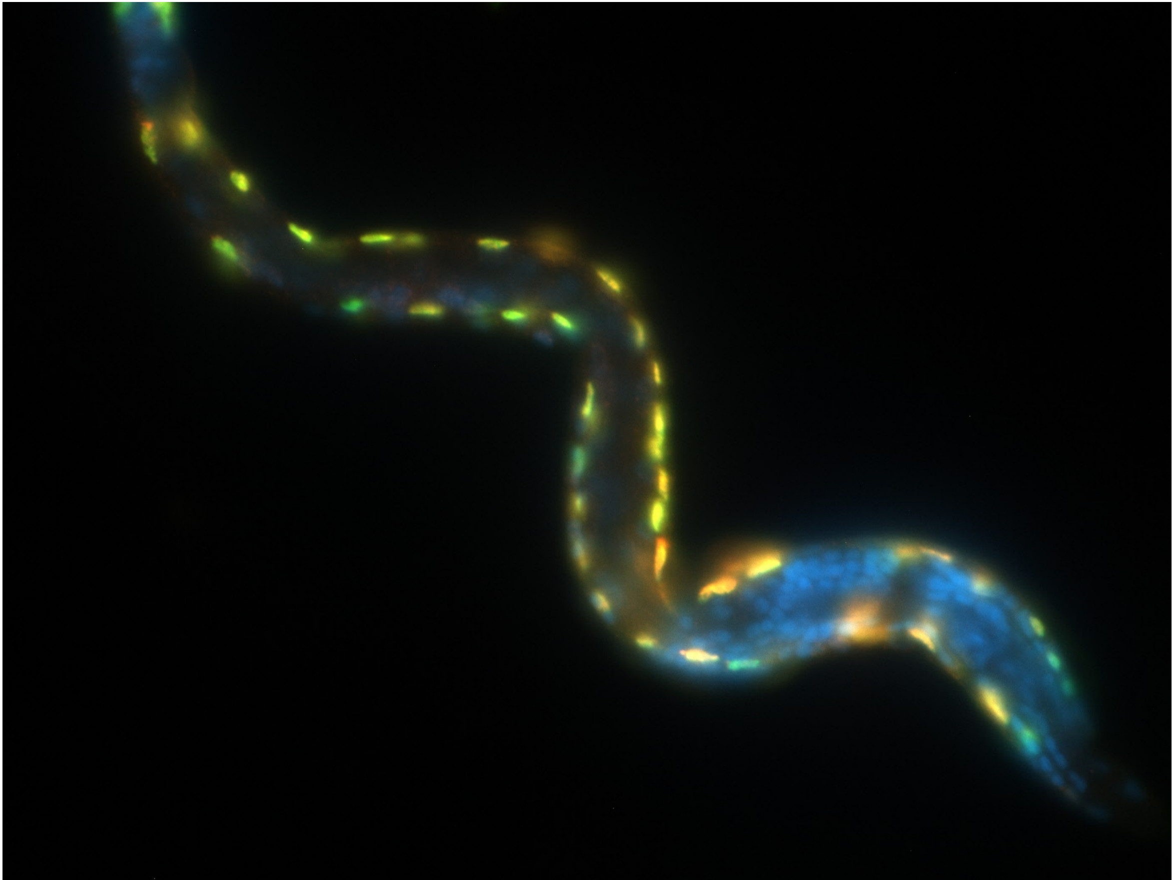
Thesis literature part.

Regulation of quiescence in the *Drosophila* neuroblast



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Regulation of quiescence in the *Drosophila* neuroblast

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The picture on the cover shows the expression of cyclin E (red) in muscle cells (green) in a *C.elegans* strain overexpressing cyclin E in muscle cells. Visualised under a fluorescence microscope. This picture is part of an experiment in the practical part of this thesis.

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Abstract

Quiescence is a state of the cell cycle in which the cell cycle does not progress. It is a cellular process that is currently poorly understood. This thesis describes processes central in the regulation of quiescence that are known at the time. Quiescence is primarily regulated in the transition between the G_1 and S phase of the cell cycle. More recent findings on how quiescence is regulated are described by using a model system. This model system is the *Drosophila* neuroblast. In the *Drosophila* neuroblast both intrinsic and extrinsic signals control quiescence. This includes nutrition, growth factors, fate determinants and spatio-temporal determinants. Some mechanisms which are described in this thesis are conserved and may prove to be valuable for understanding quiescence in mammals.

Introduction

Many processes during development can only occur when the cell does not proliferate. Generally, cells exit from the cell cycle in order to differentiate, since proliferation and differentiation are often found mutually exclusive. There are several ways in which the cell can exit the cell cycle. The exit from the cell cycle can either be permanent or temporarily which is called quiescence. Quiescence is a state of the cell cycle. There are several fields where the study of quiescence could contribute to a better understanding of the involved processes. These fields include cancer research, stem cell research, neurobiology and developmental biology.

In most cancer cells control of proliferation is dimished or absent. The reduced control often leads to continuously proliferating cells which leads to cancer. Controlling quiescence to stop this proliferation might have beneficial effects on cancer patients. By inhibiting proteins that prevent the cell from exiting the cell cycle or by stimulating genes that allow a cell to enter the quiescent state the growth of a tumour could be inhibited. Quiescence is reversible as well which would be a great advantage compared to other medication. Slowing or stopping proliferation altogether is a vital part of treating cancer (Li and Bhatia, 2011).

In stem cells quiescence takes place to initiate the differentiation process, and to preserve the self renewing function of stem cells. Therefore, quiescence is important in maintaining the stem cell niche. By controlling quiescence it could be possible to control the differentiation process of the stem cells. Forcing cells to differentiate by overstimulating genes or adding proteins under the correct circumstances could be applied to direct the differentiation of the stem cells. In addition, important proteins which are necessary to induce quiescence in stem cells can be inhibited to stop the differentiation. Being able to manipulate this process might be beneficial for stem cell therapy (Conover and Notti, 2008;Li and Bhatia, 2011).

Besides cancer and stem cell research, quiescence is important in neurobiology. The neurons in the human body are non-dividing cells for most of the time. Understanding the process of quiescence might lead to new insights in the development and functioning of the brain. This could lead to a better treatment of neurological disorders such as Alzheimer’s disease (Conover and Notti, 2008;Raina et al., 1999).

However, the mechanisms involved in entering or exiting the quiescent state are not completely understood at this moment. Therefore, it is of great importance to form an understanding of the processes involved in quiescence. To understand these complex processes it is possible to use a model system. For neurons this is usually the *Drosophila* neuroblast. The *Drosophila* neuroblast is an intensively studied model system for quiescence since most processes are likely to be completely or partially conserved in mammals. This thesis will describe the mechanisms involved in entry and exit from quiescence by using the *Drosophila* neuroblast as a model.

The literature part consists of four chapters followed by a summary and discussion. In the first chapter the cell cycle and quiescence will be discussed. In the second chapter the model system will be introduced. In the third chapter the processes and pathways that regulate quiescence that are currently known will be discussed. In the fourth chapter the regulation of quiescence in the *Drosophila* neuroblast will be compared to other model systems.
In the practical part of the thesis experiments to form more insight into cell division during development will be described. For this purpose another model system is used namely, *C. elegans*.

Chapter 1: The cell cycle and quiescence

The cell can either permanently or temporarily exit from the cell cycle. This temporary exit is called quiescence. Quiescence is a state of the cell cycle which can also be named the G_0 phase of the cell cycle. Cells can re-enter the cell cycle by being stimulated with factors that are not fully known at the time. Since it is possible to exit from the state the quiescent state can be called reversible. To enter the state of quiescence cells need to exit the cell cycle at the transition between the G_1 and S phase (Wang et al., 2010).

The stimuli required for regulation of quiescence are thought to be extrinsic and intrinsic. The most likely stimulus is nutrition since excess proliferation will deplete the resources vital for an organism to survive. There has been an indication for this in yeast where the availability of resources regulated entry and exit from quiescence (Yanagida, 2009). Besides nutrition other cell signalling is likely to regulate quiescence in development as well. (Egger et al., 2008)

The entry or exit into quiescence is controlled by several processes. This involves gene expression, apoptosis and signalling to nearby cells (Chesnokova and Pechnick, 2008;Egger et al., 2008;Litovchick et al., 2007). These processes will be discussed in the following paragraphs.

Regulation of cell cycle progression

In order to regulate cell cycle progression or entry into the quiescent state gene expression has to be altered. The two most important methods of regulating this is by chromatin remodelling which prohibits or allows the transcription of the involved genes and the second method is direct inhibition or activation of factors that are involved in the process.

To enter the G_0 phase cells must not progress into cell cycle. The key regulators of the switch between the G_1 phase and the S phase are the retinoblastoma (Rb) family of proteins and the E2F family of transcription factors. Rb inhibits E2F by binding to it. If Rb is phosphorylated by the D type cyclins in a complex with Cdk 4 or 6 it disassociates from E2F and the S phase genes will be transcribed. As well as the D type cyclins, E type cyclins phosphorylate Rb while associated with Cdk2 (Sears and Nevins, 2002). This process is initiated by growth factors such as C-Myc which induces the cyclin/Cdk activity (Blagosklonny and Pardee, 2002). There are also CKIs (Cip/Kip inhibitors) known to inhibit the cyclin/Cdk complexes such as p21 and p27. This process is illustrated in Figure 1 (Blomen and Boonstra, 2007;Sears and Nevins, 2002).

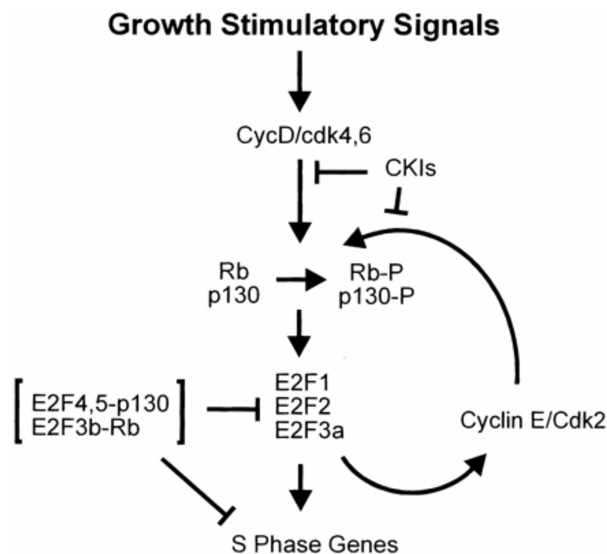


Figure 1. The G_1/S phase checkpoint
Schematic representation of the key factors involved in the transition from G_1 to S phase.
(Sears and Nevins, 2002)

More interestingly, E2F and C-Myc have shown to induce exit from quiescence when added to the cell (Litovchick et al., 2011). On the other hand high levels of the Rb family member p130 are found in cells that are in the quiescent state (Chesnokova and Pechnick, 2008). In addition, some brain regions have an elevated expression of CKIs which maintains the quiescent state in those cells. This indicates that this pathway plays a key role in regulating quiescence (Chesnokova and Pechnick, 2008).

The dREAM complex

To enter the quiescent state, genes involved in the progression of the cell cycle need to be repressed or activated. The previous example showed that Rb was involved in inhibiting the E2F family of transcription factors. Another common way to inhibit gene expression is by binding directly to the promoter of the target genes. There is one complex of proteins that is known to bind over 800 promotors of cell cycle-regulated genes. This is the *Drosophila* RBF, E2F and MYB (dREAM) complex which consists of Dp, the Rb family members p130, p107 and pRB, E2F, a MuvB like protein and four MuvB binding proteins. This complex has been discovered in *Drosophila* and homologues of these subunits are present in humans (Korenjak et al., 2004). This means the complex is highly conserved and might therefore be important in mechanisms involving quiescence (Litovchick et al., 2007).

The dREAM complex is present in quiescent cells but dissociates as soon as the cell enters the S phase. After dissociating from the former dREAM complex the MuvB like protein and the four binding proteins bind to the transcription factor BMYB. This forms the MuvB-MYB (MMB) complex which activates the expression of genes involved in the cell cycle (Litovchick et al., 2011). The switch between the dREAM complex and the MMB complex by MuvB seems to be a key mechanism that controls quiescence.

The mechanism that regulates the dissociation of the dREAM complex, allowing the formation of the MMB complex, is largely unknown. However, it has been reported that one of the MuvB binding proteins of the dREAM complex is involved. In humans this MuvB binding protein is lin-52. It is required to be phosphorylated in order to form the dREAM complex *in vivo*. This phosphorylation is caused by the protein kinase DYRK1A (Litovchick et al., 2011). Yet, it is not known whether this is the only kinase involved in this process. It is highly likely that there are other, yet unknown, factors involved in the forming and dissociation of the dREAM and MYB complexes. The pathway that regulates DYRK1A is not known either.

