# Wingless signaling directly regulates *cyclin E* expression in proliferating embryonic PNS precursor cells

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## Abstract

Cell proliferation and cell type specification are coordinately regulated during normal development. Cyclin E, a key G1/S cell cycle regulator, is regulated by multiple tissue-specific enhancers resulting in dynamic expression during *Drosophila* development. Here we further characterized the enhancer that regulates *cyclin E* expression in the developing peripheral nervous system (PNS) and show that multiple sequence elements are required for the full *cyclin E* PNS enhancer activity. We further show that Wg signaling is important for the expression of *cyclin E* in the sensory organ precursor (SOP) cells through two conserved TCF binding sites. Blocking Wg signaling does not completely block SOP cell formation but does completely block SOP cell proliferation as well as the subsequent differentiation.

## Keywords

Cyclin E; Wingless (Wg); Peripheral Nervous System (PNS); Sensory Organ Precursor (SOP); Drosophila; TCF

## 1. Introduction

Cell proliferation is coordinated with patterning and cell differentiation during the development of multicellular organisms. There is increasing evidence suggesting that this coordination is mediated through the control of key cell cycle regulators by developmental mechanisms (Cayuso et al., 2006; Coffman, 2004; Edgar and Lehner, 1996; Gutierrez, 2005; Luo and Kessel, 2004; Sukhanova et al., 2007). In *Drosophila*, dissection of the enhancers that regulate the transcription of *string* (encoding a G2-M phase regulator), *cyclin E* (encoding a key G1-S phase regulator, that binds to and activates the Cdk2 protein kinase) and *dacapo* (encoding the p21/p27/p57 Cyclin E/Cdk2 inhibitor) during embryogenesis all revealed a complex cis-regulatory region containing tissue and stage specific enhancers (Jones et al., 2000; Lehman et al., 1999; Liu et al., 2002; Meyer et al., 2002; Sukhanova et al., 2007). These observations

indicate that transcriptional control of key cell cycle regulators by developmental cues is likely to be an important mechanism that coordinates cell proliferation and patterning. However, the precise developmental mechanism that regulates the expression of these cell cycle regulators is still largely unknown.

*Drosophila* embryogenesis starts with 13 synchronous, syncytial division cycles, followed by three cycles of G2-regulated cell divisions. These early cell cycles have no G1 phase and Cyclin E activity is not limiting during these cycles. Consistent with this, maternal *cyclin E* mRNA was found throughout the embryo during cleavage cycles and was degraded in all cells except germ cells at cycle 14 while the zygotic expression of *cyclin E* was ubiquitously observed in all somatic cells of the epidermis (Richardson et al., 1993). Later, zygotic expression of *cyclin E* was abruptly down regulated in most of the cells at stage 11 but was dynamically expressed in the developing central nervous system (CNS), peripheral nervous system (PNS), and the endocycle domains (Knoblich et al., 1994; Richardson et al., 1993). The expression of *cyclin E* in the proliferating embryonic PNS cells precedes S phase in these cells, (Richardson et al., 1993) and Cyclin E is required for DNA replication in PNS cells, as revealed by the analysis of *cyclin E* mutant embryos (Knoblich et al., 1994). Furthermore, precise inactivation of Cyclin E/Cdk2 activity by Dacapo (Dap) is required for normal cell cycle exit (de Nooij et al., 1996; Lane et al., 1996), and ectopic expression of Cyclin E leads to precocious S phase entry and developmental defects (Li et al., 1999; Richardson et al., 1995). Thus, appropriate regulation of Cyclin E expression and of Cyclin E/Cdk2 activity is important for limiting cell proliferation.

The *Drosophila* PNS contains several distinct types of sensory organs, including the external sensory (es) organs and the chordotonal (ch) organs (for a review, see (Jan and Jan, 1993). A typical es or ch organ is composed of four components: a neuron and three supporting cells. The different components of the es and ch organs are derived from divisions of the sensory organ precursor (SOP) cells, which can be identified by the expression of the protein Senseless (Sens) (Nolo et al., 2000). The SOPs undergo two to three rounds of asymmetric cell division to give rise to a neuron, a sheath cell, which expresses the protein Prospero (Pros), and two accessory cells to form the es organs and the ch organs (Bodmer et al., 1989; Doe et al., 1991; Gho et al., 1999; Hartenstein and Posakony, 1989; Reddy and Rodrigues, 1999; Roegiers and Jan, 2004; Wang and Chia, 2005).

In this report, we characterize the enhancer that regulates zygotic *cyclin E* expression in the embryonic PNS precursor cells and show that multiple sequences elements are required for full PNS enhancer activity. We show that Wingless (Wg) signaling is required for *cyclin E* expression in the developing embryonic PNS precursor cells and identify two TCF binding sites that are required for full PNS enhancer activity. These results indicate that Wg signaling directly regulates *cyclin E* expression in the developing embryonic PNS precursor cells.

