition between daughter cells (Guo et al., 1996). Other asymmetrically distributed factors include Prospero (PROS), a neuralizing transcription factor that drives the receiving cell towards a more neural cell fate (Hassan et al., 1997; Reddy and Rodrigues, 1999b). Control of PROS distribution within the NB lineage has recently been the subject of intensive studies (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006). Therein the authors have described a requirement of *brain tumor (brat)*, during NB division and GMC fate determination. BRAT is a Tripartite Motif (TRIM) protein paralogous to Dappled (DPLD), a focal point of this work.

BRAT, as the name implies, leads to tumorous overgrowth within the larval brain. These tumors are lethal to the animal and can be transplanted to wildtype

animals causing their mortality (Arama et al., 2000). The NHL domains of BRAT form a  $\beta$ -propeller structure akin to WD40 domains (Edwards et al., 2003), and have been demonstrated to mediate protein-protein interactions (Sonoda and Wharton, 2001) which are key to the tumor suppressive role of BRAT (Arama et al., 2000). BRAT is capable of direct protein-protein interaction with the asymmetric determinant, Miranda, a cytoskeletal scaffolding protein (via its NHL domain). Together with Miranda, BRAT serves the asymmetric distribution of PROS from the dividing NB to the GMC. PROS, in addition to driving expression of neural specific genes also regulates the expression of Cyclin-dependent kinase inhibitor, Dacapo, which triggers cell cycle exit (Liu et al., 2002). Thus, mutation of *brat*, resulting in a lack of PROS segregation to the GMC, likely accounts for the poor differentiation and excessive proliferation observed in these cells (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006). Bello *et al.* conducted a key experiment, ectopically expressing the *pros* transgene within *brat* mutant tissue, thereby reducing tumor growth. This implies that the delivery of PROS to GMC fulfils BRAT function and as such represents arguably BRAT's primary role in this context.

In its role asymmetrically distributing PROS, BRAT presumably performs an adaptor function, mediating contact between Miranda (shown to be direct (Betschinger et al., 2006; Lee et al., 2006)) and PROS (whether this is direct is not described). Miranda cortical placement is in turn, likely driven by association with the cytoskeletal proteins Myosin II and VI (Petritsch et al., 2003), the activities of which are controlled by the BAZ/PAR/aPKC polar complex. This is accomplished via aPKC phosphorylation and inhibition of the tumor suppressor protein Lethal(2) giant larvae (LGL). LGL is normally found uniformly associated with the interior of the cell cortex (Albertson and Doe, 2003). Inactivation of LGL following aPKC phosphorylation leads to its dislocation from the membrane at the apical pole (where aPKC is localised). Meanwhile the basal fraction of LGL, distant to apically localized aPKC, remains active. There it is thought to inhibit Myosin II activity (Strand et al., 1994). The apical fraction of active Myosin II is thought to consequently push into the basal region, taking associated proteins such as Miranda with it. Thus the polar complexes, through phosphorylation and a series of protein interactions, direct asymmetric determinants, such as PROS and Numb, to one pole of the cell prior to division. This will lead to their inheritance by one of the two daughter cells, specifying it towards a more neural cell fate (Rhyu et al., 1994; Spana and Doe, 1995; Shen et al., 1997). The asymmetric

distribution of Numb during the various PNS precursor cells is summarized in Figure 2B.

### **Unequal cell division.**

As mentioned the polar complexes determine the size of daughter cells. This is achieved through influencing the position of the metaphase/anaphase plate prior to division, through a combination of centrosome (the microtubule nucleation site) displacement, forcing both centrosomes basally, away from the apical complexes, with a stimulated increase in the apical centrosomes size/activity. The activity of the aPKC kinase, in addition to G-protein coupled receptor receptor-independent signaling from the PINS complex, is implicated in this process (Albertson and Doe, 2003; Cai et al., 2003; Izumi et al., 2004) see Figure 6A.

Within the PNS precursor cell divisions, the cell size determining properties of the polar complexes are set into play in distinct ways, influencing cell divisions such that both equally and unequally sized daughter cells are generated. Within the NB-like pIIb division, both cortical signaling complexes are located on the same (apical) side of the precursor cell. The stimulation of centrosomal activity is therefore biased to one pole of the cell, resulting in an unequal “push” basally upon the metaphase/anaphase plate. Ultimately this leads to an unequal cytokinesis, generating two cells of unequal size, the smaller basal pIIIb-sibling cell and the larger pIIIb cell (Orgogozo et al., 2001; Roegiers et al., 2001) see Figure 2B. Within the SOP and pIIa cells however, these cortical complexes form at opposing cellular poles, in part due to polarity cues from the surrounding epidermis (Gho and Schweisguth, 1998) but additionally due to the lack of the Inscutable protein (INSC) in these cells. INSC, present in the NB and pIIb precursor, forms a link between the polar complexes, causing them to both to lie on the apical cortex. Consequently, in its absence, the metaphase plate receives positional cues from opposing cellular cortexes and lies centrally prior to division, giving daughter cells of equal size. Due to the asymmetric inheritance of fate determinants, these sibling daughter cells will nonetheless assume distinct cellular identities. The pIIIb cell division is again more pIIb/NB-like, with regards to spindle orientation (apical-basal) and asymmetric determinant segregation (Roegiers et al., 2001). Whether this is INSC dependent has not been directly addressed, its requirement in the preceding division complicates genetic determination of this, *e.g.* pIIIb daughter cell fate transformations are observed in the *insc* mutant background, however, these may result from inadequate INSC function in the prior pIIb

cell division, causing mis-specification of the pIIIb, rather than a subsequent role in the apical basal pIIIb division (Orgogozo et al., 2001).

It is via the coordinate action of these overlapping processes that two distinct daughter cells are generated from each cell division.

### **Coordination of cell cycle and asymmetric establishment.**

Logically, the processes controlling asymmetric cell division should be suitably imposed upon the underlying cell divisional program, such that the transcription and translation of determinants, and their subsequent asymmetric distribution, occur in a timely fashion. This is likely facilitated by both hierarchal regulatory mechanisms common to mitosis and asymmetric distribution, and/or unidirectional instructive cues from the mitotic process, rather than bi-directional crosstalk between these processes themselves. A number of examples argue for this.

The activation of Cyclin dependent kinase 1 (CDK1) triggers the entry into mitosis by phosphorylating a broad spectrum of protein targets, and its degradation via the proteasomal pathway, facilitates the end of mitosis and start of cytokinesis (Wolf et al., 2007). The activity of CDK1, and consequently the G2 to M-phase transition, is itself regulated in part by protein phosphorylation. CDK1, when phosphorylated by the WEE1/MYT1 kinases, is inactive (Price et al., 2002). The CDC25 phosphatase, String (STG) in *Drosophila*, is responsible for removal of these inhibitory phosphates and CDK1 activation. Expression of *stg*, causing inappropriate activation of CDK1, is sufficient to trigger mitosis in G2 phase arrested cells (Edgar and O'Farrell, 1990). CDK1 activity and target specificity depends additionally on the presence of its regulating partner proteins, Cyclin's, for review see (O'Farrell, 2001).

In addition to this general role in cell cycle regulation, CDK1 activity is required for the set-up or the establishment of the asymmetry of determinant proteins within the cell prior to division. Using conditional alleles of *cdk1* in which protein activity levels are lowered, although to a level sufficient for cell cycle progression, the asymmetry of protein determinants (without altering the levels of determinant expression) was lost within dividing NB cells, resulting in symmetric distribution of the determinants with consequent cell fate transformations (Tio et al., 2001). In a similar study, examining the ES precursor divisions using a *cdk1* conditional allele (in addition to mis-expression of CDK1 inhibitory regulators), cell fate transformations were observed upon temporarily blocking mitosis (Fichelson and Gho, 2004). The abnormal delay caused the SOP to assume a pIIb-like fate without an intervening cell division.

The pIIa cells were lost, leading to a lack of external socket and bristle structures. Both studies demonstrating that lowered levels of CDK1 have an impact upon the establishment of asymmetry.

This has been demonstrated in another fashion, by mis-expression of *stg*. Controlled mis-expression of *stg* during the development of macrochaetae leads to both the loss and duplication of organs, depending on the time-point at which expression is induced (Kimura et al., 1997). The loss of organs was subsequently shown to result from the precocious divisions of proneural cluster cells, interfering with the SOP fate specification. The doubling of macrochaetae, which occurred in response to ectopic expression of *stg* at a later time-point, presumably after the SOP specification event, was never examined. The SOP precursor cells of the adult ES-organs have a longer period of G2 arrest than other precursors of the lineage (Audibert et al., 2005), some of which have very short cycles, perhaps skipping phases. The long SOP G2 arrest seems to reflect the time taken to select the SOP from the surrounding proneural cells (Usui and Kimura, 1992). Additionally, the authors hypothesize that this long pause in G2 may allow the build up of determinants within the SOP cell that are relevant for daughter cell specification. These examples of the effect of cell cycle upon asymmetric distribution or its establishment, likely reflect instructive cues from regulators of the cell cycle to the machinery responsible for the establishment of asymmetry. More hierarchal examples can also be found, again involving the mitotic regulator *stg*. Here both *stg* and *insc* have been shown to be under the control of the Snail Zinc finger transcriptional repressor (Ashraf and Ip, 2001). Ectopic expression of *stg* and *insc* could rescue the *snail* loss of function NB phenotype, demonstrating the coordinated co-regulation of these two genes, one controlling cell division, the other, the asymmetric and unequal nature of the division.

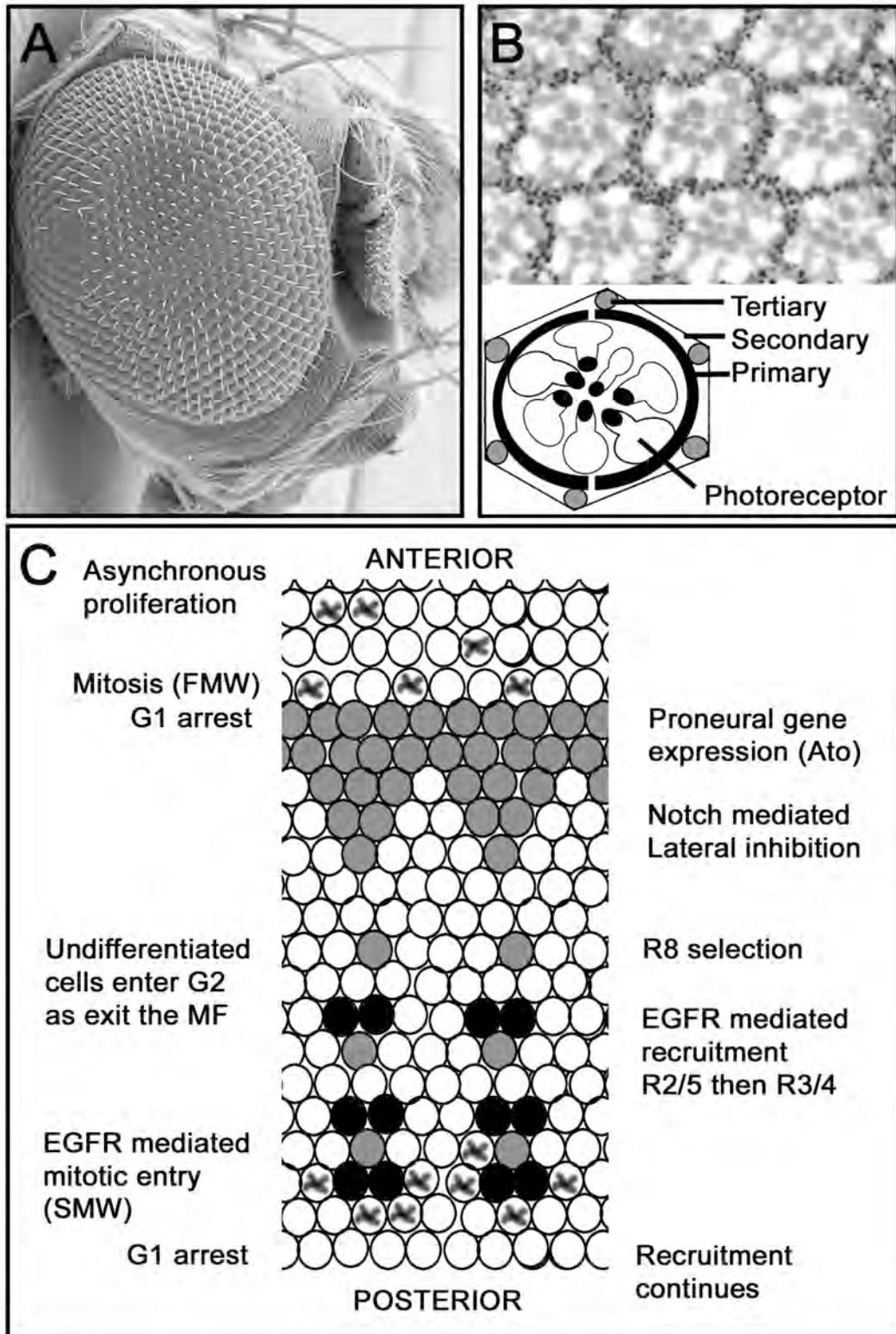
There is no evidence suggesting the presence of a direct mechanism that assesses the integrity, or level of completion, of asymmetric set-up prior to division, at least no mechanism capable of halting the divisional program upon detection of such errors. Rather, on the basis of; the cell transformation phenotypes observed from mutations affecting components of the asymmetric distribution machinery, or the factors distributed; the fact that the mutation of several of these factors results in an increase in proliferation (albeit likely indirectly *e.g.* *lgl* and *brat*); taken with the observed changes in cell fate upon mitotic deregulation described above, the circumstantial evidence speaks strongly against such crosstalk. Potentially, temporal control of the initiation of both mitosis and asymmetric establishment, along with