It is known that the dREAM complex binds to targets involved in the cell cycle including E2F target genes. It has a repressing function on the cell cycle progression as a result. However, it is not known how this complex inhibits the progression. A likely explanation would be that the complex uses epigenetic modifications to alter the gene expression. In the transition between the S and G_2 phase MuvB has shown to alter the chromatin structure in flies (Wen et al., 2008). The finding of epigenetic modification in the S and G_2 phase transition mildly suggests that the dREAM complex exhibits a similar function in the G_1 to S phase transition. Epigenetic modification would explain the regulation of multiple target genes by the dREAM complex (Korenjak et al., 2004).

The metabolic status and the cell cycle

A fundamental pathway that regulates the transition between the G_1 and S phase is by E2F family members and Rb as described before. However, this is not the only pathway involved. Growth factors such as C-Myc are influenced by other pathways that integrate signals from outside the cell.

One of those pathways is the TOR pathway. The TOR pathway governs many processes in the cell including cell growth, proliferation and survival. The TOR pathway integrates signals such as a low concentration of nutrients and growth factors into translational control and global protein synthesis (Laporte et al., 2011;Mamane et al., 2006). The TOR pathway is visualised in Figure 2.

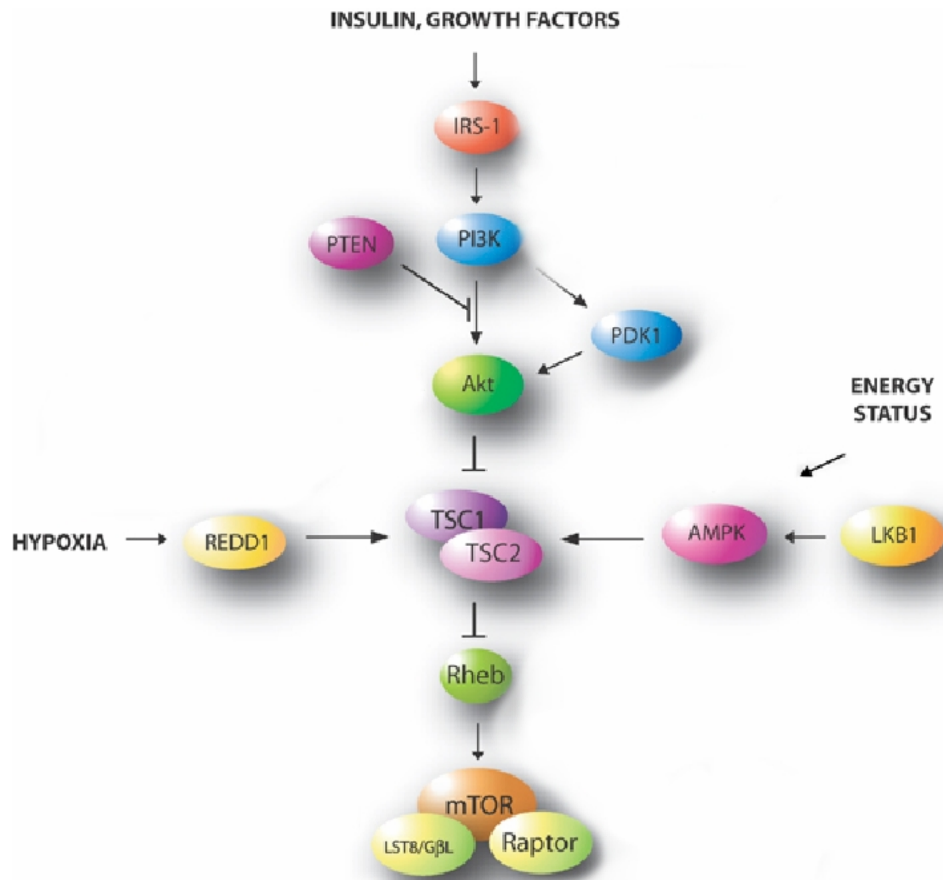


Figure 2. The TOR pathway
The TOR pathway regulates the initiation of translation by integrating the metabolic status of the cell. Growth factors activate PI3K which Phosphorylates Akt. Akt destabilises the TSC complex by phosphorylation after which Rheb can be activated. Rheb can induce mammalian TOR (mTOR) activity which promotes several translational factors by phosphorylation.
Edited from (Mamane et al., 2006).

The TOR complex is capable of initiating several eukaryotic initiation factors. This is inhibited by the TSC complex which inhibits the function of Rheb. Phosphorylation by Akt will destabilise this complex and causes activation of TOR. Akt is regulated by PI3K and PDK1 which are both kinases. PTEN inhibits the phosphorylation of Akt by PI3K. PI3K is activated by signals from growth factors, insulin or hormones. As well as growth factors, the concentration of Oxygen and the energy status influence this pathway. Both by stimulating the TSC complex. (Mamane et al., 2006).

First of all the importance of this pathway in quiescence is because of the translational control that is necessary to produce factors such as C-Myc. Those factors such as C-Myc control the cell cycle progression between the G_1 and S phase. In yeast it has been discovered that nutrients seem to affect quiescence as well. This indicates that the TOR pathway, which is known for integrating the concentration of resources into a response, is contributing to this. By depleting the carbon source the yeast exited the cell cycle from any phase of the cell cycle. The yeast entered the cell cycle after adding glucose indicating that

the metabolic status is very important in yeast (Laporte et al., 2011). Perhaps this is similar in mammals as well.

However, are energy levels and nutrition the only outside influences affecting quiescence or is there more involved? The transition between the G_1 and S phase is regulated by a large number of genes including those described before. Nevertheless, not all of the factors regulating the transition between G_1 and S are thought to have a function in entering or exiting from quiescence. These factors exhibit different functions such as apoptosis, senescence (permanent exit from the cell cycle), DNA-repair or differentiation (Blomen and Boonstra, 2007). Which leads to another question. If nutrients would be the only factor contributing to quiescence, why are cells not entering apoptosis or senescence which is another option to save resources? This will be discussed in the following paragraph.

Apoptosis and quiescence

The transition between the G_1 and S phase and the TOR pathway are mechanisms that contain well known tumorsuppressor genes and proto-oncogenes. However, it is still unclear how the decision is made that will lead to either apoptosis or quiescence. This has become more uncertain since p53 known for its function in regulating apoptosis has been found to induce quiescence in hematopoietic stem cells (HSCs) as well. The main function of p53 is to either stop the cell cycle by inducing senescence or to activate apoptosis under stress conditions. When the cell is not under stress conditions p53 has been shown to induce expression of the transcription factor Necdin which was shown to induce quiescence. In addition, Necdin showed a decreased occurrence of quiescence while being knocked out (Liu et al., 2009). Altough, this was shown in HSCs it does not have to occur in other tissue as well.

To further investigate regulation of apoptosis and quiescence, the mechanism could be examined in a developmental context in which developmental cues rather than stress induces apoptosis or quiescence. This topic will be further discussed in chapter 3.

Summary

In this chapter the mechanisms which play a central role in quiescence have been discussed. Quiescence is a state of the cell cycle otherwise known as a temporary exit from the cell cycle. Cells can enter quiescence in the G_1 phase. This is controlled by several mechanisms such as transcription of the E2F family members and Rb which both act in the dREAM complex. A factor thought to influence quiescence from outside the cell is the amount of nutritients. The TOR pathway integrates this signal into a cellular response. In addition to these pathways and mechanisms there is also a pathway which controls apoptosis and quiescence. To probe more deeply into the question of how quiescence is regulated a model system will be used. This modelsystem is the extensively studied *Drosophila* neuroblast.

Chapter 2: The *Drosophila* neuroblast

Several model systems are used in order to form an understanding of the mechanisms involved in quiescence. One of those systems are hematopoietic stem cells (HSCs) to study quiescence in humans. It does have an advantage of being able to study processes directly rather than looking for conserved processes. Nevertheless, it is harder to study developmental cues in this model and that is why the *Drosophila* neuroblast will be used. In this model system factors can be added or removed more easily. Another advantage is that there are various known intrinsic and extrinsic mechanisms in this system which control cell fate and quiescence (Egger et al., 2008).

This chapter will describe some of the processes involved in the development of the neuroblast. The processes described here are important for the development of the *Drosophila* neuroblast. In addition to the development of the neuroblast some processes have also shown to influence quiescence (Egger et al., 2008;Yanagida, 2009). First, the forming of the neuroblast from the neuroectoderm will be described. Then, the regulation of asymmetric division and important differences between neurons will be discussed briefly. After which fate determination by spatio-temporal factors will be introduced.

Formation of the neuroblast

The neuroblast arises from the neuroectoderm which is determined in the early embryo. One quarter of all the neuroectoderm cells will form neuroblasts. The other cells will become dermal cells. This fate is determined while the cell is part of the neuroectoderm. By creating a morphogen gradient of the transcription factor Dorsal the factor decapentaplegic (dpp) is inhibited. Dpp is from the family of bone morphogenic protein which is known for its function to form the polarity of an embryo. A low concentration of Dorsal induces the expression of short gastrulation (sog) which will determine the fate of the neuroectoderm. Together with this morphogen gradient segment polarity genes will subdivide the neuroectoderm into 'neural equivalence groups'. These groups contain a small amount of neuroectoderm cells, in which one cell will differentiate into a neuroblast. The other cells will differentiate into ectodermal cells (Egger et al., 2008).

So how is it determined which cell will become the neuroblast, and which cells will become dermal cells? There is an important process which governs this transition. This is lateral inhibition. Lateral inhibition is inhibiting directly adjacent cells from adapting the same fate. It is regulated by concentrations of the Delta transmembrane ligand and the Notch receptor (Egger et al., 2010). This is illustrated in Figure 3.

When Delta binds to the Notch receptor this leads to the cleavage of Notch (Kunisch et al., 1994). Cleaved Notch will enhance the expression of the enhancer of split genes (Espl) by binding to Suppressor of hairless (SuH). Espl genes encode repressors that will reduce the transcription of proneural genes (Kunisch et al., 1994). Proneural genes are genes that will determine the neural fate of a cell. Next to fate determination the Delta-Notch interaction will also lead to a reduced expression of Delta. This occurs by downregulation of the Achaete Scute complex which activates Delta expression. Therefore, there will be less signalling to nearby cells. Cells expressing Delta will eventually adapt the neuroblast fate. The cell which

contained the largest amount of Delta will then specify the nearby cells as ectodermal cells and will adapt the neuroblast fate itself. (Egger et al., 2008;Egger et al., 2010;Kunisch et al., 1994).

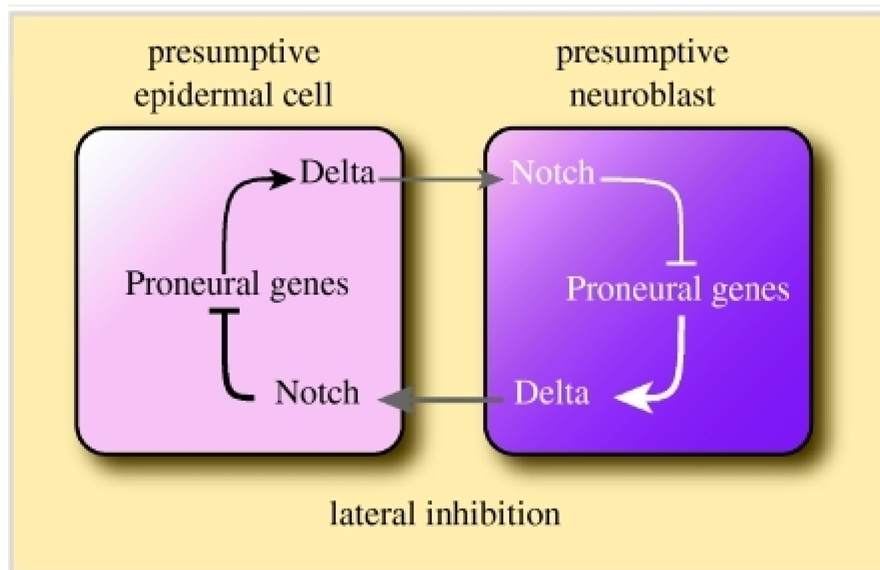


Figure 3. Lateral inhibition
Schematic representation of the lateral inhibition by the Delta-Notch pathway. The purple colour indicates a high concentration of delta. Binding of Delta to Notch will inhibit gene expression of proneural genes. This leads a reduced transcription of Delta and eventually to a different fate (Egger et al., 2008).