## 2. Results and discussion

### 2.1. Deletion analysis of the PNS enhancer of cyclin E

To begin dissection of the promoter region required for the regulation of *cyclin E* gene expression in the developing embryonic PNS, a DNA fragment (*4.6-PNS*, Fig. 1), containing the PNS enhancer defined to be within a 2.6 kb KpnI-NcoI fragment (Jones et al., 2000), was cloned into the GFP-expressing pH-Stinger vector (Barolo et al., 2000) and transgenic flies were generated. As expected, expression of the GFP reporter was observed in the PNS region of stage 12 embryos (Fig. 1 and 2A). Antibody staining revealed that all the GFP-positive cells also expressed Sens (Nolo et al., 2000), confirming that the GFP reporter is indeed expressed in PNS SOP cells (Supplemental Fig. 1). The GFP reporter was also observed in stage 13 embryos (Fig. 1 and Fig. 2B) as well as in later stage embryos when the *cyclin E* message was no longer detected. This is likely due to the extended stability of the nuclear GFP protein as

reported previously (Sukhanova et al., 2007). Deletion of the PNS enhancer from the proximal end leads to a gradual decrease in the expression of GFP reporter (Fig. 1, 3.67-PΔ, 2.77-PΔ, 1.33-PΔ and Fig. 2C–H). In contrast, deletions from the distal end completely abolished GFP reporter expression (Fig. 1, 3.6-DΔ, 2.73-DΔ, Fig. 2I–L). These results indicate that both the proximal and the distal end sequences of the *4.6-PNS* enhancer contribute to the enhancer activity, with the distal end sequences playing an essential role while the proximal end sequence augments the enhancer activity.

## 2.2. Wg regulates cyclin E PNS expression and the cyclin E 4.6-PNS enhancer activity

To determine the developmental mechanisms that regulate *cyclin E* expression in PNS, we examined the activity of *cyclin E* PNS enhancer in several mutant backgrounds. Interestingly, *4.6-PNS* GFP reporter expression in PNS precursor cells were mostly absent in the *wg* mutants, even though 4.6-PNS GFP reporter expression in the CNS cells can still be observed (Figs 3A and B). Double staining with anti Sens antibody showed that a large number of the Sens positive cells were expressing GFP reporter from the *4.6-PNS* enhancer in WT embryos, while only a few Sens positive cells express *4.6-PNS* GFP reporter in *wg* mutant embryos (Fig. 3C and D). These results suggest that Wg signaling regulates *cyclin E* expression in the developing PNS. To further test this idea, *in situ* hybridization of *cyclin E* message was carried out. As shown in Figs. 3E and F, *cyclin E* expression in the developing PNS but not CNS was significantly decreased in *wg* mutants (see also supplemental Fig. 2). We conclude from these observations that Wg signaling plays an important role in the expression of *cyclin E* in the developing PNS cells.

## 2.3. Wg signaling does not block PNS progenitor cell determination but does block PNS progenitor cell proliferation and differentiation

As described above, the *4.6-PNS* reporter specifically targets GFP reporter expression in the developing PNS precursors, which can be identified by Sens antibody. To determine if the effect of *wg* mutation on the expression of *cyclin E* was due to its effect on SOP determination, we examined the embryonic PNS SOP cells by Sens staining. As shown in Figs. 4A and B, significant number of Sens positive cells were still observed in *wg* mutants, suggesting that the failure to express *cyclin E* from the *4.6-PNS* enhancer in *wg* mutant embryos is not likely simply due to a failure in SOP determination.

To test if decreased *cyclin E* expression in *wg* mutants blocks cell proliferation in developing PNS cells, BrdU incorporation in the developing PNS cells was determined. As shown in Fig. 4C, D, BrdU incorporation in the developing PNS cells were completely blocked in *wg* mutants while BrdU incorporation in the developing CNS and the head region can still be observed in *wg* mutant embryos (Fig. 4C, D). These observations are consistent with the observation that *wg* mutation does not affect *cyclin E* expression in these regions (Fig. 3E, F).

To further characterize the effect of *wg* mutation on PNS development, we examine the expression of Pros and Elav in developing PNS. Expression of Pros in the PNS, which labels the sheath cell of the es organ and the scolopale cell of the ch organ (Doe et al., 1991; Vaessin et al., 1991), was significantly decreased in *wg* mutant embryos as compared with those in WT embryos (Figs. 4E and F). Similarly, expression of Elav in PNS, which labels the PNS neurons, was also largely absent in the PNS region (Fig. 4G and H). These observations showed that Wg signaling is required for the proliferation of PNS progenitor cells and is required for the proper differentiation of multiple cell types in the developing PNS.

### 2.4. Two conserved TCF-binding sites identified in the 4.6-PNS enhancer are required for PNS enhancer activity

To examine the possibility that Wg signaling regulates *cyclin E* PNS expression directly, we used bioinformatics tools to identify putative binding sites for the Wg pathway transcription factor TCF in the *4.6-PNS* enhancer that are conserved in different *Drosophila* species. Two *TCF*-binding sites (−5101 to −5110 and −3768 to −3777) were identified that are conserved between different species of *Drosophila* (Fig. 5A). To determine if dTCF protein can bind to the observed *TCF*-binding sites, gel shift assays were carried out using purified His-tagged dTCF protein. As shown in Fig. 5B, probes containing either binding site *TCF-1* or *TCF-2* can bind strongly to dTCF protein. Furthermore, mutations in the conserved *TCF*-binding sites completely abolished dTCF binding, indicating that dTCF protein bound specifically to *TCF-1* and *TCF-2* sites.