instructive cues from the mitotic regulators, represents the only coordination events between these two superimposed processes.

### **Making an Eye.**

The compound eye of *Drosophila* is composed of approximately 800 individual unit eyes known as ommatidia, arranged in a highly organized crystal lattice-like structure (Figure 5A). Each ommatidia is comprised of eight photoreceptors, four cone cells and two primary pigment cells, surrounded by twelve interommatidial cells, which include mechanosensory bristle organs (three in common with neighboring ommatidia) (Ready et al., 1976). The photoreceptors are organized in a trapezoidal pattern, with six outer photoreceptors (R1-6) surrounding the inner two (R7/R8 that are stacked upon each other, the R7 lying uppermost). Each photoreceptor cell develops a light sensitive rhabdomere structure, composed of an array of microvilli held out from the photoreceptor cell body to the centre of the ommatidial unit (Figure 5B), beneath the lens, deposited by the cone cells. Photoreceptors express various forms of Rhodopsin (light sensitive G-protein coupled receptors) which render individual photoreceptors sensitive to either broad spectrums of light (R1-6) or specific wavelengths/colors (R7/8) (Mollereau and Domingos, 2005). The activation of Rhodopsin indirectly stimulates TRP channels within the rhabdomere, triggering ion influx and firing of the photoreceptor, reviewed in (Venkatachalam and K. Montell, 2007).

Cells that will give rise to the adult eye are specified during embryogenesis. The eye anlagen is composed of a stratified monolayer of cells, physically coupled to the antennae anlagen, referred to collectively as the eye-antennal disc. During larval development the eye-antennal disc proliferates without differentiation with the size of the disc increasing dramatically, although retaining its monolayer form. During the 3<sup>rd</sup> larval stage, retinal differentiation begins in a wave like fashion, traveling from the posterior to the anterior end of the disc, a passage that takes about two days. This wave, known as the morphogenetic furrow, forms a physical indent in the monolayer as cells entering the furrow synchronously arrest and contract towards the basal layer. The furrow spans the entire dorsal ventral axis of disc. Anterior to it, cells proliferate in an asynchronous manner, however, upon arrival of the furrow they synchronously exit mitosis and enter G1, the first mitotic wave (FMW) has



**Figure 5. Development of the adult compound eye.**

(A) SEM of an adult eye. (B) Semi-thin section through the adult retina. Seven photoreceptors are visible at the center of each ommatidial unit, demarked by pigment cells (dark granular staining). Inset show schematic representation of an ommatidia, R8 and the four cone cells are not depicted. (C) Illustration of the photoreceptor selection process. Atonal positive cells are grey, including the R8. Recruited cells are black. Mitotic bodies are indicated as  $\times$ . Mitotic profile of cells prior to and post furrow lies to the left, stages in the selection process to the right.

occurred, see Figure 5C. The FMW is elicited in response to prepatterning gene product expression (DPP and Hedgehog), which both promote cell cycle exit (Horsfield et al., 1998; Firth and Baker, 2005) and induce *ato* expression within cells of the furrow (Jarman et al., 1994; Dominguez, 1999). From these proneural cells, the founding photoreceptor, R8, is specified via lateral inhibition (Baker and Zitron, 1995). It subsequently recruits additional photoreceptors in a sequential order (R2/5, which cooperates with R8 to recruit R3/4), via secretion of the EGFR ligand Spitz (Freeman, 1996). Subsequently, as the differentiating photoreceptor cluster exits the furrow, the surrounding non-differentiated cells are stimulated to pass through G1 and S-phase, in response to Notch signals, and arrest in G2 upon furrow exit (Baonza and Freeman, 2005). The reception of EGFR signal within these G2 arrested cells, from the photoreceptor group expressing the Spitz ligand, now, rather than signaling for recruitment, signals instead for mitosis (Baker and Yu, 2001). This leads to another synchronous round of division after the furrow referred to as the second mitotic wave (SMW). This is thought to be required to ensure adequate cell numbers for eye development (de Nooij and Hariharan, 1995). Following this division, the cells again arrest in G1 and await recruitment signals from the growing photoreceptor cluster before differentiation and inclusion into a growing ommatidial unit (Freeman, 1996).

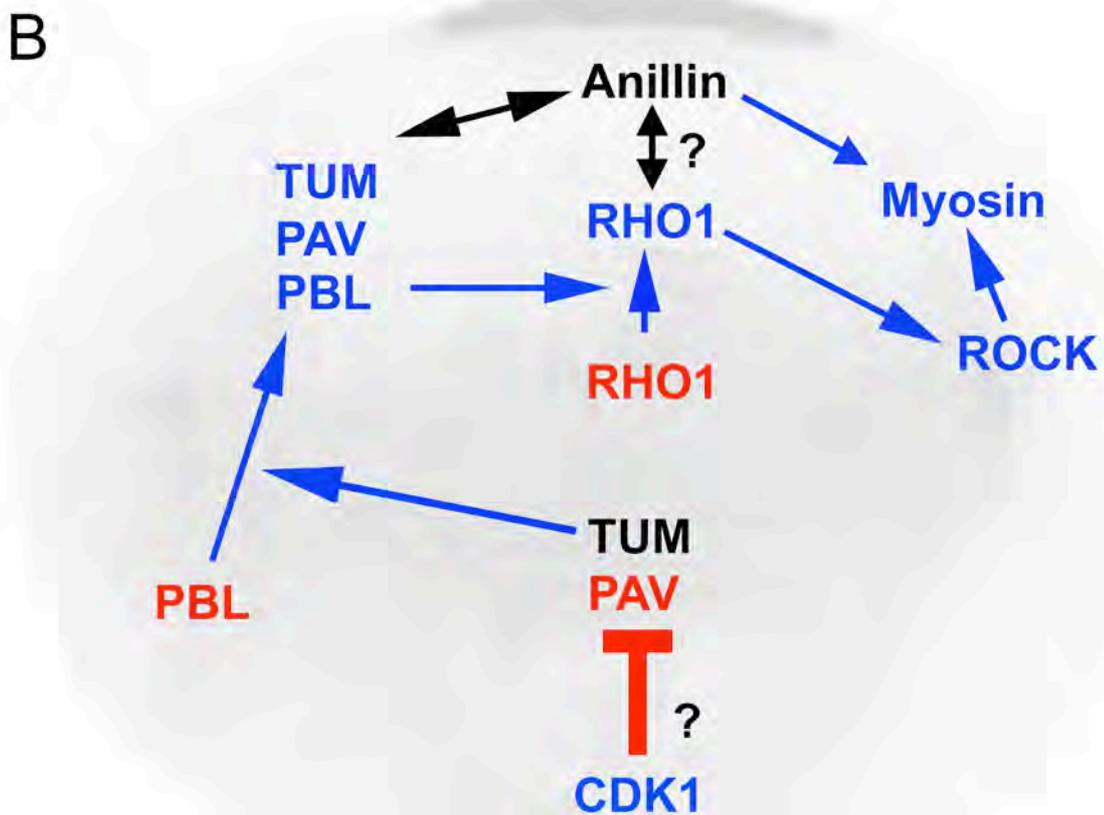
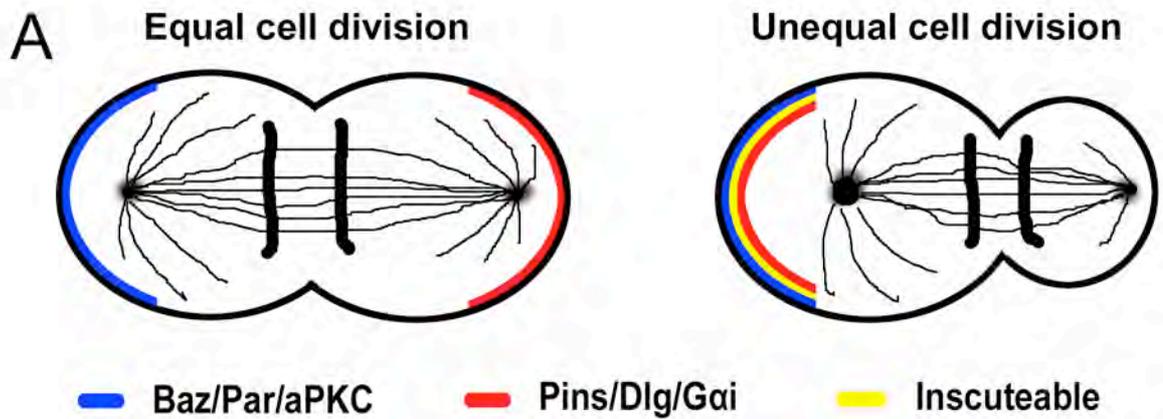
Some parallels between eye development and that of other PNS organs are evident *e.g.* the use of the *ato* proneural gene to generate proneural clusters from which the first photoreceptor cell (R8) is selected, and moreover that this selection process is mediated by Notch. Furthermore, the remaining photoreceptor cells of each ommatidium are recruited in an EGFR mediated fashion, similarly to the Ch-organ recruitment process. Cellular differentiation continues during pupation, during which the eye disc everts, photoreceptor and accessory cells elongate and the lens is secreted to the surface of the ommatidial unit.

These well-described developmental processes involved in the generation of the peripheral nervous system organs, *i.e.* prepatterning, lateral inhibition, cell cycle regulation, asymmetric cell division and cellular differentiation, make them an attractive model in which to study the function of genes found to be expressed therein.

## **Cytokinesis and Anillin.**

The final stage of the cell cycle involves cytokinesis, the physical separation of the two daughter cells. As mentioned, the BAZ/PAR/aPKC and PINS/DLG/Gai complexes control the size of daughter cells via positioning of the metaphase plate, and hence the positioning of the cleavage furrow, which interprets cues provided by the metaphase plate during anaphase. Cytokinesis begins with the build up of the contractile ring underlying the cellular membrane. This heterogeneous protein ring structure constricts during cytokinesis, drawing the membrane with it, forming a physical invagination that furrows inwards (the cleavage furrow), extending towards the mid-plate (derived from the metaphase plate). Eventually the membrane is drawn tightly together and subsequently cleaved (abscission) to release two independent daughter cells, see (Glotzer, 2005). Numerous protein components of the ring have been identified, including Actin and Myosin II, which play mechanical roles, and Septins and Anillin which play various roles including Actin and Myosin filament formation (Fares et al., 1995; Field and Alberts, 1995; Kinoshita et al., 2002). Notably, while many components of the ring structure are conserved, others are not, leading to the surmise that organism specific variations of cytokinesis may have evolved (Eggert et al., 2006).