After specifying the fate of the neuroblast and ectodermal cells, the neuroblast will delaminate from the epithelium and will move more basal into the embryo. This occurs by asymmetric division (Egger et al., 2008). This is illustrated in figure 4A.

Asymmetric division

It is necessary for the neuroblast to divide asymmetrically to maintain the identity as a stem cell. Since there is no stem cell niche that specifies which cells will differentiate the process of asymmetric division is used to specify the fate of a cell. Notch seems to be involved in the process of switching between symmetric and asymmetric division. The mechanism behind this is still unclear but it is thought to be initiated by the delamination of the neuroblast. Delamination is the migration of the neuroblast from the epithelium into the embryo. Notch loss of function mutants show that the neuroectoderm can delaminate prematurely. Which indicates that Notch is required to maintain the position of the neuroblast at the epithelium (Egger et al., 2010).

When a neuroblast divides asymmetrically, it gives rise to two different daughter cells. One cell is the neuroblast which will self-renew and will divide about 10 to 20 times before entering quiescence (Bello et al., 2008). The other cell is the ganglion mother cell (GMC), which contains different fate determinants and is significantly smaller. This cell type will divide only once to form terminally differentiated cell types such as neurons or glia cells.

The difference in size between the neuroblast and the GMC results from a division plane that is located slightly more to the basal side of the neuroblast. The determinants are regulated by the polarity of the cell (Choksi et al., 2006;Kitajima et al., 2010). The process is illustrated in Figure 4B.

There are a large number of proteins and interactions involved in the regulation of the processes controlling asymmetric division beyond the scope of this thesis. Therefore, only a few factors which also control quiescence will be discussed.

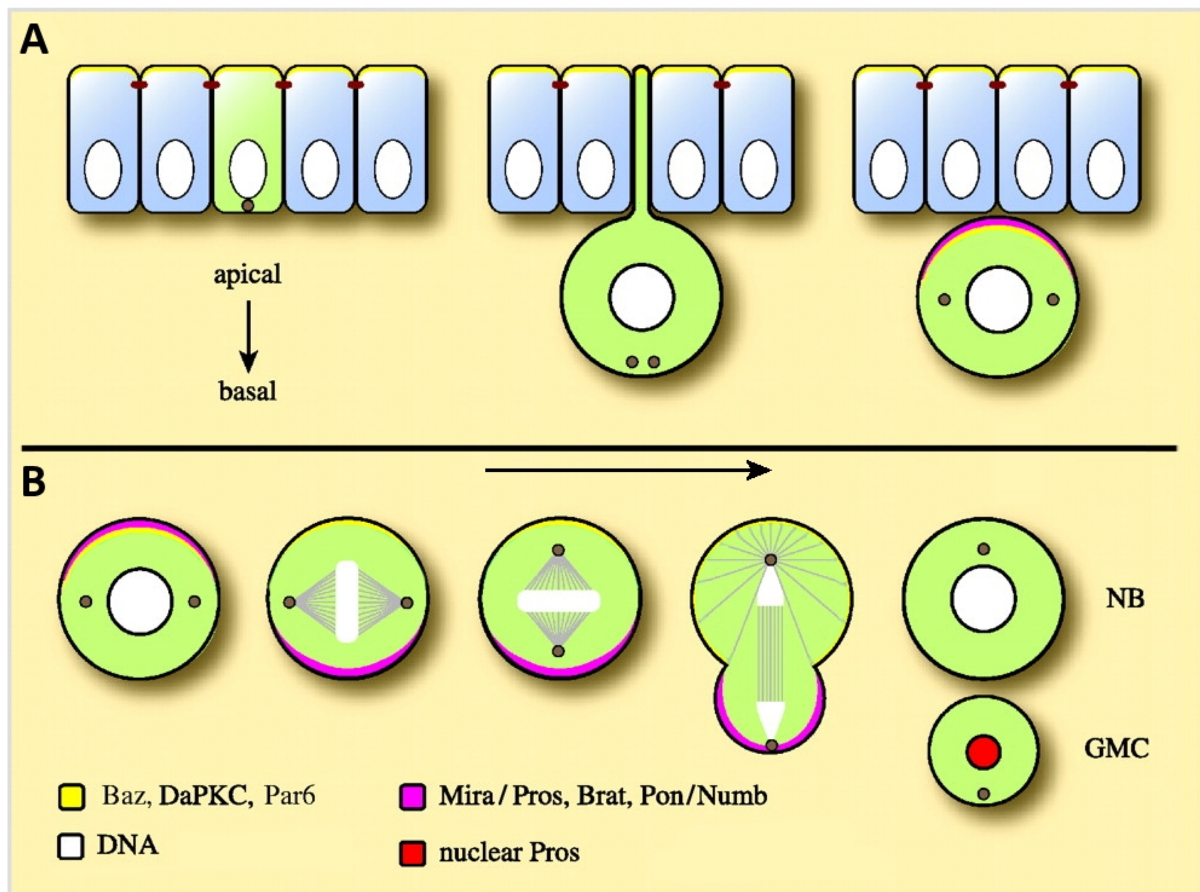


Figure 4. Segregation of basal determinants

A. Delamination and distributution of determinants in the *Drosophila* neuroblast B. Schematic representation of the asymmetric division of the neuroblast. The asymmetric localisation of the spindle poles leads to the segregation of basal determinants into the GMC. After segregation Miranda is degraded allowing Pros to enter the nucleus. Basal determinants are colored purple, apical determinants are colored yellow and DNA is colored white. Edited from (Egger et al., 2008).

To establish the apical/ basal polarity axis a gradient of par proteins is formed. At the apical side of the cell a complex of Bazooka, DmPar6 and DaPKC forms. This complex restricts the localization of the adaptor protein Miranda (Mira). Mira localises basal determinants such as Prospero and Brat to the basal cortex. These determinants will segregate into the GMC together with Numb and the protein partner of numb (PON). The proteins segregate into the GMC by aligning to the spindle pole (Choksi et al., 2006;Kitajima et al., 2010).

After the GMC is formed the transcription factors Brat, Prospero and Numb will regulate the gene expression leading to the GMC fate. However, it is not entirely clear what the function of each factor is in the GMC (Conover and Notti, 2008). Prospero has been studied the most out of all the determinants segregating into the GMC. After being localised by Miranda to the cortex it will be kept there until Miranda is degraded. This occurs shortly after the GMC formation. After Prospero is released it enters the nucleus to repress neuroblast specific genes such as genes required for self-renewal. Besides repressing the function of neuroblast specific genes, Prospero activates the gene expression of the GMC fate (Bello et al., 2008). There are results that support this function, GMCs of Prospero mutants have shown to adapt the neuroblast fate (Choksi et al., 2006).

Intermediate progenitors

The formation of the GMC described above does not result from asymmetric division directly in every neuroblast. In type 1 neuroblasts, which is the majority of the neuroblasts, a GMC is formed directly. However, type 2 neuroblast, which form less than 10% of the adult larva brain, is a type of neuron in which the GMC is formed indirectly. Type 2 neuroblasts form an intermediate progenitor (INP) cell before eventually forming the GMC. In the INP some proteins are not present such as Deadpan (Dpn), Asense (Ase) and Prospero. The fate determinant Dpn is necessary to form the GMC fate. Asense is a nucleair protein which is part of the Achaete Scute complex in the Notch pathway (Bayraktar et al., 2010;Bello et al., 2008;Bowman et al., 2008). The formation of neurons by type 2 neurons is illustrated in Figure 5.

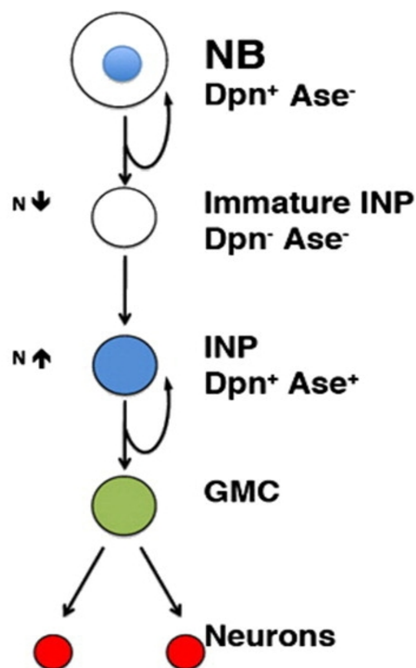


Figure 5. Differentiation of Neuroblast type 2.
Schematic representation of the steps required for differentiation of neuroblasts type 2. The neuroblast will divide asymmetrically, mature, divide asymmetrically again and will form a GMC. The GMC will form two terminally differentiated neurons. Prospero is only present in the GMC and Neurons. N stands for Notch signalling. Arrows pointing up illustrate de-differentation (San-Juan and Baonza, 2011).

The factors Dpn, Ase and Prospero absent in the INP will eventually be expressed under influence of Notch signalling. This signalling is redundant in function (San-Juan and Baonza, 2011). The redundancy suggests regulation of Notch expression by other factors such as Brat and Numb. Besides Brat and Numb, there is also earmuff (Erm) which represses Notch signalling and induces Prospero expression (Weng et al., 2010). Perhaps there are other outside influences besides Notch that control the expression of the factors absent in INP cells as well.

An interesting aspect of INPs is the capability to de-differentiate. De-differentation is the opposite of differentation, this means a cell will become less specified and can adapt a larger variety of fates. De-differentation can occur in the INP which forms a neuroblast or in the GMC which forms an INP. The process causing de-differentation indirectly controls the amount of neuroblasts since non-maturing neuroblasts will enter apoptosis (San-Juan and Baonza, 2011). Apoptosis is a known mechanism that controls the amount of cells. Perhaps quiescence has a similar function in development. It might be possible that the process of de-differentation and formation of INPs is influencing quiescence and apoptosis to regulate the amount of cells. This will be discussed further in the next chapter.

Cell fate

After formation the GMC in both type 1 and type 2 neurons the fate will be determined. This occurs through several processes of which two important processes are temporal and spatial determination. The process of temporal determination partially occurs through temporal identity genes. Temporal identity is the fate of a cell established by time. The genes Hunchback (Hb), Kruppel(Kr), Pdm1/Pdm2 (Pdm), Seven-up (Svp) and Castor (Cas) are known to influence temporal identity (Isshiki et al., 2001). The process of spatial determination partially occurs through Homeobox (Hox) genes. Spatial determination determines the fate of a cell based on position. An example of these genes is Abdominal-A (Abd-A) a known factor for stimulating apoptosis in ventral neuroblasts (Tsuji et al., 2008).

Temporal identity genes and Hox genes influence several processes in the cell by regulating transcription factors such as cell fate, apoptosis and proliferation (Isshiki et al., 2001;Tsuji et al., 2008). Therefore, it is likely that the processes of spatial and temporal determination control quiescence as well. This will be discussed in the next chapter.

Summary

The *Drosophila* neuroblast is formed from the neuroectoderm of the fly. One quarter of all the neuroectoderm cells will form neuroblasts. The other cells will become dermal cells.
The *Drosophila* neuroblast will divide asymmetrically to maintain itself since there is no stem cell niche involved in this process. Which means there are no extrinsic signals necessary to maintain the stem cells. Asymmetric division can occur through intermediate progenitors as well. The daughter cells will differentiate into various neurological structures. These include the central nervous system, the brain and peripheral structures. This is influenced by spatial and temporal determinants. The development of the *Drosophila* neuroblast requires a complex interaction of regulators to form these types of neurological tissue correctly. One of these regulations is entering quiescence in later stages of development and exiting from quiescence in the early larval stage.

Chapter 3: Quiescence in the *Drosophila* neuroblast

The development of Drosophila neurons occurs through several complex mechanisms. In chapter 2 some important mechanisms were discussed. This chapter will focus on a different aspect of the neurological development, namely quiescence in the neuroblast. Quiescence, a temporary exit of the cell cycle discussed in chapter 1, regulates the amount of proliferating cells (Wang et al., 2010). The quiescent state of the cell cycle needs to be regulated by several pathways to create a balance between the amount of proliferating cells and the amount of cells that are in the quiescent state. So how is the balance between quiescence and proliferation regulated in the neuroblast? In this chapter processes that might provide an answer to this question will be discussed.

First, the influence of extrinsic factors (signals from outside the cell) in regulation of quiescence will be discussed. Followed by some known intrinsic factors (signals from inside the cell) which are important for the development of the neuroblast. Then the balance between apoptosis and quiescence will be discussed. And finally, the influence of genes involved in the formation of neuronal identity will be discussed briefly.