To determine if the observed *TCF*-binding sites are important for regulating *cyclin E 4.6-PNS* enhancer activity, the effect of mutating the *TCF*-binding sites on the activity of the *4.6-PNS* enhancer was determined. As shown in Fig. 6, mutations of either the *TCF-1* (4.6-mut1) or the *TCF-2* sites (4.6-mut2) partially reduced *4.6-PNS* enhancer activity (Figs. 6A–D). Furthermore, mutation of both *TCF*-binding sites (4.6-mut1-2) completely abolished *cyclin E 4.6-PNS* enhancer activity (Fig. 6E). Taken together, these results indicate that Wg signaling directly regulates the expression of *cyclin E* in the developing embryonic PNS cells through these two *TCF*-binding sites.

### 2.5. Conclusions and Discussion

Taken together, our results indicate that Wg signaling is required for the expression of *cyclin E* in proliferating PNS precursor cells. In the absence of Wg signaling, the expression of *cyclin E* is dramatically reduced, resulting in an inhibition of PNS precursor cell proliferation and the determination of different cell types in the developing PNS.

Our results also revealed that *cyclin E* expression in developing PNS precursor cells is regulated by a large enhancer containing multiple sequence elements, including two *TCF*-binding sites that mediate the regulation by Wg signaling. While these TCF-binding elements are essential for the activity of the PNS enhancer, proximal and distal elements in the *4.6-PNS* sequence appear to be important for full activity. The importance of Wg in the regulation of the PNS expression of *cyclin E* is supported by the fact that *wg* mutant embryos displayed decreased *cyclin E* expression in the developing PNS cells. This reduction in *cyclin E* expression in *wg* mutant embryos was accompanied by an inhibition of BrdU incorporation in the developing PNS, and an inhibition of the determination of the Pros and Elav expression cells in the developing PNS. It is possible that the block in differentiation into the Pros and Elav positive cells is a consequence of the inhibition of *cyclin E* expression or perturbations to the cell proliferation as has been previously observed (reviewed by Chia and Prokopenko, 2005). However it is also possible that the observed differentiation block in PNS is due to a function of Wg that is independent of PNS cell proliferation. Further studies will be needed to resolve this issue.

In addition to *wg*, a number of other mutations such as *achaete/scute* (*ac/sc*) complex and *da* have also been reported to block PNS precursor proliferation and affect the expression of several cell cycle genes (Bodmer et al., 1989; Hassan and Vaessin, 1997). Ac/Sc complex proteins and Da are bHLH proteins that are important in all aspects of es-PNS precursor differentiation while bHLH protein Atonal (ato) and Da are required for all aspects of ch-PNS precursor development. Recent studies of the expression of the Cdk inhibitor Dap during cell type specification revealed that Dap expression is directly regulated by the same developmental mechanisms that control the differentiation of these cell types (Sukhanova et al., 2007).

Therefore it will be interesting to test if bHLH proteins such as Da also directly regulate *cyclin E* expression in the developing PNS cells.

## 3. Experimental procedures

### 3.1. Fly stocks

$wg^{1-17}$ mutant flies, a molecular null mutant described previously (van den Heuvel et al., 1993), was obtained from the Bloomington Stock center and used.

### 3.2. Gel shift assay

The HMG (High Mobality group) DNA binding domain of dTCF was amplified by PCR (van de Wetering et al., 1997), and cloned into the pET15b vector. The pET15b-dTCF plasmid was transformed into BL21(DE3) to produce His-tagged dTCF protein. His-tagged dTCF protein was purified by Ni-NTA column and was used in gel shift experiments as described previously (Duman-Scheel et al., 2002). WT or TCF-binding site-mutated nucleotides were used as probes in gel shift experiments, and the sequences of wild type (WT) and TCF-binding site mutated probes are:

WT TCF1: 5' GCATGCC<u>AGGACAAAGG</u>AGCCGGACCAG 3'

Mut TCF1: 5' GCATGCC<u>AG*CAC*T*G*A*GG</u>AGCCGGACCAG 3'

WT TCF2: 5' GGT TTG T<u>CCTTTGTCCA</u>CTTACCCTCC 3'

Mut TCF2: 5' GGT TTG T<u>CCT*GAG*TCCA</u>CTTACCCTCC 3'

The dTCF-binding sites are underlined and the mutated bases are shown in italics.