The localized activity of the *Drosophila* RHO1 small GTPase (RHOA in other systems) at the cellular equator is required for the build up of the contractile ring in addition to its constriction at the end of anaphase. This function is conserved to RHO1/A in both vertebrate and invertebrate systems. When active, RHO1 is capable of coupling to several target proteins and stimulating their activity, *e.g.* *Drosophila* Diaphanous (Formin), which stimulates Actin polymerization and RHO kinase, (ROK or ROCK), which activates Myosin motor function (Piekny et al., 2005) (see Figure 6). RHO1 is widely dispersed throughout the cytoplasm yet activation only occurs at the prospective contractile ring formation site (Bement et al., 2005). This is accomplished by delivery of the activating GDP-GTP exchange factor Pebble (PBL RHOGEF) to the prospective furrow site. *Drosophila* Tumbleweed (TUM or RacGAP50C) in combination with the spindle interacting kinesin, Pavarotti (PAV, KLP in other systems), is thought to activate and deliver PBL to membrane regions in juxtaposition to the central spindle (Zavortink et al., 2005; D'Avino et al., 2006). In some systems the activity of PAV is negatively regulated by CDK1 (Mishima et al., 2004), providing a link between the end of mitosis when CDK1 is degraded, and the start of cytokinesis when active PAV travels along spindles that radiate towards the cellular cortex.



**Figure 6. Modes of cell division and a model of Cytokinesis.**

(A) The positioning of the cytokinetic furrow is dependent on localization of the Polar complexes. The presence of Inscuteable will, through interaction with both complexes, dictate whether cytokinesis occurs on- or off-center. (B) A model of the interactions leading to cytokinesis. Blue arrows indicate activation and red inhibition. Black arrows indicate protein interactions that result in localization to the cortex. Blue text indicates active states where relevant and red inactive states. Black text: TUM, may be active but kept from cortex until PAV repression is relieved; Anillin interactions reflect positive localization to the cortex. ? from a vertebrate system.

Recent studies have shown that Anillin is involved in a complex set of interactions with several factors required for the initiation of cytokinesis (Gregory et al., 2008; Piekny and Glotzer, 2008). Piekny and Glotzer report that in vertebrate cell culture, Anillin interacts directly with RHOA and that this is required for Anillin build-up at the presumptive furrow site. When there, Anillin reciprocally stabilizes RHOA's location, maintaining its presence after furrowing has commenced. While depletion of Anillin within this system does not block contractile ring formation, or the first stages of contraction, simultaneous depletion of Anillin and the PAV ortholog (MKLP1) blocks both (Piekny and Glotzer, 2008), giving a strong enhancement of MKLP1 depletion phenotype alone. The authors speculate upon redundant mechanisms of contractile ring formation, suggesting Anillin is required for ring formation in a parallel pathway, only recognizable under certain conditions. Within the *Drosophila* systems examined by Gregory et al. Anillin was found to interact directly with TUM. This was reciprocally required for localization of both proteins during cytokinesis. The authors suggest that the association of the TUM/PAV/PBL complex with the contractile ring components, mediated by Anillin, will lead to sustained activation of RHO1 during contractile ring formation and constriction. Anillin may mediate the interaction of the contractile ring with the intracellular membrane having a lipophilic PH domain (Haslam et al., 1993).

In *C. elegans*, Anillin is required for extrusion of polar bodies via unequal cytokinesis (a form of unequal cell division) and asymmetric furrowing (ingression of the cytokinetic furrow from one cellular cortex) of the embryonic cell cleavages (Maddox et al., 2005; Maddox et al., 2007). In fission yeast, Anillin is represented by two proteins, MID1 and MID2, where MID1 designates the site of contractile ring formation in response to cues from the polar cortex (Bahler et al., 1998; Celton-Morizur et al., 2006; Huang et al., 2007), while MID2 functions in contractile ring stabilization (Berlin et al., 2003).

The *Drosophila scraps* gene (*scra*) encodes for the Anillin protein (Thomas and Wieschaus, 2004) and was first isolated in a screen for maternal effect alleles (Schupbach and Wieschaus, 1989). *scraps* alleles generated in this screen were homozygous female sterile. However, these alleles were additionally transheterozygous lethal in combination with genomic deficiencies uncovering the locus, indicating that these alleles were hypomorphic and moreover that *scraps* is required for normal developmental progress. Furthermore, null alleles are embryonic lethal, indicating that maternally contributed *scraps* expires prior to the end of embryogenesis and that

zygotic *scraps* transcription is initiated by this time (Heitzler et al., 1993). Elegant studies by Field *et al.* revealed specific roles of maternal *scraps* during cellularization and pole cell segregation in developing *Drosophila* embryos (Field et al., 2005). This work demonstrated that Anillin was responsible for the correct localization of *Drosophila* contractile ring proteins and moreover, that Anillin had a hitherto unknown role in stabilizing newly deposited plasma membrane within the newly formed daughter cells.

## **miRNA.**

A new and rapidly expanding field, microRNA (miRNA) are predicted to regulate vast numbers of genes involved in a broad spectrum of biological processes, with some predictions estimating that 30% of mammalian protein coding genes could be targeted by miRNA (Filipowicz et al., 2008). Found in both plants and animals (so far), miRNA are short stretches (approximately 22 nucleotides long) of anti-sense RNA, capable of recognizing and binding to specific semi-complementary target regions of an mRNA, causing a reduction to protein levels of the corresponding targeted gene (Bartel, 2004).

miRNA are encoded in the genome and transcribed as longer precursor RNA strands that form double stranded hairpin loop secondary structures. Loci frequently contain more than one miRNA which are transcribed into the same RNA. These primary miRNA transcripts (pri-miRNA) are sequentially processed down to their active state via the action of RNase enzymes. This occurs via a two step catalysis of the pri-miRNA, mediated first by the Drosha complex in *Drosophila*, cleaving to a shorter precursor miRNA fragment (pre-miRNA) containing the hairpin loop portion (Lee et al., 2003). This is exported from the nucleus to the cytoplasm where the double stranded RNA is recognized by the RNase activity of Dicer. After Dicer processing the ~22 nucleotide miRNA, and its complementary strand, are released. The miRNA then complexes with the RNA Induced Silencing Complex (RISC) before selectively binding to the semi- or fully-complementary target sequences, typically located within the 3'UTR of the relevant mRNA. Formation of the miRNA::RISC::mRNA complex prevents translation, either by blocking translation (Pillai et al., 2005), or selectively degrading the transcript (Bagga et al., 2005). This choice may reflect the degree of complementary binding between the miRNA and its target where imprecise pairing leads to repression, whereas perfect (or near perfect) base pairing are more likely to be degraded, see (Wu and Belasco, 2008). The net result in either case is a decrease in the

protein level of that specific gene. While in the first case, a translational block represents a reversible step, degradation of the target represents an irreversible one. This difference may represent a biologically significant distinction between the two modes of repression. In both cases, strong 5' complementation is considered a prerequisite for significant miRNA duplex formations, in addition to a list of other experimentally determined sequence requirements, see (Filipowicz et al., 2008).

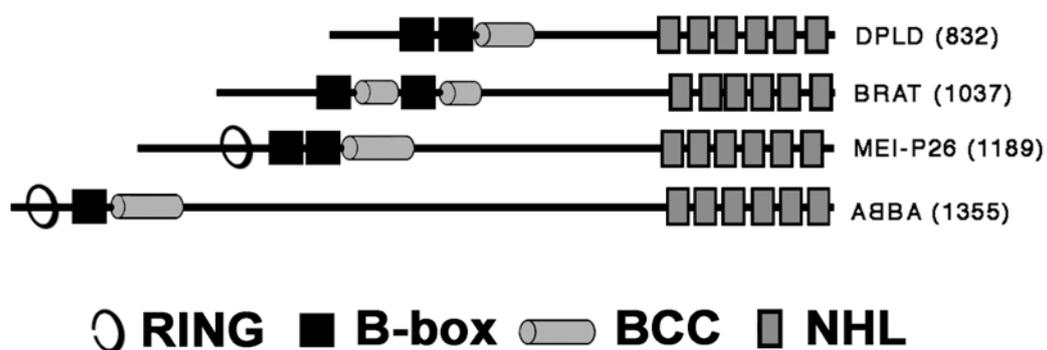
While repression of target mRNA, via degradation or translational inhibition, has been the “dogma” for the past decade, new and exciting data shows that this repressive effect may in fact be altered, even reversed, under specific conditions (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). Specifically, these studies detail the translational activation of specific genes by miRNA in response to cell cycle arrest or cellular stress, whereas under normal or proliferating conditions the same miRNA elicits its more dogmatic repression of the targeted mRNA. As the use of miRNA has been described as a selective approach towards the treatment of certain disorders, including cancer (Slack and Weidhaas, 2006), these findings may affect the feasibility of this treatment. It will be very interesting to find out what confers this differential response to the transcripts.

As mentioned, miRNA regulation has impacted upon numerous biological fields, and neural development is no exception. Li *et al.* have reported the role of miRNA-9a during SOP cell selection (Li et al., 2006). There, miRNA-9a specifically targets and represses *senseless (sens)* mRNA, preventing accumulation of Sens. Sens acts in a proneural gene auto-regulatory loop in which activation of *sens* transcription by proneural gene products will enhance their expression (Nolo et al., 2000) see Figure 4. The presence of miRNA-9a specifically in the non-SOP cells and its absence in the presumptive SOP cells during lateral inhibition is thought to augment differences in Notch signaling. Corresponding negative regulation of the Notch pathway within the presumptive SOP by miRNA has also been proposed (Lai et al., 2005). Here, several miRNA target and repress translation of reporter constructs containing the Brd genes 3'UTR's. Brd proteins, as mentioned, are Notch effectors encoded in the E(Spl) complex, which is transcriptionally activated by Notch signaling. Translational repression by miRNA will thus block the effect of Notch signaling downstream of transcriptional activation of its target effectors (see Figure 4). During lateral inhibition this will indirectly promote proneural gene expression and interfere with SOP cell selection. In line with this, the authors note many Notch-like mutant phenotypes, including the development of excess macro- and microchaetae on the

dorsal thorax of the adult, upon mis-expression of the relevant miRNA (Lai et al., 2005).

### **Tripartite Motif (TRIM) proteins.**

As the name suggests TRIM proteins typically contain three major defining protein domains; a RING (Really Interesting New Gene) domain, which possesses E3 ligase activity; one or two B-box domains (the RING domain was once referred to an a A-box, the second domain a B-box and this phrase has since persisted), which are Zinc-finger domains involved in protein-protein interactions, *e.g.* with bHLH transcription factors (Bloor et al., 2005); and a Coiled-coil domain (CC), also thought to mediate protein-protein interactions (Reymond et al., 2001) and see (Torok and Etkin, 2001). These domains are typically found in the N-termini of TRIM proteins, occurring invariantly in the order Ring-B-box-CC without intervening domains (family members are also referred to as RBCC proteins). Frequently, TRIM proteins contain C-terminal domains, the most relevant herein being the series of NHL repeats found in DPLD and BRAT. These fold to form a six bladed  $\beta$ -propeller structure (Edwards et al., 2003), demonstrated to mediate protein-protein interactions (Sonoda and Wharton, 2001; Lee et al., 2006). Sub-cellular localization of the mammalian family members has been investigated and found to vary dramatically, being found everywhere from the cytoplasm (where they appear to localize to distinct but unidentified cytoplasmic compartments) to the nucleus (again with distinct sub-compartmental localizations) (Reymond et al., 2001).



**Figure 7. *Drosophila* NHL containing TRIM proteins.**  
The length of each protein (amino acid residues) is indicated.