The influence of metabolism

In chapter 1 the TOR pathway was described. The TOR pathway has shown to be an important pathway which integrates the energy status of a cell into a cellular response. The fact that nutrients and energy levels are essential for both proliferation and quiescence is underscored by an experiment which showed that adding nutrients to yeast lead to exit from the quiescent state. This response is likely caused by TOR signalling (Laporte et al., 2011). Is this the case in *Drosophila* as well?

Drosophila larvae need to consume a large amount of nutrients in order to grow. In this stage of development the availability of nutrients plays an important role in the regulation of quiescence (Britton and Edgar, 1998). It was shown that *Drosophila* larva neuroblast cells were entering quiescence after starvation while they were able to re-enter the cell cycle when E2F or Cyclin E was added to the cell (Britton and Edgar, 1998). Next to adding E2F or Cyclin E, feeding the larvae sucrose resulted in cell cycle re-entry. This indicates that the cell cycle of developing *Drosophila* neuroblasts is influenced by nutrients (Britton and Edgar, 1998;Chell and Brand, 2010). These results could also indicate that nutrients act in the same pathway as E2F, suggesting that the TOR pathway is involved.

However, not all cells were influenced by starvation. The cells that were not influenced were cells necessary for survival such as the gut, muscle cells, epidermis and glands. This suggests that there is another mechanism for the control of quiescence in those cells. This is believed to be the fat body which acts as a sensor for the concentration of nutrients in the *Drosophila* liver (Britton and Edgar, 1998;Chell and Brand, 2010). The *Drosophila* fat body secretes mitogens in response to nutrition that permits cells that are necessary for survival to re-enter the cell cycle.

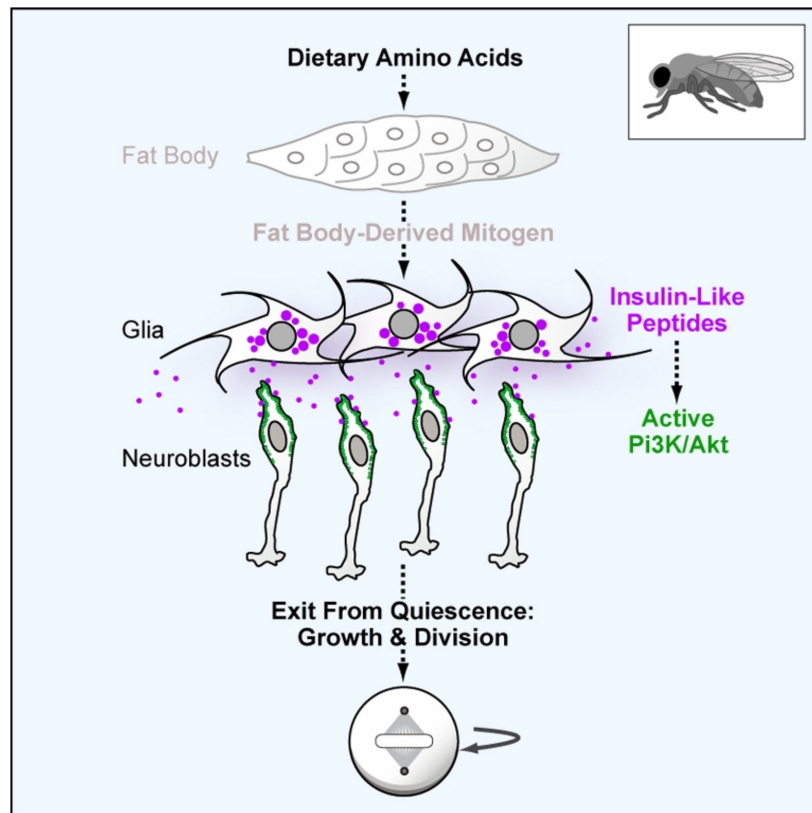


Figure 7. Regulation of quiescence by nutrition
Picture depicting the process of cell cycle re-entry by adding nutrients. Dietary amino acids activate fat bodies to secrete FBDM which triggers glia cells to form dILPs. This will stimulate the TOR pathway (Chell and Brand, 2010).

Figure 7 illustrates the process required for the activation of neuroblasts by nutrition. The presence of dietary amino acids stimulates the fat bodies to secrete a growth factor called Fat Body-Derived Mitogen (FBDM)(Chell and Brand, 2010). This mitogen is formed by activation of the TOR pathway. This is coupled to amino acids binding to the Slimfast receptor of the fat body. By knocking this receptor out the TOR pathway is downregulated (Sousa-Nunes et al., 2011). Glia cells around some of the neuroblasts are secreting *Drosophila* Insulin like peptides (dILPs) in response to FBDM. These dILPs will then act as a growth factor on neuroblast cells to activate proteins like PI3K or Akt which control the TOR pathway. The TOR pathway then activates transcription factors which will allow cell cycle progression as described before (Chell and Brand, 2010;Sousa-Nunes et al., 2011).

The glia cells are thought to function as a relay between the fat body and the neuroblast. This relay might be important to establish the timing of entering quiescence in different kind of neurons. There is a possibility that other signals apart from nutrition could induce the release of dILPs and therefore stimulate the exit from quiescence. Induction of PI3K signalling in the absence of nutrition could explain why important cell types do not enter quiescence in the absence of nutrients (Chell and Brand, 2010). Evidence for this theory is provided by an experiment which overexpressed genes coding for dILPs in the absence of nutrients. This resulted in reactivation of neuroblasts. Altough this effect was smaller than

adding nutrients to the cell it does indicate that dILPs are responsible for the exit from quiescence (Chell and Brand, 2010).

The decreased effect of dILPs compared to nutrition indicates that other extrinsic or intrinsic factors are dependent on nutrients in regulating the re-entry into quiescence. Currently, it is unknown which factors are regulated by nutrients except for the TOR pathway. Another common theory is that cell growth rather than molecular pathways determine the entry into quiescence. It has been shown that cells below a certain volume do not divide anymore and have likely entered quiescence. This is altered by the concentration of nutrients, since this affects cell growth, and through intrinsic mechanisms which affect divisions. Neuroblasts become smaller every division by budding of the GMC (Kitajima et al., 2010). This process seems to be occurring prior to entry into quiescence. To understand more about the extrinsic regulation of neuroblast quiescence other growth factors will be discussed.

Growth factors

In the previous paragraph it was shown through experiments that nutrients seem to be influencing quiescence in the neuroblast. Another factor that is likely to be involved in the process of quiescence is a gene named Terribly reduced optic lobes (Trol). Knocking out this gene resulted in a phenotype of the fly where a significant amount of cells did not re-enter the cell cycle after quiescence. This could be rescued by adding cyclin E which indicates that the product of Trol regulates the cell cycle (Park et al., 2003;Voigt et al., 2002).

Trol encodes for the *Drosophila* Perlecan receptor (Park et al., 2003). This receptor is known to bind to Fibroblast growth factor (FGF) which plays an important role in cell cycle regulation. The receptor was shown to interact with Hedgehog (Hh) as well. This protein is known for its function in regulating many growth related developmental processes. By knocking Hedgehog out less neuroblasts were found to be dividing. This suggests that both FGF and Hh play a role in regulating quiescence. Nevertheless, it is unclear if this is mediated by just the Perlecan receptor or by other factors as well (Voigt et al., 2002).

There is a factor known that acts upstream of Trol. This is the gene Anachronism (Ana).
Just like nutrient dependent regulation described before Ana is expressed in the glial cells rather than in the neuroblast. Ana encodes for a secreted glycoprotein that is thought to be regulating the quiescent state of the cell. However, it has been found that the concentration of Ana does not decrease when the cell re-enters the cell cycle. This stable concentration of Ana indicates that a currently unknown factor or mechanism is regulating Ana (Ebens et al., 1993). The extrinsic regulation by Ana and Trol is illustrated in Figure 8.

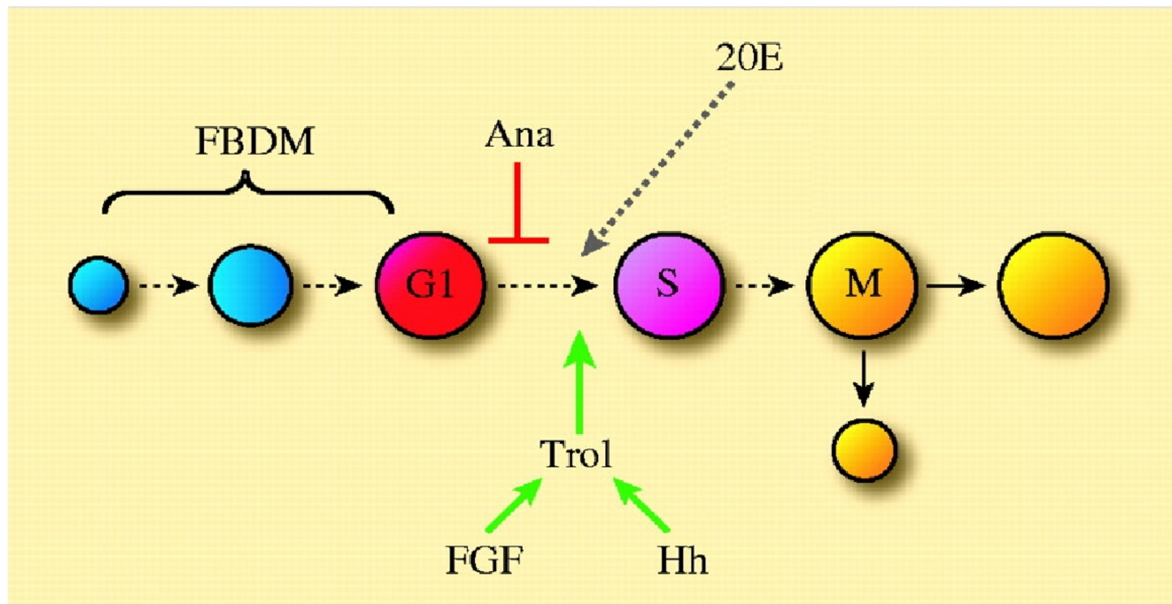


Figure 8. Extrinsic regulation by Ana and Trol
Several factors are known to regulate quiescence. FBDM in response to nutrition which could be regulated by TOR signalling, both FGF and Hh that bind to the receptor encoded by Trol and the hormone 20E (ecdyson) are currently known extrinsic factors. The gene Ana is known to keep the cell in the quiescent state. How these factors are integrated is unknown (Egger et al., 2008).

This regulator of Ana could be another factor with an unknown function that is part of this mechanism. This is the hormone ecdyson. Ecdyson stimulates Trol expression by being added to the cell. The function of ecdyson can be stimulated by overexpressing the Even skipped (Eve) gene as well. Eve is a homeobox gene which is known for its function in establishing segment polarity (Voigt et al., 2002). This means it is influenced by temporal identity factors since segment polarity is partially regulated by spatial and temporal determinants. The involvement of this factor connects this pathway to intrinsic regulators. These intrinsic regulators will be discussed later.

Currently the function of both ecdyson and Eve in the context of quiescence are not completely understood. It is only known that they stimulate the exit from quiescence by stimulating Ana. However, it is not known how. To put it briefly, there are several extrinsic factors which regulate proliferation, yet it is unknown if the factors act in the same pathway or that separate pathways are involved (Voigt et al., 2002).

But this does provide answers on questions such as how the neuroblast stops dividing at the right time and how the cell knows it has the right size to stop dividing. Since there are still some questions left unanswered we will now turn to the intrinsic mechanisms that controls the cell cycle.

Asymmetric divisions and quiescence

Neuroblasts divide asymmetrically to form differential progeny. This involves localisation of proteins to both sides of the cell. One of these factors, named Prospero, will be segregated into the GMC daughter cell. There it will establish the fate of the GMC (Choksi et al., 2006;Kitajima et al., 2010).

Although this function of Prospero is interesting, more important for this thesis is that Prospero has an effect on the cell cycle as well. In a Prospero mutant cells do not enter the quiescent state. By adding Prospero to Prospero mutants the phenotype can be rescued which means cells are capable of entering quiescence again (Choksi et al., 2006). In addition to not entering quiescence Prospero mutants formed tumors. This indicates that Prospero is a tumor suppressor gene (Choksi et al., 2006). So which genes are regulated by Prospero that it has such a profound effect on the cell cycle?