### 3.3. Generation of transgenic flies

The 4.6 kb enhancer (*4.6-PNS*) and the different deletions of the *cyclin E* PNS enhancer region were amplified by PCR from the P1 clone DS07108 (BDGP Resources) and were cloned into the BamH1 site of the pH-Stinger vector (Barolo et al., 2000) to generate transgenic lines using standard P-element mediated germ line transformation (Spradling and Rubin, 1982). To generate the *4.6-PNS* enhancer with mutated dTCF binding sites, the same TCF-binding site mutations used in the gel shift experiments were introduced into the *4.6-PNS* enhancer. The WT and the TCF-binding site-mutated constructs were confirmed by DNA sequencing and cloned into the pH-Stinger vector to generate transgenic lines. At least two different transgenic lines were examined for each construct with enhancer deletions and mutated TCF-binding sites. No significant variations were observed between different insertions of the same constructs.

### 3.4. in situ hybridization, BrdU incorporation, and antibody staining

The antibodies used in this paper are: rabbit anti β-gal antibody (Cappel), rat anti Elav (7E8A10), 22C10, and mouse anti Prospero antibodies (Hybridoma Bank), mouse anti BrdU (Becton Dickinson), and guinea pig anti Senseless antibody (Nolo et al., 2000). *wg* mutant embryos were identified by the lack of β-gal antibody staining. All embryos were mounted in 80% glycerol in 1xPBS.

For *in situ* hybridization, Digoxigenin-labeled *DmcycE* antisense RNA probe was prepared by *in vitro* transcription reaction. Properly aged embryos were fixed with 4% Paraformaldehyde over heptane and devitellinized with methanol. Hybridization was carried out at 65°C as described (Du and Dyson, 1999).

For BrdU incorporation, properly aged embryos were pulse-labeled for 15 min in Schneider's medium containing 1 mg/ml BrdU as described previously (Du and Dyson, 1999). BrdU was

incorporated into replicating nuclei and the pulse was subsequently fixed and detected by anti-BrdU mouse monoclonal antibody (Becton Dickinson, 1:50 dilution).