Within mammals there are approximately 70 identified TRIM genes to date. Of these, several are implicated in cancers and disease states such as breast cancer (Urano et al., 2002), lymphoma (Kosaka et al., 2007), specific forms of muscular dystrophy (Kudryashova et al., 2005) and Opitz syndrome (Cho et al., 2006) and see (Meroni and Diez-Roux, 2005). TRIM proteins are found in all metazoans where they function in a diverse array of cellular processes such as apoptosis, proliferation, differentiation and immune response (Evans et al., 2004; Bloor et al., 2005; Meroni and Diez-Roux, 2005; Lee et al., 2006). Potentially, this diversification of function partially reflects the RING domains E3 ligase activity.

E3 ligases are a component of the ubiquitin pathway, typically known for its role in the controlled degradation of proteins via the proteasome. The E3 component of the pathway confers specificity to the ubiquitination process, by binding to the protein target and mediating the transfer of ubiquitin from the E2 carrier ligase to the protein target substrate (Sun, 2006). Hundreds of E3 ligases are present in mammals, each thought to have a unique subgroup of protein targets and likely a specific E2 partner (although E2 ligases, fewer in number than E3, likely can partner with several E3's). Protein-protein interaction domains present in other regions of the E3 ligase proteins, or in a binding partner (many E3 ligases form larger protein complexes *e.g.* the anaphase promoting complex), confer the substrate specificity. Recent findings demonstrate novel roles for ubiquitination in the regulation of cellular events other than protein degradation, *e.g.* vesicular trafficking in the case of NEUR, the RING containing ligase, mentioned previously (Bardin and Schweisguth, 2006).

In the fly there are at least nine TRIM encoding genes (PAPER IV this work). Of these, three have been previously noted to contain C-terminal NHL repeat regions (Sonoda and Wharton, 2001). While Meiotic Protein 26 (MEI-P26) and Another B-box Affiliate (ABBA, CG15105, a novel NHL TRIM described in PAPER IV) possess all three TRIM motifs, BRAT and DPLD lack the N-terminal most RING domain, and are therefore atypical members of the TRIM family (see Figure 7). MEI-P26 has been assigned a role in meiotic exchange and mutations affecting its function lead to ovarian tumors (Page et al., 2000). In addition to its role in asymmetric distribution (Lee et al., 2006), BRAT is crucial during embryonic segmentation, where in combination with Pumilio, an RNA binding protein (Murata and Wharton, 1995), BRAT regulates the translation of hunchback mRNA (Sonoda and Wharton, 2001). Additionally a role for BRAT, and its worm ortholog, in the regulation of rRNA and

cellular growth has been described (Frank et al., 2002) and as mentioned, during asymmetric cell divisions.

*dappled* was first recovered during a screen for immune system affecting genes and was described as causing a melanotic tumor phenotype in both larvae and flies (Rodriguez et al., 1996). Melanotic tumors are generally free floating melanized masses, visible due to their dark brown appearance beneath the cuticle. The color results from melanization by the phenol oxidase cascade, an immune response to tissues considered non-self, typically invading pathogens (Tang et al., 2006). Therefore melanotic tumor formation may result in response to either immune dysfunction or tissue abnormalities. The *dappled* mutations isolated in this screen were shown not to perturb normal immune function and on this basis classified as mutations likely affecting cell growth or differentiation. The observation that each of the characterized NHL containing TRIM genes in flies is involved in tumor suppression, suggests conservation of this function between these paralogous proteins.

Apart from an ascribed role in tumor suppression, data available on *dappled* is scant. Screens to determine the gene expression profile of the developing CNS find *dpld* to be expressed therein (Brody et al., 2002; Kearney et al., 2004). An RNAi screen performed by (Pilot et al., 2006), searching for genes affecting embryonic cellularization recovered *dpld*. They note *dpld* as being important for trafficking lipid-droplets. Transportation of these large protein filled vesicles is microtubule based (Cermelli et al., 2006), indicating potential interactions of *dpld* with the cytoskeletal machinery. Laviolette *et al.* have screened for suppressors of a dominant neuromuscular junction overgrowth defect, mis-expressing candidate genes specifically in the neuron (Laviolette et al., 2005). Via this approach they isolated mis-expression alleles of *dpld* (and *brat*) that could strongly suppress this phenotype. While preparing this thesis, it has been reported that *dpld* (referred to as *wech* therein) plays a vital role in the attachment of the larval muscles to the epidermis (Loer et al., 2008). The authors describe DPLD localization specifically within the tendon (muscle attachment cells) and muscle cells, where it is proposed to mediate protein contacts at Integrin cellular adhesion sites. Furthermore, this interaction is mediated by interactions of the B-box, CC and NHL region of DPLD with known Integrin associated proteins, Talin and ILK. The loss-of-function studies detailed therein are partially based upon the embryonic lethal phenotype of the *dpld*<sup>k08815</sup> chromosome (detailed in Results and Discussion) and a novel P-element mediated micro-deletion that fails to complement this chromosome and moreover, reproduces the embryonic lethality. The micro-deletion removes a

portion of the 5'UTR (leaving the protein coding region intact), which the authors show destabilizes *dpld* mRNA transcripts, to the extent that they consider the mutation to represent a null or amorphic allele. Our own findings concerning the *dpld*<sup>k08815</sup> allele, detailed in the Results and Discussion section, are worth consideration when interpreting these findings.

## AIMS

The initial aim of this project was to identify novel genes that could affect cell fate determination during the development of the PNS. The relevance of individual candidate genes recovered in our forward genetic screening approach would be assessed subsequently and a selection of those deemed pertinent would be studied further. In the latter regard, an emphasis was placed upon candidate genes that were implicated in cell cycle regulation.

Subsequent work was directed towards gaining an understanding of the involvement of two candidate genes (*string* and *dappled*) in PNS development.

Initial characterization of *dappled* began with the use of pre-existing mutant alleles, one of which revealed intriguing nervous system phenotypes suggestive of a role during asymmetric cell division. However, subsequent work would show that mutation of the *dappled* locus did not account for the phenotypes observed. Thus, effort became focused upon identification of the genetic loci responsible for the described cell division phenotype and tumor suppression.

Subsequent attempts to generate “true” *dappled* alleles were instigated in an effort to investigate *dappled*'s role during PNS organogenesis.

## METHODS

Standard techniques are detailed in PAPERS I-IV, as are fly strains used in these studies. Reverse transcriptase PCR based identification of expressed transcripts resulting from P{GS} screening was as described in (Cermelli et al., 2006). Fly strains detailed herein are available from the Bloomington stock center unless otherwise stated.

P-element mobilizations detailed herein were performed by adding a source of transpose ( $\Delta 2-3$ , 3<sup>rd</sup> chromosome) and, when possible, scoring the P-element eye color marker (the mini-white gene). In reversion and imprecise excision experiments, typically progeny minus the eye color marker were selected, out-crossed, and the chromosome was assessed for lethality when transheterozygous to either the starting stock or a deficiency of the area. When reversion of the lethality was desired (precise excision of the P-element) viability was selected for in this manner. Imprecise excision of a P-element (which occurs at a lower frequency) potentially deletes part or all of a flanking gene(s). When attempting to recover such mutations, lethality was selected for as described above. Lethality determinations were made using balancer chromosomes marked with GFP (typically CyO, Actin-GFP for 2<sup>nd</sup> chromosome mutations) useful for differentiating homozygous and heterozygous embryos, larvae and adults.

Criteria for recovering TILL alleles are described at the TILLing project web interface. In short, factors to consider include;

- the likelihood of generating a stop codon within each 1000bp spanning target region.
- coverage of protein domains; it is advantageous to recover nucleotide substitutions within conserved domains, particularly those of known functional importance.
- nucleotide substitutions resulting in the introduction of stop codons in the 5' region are advantageous over such substitutions closer to the 3' end of gene.
- Cost depends on the number of 1000bp regions screened.

## RESULTS AND DISCUSSION

### **A screen for PNS affecting genes identifies *dappled* and *string*.**

This work began with a screen for genes affecting PNS development (PAPER I). This was a forward screening approach designed to identify novel genes using the UAS/GAL4 binary system (Brand and Perrimon, 1993). Here, mis-expression of random loci utilizing the GeneScreen (GS) P-element (Laviolette et al., 2005) that, when coupled to a GAL4 source, drives the expression of proximal genes. Expression driven by a GAL4 line expressed in the scabrous gene pattern (*sca-GAL4*, expressed in and around the SOP cells and the derived precursor cells), will direct expression of candidate genes within PNS organs during their development. The P-element was mobilized and novel insertions whose mis-expression resulted in visible phenotypes to the external socket and shaft cells of the ES-organs were recovered.

Screening in this manner should specifically recover genes that affect PNS development after prepattern establishment, *i.e.* candidate genes affecting both the lateral inhibition process and, of more interest to us, the PNS precursor cell divisions. In an effort to bias this approach towards the selection of genes affecting specifically divisions of the PNS precursor cells we reasoned that mis-expression of such genes would likely affect the output from individual organs causing a loss or transformation of external cell types. In this manner, the consequences of mis-expression of various novel insertions to the developing ES macro- and microchaetal organs were scored for directly. Those deemed relevant by our phenotypic criteria were kept for identification and further characterization. The ease of this approach facilitated a rapid throughput. The relevance of candidate genes to PNS development would be assessed after their identification.

Approximately 20,000 potential mutants were screened from which 21 candidate lines causing potential cell fate transformations were selected. After identification of a handful of these (PAPER I, Table 1) efforts became focused upon qualifying the candidates relevance and study of those considered the most pertinent. From this, three were selected for further study, *geminin*, *string* and *dappled* on the basis of published data or available loss-of-function alleles.

### *geminin*

The function of a *Drosophila geminin* ortholog had not been described at the time of its retrieval from our screen. We observed a severe reduction of macrochaetae upon *geminin* mis-expression, both bristle and socket cells were absent. We tentatively interpreted this to represent either the loss or the transformation of pIIa cells. Studies in *Xenopus* indicated *geminin* to have a neuralizing function and cell cycle regulatory role (Kroll et al., 1998; McGarry and Kirschner, 1998). On these bases *geminin* was selected for further study.

In an effort to determine the fate of the pIIb daughter cells after exposure to altered levels of *geminin*, we visualized the neuronal cells, via directed expression of a membrane targeted GFP in a *geminin* mis-expression background (non-submitted work). This revealed both cellular fragmentation and increased dendritic branching of the remaining ES-neurons. Work performed in the Richardson lab around this time revealed a conserved role for *geminin* in flies, regulating both cell cycle (S-phase by inhibiting replication licensing factors) and neuronal differentiation (mis-expression resulted in both the formation of ectopic neurons in addition to a reduction of the normal complement of neurons elsewhere within the embryo) (Quinn et al., 2001). Additionally, their mis-expression studies revealed an increase in apoptosis. Thus, the loss of organs resulting from mis-expression of *geminin* in the thorax, likely corresponds to an induced block in the cell cycle and/or increase in apoptosis and was not studied further.