In Figure 9 the genes that are downregulated by Prospero are shown. This figure strongly suggests that Prospero has a role in regulating quiescence. The data was obtained by genome wide expression profiling in developing neuroblasts of both wildtype flies and Prospero mutants. Especially the expression level of cyclin E which is over a 100 times higher in wildtype flies indicates that Prospero has a strong effect on cyclin E expression and therefore cell cycle progression. Besides downregulating the cell cycle regulator cyclin E it also downregulates other cell cycle regulators such as string (cdc25) and E2F. Prospero also downregulates factors important in the division of type 2 neuroblasts such as Asense and deadpan. This indicates that the presence of Prospero suppresses the fate of INPs. Finally, Prospero seems to downregulate grainyhead, which is a factor regulating apoptosis as well as quiescence. This will be discussed later on in the chapter (Choksi et al., 2006).

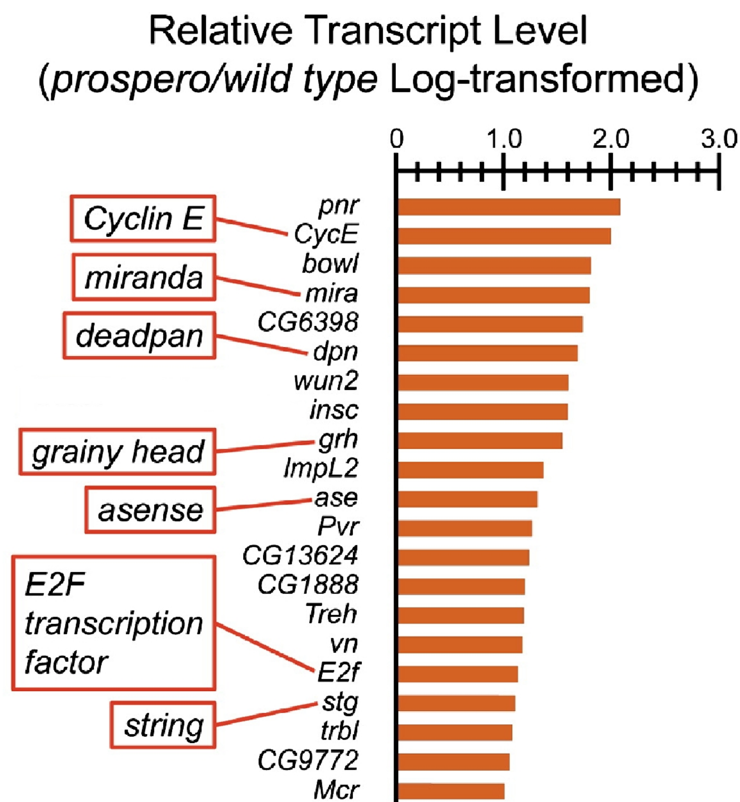


Figure 9. Genes downregulated by Prospero
Results of gene expression profiling of prospero. The bars indicate the levels of transcripts in Prospero mutants relative to the levels of transcript in wildtype *Drosophila*. Genes shown here have a larger amount of transcript in wildtype flies compared to the Prospero mutants. Genes that are thought to be important for neuroblast proliferation are annotated in red. Edited from (Choksi et al., 2006).

It has been shown that in Prospero mutants tumors arise. But these tumors did not arise in INPs (Bowman et al., 2008). Thus, there are other tumorsuppressor genes active in INPs and in GMCs as well.

The factors Brat and Numb function in both INPs and GMCs. Numb functions by promoting the endocytosis of the Notch receptor. As discussed in chapter 2 Notch plays an important role in specifying the fate of the neuroblast. But inhibiting the action of the Notch receptor seems important to enter quiescence. It suggests that Notch is necessary for the cell to self-renew and that it needs to be downregulated in order for the cell to differentiate (Bowman et al., 2008).

Another factor shown to have an effect on quiescence is Brat which is also segregated into the GMC along with Prospero. Brat seems to have a function in arresting genes required for cell growth. Altough this seems unrelated, the size of the neuroblast is relevant for entering quiescence. This was also seen in the extrinsic pathway regulated by nutrition. By knocking out Brat an excess of neuroblasts will arise with a significantly lower amount of neurons (Bowman et al., 2008). This suggests that Brat and Numb have a function in regulating quiescence much like Prospero.

Besides Brat and Numb there is also Dpn which plays a role during the development of type 2 neurons. Dpn mutants show a decreased amount of neuroblasts that are formed and no type 2 neurons. However, it has also been shown that there is an increase in Prospero expression when Dpn is downregulated (San-Juan and Baonza, 2011). These results are contradicting since Prospero limits cell division and Dpn which has shown to inhibit Prospero expression shows a decreased amount of neuroblasts. It remains unclear what causes this.

So how are those processes coupled? INPs eventually express Prospero which is thought to occur by the upregulation of Ase. Ase is not present in INPs next to Prospero and Dpn. But later in development all are expressed (Bayraktar et al., 2010). The exact reason why they are all expressed and how they are expressed remains unclear.

Notch signalling is thought to be initiator of this process. Notch activates Ase and Dpn which both repress the function of Prospero. Numb and Brat are thought to regulate the expression of Notch and therefore Ase and Dpn expression to regulate the maturation of the INP. When both Brat and Numb are knocked out maturation does not occur and both Ase and Dpn will not be upregulated (Bowman et al., 2008). Brat seems to localise Prospero but currently it is unclear which factor promotes the expression of Prospero (Bayraktar et al., 2010;Choksi et al., 2006).

One theory is that the Erm which functions by suppressing the de-differentiation of the INP localises Prospero in the nucleus and promotes the activity of Prospero. Real time PCR has shown that in Erm mutants Prospero RNA has been reduced by 60%. In addition to this experiment another experiment shows that erm can indusce Prospero expression. By briefly overexpressing erm in an erm mutant by using a Gal4/UAS transgene was shown to induce Prospero expression (Weng et al., 2010). Gal4/UAS is conditionally expressing a gene by adding the yeast protein Gal4 to it. This protein will bind to the UAS sequence of the transgene to induce the expression.

Thus, it seems likely that quiescence in type 2 neuroblasts is regulated by Numb and Brat, which act through Notch signalling to induce Prospero by factors such as Erm. Prospero inhibits the expression of factors such as Dpn and Ase, while Dpn and Ase inhibit Prospero. This would suggest that there is some sort of switch between quiescence and cell cycle progression based on the expression of Ase, Dpn, and Prospero and the concentration of proteins encoded by the genes.

Altough the processes described above explain various aspects of quiescence in both type 1 and type 2 neurons it is likely that there are some factors involved that are currently unknown. For instance, the factors that regulate Prospero and cues that stimulate that function of Brat and Numb. In addition, the process described here does not answer questions such as how the cell "knows" when to enter quiescence and which cells should enter quiescence. However, some cells do not enter quiescence but enter apoptosis instead such as the neuroblasts in the abdomen of the *Drosophila* (Siegrist et al., 2010). Perhaps there is a mechanism which controls the occurrence of quiescence and apoptosis. Therefore, apoptosis will be discussed briefly after which this will be coupled to quiescence.

Quiescence and apoptosis

During the development of the *Drosophila* some cells enter quiescence while others enter apoptosis. It has been shown by fluorescence microscopy that cells reduce their mitotic activity and cell size prior to apoptosis. An example of occurring apoptosis is the brain area of the *Drosophila* when the neurogenesis ends (Siegrist et al., 2010).

Apoptosis of adult neuroblasts occurs through reaper(rpr). Rpr is a protein that induces apoptosis together with the factors hid and Grim together known as (RHG). The RHG proteins induce caspase-mediated cell death (Siegrist et al., 2010). It is thought that these proteins are expressed by the action of both Abdominal-A (Abd-A) and Grainyhead (Grh). Abd-A is a Hox gene as discussed in chapter 2 and Grh a transcription factor transcribed by temporal identity genes which inhibits Prospero expression (Maurange et al., 2008). However, knocking out RHG does not prevent apoptosis. In neuroblasts of RHG knock-out flies caspase mediated cell death still occurred. There seems to be another mechanism that controls the occurrence of apoptosis as well.

Apoptosis was also inducible by over expressing PI3K kinase signalling which is part of the TOR pathway. This would suggest that the TOR pathway does function in both apoptosis and quiescence. Similar to the TOR pathway p53 has shown to regulate both apoptosis and quiescence as well (Liu et al., 2009). This was discussed in chapter 1. It seems that apoptosis and quiescence are controlled by similar pathways but that slight differences caused by extrinsic factors in addition to the identity of the neuron determines whether the cell enters apoptosis or quiescence.

Neuronal identity, apoptosis and quiescence

Neuroblasts enter quiescence in a predictable order (Choksi et al., 2006). This occurs from anterior, which is formed first, to posterior, which is formed later (Maurange et al., 2008). It suggests that the identity of the neurons is involved in the process of quiescence. In addition, it is known that after embryogenesis abdominal neuroblasts enter apoptosis (Tsuji et al., 2008). Is spatio-temporal determination regulating whether the cell enters apoptosis or quiescence?

Besides the expression of factors such as Dpn, Ase, and Prospero there are also other intrinsic factors that control quiescence of the neuroblast. Temporal identity factors such as Hunchback (Hb), Kruppel(Kr), Pdm1/Pdm2 (Pdm), Seven-up (Svp) and Castor (Cas) are transcription factors that encode for many genes necessary for development. This includes the factors Chinmo and Broad complex (Br-C) which are functioning to determine the size and fate of the cell. Chinmo is present in earlier stages of development and Br-C in later stages (Maurange et al., 2008). The presence of those factors seems to mark the stage of development and could thereby contribute to the decision to enter quiescence. But there has been no proof yet that this is the case.

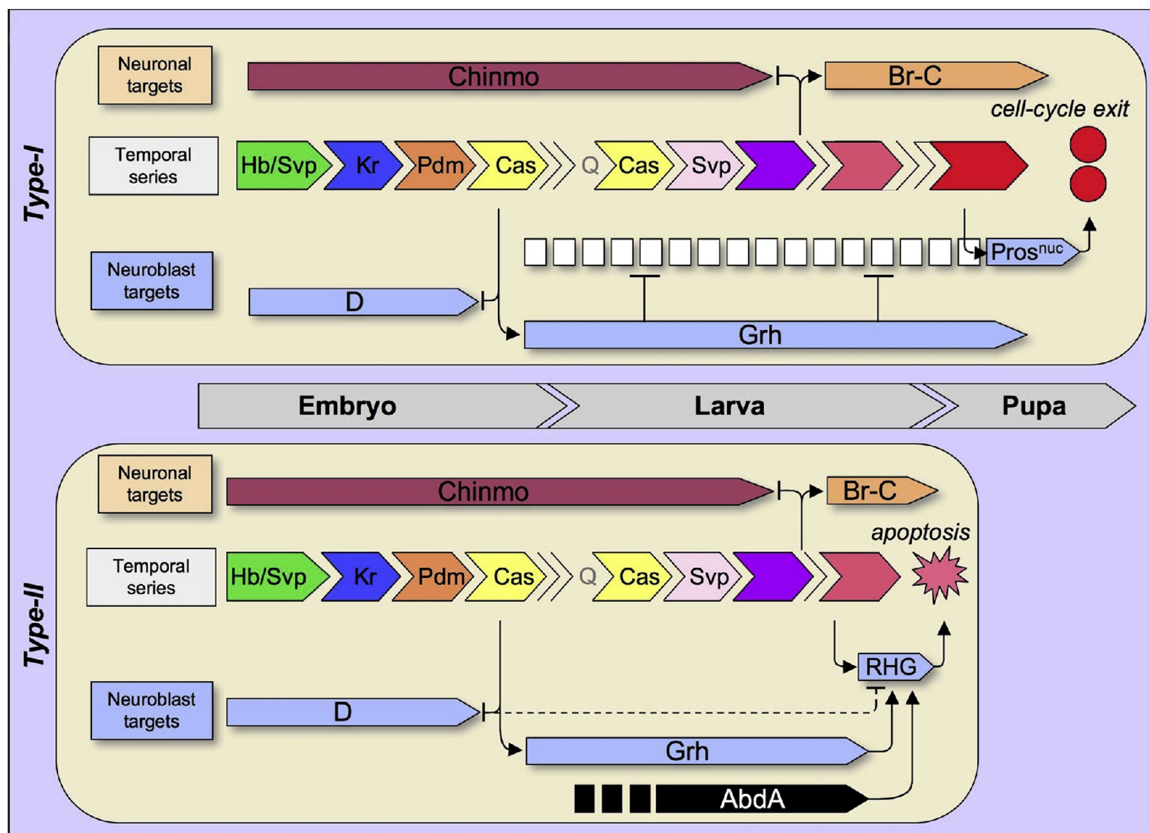


Figure 10. Regulation by temporal identity
At both type 1 and type 2 neuroblasts temporal identity genes (Hb, Seven-up (Svp), Kr, Pdm and Cas) control the expression of several genes. Chinmo and Br-C are functioning in fate determination and maturation of the neuroblast while D and Grh limit the number of neuroblasts by apoptosis (RHG) or quiescence (Pros). This figure shows a timeline in which each temporal identity gene is activated in both type 1 and type 2 neuroblasts. In type 2 neuroblasts AbdA is present which means the cell can enter apoptosis. In type 1 neuroblast cells enter quiescence (Maurange et al., 2008).