## Supplementary Material

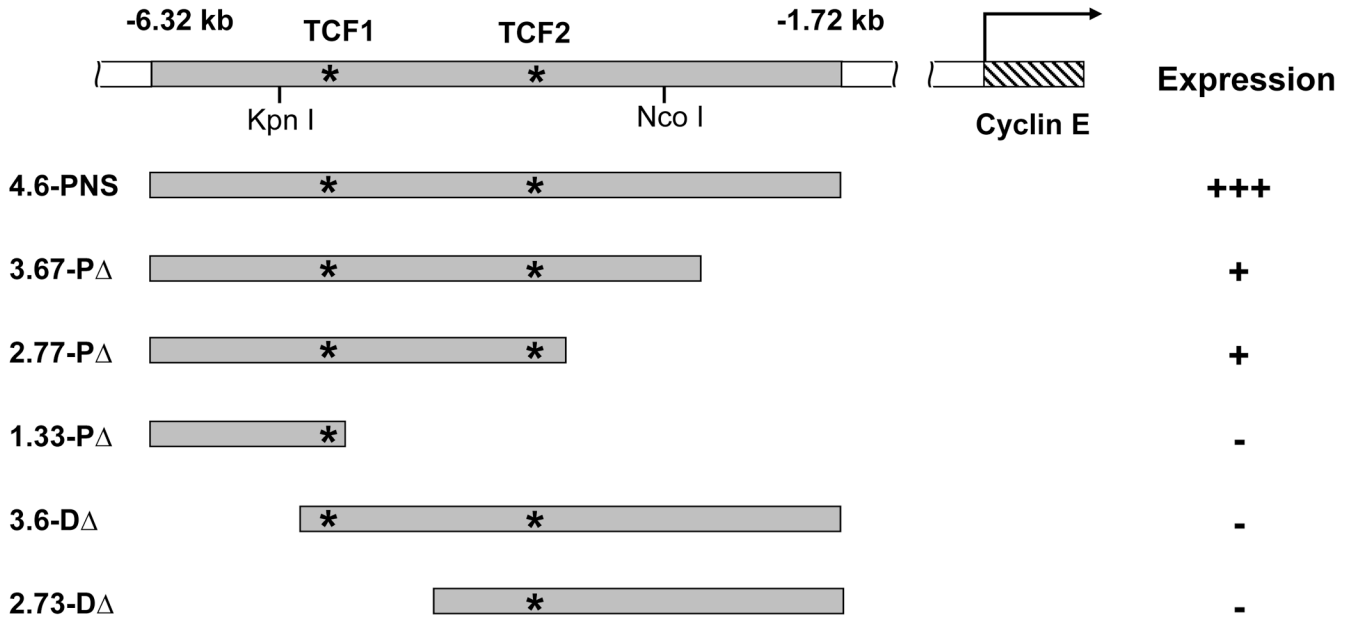Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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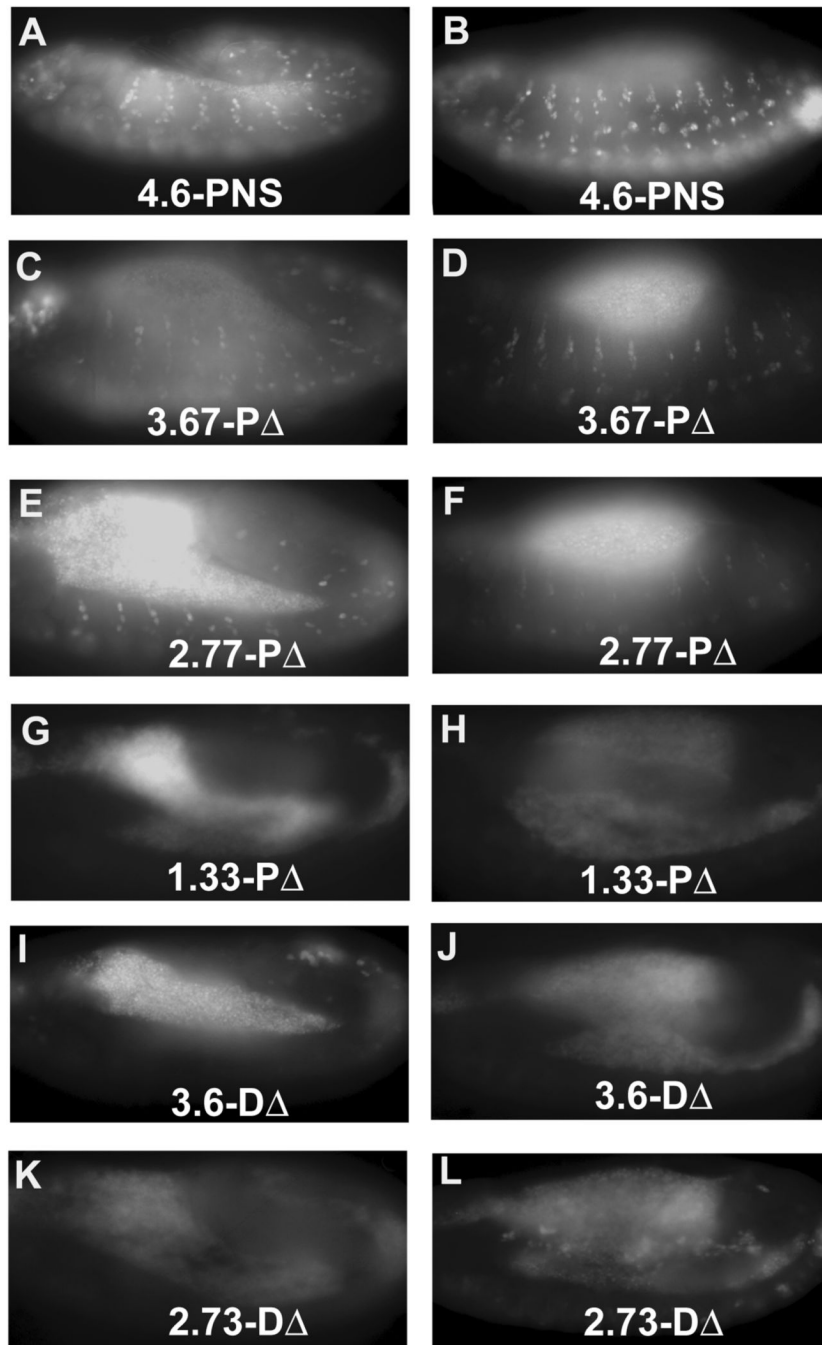
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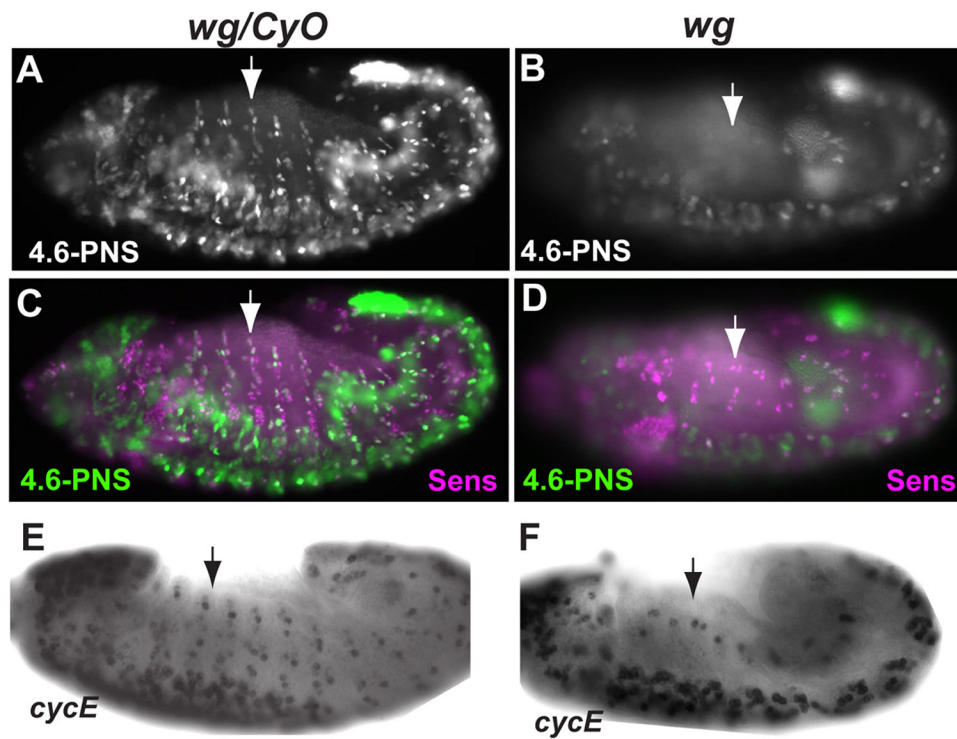
**Fig 1. A diagram of the *cyclin E* genomic region and of the different *cyclin E* PNS enhancer constructs**