### *string*

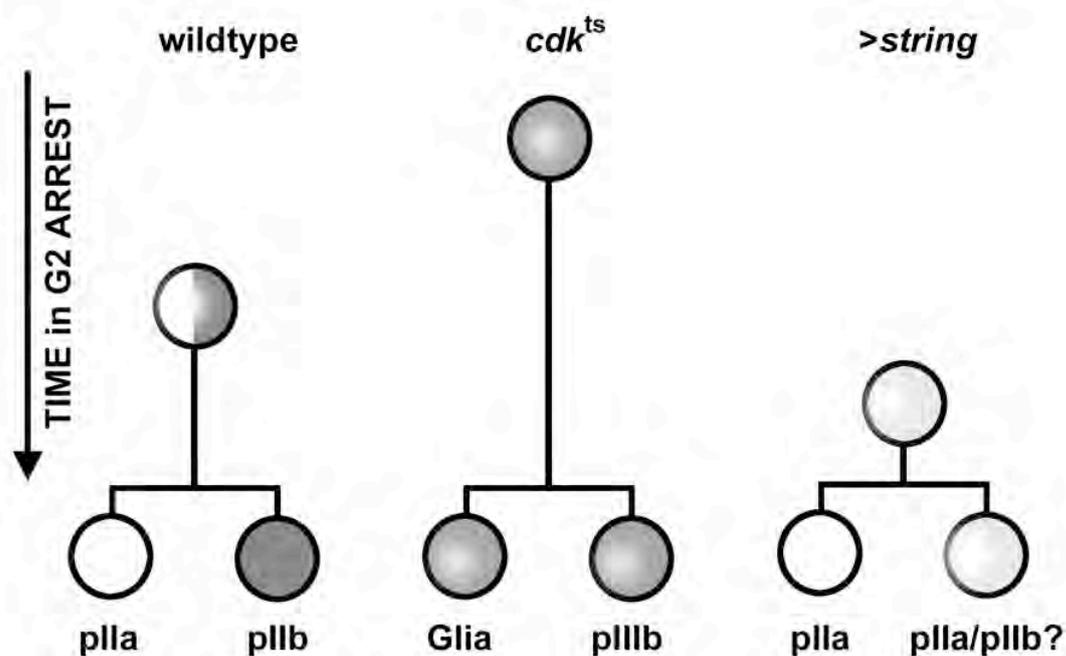
Mis-expression of the *stg* gene lead to extensive loss of macrochaetae in addition to a curious bristle “twinning” phenotype, more predominant in microchaetae. Twinning refers to the appearance of the organ, having two bristles emerging from a single socket cell, presumably resulting from the generation of three pIIa daughter cells. Pre-existing literature indicated a role of *stg* in the developing macrochaetal organs with a specific allele of *stg*, *stg*<sup>hwy</sup>, causing bristle loss (Mozer and Easwarachandran, 1999). Moreover, expression of a negative regulator of *stg*, *tribbles*, via *sca-GAL4*, causes a loss of both macro- and microchaetae (non-submitted data and (Fichelson and Gho, 2004)). *String*, *Drosophila* CDC25, promotes the G2 to M-phase transition via activation of CDK1 (see Introduction). As *stg* expression is generally required for G2 exit, it is unsurprising that it functions during PNS development, however, the finding that mis-expression caused cell fate transformations was largely novel and unexplored.

The most reasonable interpretation of the cell transformation phenotype was an early triggering of mitotic entry resulted in a cell fate change within the ES-lineage. That two bristles and seemingly just one socket cell were generated was mysterious and we delved (quite literally) deeper.

Beginning with the adult microchaetal bristle organs, the effect of *stg* mis-expression to the cells that lay beneath the cuticle was investigated, using a combination of enhancer trap (detecting sheath cells) and immunohistochemical staining (labeling neurons). A positive side effect to the treatment of the adult epidermis in this manner (staining using DAB) is a non-specific labeling of the socket cell body, found under the cuticle. Comparison of wildtype and the twinned bristles resulting from *sca-GAL4* driven expression of P{GS}*stg* (*sca-stg*<sup>GS</sup>), lead to the identification of an extra socket cell beneath the cuticle of the aberrant twinned bristle organ, buried deeper than its twin (PAPER I, Figure 3). This indicates the presence of two pIIa precursor cells within this lineage, both of which divide to generate socket and bristle cells. After close examination of several such organs, we determined that a lone neuron was associated to the four structural cells. Moreover, the neuronal sibling cell, the sheath, could not be detected in any of the organs examined. Determining the relative levels of sheath cells within the entire notum of *sca-stg*<sup>GS</sup> thoraxes revealed an overall decrease in sheath number, consistent with observations made in individual twinned organs (PAPER I, Figure 3). The ectopic pIIa cell appeared to be generated at the expense of a sheath cell. The most expected transformation would be a pIIb to pIIa cell transformation, as shown previously (Rhyu et al., 1994). However, the presence of a neuronal cell associated with these twinned organs deters this model.

The precursor cells divide in a stereotypical fashion (see Introduction and PAPER I) in which the time-points of each division have been documented (Audibert et al., 2005). We next attempted to determine the nature of the observed precursor cell fate transformation via timed induction of *stg* (via the application of heatshock to *hs-stg* animals). Using this approach we asked which precursor cell divisions are affected by ectopic *stg* expression? Surprisingly, the induction of *stg* prior to division of the SOP cell gave rise to the highest penetrance of twinning (PAPER I, Figure 3) *i.e.* early division of SOP cells resulted in a duplication of pIIa daughter cell fates. To reconcile these observations with the described ES-lineages we propose a model in which the premature division of the SOP cell disturbs the asymmetric establishment of cellular determinants. Consequently mis-specified cells are born, which on the basis of the cell types found in the mature organs, we reason to be a pIIa cell and a pIIa/pIIb hybrid.

This hybrid cell subsequently divides, giving an ectopic pIIa cell and a neuronal cell. This model takes into account the wildtype number of cell divisions that occur within the lineage and the observed cell types composing the aberrant organs. Reciprocal experiments in which *stg* function is blocked, increasing the G2 arrest period, in developing ES-SOP cells leads to a loss of both bristle and sheath cells (Fichelson and Gho, 2004). These SOP cells switch fate, becoming pIIb-like, the pIIa daughter structural cells are lost from the lineage. Collectively, the data on *stg* suggest a tight temporal coupling of the processes of cell division to asymmetry establishment prior to precursor cell division, see Figure 8.



**Figure 8. Temporal effects on the microchaetal SOP asymmetric cell division.** Shaded portion indicates cellular distribution or inheritance of an asymmetric determinant such as Numb, normally distributed to the pIIb, biasing it towards a more neural fate. In conditional *cdk* mutants, the long period in G2 results in a loss of structural cells. The opposite effect is observed upon shortening the G2 period, extra structural cells emerge at the expense of pIIb daughter cells. Potentially this reflects less time granted the SOP to coordinate fate determinant transcription or distribution.

We next attempted to investigate the consequences of premature mitotic entry within the embryonic Ch-organ precursor cells. These lineages are well-described, analogous to adult ES-lineages, generate terminally differentiated cells that are readily distinguishable and the divisional times of the precursor cells are roughly established (Bodmer et al., 1989; Brewster and Bodmer, 1996; Inbal et al., 2004). However, the results obtained therein were not entirely congruent with observations made in the adult ES-lineages. Here, timed induction of *stg* was used to assess the

effect of mitotic de-regulation on Ch-precursor cells. Similarly to the ES-organs, induction of *stg* prior to division of the SOP cell had the greatest effect upon the Ch-organs. Thus, particular sensitivity of the SOP to mitotic deregulation is conserved between these lineages. However, no cell fate transformations were observed within the Ch-lineage. Instead we frequently observed the loss of entire organs (PAPER I, Figure 4) in addition to a mild loss of pIIb daughter ligament cells. While many aspects of the SOP selection process are conserved between Ch and ES-SOP cells, differences exist (see Introduction for a description of EGFR mediated Ch-SOP recruitment). Normally, the 1<sup>st</sup> of the three SOP cells selected by lateral inhibition has divided by the time the recruited SOP cells begin to show proneural gene expression (zur Lage et al., 1997). This is an indication of the time constraints in which this recruitment process occurs. Potentially, its disturbance, via hastened division of the SOP, does not allow time for proper recruitment to occur.

The differing susceptibilities of ES- and Ch-lineages to switch cell fate, as a consequences of premature division, may reflect more robust temporal coupling of the mitotic and asymmetric machineries within the Ch-lineage. That differences per se are observed between these lineages is not entirely surprising, the terminal cell fates are very different. Reflecting this, the specifying proneural genes of each SOP (ES and Ch) are distinct. It has been proposed on the basis of the relationships between *cut*, *ato* and *ac-sc*, that the Ch-organ represents the “base model” of a PNS organ (Jarman and Ahmed, 1998). The observation that Ch-precursors seem more robust to precocious mitosis may reflect this. ES-lineages perhaps require additional factors, which may come at a cost to the “robustness” of divisions within these lineages. Alternately, divisions of all embryonic PNS precursors may be “generally” more robust than adult precursors.

### *dappled*.

*dappled* mis-expression in the developing adult ES-lineages frequently lead to the formation of ectopic organs and, with considerably lower frequency, twinning of bristles. This latter phenotype, together with its described tumorous phenotype, prompted our characterization of this gene. We documented the expression of *dpld* during embryonic development and found it expressed within the developing PNS organs, with specific expression in neuronal cells at the end of PNS development (non-submitted data and PAPER IV, Figure 3). This, together with the observation that a pre-existing *dpld* allele (*dpld*<sup>k08815</sup>) caused an embryonic PNS phenotype, guided our

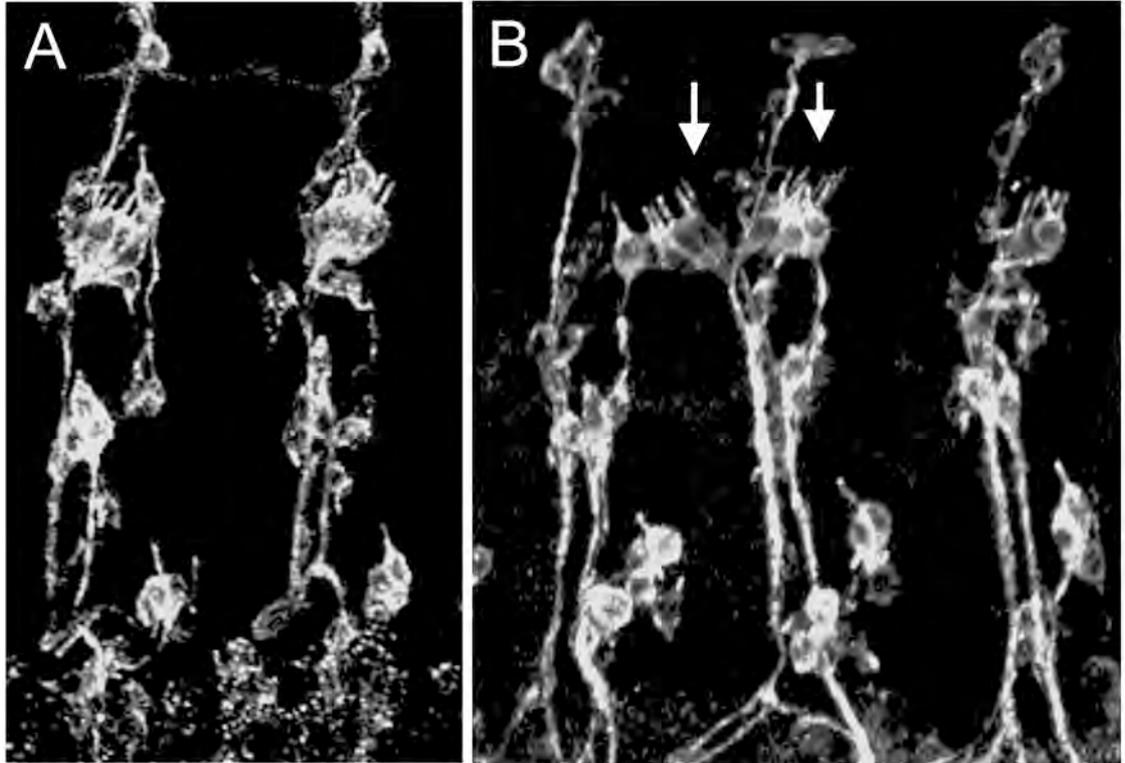
focus towards the embryonic PNS (*dpld* expression within the adult bristle organs is not documented). We provide a brief description of these mis-expression phenotypes in PAPER I.

Mis-expression of *dpld*<sup>GS</sup> within the developing PNS (directed via *sca-GAL4*, referred to as *sca-dpld*<sup>GS</sup>) resulted in pleiotropic phenotypes (PAPER I, Figure 2), frequently defects in organ positioning and neuronal guidance. Mis-guidance resulting from ectopic expression could occur in several ways, and need not represent an autonomous role for *dpld* in the neuron. Mis-expression in tissues surrounding the developing PNS, via *sca-dpld*<sup>GS</sup>, could just as easily cause guidance defects. As such these observations alone are not very descriptive. However, at the time, the *dpld*<sup>k08815</sup> allele also displayed mis-positioning of organs, indicating this phenotypic trait to be relevant. More recently we have isolated and characterized a novel *dpld* allele (*dpld*<sup>802:5</sup> described in PAPER III), which also displays mis-positioning of PNS organs and neuronal guidance defects, indicating that this aspect of the mis-expression phenotype may in fact be relevant (Figure 9, this work). As we documented expression of *dpld* in the developing neuron, the loss of function phenotype advocates for an autonomous role of *dpld* in neuronal guidance, although the significance of this has yet to be investigated.