Besides the factors Chinmo and Br-C temporal identity genes regulate the expression of both the factor D and Grainyhead (Grh). Both Grh and D seem to be important for the decision between apoptosis and quiescence. D is a protein that inhibits RHG mediated apoptosis, while Grh is a protein that stimulates RHG mediated apoptosis. However, Grh cannot function if AbdA is not present (Maurange et al., 2008;Tsuji et al., 2008). Thus, it might be possible that in order to induce apoptosis D has to be inhibited, Grh and the Hox protein AbdA have to be present and there has to be TOR signalling, as was discussed before.

If the cell does not have all the requirements for entering apoptosis it is largely unknown what happens to the cell. It seems that cells lacking AbdA enter quiescence instead of apoptosis but this has not been proven in all cell types. In type 1 neuroblasts this has been shown by expression profiling and fluorescence microscopy. In early stages Grh with a large amount of Grh present Prospero was localised at the cortex and thus inactive. In later stages of development Grh expression subsided and Prospero was able to enter the nucleus and induce quiescence. In the tested type 2 neuroblasts this did not occur. When AbdA was present the neuroblasts entered quiescence instead. This is shown in Figure 10 (Maurange et al., 2008).

The spatio-temporal determinants might be important to understand the process of quiescence. But there is still a significant amount of research to be done. The exact function of the temporal regulators remains largely unknown as well as the extrinsic factors that influence the process. In addition, the difference in entering quiescence between type 1 and type 2 neurons could be involved, but to what extend? In the final chapter this will be discussed further.

Summary

Both intrinsic and extrinsic factors regulate quiescence in the *Drosophila* neuroblast. Nutrients stimulate the secretion of dILPs in fat bodies which signal the neuroblast to exit from quiescence through the TOR pathway. Apart from nutrients there are other extrinsic factors which generate a similar response. Besides extrinsic regulation there are also intrinsic factors regulating quiescence. An important factor seems to be Prospero which is influenced by other factors which are segregated into the GMC or progenitor cell as well. In addition to these factors there is also spatio-temporal determination which seems to influence the expression of Prospero. Apart from this function spatio-temporal determinants control apoptosis through a similar regulatory mechanism as well. The next chapter will describe some processes, discussed in this chapter, which are conserved in other species.

Chapter 4: Conserved mechanisms

In the previous chapter the regulation of quiescence in the *Drosophila* neuroblast was discussed. Nevertheless, it might very well be that other mechanisms contribute to the regulation of quiescence in mammals. Therefore, other model systems have to be used to understand which process occurs in higher organisms such as mice and perhaps even humans. In this chapter some conserved mechanisms will be discussed that are known to be conserved. Not all of the mechanisms will be discussed here. For some processes described before it is unknown whether the process itself is conserved, the function in quiescence is conserved or neither is conserved. This will not be discussed further in this chapter.

First Notch signalling will be discussed in several organisms, after which the influence of the metabolic status on quiescence will be discussed. Followed by a brief description of the intrinsic factor Prospero that is likely to be conserved.

Notch signalling

In the *Drosophila* neuroblast Notch signalling has also been found to control the expression of Ase and Dpn which were regulators of quiescence. Notch signalling occurs in multicellular organisms to define the cell fate of adjacent cells. It is known that Notch signalling is highly conserved but is the control of quiescence by Notch conserved as well?

Notch signalling has shown to influence quiescence in the zebrafish, in several birds and more importantly in human fibroblasts (Chapouton et al., 2010;Daudet et al., 2009;Sang et al., 2008). In the zebrafish Notch activity has shown to regulate the neurogenesis in the telencephalon. This occurred through a similar mechanism as the INPs in the *Drosophila* brain. Activation of the Notch pathway resulted in entry into quiescence and blocking of Notch signalling resulted in exit from quiescence (Chapouton et al., 2010). This is also the case in the auditory epithelium of birds. In birds Notch regulates progenitor cells of the auditory epithelium. This occurred by upregulation of the downstream target (Hairy Espl) Hes5. However, this was not the only factor that regulated quiescence since blocking Notch signalling did not result in more dividing cells or cells in the quiescent state. It is unknown what other mechanism controls this (Daudet et al., 2009).

It seems that Notch signalling is an important regulator of quiescence in birds, zebrafish and *Drosophila* but is it relevant in the human regulation of quiescence as well? Notch signalling upregulates the expression of downstream targets which are similar to those found in birds in mammals as well. Interestingly, one of the downstream targets of Notch, Hes1, has shown to be important in the regulation of quiescence. This occurred by the regulation of p21 levels a Cdk inhibitor which was also described in chapter 1 (Sang et al., 2008). Cells with a high amount of p21 were shown to enter quiescence while cells with a low amount of p21 did not (Sang et al., 2008). This shows that Notch is a conserved regulator of quiescence.

Metabolic status

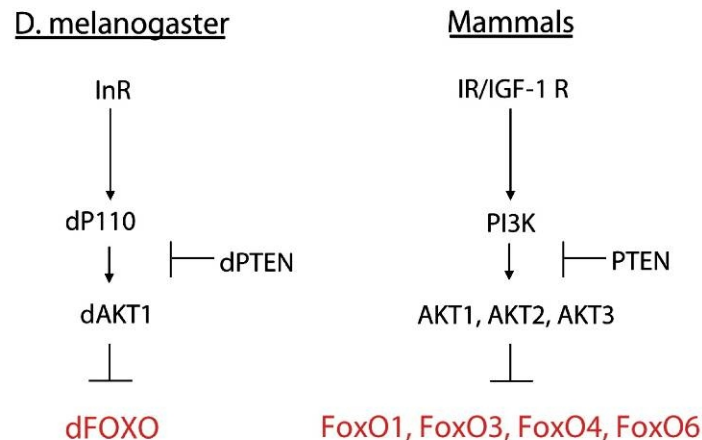


Figure 11. Conserved PI3K signalling.
In both mammals and Drosophila signals from the insulin or insulin like receptor activate a kinase named p110 or PI3K. The kinase activates Akt or dAkt. Pten or dPTEN inhibits this activation. In both Drosophila and mammals Akt leads to activation of FoxO genes which regulate processes like quiescence and growth (Tothova and Gilliland, 2007).

Nutrition was shown to be necessary and sufficient to regulate quiescence in yeast (Laporte et al., 2011;Yanagida, 2009). In *Drosophila* nutrition was shown to be important in controlling both intrinsic regulation and extrinsic signals. Is the regulation of quiescence by nutrition conserved and present in humans as well?

The TOR pathway is conserved in mammals. In the *Drosophila* homologs with the same function as those in humans have been found. It might very well be that this pathway controls the same processes which were observed in the *Drosophila* neuroblast. In Figure 11 the difference between the PI3K signalling, which is part of the TOR pathway, is illustrated (Tothova and Gilliland, 2007).

The figure suggests that the PI3K pathway is roughly the same. The function has also shown to be similar in regulating quiescence. In Foxo deficient mice it has been shown that cells did not re-enter the cell cycle. In addition, in human hematopoetic stem cells (HSCs) loss of FoxO1, FoxO3 and FoxO4 resulted in a significant increase of cells in the S and G_2 phase (Tothova and Gilliland, 2007). This suggests that a similar regulation of quiescence exists in mammals. However, this has not been researched in the development of mammalian neurons and it is not known which other factors contribute to this. Regulation of quiescence could very well be through a glia insulin relay as was seen in the *Drosophila* but it could be a different mechanism as well.

Conservation of intrinsic mechanisms

Mammalian development of neurons and in particular the brain is considerably different from *Drosophila* development. It is proposed that mammalian brain development uses a system similar to type 2 neurons in *Drosophila*. However, it is unknown if the molecular composition of both neurons is conserved (Bello et al., 2008). It seems that Prox1 the human

homolog of Prospero has the same function. Prox has shown to inhibit cell division and initiate differentiation in other vertebrates. Thus, it seems likely that a similar mechanism exists in mammals (Choksi et al., 2006). Nevertheless, this is based on speculation and mammalian development can prove to be more complex. It does seem likely that at least Notch signalling, nutrition, and intrinsic factors play a role in regulating quiescence just as in *Drosophila*. To understand the entire process more research has to be done in *Drosophila* and mammals alike.

Summary

Several mechanisms, involved in the regulation of quiescence in the *Drosophila* neuroblast, were found to be conserved in mammals and other species. Notch signalling was shown to be conserved in both the process and function of regulating quiescence. Nutrition was found to be regulating quiescence in mammals as well, but it is still unknown if this occurs through the same processes described in *Drosophila*. Finally, Prospero was discussed which could have the same function in mammals as in *Drosophila*. However, this cannot be proven yet.

Discussion and summary

Quiescence is a state of the cell cycle in which the cell cycle does not progress. This state is reversible and is controlled by many different and largely unknown mechanisms. To understand the mechanisms involved in quiescence the *Drosophila* neuroblast was used.

Some aspects of the neuroblast development were discussed. This includes lateral inhibition by Notch signalling which specifies the fate of the neuroblast. After that, the process of asymmetric division was discussed in which several factors are segregated to form two different cells. This process was shown in two types of neuroblasts in which the second type forms a progenitor cell before it forms the eventual GMC. Finally, the temporal identity factors and Hox genes were discussed which are important in specifying the fate of the neuroblast.

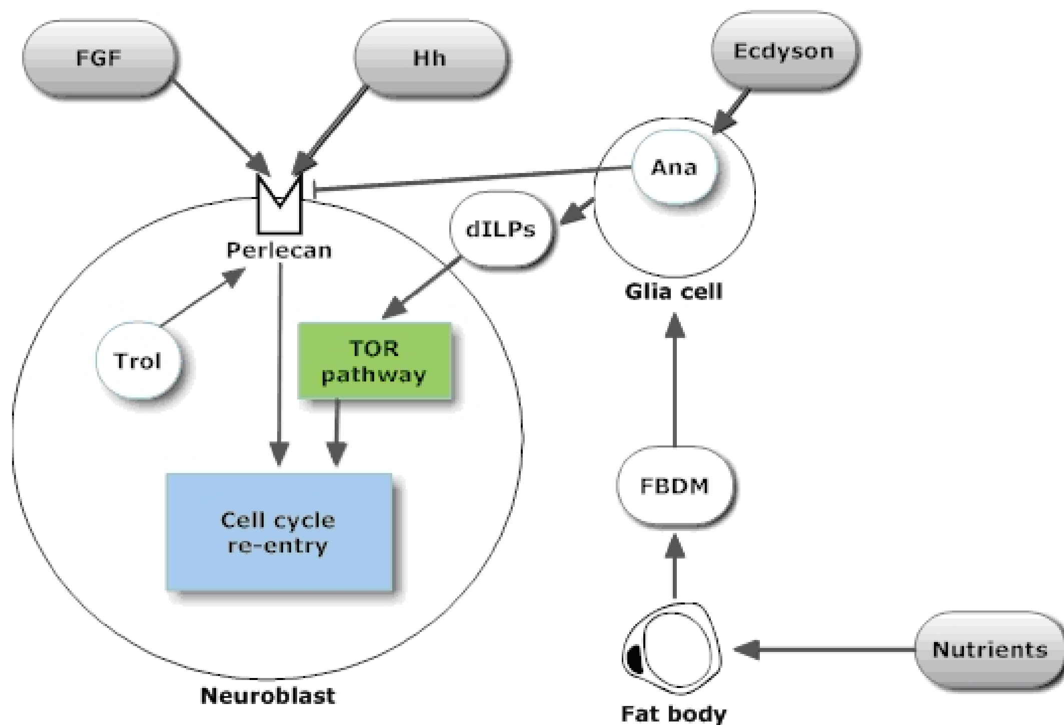


Figure 12. Extrinsic regulation
Schematic representation of extrinsic signals influencing cell cycle re-entry. Nutrients stimulate fat bodies to secrete FBDM which stimulate glia cells to secrete dILPs. dILPs activate the TOR pathway which leads to cell cycle re-entry. Besides nutrition FGF and Hh stimulate cell cycle re-entry by binding to the perlecan receptor. Trol is a gene which encodes for perlecan and is inhibited Ana. It is unknown which factor stimulates Ana but it is thought to be the hormone ecdyson. Outside influences are shown as grey, pathways are shown as green and resulting processes in blue.