*cyclin E* genomic fragments were used to generate a series of nuclear GFP (nGFP) reporter constructs. The numbers on top represent the locations of the *cyclin E* genomic sequence relative to the translation start of the type I *cyclin E* transcript. The location of the Kpn I and Nco I restriction sites, which defines the PNS enhancer in the work of Jones et al. (2000) are indicated. Asterisks indicate the locations of the two TCF-binding sites in the *cyclin E* PNS enhancer. The presence or absence of GFP expression is indicated by plus or minus signs, respectively. The number of plus signs indicates the level of GFP expression relative to the full-length *4.6-PNS* enhancer.
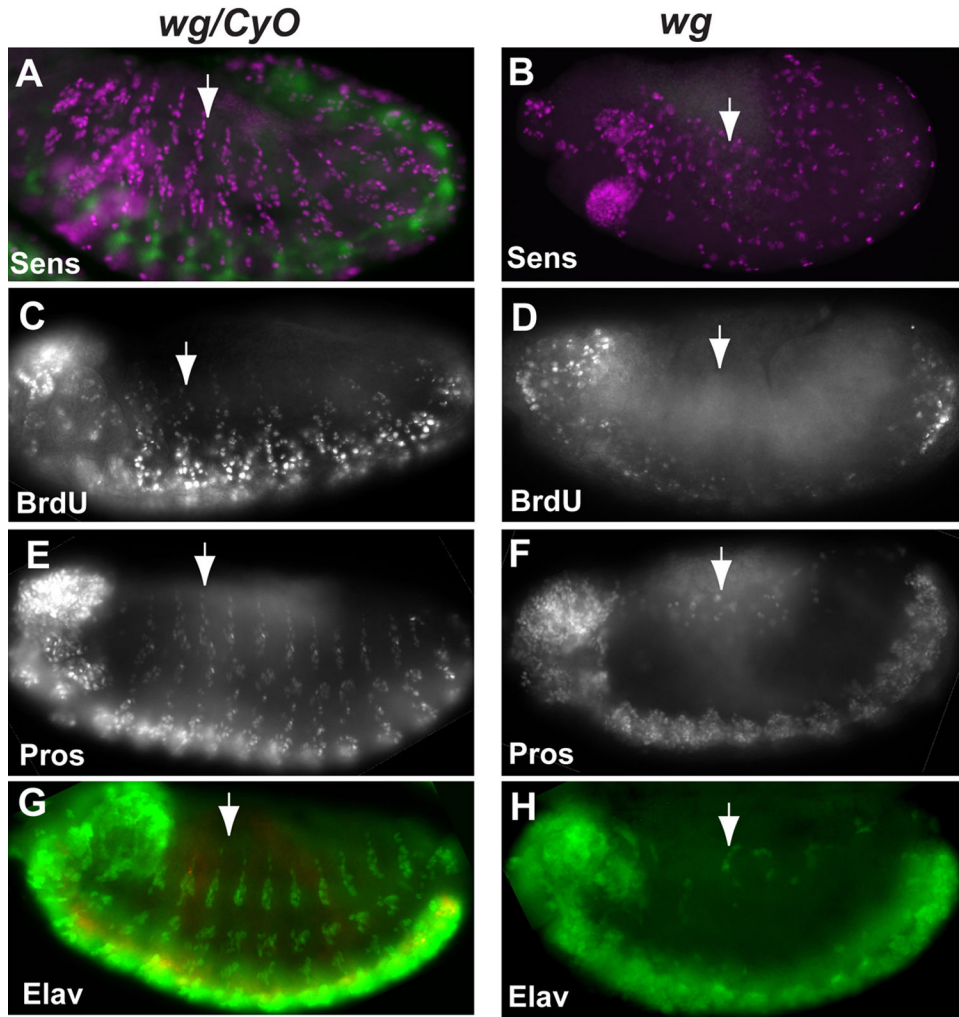
**Fig. 2. GFP reporter expression from different *cyclin E* PNS enhancer constructs**
GFP reporter expression of the indicated *cyclin E* transgenic constructs at embryonic stages 11/12 (A, C, E, G, I, and K) or stages 12/13 (B, D, F, H, J, and L) are shown. Multiple insertions of each deletion construct were examined and no significant differences were observed between insertions of the same constructs. The level of GFP expression is summarized in Fig. 1.