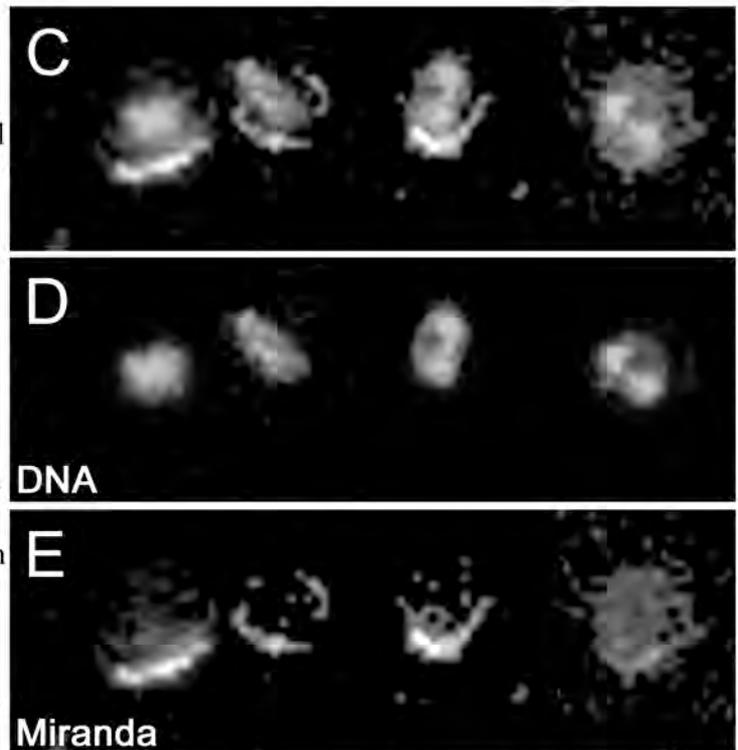
Additionally, within *sca-dpld*<sup>GS</sup> embryos, organs of the Lch5 were lost in a manner similar to that observed in *sca-stg*<sup>GS</sup>. Here also, interference with the recruitment process inherent to these Ch-organs may underlie this phenotype. We did not observe any cell fate transformations within *sca-dpld*<sup>GS</sup> embryos (PAPER I) nor could they be induced by mis-expression using either general or neural specific GAL4 drivers (non-submitted data). However, entire organ duplications of the Lch1 lineage were observed in *sca-dpld*<sup>GS</sup> embryos (PAPER I, Figure 2). This, like the ES-organ duplications observed in the adult, likely reflect interference with lateral inhibition, resulting in the selection of more than a single SOP. From our observations in both adult ES- and embryonic Ch-organs we conclude that *dpld* may play a role in SOP selection in addition to neuronal pathfinding.

### **DPLD belongs to the LIN-41 sub-clade of the TRIM superfamily.**

After the recovery of *dpld* from our screen we began to assess the relevance of it during PNS development. In-situ hybridization experiments directed against *dpld*, found it to be expressed in the developing embryonic and adult PNS (and CNS) (PAPER IV, Figure 3). This would guide our focus towards study of *dpld*



**Figure 9. PNS phenotypes.** Labeling wildtype (A) and *dpld*<sup>802:5</sup> maternal/zygotic null (B) embryos with MAb22c10 directed against the neuron and its projections revealed guidance phenotypes and entire organ mis-positioning. Arrows indicate two Lch5 organs that have fasciculated together. A corresponding loss of an Lch5 is observed in the anterior (left) hemisegment. This loss of function phenotype is very similar to that observed from *dpld*<sup>GS</sup> mis-expression (PAPER I), collectively arguing for a role during neural development.



(C-E) Visualization of pIIb/pIIIb precursor cells mitoses of *scra* mutant embryos, using the asymmetric determinant Miranda as a marker for pIIb/pIIIb divisions (E) and anti-phosphohistone H3 as a marker for condensed mitotic chromosomes (D). (C) Overlay. *scra* embryonic neural precursor cells (pIIb/pIIIb) expressed mitotic markers at much later stages of embryonic development than wildtype. We interpret this as prolonged/delayed divisions, specifically in pIIb/pIIIb cells. The wildtype complement of pIIa daughter cells were observable at the end of PNS organogenesis, while pIIb daughter cells were predominantly lost. Note, the image represents independent cells in close proximity.

function during both development of the embryonic PNS (inadvertently leading to PAPER II) and the eye (PAPER III).

DPLD is an NHL containing B-box protein, a subgroup of proteins belonging to the Tripartite Motif (TRIM) family. We wished to establish the relationship of *Drosophila* NHL containing TRIM proteins to those of other species. Interestingly, we noted novel mammalian TRIM's which associated with the DPLD/LIN-41 sub-clade, subsequently reported by (Lancman et al., 2005; Schulman et al., 2005; Kanamoto et al., 2006). Phylogenetic reconstruction placed BRAT and MEI-P26 to sub-clades, distinct from both TRIM2/3 and LIN-41 that appear not to contain vertebrate orthologs. Via these analyses we discovered a new *Drosophila* TRIM, which we refer to as Another B-box Affiliate, ABBA, which encodes for the most orthologous fly protein to mammalian TRIM2/3 (PAPER IV Figure 2). We characterized the expression pattern of *abba* and found it to be specifically transcribed in larval muscles (PAPER IV Figure 3). Thus, the four *Drosophila* TRIM genes are expressed in a non-redundant fashion, with the exception of *dpld* and *brat* which are both transcribed in the embryonic CNS and PNS (PAPER IV Figure 3 and (Arama et al., 2000)).

### **A Pre-existing *dappled* allele displays PNS phenotypes.**

As mentioned, a deciding factor in selecting *dpld* for further study included the availability of *dpld* loss of function alleles, crucial in functional studies. These alleles however, would ultimately lead us astray. The embryonic PNS was our first choice of system in which to study *dpld* function on the bases mentioned above.

The 1(2)k08815 chromosome is stated to contain a P-element in the *dpld* locus (consult Flybase for details). This allele is referred to as *dpld*<sup>k08815</sup> and is recessive embryonic lethal. The mutation was identified on the basis of sequence recovered from the genomic region surrounding a lethal P-element insertion (plasmid rescue). Our own plasmid rescue experiments (several independent experiments) confirmed this finding. No other insertion sites were identified, despite that this chromosome is also stated to harbor an additional P-element insertion approximately 200kb away (See Figure 10, this work) in the unstudied locus CG2064. Using genomic deletions we could discriminate the contribution of each insertion to the embryonic lethality and associated phenotypes, ruling out a contribution of CG2064 (See Table 1). Characterization of the embryonic phenotype was performed using a transheterozygous combination of *dpld*<sup>k08815</sup> and a deficiency not disrupting CG2064. The *dpld*<sup>k08815</sup> phenotype included the loss of specific cells stemming from the pIIb and pIIIb

precursor cells, whilst divisions of the SOP and pIIa precursors were not affected. This appeared to be caused by a failure of the pIIb precursor cells to divide (Figure 9. this work), leading to multinucleation and an aberrant increase in mitosis observed in the late stages of embryonic PNS development (similar to that shown in PAPER II).

In order to irrefutably demonstrate that this phenotype resulted from loss of *dpld* function we attempted genetic rescue experiments, supplying the animal with an independent copy of the *dpld* gene. This was attempted without success using several approaches. Flies containing the *dpld* transgene, either full length *dpld* cDNA or the entire *dpld* locus (detailed in PAPER IV) under the control of the UAS promoter, were created and used to express *dpld* during embryogenesis in either a ubiquitous (*armadillo*, *daughterless*), segmental (*engrailed*, *hairy*), neuronal (*elav*) or PNS (*scabrous*) pattern (non-submitted work). This approach failed to rescue either the embryonic lethality or PNS phenotype, although frequently caused severe embryonic phenotypes, indicating the transgenes to be expressed.

### ***Scraps? Not dappled?***

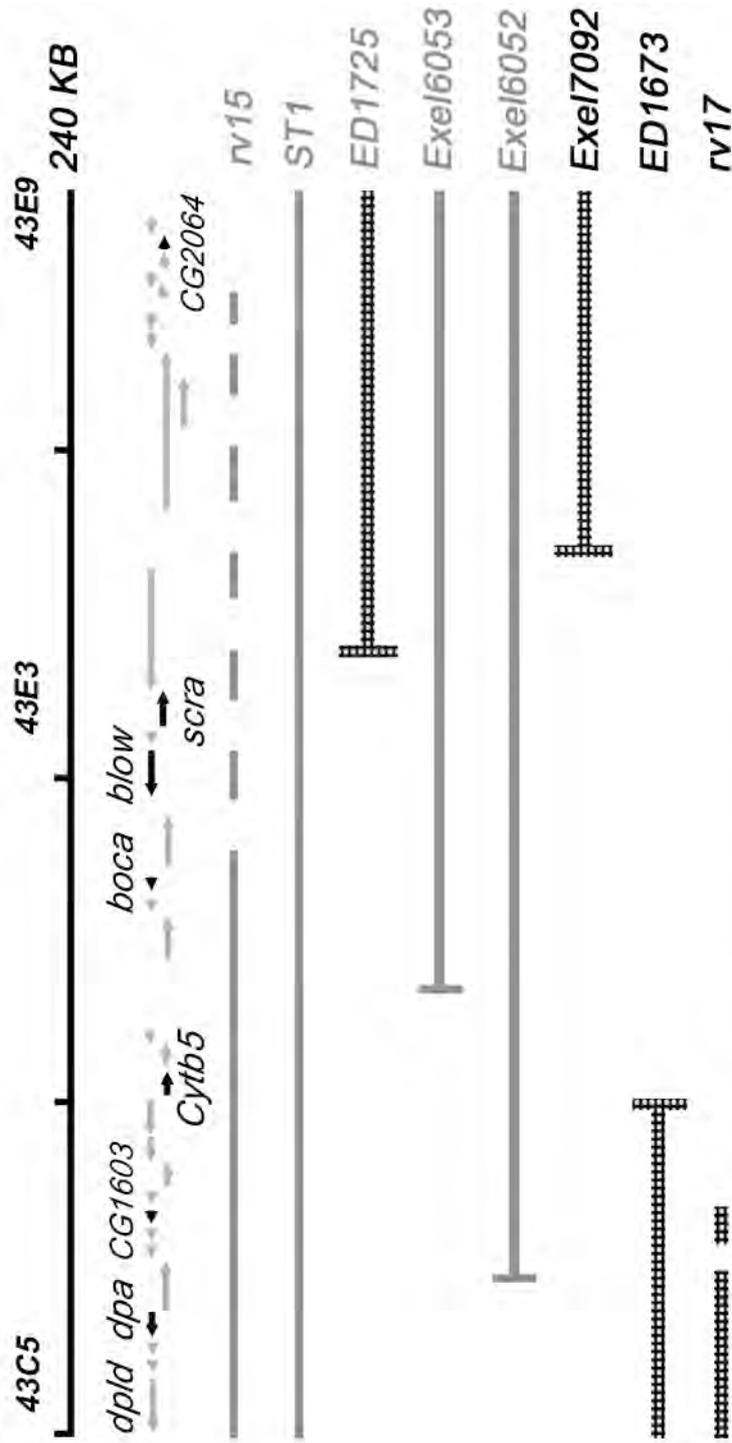
Around this time, another experiment was giving way-finding results. Several attempts to revert the *dpld*<sup>k08815</sup> PNS phenotype and lethality were performed, screening in excess of 200 excision or “jump-out” events, looking for reversion of the lethality (see methods). None of the excision events recovered gave complete restoration of the chromosome *i.e.* viable homozygous adults. As this chromosome is stated to harbor an additional P-element insertion (CG2064), the reversion potential of this insertion was questioned, and, this P-element mediated mutation proved to be revertible. This particular CG2064 reverted chromosome retained the embryonic lethality and PNS/CNS phenotype. Assessing the lethal stage of 50 of these non-reverted “jump-out” events indicated that although lethality was not reverted in any case, the stage of lethality in some cases shifted from embryonic to larval (Table 1, this work). One implication of this was that the embryonic lethality was caused by a revertible P-element insertion. However, this chromosome potentially harbored more than a single lethal mutation, in fact this proved to be the case.