The processes described were shown to be important for development and for the regulation of quiescence as well. First extrinsic factors such as nutrients and growth factors were shown to influence cell cycle re-entry and quiescence. This is schematically visualised in Figure 12. The figure shows that at least four different external factors influence the process of cell cycle re-entry. Nutrients through glia cells and eventually through the TOR pathway, the growth factors fibroblast growth factor (FGF) and Hedgehog (Hh) through the perlecan receptor and the hormone Ecdyson supposedly through Anachronism (Ana).

In addition to extrinsic signals, intrinsic mechanisms were discussed as well. These mechanisms were shown to influence apoptosis and cell cycle exit. This is shown in Figure 13.

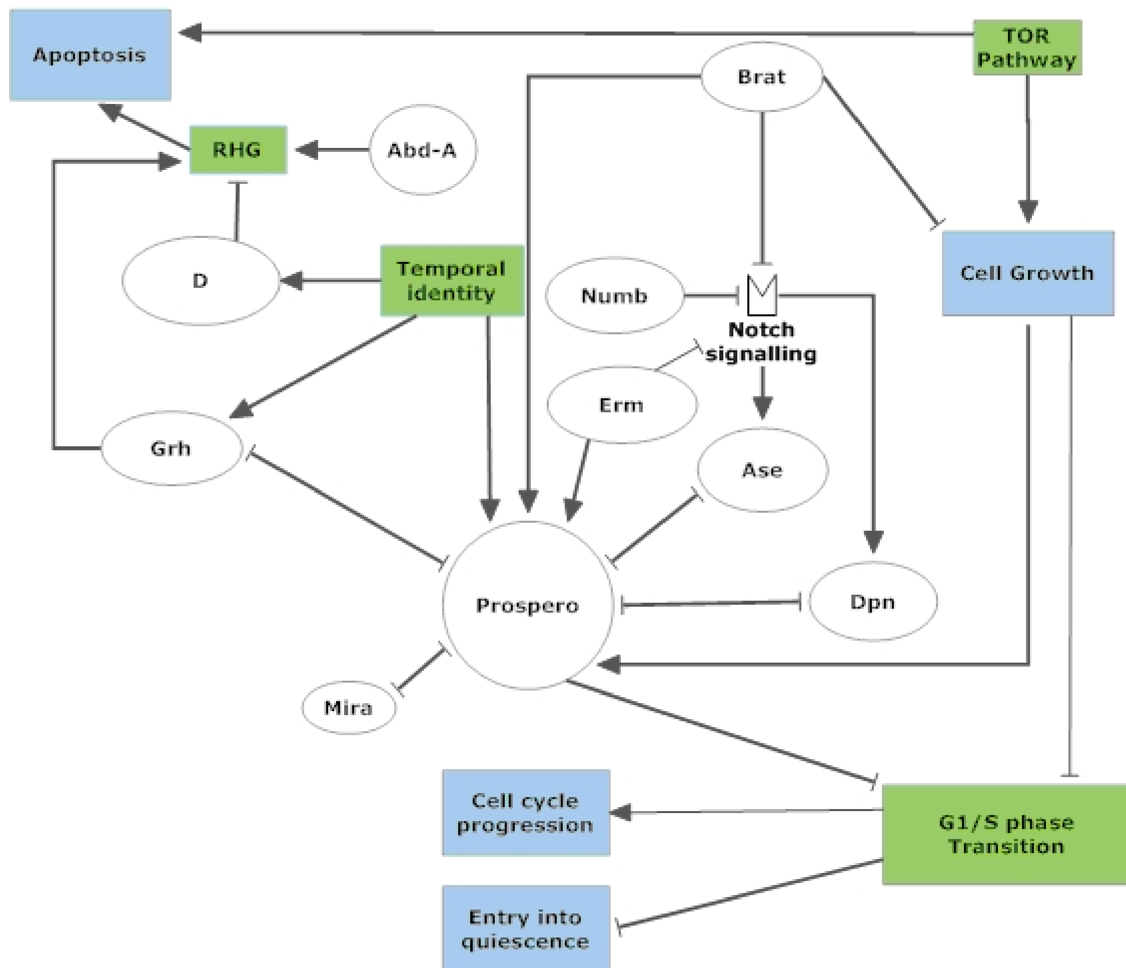


Figure 13. Intrinsic regulation

Schematic representation of intrinsic factors influencing cell cycle exit and apoptosis.

The segregated factors Prospero, Mira, Brat, and Numb are important for cell cycle exit. Prospero promotes entry into quiescence by inhibiting several factors stimulating the G_1/S transition. Brat and Numb control Notch signalling to regulate the expression of Ase and Dpn. Ase and Dpn inhibit Prospero expression. Erm stimulates Prospero. Mira localises Prospero at the cortex limiting its function. Temporal identity genes stimulate Prospero expression as well, but limit the action by stimulating Grh expression. Temporal identity links quiescence to apoptosis by the factors D and Grh which regulate RHG mediated apoptosis. Apoptosis is regulated by the factors Abd-A and TOR signalling as well. Pathways are shown as green and resulting processes in blue.

The intrinsic mechanisms only seem to control entry into quiescence by Prospero. This is influenced by at least two different mechanisms. Temporal identity and Notch signalling. Temporal identity genes code for transcription factors that influence both apoptosis and quiescence. This occurs through regulation of D and Grh. However for apoptosis to occur Abd-A needs to be present. The temporal identity factors are expressed sequentially which regulates the timing at which either apoptosis or quiescence occur. The other mechanism is initiated by asymmetric division and through lateral inhibition. Brat and Numb control Notch signalling and Notch signalling controls the expression of Ase and Dpn. Ase and Dpn limit the

function of Prospero, while Prospero downregulates the expression of both Ase and Dpn. Erm has shown to stimulate the function of Prospero but it is unknown in which pathway this factor acts. Besides temporal identity and Notch signalling the size of the cell seems to influence quiescence as well. This is regulated by the TOR pathway.

Yet, it seems that some important factors are missing. The most important part that is missing is the feedback between intrinsic and extrinsic mechanisms. Now only the TOR pathway is known to act in both, but it is highly unlikely that there is no direct feedback between intrinsic and extrinsic mechanisms. Nevertheless, it will be difficult to find these feedback mechanisms with several major developmental pathways involved. Perhaps ecdyson which is influenced by Eve will provide another link between intrinsic and extrinsic mechanisms.

Besides feedback between mechanisms it seems that some important steps in feedback between factors are absent. It seems that Ase needs to be expressed prior to functional Prospero in the nucleus. However, Ase has shown to inhibit Prospero expression while Prospero has shown to inhibit Ase expression. Thus, it seems likely that there is a mechanism which controls this expression. Perhaps there is a factor stimulated by Ase expression which promotes the function of Prospero, by either inhibiting the anchoring of Mira or by localising the factor itself. The existence of such a factor would indicate a threshold that controls the expression of Prospero.

Another mechanism where a threshold should be involved is cell growth. In several papers it has been shown that cells entered quiescence when the size of the cell was small enough. This was usually after 10-20 rounds of division in type 1 neuroblasts (Britton and Edgar, 1998;Isshiki et al., 2001). This threshold of cell size is unknown as well. Perhaps this threshold is coupled to other pathways. A decrease in size could lead to a decrease of protein levels in the cell which might trigger signalling to nearby cells. Perhaps this is a feedback mechanism between the glia cells and the neuroblast to regulate perlecan. On the other hand it could be through Notch signalling as well, signalling to nearby cells to differentiate. Nevertheless, this is purely hypothetical.

Thus, it seems that a large amount of pathways and processes are involved and perhaps this number will increase even further. Factors such as Br-C and Chinmo, which controls the GMC fate, could prove to be involved or perhaps others which have not been discussed here.

Currently, the understanding of most processes involved in quiescence is insufficient for use in current biological problems such as in cancer biology, stem cell biology and neurology. The lack of information about how the mechanisms are connected limits the potential of using quiescence in these fields. In addition, it is not clear if all the mechanisms described in this thesis are conserved in mammals. It is possible that the factors described here could be completely absent or substituted by a more complex mechanism. This is not unlikely since mammalian development is considerably more complex as well. Once the mechanisms regulating quiescence have been found out, a large amount of targets for either therapy or research will become available. More research on quiescence could lead to a better understanding of cancer biology, stem cell biology and neurology and could perhaps lead to new treatments of the associated diseases.

References

Bayraktar, O. A., Boone, J. Q., Drummond, M. L. and Doe, C. Q. (2010). Drosophila Type II Neuroblast Lineages Keep Prospero Levels Low to Generate Large Clones that Contribute to the Adult Brain Central Complex. *Neural Dev.* **5,** 26.

Bello, B. C., Izergina, N., Caussinus, E. and Reichert, H. (2008). Amplification of Neural Stem Cell Proliferation by Intermediate Progenitor Cells in Drosophila Brain Development. *Neural Development* **3,**.

Blagosklonny, M. V. and Pardee, A. B. (2002). The Restriction Point of the Cell Cycle. *Cell. Cycle* **1,** 103-110.

Blomen, V. A. and Boonstra, J. (2007). Cell Fate Determination during G1 Phase Progression. *Cell Mol. Life Sci.* **64,** 3084-3104.

Bowman, S. K., Rolland, V., Betschinger, J., Kinsey, K. A., Emery, G. and Knoblich, J. A. (2008). The Tumor Suppressors Brat and Numb Regulate Transit-Amplifying Neuroblast Lineages in Drosophila. *Dev. Cell.* **14,** 535-546.

Britton, J. S. and Edgar, B. A. (1998). Environmental Control of the Cell Cycle in Drosophila: Nutrition Activates Mitotic and Endoreplicative Cells by Distinct Mechanisms. *Development* **125,** 2149-2158.

Chapouton, P., Skupien, P., Hesl, B., Coolen, M., Moore, J. C., Madelaine, R., Kremmer, E., Faus-Kessler, T., Blader, P., Lawson, N. D. et al. (2010). Notch Activity Levels Control the Balance between Quiescence and Recruitment of Adult Neural Stem Cells. *J. Neurosci.* **30,** 7961-7974.

Chell, J. M. and Brand, A. H. (2010). Nutrition-Responsive Glia Control Exit of Neural Stem Cells from Quiescence. *Cell* **143,** 1161-1173.

Chesnokova, V. and Pechnick, R. N. (2008). Antidepressants and Cdk Inhibitors: Releasing the Brake on Neurogenesis? *Cell. Cycle* **7,** 2321-2326.

Choksi, S. P., Southall, T. D., Bossing, T., Edoff, K., de Wit, E., Fischer, B. E., van Steensel, B., Micklem, G. and Brand, A. H. (2006). Prospero Acts as a Binary Switch between Self-Renewal and Differentiation in Drosophila Neural Stem Cells. *Dev. Cell.* **11,** 775-789.

Conover, J. C. and Notti, R. Q. (2008). The Neural Stem Cell Niche. *Cell Tissue Res.* **331,** 211-224.

Daudet, N., Gibson, R., Shang, J., Bernard, A., Lewis, J. and Stone, J. (2009). Notch Regulation of Progenitor Cell Behavior in Quiescent and Regenerating Auditory Epithelium of Mature Birds. *Dev. Biol.* **326,** 86-100.

Ebens, A. J., Garren, H., Cheyette, B. N. and Zipursky, S. L. (1993). The Drosophila Anachronism Locus: A Glycoprotein Secreted by Glia Inhibits Neuroblast Proliferation. *Cell* **74,** 15-27.

Egger, B., Chell, J. M. and Brand, A. H. (2008). Insights into Neural Stem Cell Biology from Flies. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363,** 39-56.

Egger, B., Gold, K. S. and Brand, A. H. (2010). Notch Regulates the Switch from Symmetric to Asymmetric Neural Stem Cell Division in the Drosophila Optic Lobe. *Development* **137,** 2981-2987.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). Drosophila Neuroblasts Sequentially Express Transcription Factors which Specify the Temporal Identity of their Neuronal Progeny. *Cell* **106,** 511-521.