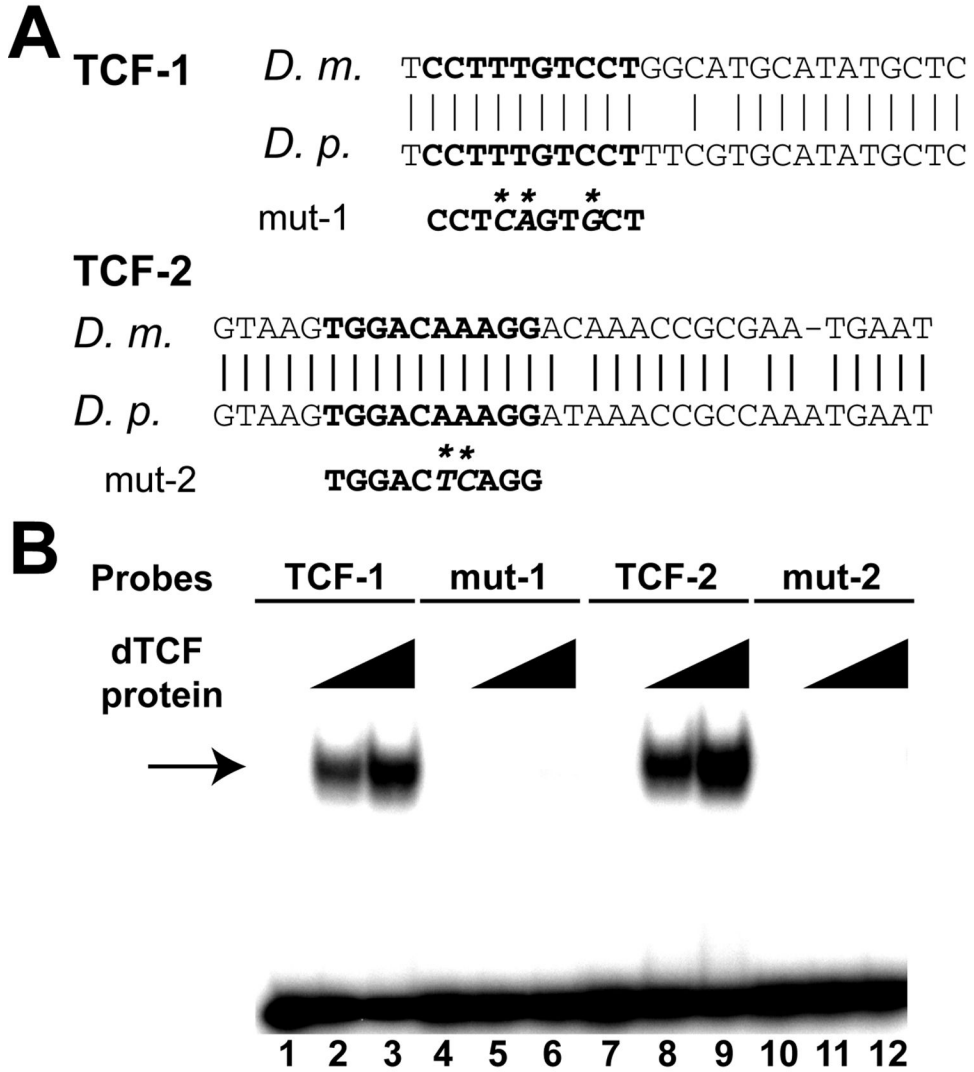
**Fig. 3. The effect of *wg* mutation on *cyclin E* expression in the embryonic PNS**
(A, B) Expression of the GFP reporter from the *4.6-PNS* constructs was significantly reduced in the *wg* mutant embryos (B) as compared to that in the control (*wg/CyO*) embryos (A). (C, D) Double labeling of *4.6-PNS* GFP reporter expression and Sens revealed large number of Sens positive cells express GFP reporter in *wg/CyO* embryos (C) while very few Sens positive cells express GFP reporter in *wg* mutant embryos (D). (E, F) Whole-mount *in situ* hybridization showing expression of zygotic *cyclin E* in the developing PNS. (E), *wg/CyO* embryos show normal *cyclin E* expression in the PNS. (F), *wg* mutant embryos exhibit significantly reduced PNS *cyclin E* expression.
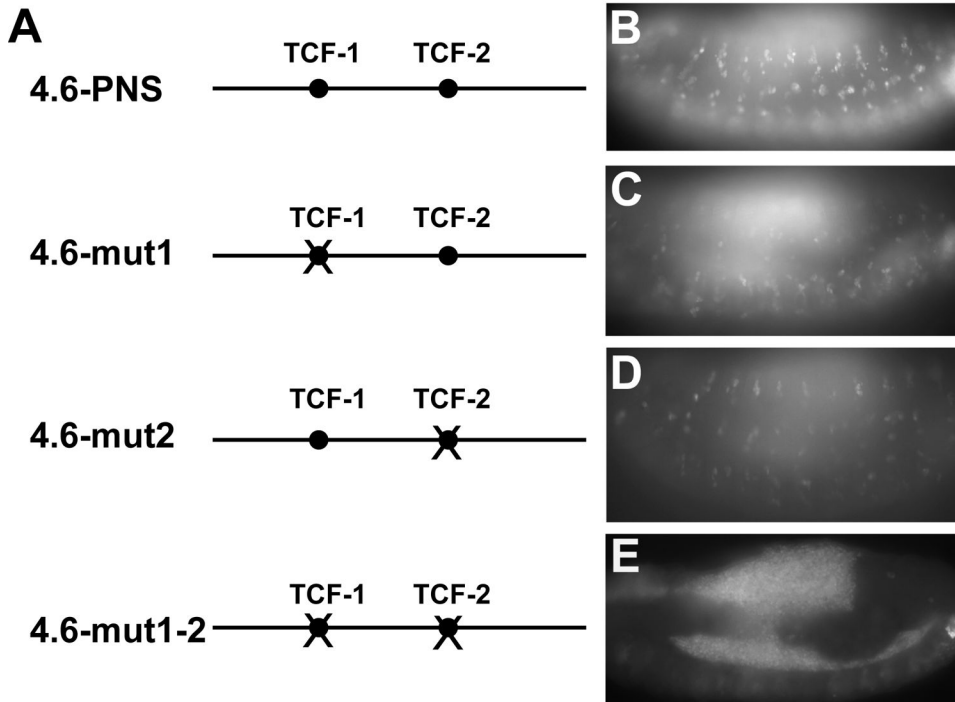
**Fig. 4. Wg signaling is required for cell proliferation in the developing PNS and for the development of neurons and Pros expressing cells in the PNS**