Complementation studies, using both genomic deletions and described alleles within the region (cytological location 43, see Figure 10), were performed in an effort to determine the location of the revertible P-element responsible for the embryonic phenotype. Deletion coverage of the *Drosophila* genome increases with

**Table 1. A summary of Complementation studies mapping mutations in the *dappled* gene region.**

| Gene/Allele                   | Comp.<br><i>dpld</i> <sup>k08815</sup> | Comp.<br><i>dpld</i> <sup>k14202</sup> | Comp.<br><i>scra</i> <sup>k08255</sup> | (No. of alleles used)<br>Comments            |
|-------------------------------|--|--|--|--|
| <i>dpld</i> <sup>k08815</sup> | N (E.L)                                | N (L.L)                                | N (E.L)                                | Not Revertible?                              |
| <i>dpld</i> <sup>k14202</sup> | -                                      | N (L.L)                                | Y                                      | Reversion ND                                 |
| <i>scra</i> <sup>k08255</sup> | -                                      | -                                      | N (E.L)                                | Revertible                                   |
| <i>dpa</i>                    | N                                      | Y                                      | Y                                      | (2) Revertible                               |
| <i>CG1603</i>                 | N                                      | Y                                      | Y                                      | (3) <sup>X</sup> Revertible                  |
| <i>Cytb5</i>                  | N <sup>T</sup>                         | N <sup>T</sup>                         | Y                                      | (2) Not Revertible?<br>Tumors!               |
| <i>scra</i>                   | N                                      | Y                                      | N                                      | (2) Revertible                               |
| <i>boca</i> *                 | N                                      | ND                                     | ND                                     | (1) Reversion ND                             |
| <i>blow</i> *                 | N                                      | ND                                     | ND                                     | (1) Reversion ND                             |
| <i>CG2064</i>                 | NA                                     | ND                                     | ND                                     | (NA) Revertible                              |
| <i>dpld</i> <sup>802:5</sup>  | Y                                      | Y                                      | Y                                      | Represents a micro-deletion of <i>dpld</i> . |

The lethality stage is given where determined, E.L = embryonic lethal, L.L = larval lethal. N= failure to complement; Y= complementation. <sup>X</sup> Indicates two previously unmapped alleles referred to as Da and Cc which failed to complement each other and both *dpld*<sup>k08815</sup> and an allele of CG1603. \* A *dpld*<sup>k08815</sup> “jump-out” (see text) variant was used; the embryonic phenotype and all mutations mapped to *dpld*<sup>k08815</sup> were present, except *dpa*, which was reverted. NA = independent allele not available. ? No reverted lines recovered. 50 potentials “jump-out” events were assessed. <sup>T</sup> indicates the presence of melanotic tumors.



**Figure 10. A summary of the l(2)k08815 mutations mapping to cytological location 43.**

The l(2)k08815 chromosome is multiply mutated. Genes are denoted with arrows, names of those found to be mutated in the l(2)k08815 background are depicted. Genomic deficiencies useful for mapping of the various lethalitys on this chromosome are given. Breakpoints when known are indicated. Solid grey bars indicate deficiencies which uncovered the embryonic lethality. See accompanying table for further details.

time thanks to the efforts of the *Drosophila* Genome Deletion Project, making finer mapping of the *dpld* genomic region possible. This approach led to two conclusions; several lethal mutations were present on the *dpld*<sup>k08815</sup> chromosome (Table 1, this work); the embryonic lethality mapped outside of the *dpld* locus. For some loci the developmental stage in which lethality occurred was assessed. In this way an embryonic lethality was uncovered when the *dpld*<sup>k08815</sup> chromosome was transheterozygous with an allele of *scraps* (*scra*<sup>8</sup>), a significant finding. The reversion potential of the individual I(2)k08155 mutations was assessed utilizing the I(2)k08815 “jump-out” lines described above, and the non-complementing independent alleles. Several of these insertions were revertible (Table 1, this work). This indicates that a large chromosomal break, deleting numerous genes, did not account for the mutation of multiple genes in this region.

In addition to using mapped mutations for complementation studies, lesions partially mapped to the relevant cytological location that were stated to be lethal were also examined. Several of these unmapped alleles were also lethal with the I(2)k08815 multiply mutant chromosome (Table 1, this work and non-submitted data), including the partially mapped P-element insertion I(2)k08255. This P-element insertion was found to be embryonic lethal, both homozygously and when transheterozygous to the I(2)k08815 chromosome. Plasmid rescue placed this insertion in the *scra* locus (PAPER II). This allele failed to complement the *scra*<sup>8</sup> allele, moreover, subsequent experiments showed that the combinations I(2)k08815/*scra*<sup>8</sup>, I(2)k08815/I(2)k08255 and *scra*<sup>8</sup>/I(2)k08255 (not shown and PAPER II) recreated the embryonic phenotype. Precise excision and a consequent reversion of the I(2)k08255 chromosome to wildtype indicated that the P-element insertion was the source of the lethality and phenotype, we dubbed it *scra*<sup>k08255</sup> (PAPER II).

Collectively, this indicated *scra* to be both the source of the PNS phenotype and embryonic lethality we originally thought caused by mutation of *dappled*. Genetic rescue of the *scra* mutation using the full length *scraps* cDNA (detailed in PAPER II) proved successful, as assessed by both rescue of the embryonic lethality (animals survived till pupation) and a partial rescue of the PNS phenotype (PAPER II, Figure 2 and non-submitted data). That complete rescue to adulthood was not obtained potentially reflects broad *scraps* expression patterns not fully represented by the GAL4 expression pattern (which can be mosaic). Additionally, we observed nervous system phenotypes and semi-lethality consequent to *scra* mis-expression in a wildtype background, compounding this issue (non-submitted data). However, in

various *scra* backgrounds we note a return to wildtype position of many neurons in addition to a restoration of many previously missing cell types (PAPER II and non-submitted data). Additionally, transheterozygous combinations of *scra* alleles and the l(2)k08815 chromosome were rescued (non-submitted data). Based on this we attributed the PNS phenotype to the *scraps* locus. In PAPER II no data using the l(2)k08815 chromosome is presented, instead the *scra*<sup>8</sup> and *scra*<sup>k08825</sup> alleles were exclusively used. This was largely due to the complicated background of the l(2)k08815 chromosome which we consider to be multiply mutated.

During the complementation studies we noted the l(2)k08815 (multiply mutated) chromosome failed to complement alleles of the *Cytochrome b5* (*Cytb5*) gene (Table 1, this work). Moreover these allelic combinations gave rise to larval tumors. Complementation testing between *Cytb5* and the other pre-existing *dpld* allele, *dpld*<sup>k14202</sup>, revealed that it too harbored a mutation in the *Cytb5* locus (Table 1, this work). Additionally, deficiencies uncovering the *dpld* locus of *dpld*<sup>k14202</sup> chromosome, but not the *Cytb5* locus, were not lethal (Figure 10, this work). Cumulatively, these results suggest that a larval lethality and the tumorous phenotype maps to the *Cytb5* locus.

In an effort to isolate a true *dpld* allele we identified the *dpld*<sup>802:5</sup> micro-deletion (PAPER III and discussed below). This allele is homozygous lethal, however it complements both of the pre-existing *dpld* alleles (Table 1, this work). Thus the data indicates that the *dpld* mutations on these chromosomes, at best represent weak hypomorphic alleles, that are not responsible for the tumorous phenotype. Consequently, all conclusions drawn from these alleles regarding *dpld* should be treated with caution.

### **Anillin is required for the unequal divisions of the PNS.**

As stated, *scraps* encodes for *Drosophila* Anillin, a protein required for cytokinesis. Having already documented PNS defects associated to the “*dpld*” l(2)k08815 chromosome, we have now reproduced the key findings using the genetically cleaner alleles, these being; independently generated; the P-element allele was revertible; and the phenotype and lethality of the combination of these alleles was genetically rescued. Using this genetic combination we found that *scra* was specifically required within the pIIb lineages of both Ch- and ES-lineages (PAPER II, Figure 3).

As a consequence of *scra* mutation, neuronal cells (that developed, ES-neurons were frequently lost) were enlarged and multinucleated. Surprisingly, we noted

that a subset of such aberrant neuronal cells (which should be mitotically inactive) displayed cell cycle markers indicative of G1, G2 and mitosis, demonstrating a loss of cell cycle control (PAPER II, Figure 4 and non-submitted data). On the basis of Anillin function, we ascribe these traits to a divisional failure of the precursor cells. Indeed, data stemming from the I(2)k08815 indicated this fault to occur within the pIIb/pIIIb cell (Figure 9C-E, this work). Anillin depletion is known to result in cell division failure, leading to enlarged multinucleate cells (Echard et al., 2004). Polyploidy has been shown to lead to aberrant cell cycling (Fujiwara et al., 2005). Hence these aspects of the PNS represent well-characterized defects resulting from failure of cellular division. However, an odd (but interesting) facet of the mutant phenotype was the biased requirement for *scra* within the PNS lineages. We have not investigated this further but provide potential explanations, the most exciting (and speculative) of which is based upon the intrinsic differences between the cell types affected. As described in the Introduction, the pIIb and pIIIb precursor cells undergo asymmetric cell division in a manner distinct to that of the SOP and pIIa cells. The division of the pIIb cell is unequal, generating differently sized daughter cells. Potentially there is an added requirement for *scra* within these unequal divisions. An alternate explanation of the unbalanced requirement for Anillin during PNS organogenesis is that degradation of Anillin occurs preferentially, or more readily, within the pIIb cell. In other systems Anillin has been shown to be selectively degraded via ubiquitination (Zhao and Fang, 2005).

A drawback of using *Drosophila* as a model system is the maternal contribution of gene products to the embryo. Here, the mother supplies the embryo with protein and RNA to enable it to progress through the first stages of embryonic development without requiring gene transcription. This expires at various times during development depending on the gene in question. While maternal contribution of Anillin to the embryo overshadows our speculation, as we pointed out in PAPER II, the pIIa cell (which is unaffected) divides after the (affected) pIIb cell. This argues against expiration of maternal contribution accounting for the differences we observe.

### ***dappled* regulates proliferation during adult eye formation.**

As described above, we encountered difficulties with pre-existing *dappled* alleles. To obtain gene mutations in *Drosophila*, a common approach is to use a P-element to create imprecise excisions, resulting in deletions of the gene of interest.

We attempted this while under the impression that *dpld* was embryonic lethal. After extensive screening (>300 jump-out events), no such mutations were recovered. We determined to take another (non-P-element) approach towards obtaining a *dappled* allele. The Tilling project represents a collection of 5600 2<sup>nd</sup> chromosome EMS mutagenized stocks (Winkler et al., 2005), the vast majority of which are unmapped (see methods for a brief description of the criteria by which this collection can be screened). We obtained a five-nucleotide deletion within the 5' coding region of the *dappled* gene, this resulted in a frame shift mutation introducing numerous stop codons, the closest being two codons away (PAPER III, Figure 1). This likely represents a molecular null. This allele, referred to as *dpld*<sup>805:2</sup>, is lethal, however, it is not embryonic lethal, nor has it an associated embryonic PNS or tumorous phenotype (PAPER III). Rather *dpld*<sup>802:5</sup> is larval lethal with a low frequency (below 0.1%) of adult escapers. Expression of the *dpld* transgene via ubiquitously expressed *armadillo*- or *daughterless-GAL4* drivers successfully rescued the lethality, giving viable fertile adults.