(A, B) Significant numbers of SOP cells were observed in *wg* mutant (B) embryos. PNS SOP cells were identified by Senseless staining shown in magenta. Homozygous *wg* mutant embryos were identified by the absence of β-gal expression from the *ftz-lacZ* construct on the *CyO* balancer shown in green. (C, D) *wg* mutation blocks cell proliferation in the developing PNS. BrdU incorporation was not observed in the PNS region of the *wg* mutant embryos (D) but was observed in the *wg/CyO* embryos (C). It should be noted that BrdU incorporation in the head and the ventral CNS region was still observed in *wg* mutant embryos. (E–H) *wg* mutation blocks the differentiation of neurons and Pros expression cells in the developing PNS. Sheath cells in the es and scolopale cells in the ch organs are the PNS cell types that accumulate high levels of Pros. Very few Pros-expressing cells were observed in *wg* mutants (F) as compared to *wg/CyO* embryos (E). Similarly, very few neurons identified by Elav staining were observed in *wg* mutant (H) as compared with that of the *wg/CyO* embryos (G). Homozygous *wg* mutant embryos were identified by the absence of β-gal expression from the *ftz-lacZ* construct on the *CyO* balancer shown in red in G and H. Arrows point to a PNS segment in the embryos.

**A**

**TCF-1**

*D. m.* TCCTTTGTCCTGGCATGCATATGCTC

|||||||||||| | |||||||||||||

*D. p.* TCCTTTGTCCTTTCGTGCATATGCTC

                       * *   *

mut-1      CCTCAGTGCT

**TCF-2**

*D. m.* GTAAG**TGGACAAAGG**ACAAACCGCGAA–TGAAT

||||||||||||||||| ||||||| || |||||

*D. p.* GTAAG**TGGACAAAGG**ATAAACCGCCAAATGAAT

                      * *

mut-2      TGGACTCAGG

**B**

**Fig. 5. Two conserved TCF-binding sites in the *4.6-PNS* enhancer**
(A) Alignment of sequences around the conserved TCF-binding sites between *D. melanogaster* and *D. pseudoobscura*. The bold letters represent the TCF-binding sites in the *4.6-PNS* enhancer. The sequences of the mutant TCF sites are shown below the binding sites and base pair changes are indicated by "*". (B) Gel shift assay showing that the *Drosophila* TCF protein bound strongly and specifically to the wild type TCF1 (lanes 2 and 3) and TCF-2 (lanes 8, 9). In contrast, no binding was observed when the TCF sites were mutated (mut-1 and mut-2, lanes 5, 6 and 11, 12). No dTCF protein were added in lanes 1, 4, 7, and 10; half the amount of dTCF protein was added to lanes 2, 5, 8, 11 compared to lanes 3, 6, 9, and 12. An arrow points to the dTCF-DNA complex. The sequences of TCF and mutated TCF probes are shown in Materials and Methods.

**Fig 6.**
(A) A diagram of the *4.6-PNS* enhancer and the *4.6-PNS* enhancers with each of the TCF binding sites mutated either alone or in combination. Filled circles represent the TCF binding sites, and crossed (x) circles indicate mutated TCF binding sites in the PNS enhancer. (B–E) Both TCF binding sites contribute to the full activity of the *4.6-PNS* enhancer. Transgenic lines carrying the *4.6-PNS* enhancer with mutated TCF-1 binding site (C) or the TCF-2 binding site alone (D) show decreased GFP reporter expression when compared to WT *4.6-PNS* enhancer (B). Transgenic lines carrying the *4.6-PNS* enhancer with mutations of both TCF binding sites only show background levels of GFP reporter expression (E).