The adult escapers, when self-crossed, lay eggs that do not hatch. However, when females were out-crossed to either *dpld*<sup>802:5</sup> homozygous flies carrying the transgene (and a source of GAL4) or *dpld*<sup>802:5</sup> transheterozygous flies, viable progeny are obtained. This indicates that *dpld*<sup>802:5</sup> females are not sterile and moreover that parentally supplied *dpld* (either wildtype zygotic or transgene) is sufficient for embryonic development. Therefore maternal contribution appears not to be strictly required. As mentioned, the embryos of homozygous parents display guidance defects within the PNS (Figure 9, this work). Having a bona fide *dpld* mutation in hand we have begun to question its role during development.

Having documented the pheno-critical time point (late larval stages) and having noted *dpld* expression in the developing eye (amongst other tissues, PAPER IV) during this stage, we began investigating the role of *dpld* mutation during eye development. *dpld* is broadly expressed around the morphogenetic furrow (MF) and in a cell specific fashion within the differentiating photoreceptors (PAPER IV, Figure 3). The imaginal discs of *dpld*<sup>802:5</sup> homozygous larvae are frequently mis-shaped and smaller than wildtype. Within the developing retina of *dpld* mutants we observed a striking increase in the levels of actively proliferating cells when compared to wildtype (as judged by anti-Cyclin B (CycB) and anti-Phosphohistone H3 (PH3) staining, PAPER III, Figure 2). This was most evident behind the morphogenetic furrow (MF), within the second mitotic wave (SMW). The MF appeared narrower than wildtype,

indicating a reduction in the numbers of cells arrested in G1. Occasionally, mitoses were even noted within the furrow itself. We interpret this as a deregulation of the cell cycle leading to an increase in proliferation in the eye. Mis-expression studies, expressing *dpld* specifically behind the furrow (via *GMR-GAL4*, referred to as *GMR>dpld*), caused a suppression in the number of actively proliferating cells, reflected by a sharp drop in the levels of CycB and a reduction in the number of cells labeling with anti-PH3 (PAPER III, Figure 2). Mis-expression of *dpld* in this fashion did not cause an increase in the levels of apoptosis within the developing retina (non-submitted data). Mis-expression of *dpld* during the earlier stages of eye development (via *eyeless-GAL4*) lead to a decrease in the size of the adult eye and could induce transformations of eye to antennal tissue, a phenomenon known as transdetermination (PAPER III). Both the reduction in eye size and transdetermination are known to result from decreased proliferation (Duong et al., 2007) and see (McClure and Schubiger, 2007).

We questioned the consequence of the increased proliferation observed in *dpld*<sup>802:5</sup> eye discs upon cell types of the developing retina. Examination of both photoreceptors and cone cells demonstrated that these cells frequently failed to develop (PAPER III, Figure 3). While we have not yet examined this in detail we postulate that this may represent a similar situation to *stg* mis-expression effect in the developing macrochaetae (see Introduction), where shortening of the cell cycle perturbs SOP selection. We observe decreased time spent in G1 and the presence of mitotic cells in the furrow. Alternatively, uncontrolled mitosis within a specified photoreceptor cell prior to its proper differentiation, could deter its specification.

Within the *GMR>dpld* eye discs, both neuronal and structural cells (or more specifically the nuclei of these cells, as the markers used, ELAV and PROS, are both nuclear) of the developing retina become displaced from the wildtype apical position. Within the plane of the eye disc these cells moreover appear enlarged (PAPER III, Figure 5). Sections of adult *GMR>dpld* eyes revealed severe morphological defects; Rhabdomeres fail to elongate and structural cells are aberrantly localized, defects most likely due to interference of *dpld* with the differentiation of these cell types. The eyes of *dpld*<sup>802:5</sup> adult escapers were sectioned in parallel, and apart from the appearance of gaps between ommatidia these appeared normal (non-submitted data).

The eye phenotype resulting from *GMR>dpld* has been used to screen candidate genes for interaction with *dpld*, assessing enhancement or suppression of the

*dpld* eye phenotype (PAPER IV, Figure 5). In this manner approximately 150 genes, have been tested. These represent a broad spectrum of genes for the most part involved in processes relevant to eye development, such as prepatterning, photoreceptor recruitment, general cell cycle regulators. Others were selected on the basis of their recovery from a yeast-2-hybrid screen, searching for interaction with the NHL domain of DPLD (non-submitted data). This was in an effort to place *dpld* in a genetic pathway. Few candidates were obtained in this approach. The cell cycle affect of *GMR>dpld* is relatively minor, requiring two copies of the transgene to exert a measurable effect on the SMW (PAPER III, Figure 2), potentially reflecting the GMR expression pattern and strength, rather than a weak anti-proliferative affect of *dpld*. The mis-expression effect on differentiation is much more striking. Hence, this enhancer suppressor approach was more likely to recover interaction with genes involved in cell differentiation.

The genetic interaction with *lola*, which encodes for a BTB type transcriptional repressor, is presented in (PAPER III, Figure 6). This interaction results from mis-expression of *dpld* in a *lola* heterozygous background and presents as an enhancement of the *GMR>dpld* rough eye phenotype. The shape of the entire eye is additionally modified (in approximately 50% of cases), becoming strikingly prominent. This interaction was reproducible with two independent *lola* alleles and most likely represented an enhancement of the differentiation defects post furrow. This assessment is made on the basis of the above argument (*dpld* mis-expression primarily affects differentiation) and the observation that *lola* is involved in retinal differentiation (Zheng and Carthew, 2008). The most likely interpretation of this genetic interaction is that lowered levels of *lola* resulted in increased *dpld* expression, implying that *lola* normally negatively regulates *dpld*, presumably on a transcriptional level given the nature of LOLA. However, a negative regulation of LOLA by DPLD is also possible. In this model, increased levels of DPLD (presumably on a protein-protein level in this case, but otherwise indirectly) acting negatively upon LOLA results in an enhancement of the phenotype upon removal of one gene copy of *lola*. In an effort to clarify this, we have probed for direct protein-protein interactions with domains of LOLA and DPLD and we find that the NHL domain of DPLD are capable of interaction with the LOLA Zinc-finger domains, speaking for the second proposed epistatic relationship. While the relevance of this interaction needs to be probed more deeply, it currently offers an explanation for the *dpld* effect in the eye, *i.e.* an interaction with a global transcriptional regulator capable of influencing multiple aspects of development, could account for the

severe and complex phenotypes observed in both gain and loss of function *dpld* eye discs.

### ***dappled* is targeted by miRNA.**

The observation that DPLD belongs to the LIN-41 sub-clade of TRIM proteins (described above and in PAPER IV) combined with the prior studies of *C. elegans lin-41* regulation by miRNA (Slack et al., 2000), spurred our interest in assessing the relevance of this mode of regulation to the fly gene, *dpld*. Using initially an in-silico approach, we combined use of multiple sequence alignment of predicted Drosophilidae *dappled* 3'UTR's, to search for highly conserved regions, and miRNA target prediction software (Enright et al., 2003; Rehmsmeier et al., 2004), to determine relevant miRNA target sites (PAPER IV Figure 4). The regions that overlapped, *i.e.* miRNA target sites within conserved regions, were deemed interesting for further study. Using the *GMR>dpld* eye phenotype we screened for an enhancement or suppression of phenotype when transheterozygous to deficiencies uncovering miRNA loci. This approach uncovered several genetic interactions with large regions of the genome encoding miRNA (PAPER IV, Table 1 and Figure 5). As *let-7* regulation had been shown to regulate *dpld* protein orthologs, whereas the other candidates appeared to be *Drosophila* specific, we chose to examine the *let-7* interaction further using a previously established *Drosophila* cell culture assay (Burgler and Macdonald, 2005). Using this luciferase approach, *dpld* 3'UTR's, in addition to UTR variants in which the *let-7* target sites were deleted, were tested for activity in the presence of co-transfected *let-7* or an unrelated miRNA as a control. This approach revealed that removal of specific *let-7* target sites within the *dpld* 3'UTR altered the sensitivity of the UTR containing reporter construct to *let-7* expression (PAPER IV Figure 6). We noted that the *let-7* target site most proximal to the translational stop site was the most significant of the three candidate target sites. This final observation lead to the hypothesis that miRNA regulation of this sub-clade of the TRIM family is likely widely conserved.

## CONCLUSIONS

We performed a screen directed towards the recovery of genes affecting PNS development, with an underlying emphasis being placed upon genes regulating the cell cycle. This screen, although small, was moderately successful. The subsequent characterization of *dappled* and *string* revealed them both to be relevant during PNS development and in a cell cycle regulatory context. *stg* was capable of causing cell fate changes upon mis-expression. This lead us to conclude that a long G2 arrest of the SOP cell is required, likely reflecting temporal coupling of the cell cycle with asymmetric establishment. This was detailed in both the developing embryonic and adult PNS, allowing comparison between the two, the results of which dictated that the lineages therein are not equally susceptible to cell fate changes.

Detailed examination of pre-existing *dappled* alleles uncovered the presence of multiple mutations in their genetic backgrounds. Surprisingly, both these alleles had at least one (additional) mutation in common, in the nearby *Cytb5* locus. This mutation was responsible for the “*dpld*” larval lethality and tumorous phenotype. Hence we have dissociated the tumorous phenotype from *dpld*. One of the pre-existing allele additionally harbored a mutation of *scraps*, *Drosophila* Anillin. Mutation of *scraps* bestowed an embryonic lethality to the chromosome and moreover caused a severe PNS phenotype. We could attribute this to the failure of specific neuronal precursor cells to divide. We interpret this to reflect a biased requirement for Anillin within the divisions of the embryonic PNS.

DPLD is a TRIM gene, despite the lack of a RING finger domain. It is orthologous to the LIN-41 proteins, found in both vertebrate and invertebrate species. Moreover, a mode of regulation, miRNA, is potentially broadly conserved throughout the LIN-41 sub-clade.

We identified a novel NHL-TRIM fly gene, expressed in muscle.

We have recovered a *bona fide* *dpld* allele and our preliminary work indicates that, much in line with the original hypothesis, *dpld* regulates proliferation. Specifically, loss of function leads to excess proliferation while mis-expression curbs normal cellular proliferation. *dpld* moreover affects cellular differentiation in both gain and loss of function backgrounds.

Now knowing the developmental processes to which *dpld* is integral, being able to disregard the previous mutant data, and having a vital genetic tool in the form of the *dpld*<sup>802:5</sup> EMS allele, the more interesting task, characterization can begin.

## FUTURE PERSPECTIVES

Some of the issues raised by this work have been addressed along the way, while others not. Some of the more interesting remaining questions and possible approaches to answer them include;

Does Anillin function in positioning and/or buildup of the contractile ring? Recent publications suggest that this may be the case in certain systems. Is there a biased Anillin requirement within unequal cell divisions as we suggest? Previous data indicates that Anillin is not required for contractile ring assembly in *Drosophila* but a null allele has not been examined. Null clones of Anillin using the alleles we describe may be useful to address both these issues, preferentially examining the asymmetric cell divisions within developing larvae CNS or adult PNS, developmental stages by which time maternally contributed Anillin has expired.

What is *dappled*'s normal function during eye development? Is it a suppressor of proliferation as our data suggests? Our preliminary data suggests that *dpld* function is not limited to the developing eye, and that maternal contribution overshadows an earlier role in embryogenesis. This warrants further detailed investigation. The germline clone technique will overcome issues arising from maternal contribution and permit examination of embryonic neural tissue development in the absence of *dappled*, while clonal analysis in developing adult tissue will help confirm the proliferation (and differentiation) effects observed in the eye. Further examination of the DPLD-LOLA biochemical interaction, challenging its relevance is required.

TRIM genes are found in all metazoans and are implicated in several human diseases including muscular dystrophy. The fly genes described thus far, all play roles in control of proliferation and/or tissue differentiation, as do many of their vertebrate homologs. Our identification of a muscle specific *Drosophila* TRIM (*abba*) could provide an accessible, genetically tractable and well characterized system in which to study the role of TRIM proteins in muscle development. No mutations of *abba* are currently available, therefore the generation of a loss of function mutant and its subsequent characterization should take precedence.

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