Kitajima, A., Fuse, N., Isshiki, T. and Matsuzaki, F. (2010). Progenitor Properties of Symmetrically Dividing Drosophila Neuroblasts during Embryonic and Larval Development. *Dev. Biol.* **347,** 9-23.

Korenjak, M., Taylor-Harding, B., Binne, U. K., Satterlee, J. S., Stevaux, O., Aasland, R., White-Cooper, H., Dyson, N. and Brehm, A. (2004). Native E2F/RBF Complexes Contain Myb-Interacting Proteins and Repress Transcription of Developmentally Controlled E2F Target Genes. *Cell* **119,** 181-193.

Kunisch, M., Haenlin, M. and Campos-Ortega, J. A. (1994). Lateral Inhibition Mediated by the Drosophila Neurogenic Gene Delta is Enhanced by Proneural Proteins. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10139-10143.

Laporte, D., Lebaudy, A., Sahin, A., Pinson, B., Ceschin, J., Daignan-Fornier, B. and Sagot, I. (2011). Metabolic Status rather than Cell Cycle Signals Control Quiescence Entry and Exit. *J. Cell Biol.* **192,** 949-957.

Li, L. and Bhatia, R. (2011). Stem Cell Quiescence. *Clin. Cancer Res.*

Litovchick, L., Florens, L. A., Swanson, S. K., Washburn, M. P. and Decaprio, J. A. (2011). DYRK1A Protein Kinase Promotes Quiescence and Senescence through DREAM Complex Assembly. *Genes Dev.* **25,** 801-813.

Litovchick, L., Sadasivam, S., Florens, L., Zhu, X., Swanson, S. K., Velmurugan, S., Chen, R., Washburn, M. P., Liu, X. S. and DeCaprio, J. A. (2007). Evolutionarily Conserved Multisubunit RBL2/p130 and E2F4 Protein Complex Represses Human Cell Cycle-Dependent Genes in Quiescence. *Mol. Cell* **26,** 539-551.

Liu, Y., Elf, S. E., Miyata, Y., Sashida, G., Liu, Y., Huang, G., Di Giandomenico, S., Lee, J. M., Deblasio, A., Menendez, S. et al. (2009). P53 Regulates Hematopoietic Stem Cell Quiescence. *Cell. Stem Cell.* **4,** 37-48.

Mamane, Y., Petroulakis, E., LeBacquer, O. and Sonenberg, N. (2006). MTOR, Translation Initiation and Cancer. *Oncogene* **25,** 6416-6422.

Maurange, C., Cheng, L. and Gould, A. P. (2008). Temporal Transcription Factors and their Targets Schedule the End of Neural Proliferation in Drosophila. *Cell* **133,** 891-902.

Park, Y., Rangel, C., Reynolds, M. M., Caldwell, M. C., Johns, M., Nayak, M., Welsh, C. J., McDermott, S. and Datta, S. (2003). Drosophila Perlecan Modulates FGF and Hedgehog Signals to Activate Neural Stem Cell Division. *Dev. Biol.* **253,** 247-257.

Raina, A. K., Monteiro, M. J., McShea, A. and Smith, M. A. (1999). The Role of Cell Cycle-Mediated Events in Alzheimer's Disease. *Int. J. Exp. Pathol.* **80,** 71-76.

Sang, L., Coller, H. A. and Roberts, J. M. (2008). Control of the Reversibility of Cellular Quiescence by the Transcriptional Repressor HES1. *Science* **321,** 1095-1100.

San-Juan, B. P. and Baonza, A. (2011). The bHLH Factor Deadpan is a Direct Target of Notch Signaling and Regulates Neuroblast Self-Renewal in Drosophila. *Dev. Biol.* **352,** 70-82.

Sears, R. C. and Nevins, J. R. (2002). Signaling Networks that Link Cell Proliferation and Cell Fate. *J. Biol. Chem.* **277,** 11617-11620.

Siegrist, S. E., Haque, N. S., Chen, C. H., Hay, B. A. and Hariharan, I. K. (2010). Inactivation of both Foxo and Reaper Promotes Long-Term Adult Neurogenesis in Drosophila. *Curr. Biol.* **20,** 643-648.

Sousa-Nunes, R., Yee, L. L. and Gould, A. P. (2011). Fat Cells Reactivate Quiescent Neuroblasts Via TOR and Glial Insulin Relays in Drosophila. *Nature* **471,** 508-512.

Tothova, Z. and Gilliland, D. G. (2007). FoxO Transcription Factors and Stem Cell Homeostasis: Insights from the Hematopoietic System. *Cell. Stem Cell.* **1,** 140-152.

Tsuji, T., Hasegawa, E. and Isshiki, T. (2008). Neuroblast Entry into Quiescence is Regulated Intrinsically by the Combined Action of Spatial Hox Proteins and Temporal Identity Factors. *Development* **135,** 3859-3869.

Voigt, A., Pflanz, R., Schafer, U. and Jackle, H. (2002). Perlecan Participates in Proliferation Activation of Quiescent Drosophila Neuroblasts. *Dev. Dyn.* **224,** 403-412.

Wang, C. Q., Jacob, B., Nah, G. S. and Osato, M. (2010). Runx Family Genes, Niche, and Stem Cell Quiescence. *Blood Cells Mol. Dis.* **44,** 275-286.

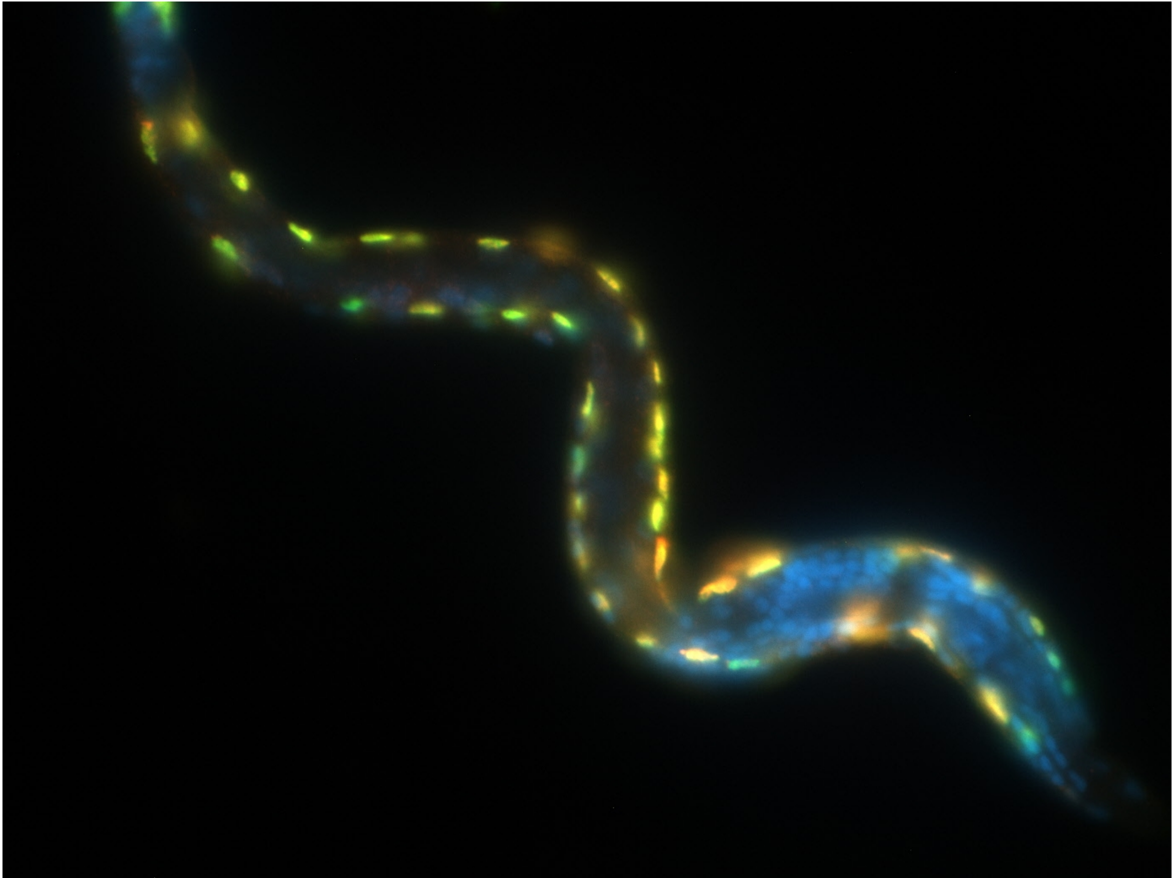
Wen, H., Andrejka, L., Ashton, J., Karess, R. and Lipsick, J. S. (2008). Epigenetic Regulation of Gene Expression by Drosophila Myb and E2F2-RBF Via the Myb-MuvB/dREAM Complex. *Genes Dev.* **22,** 601-614.

Weng, M., Golden, K. L. and Lee, C. Y. (2010). DFezf/Earmuff Maintains the Restricted Developmental Potential of Intermediate Neural Progenitors in Drosophila. *Dev. Cell.* **18,** 126-135.

Yanagida, M. (2009). Cellular Quiescence: Are Controlling Genes Conserved? *Trends Cell Biol.* **19,** 705-715.

Thesis practical part.

Expressing cyclin E in *C. elegans*



01-07-2011

Expressing cyclin E in *C. elegans*

By Sander Lambo

Supported by Suzanne Ruijtenberg

Bachelor Biomedische wetenschappen

01-07-2011

The picture on the cover shows the expression of cyclin E (red) in muscle cells (green) in a *C.elegans* strain overexpressing cyclin E in muscle cells. Visualised under a fluorescence microscope.

Abstract

Cyclin E is an important factor in cell cycle regulation. Although this protein has been extensively studied, some regulations are still unclear in development. In this part of the thesis the regulatory effect of cyclin D and the expression of cyclin E during development will be examined. For the purpose of examining the regulatory effects of cyclin D on the expression of cyclin E immunostaining was used. The immunostaining showed no increase in cyclin E expression while cyclin D was overexpressed suggesting that there is no stimulating effect of cyclin D on cyclin E expression. To visualise cyclin E expression during development a GFP expressing cyclin E line was made. This was shown to be expressed in at least seam cells, the ventral cord and the intestine of the worms.

Expressing cyclin E and cyclin D

Introduction

Cyclin E is a well known regulator of the cell cycle. In the first part of this thesis cyclin E was described as a factor which is important for the progression of the cell cycle between the G_1 and S phase. Cyclin E is thought to be indirectly stimulated by cyclin D which inhibits Rb, a known inhibitor of cyclin E. However, recent microarray data showed that cyclin E and cyclin D expression are not present in the same cell. This was found in muscle cells of the *C. elegans*. This finding contradicts the current model and therefore the expression pattern of cyclin E will be examined in vivo. In this experiment muscle cells of the *C. elegans* will be examined for simultaneous expression of cyclin D and cyclin E.

Materials and methods

The expression of cyclin E and cyclin D in body wall muscle cells of the *C. elegans* was determined by using immunostaining. In order to obtain these results three strains were used. The first strain was expressing *myo-3::H2B::GFP* and *myo-3::cyd -1/Cdk4::venus* which means that muscle cells are overexpressing Cyclin D/Cdk4 and H2B bound to GFP. The second line expressed *myo-3::H2B::GFP* and *myo-3::cye -1/Cdk -2AF* Which means cyclin E and H2B bound to GFP is overexpressed in muscle cells. The third line only expressed *myo-3::H2B::GFP* and is used as a negative control. Worms were synchronized by bleaching which is killing all the worms on a plate except for the eggs. Worms where then starved for approximately 16 hours. L1 stage larvae were put on food for 8 hours at 20°C or for 16 hours at 15°C after which they were mounted on slides and fixed with methanol aceton. Next the slides were blocked by using blocking buffer (0,1% BSA, 10% goat serum diluted in PBS +0,1% Tween). After blocking slides were washed with PBST 0,1% tween. Next, the primary antibodies monoclonal mouse anti cye-1 (Invitrogen 17C8 both 1:1000 and 1:500 diluted in blocking buffer) and polyclonal rabbit anti GFP (ABCAM AB6556 1:200 or 1:300 diluted in blocking buffer) were added. After incubating the secondary antibodies were added. These were red fluorescent (568 nm) anti mouse (Invitrogen alexa 568 1:500 diluted in blocking buffer) and green fluorescent (488 nm)anti rabbit (Invitrogen alexa 488 1:500 diluted in blocking buffer). After incubating the second antibodies the Anti-fade Gold and DAPI (1:1000) were added to the slides and the slides were fixed and stored at -20°C. The slides were examined under a non-confocal and confocal fluorescence microscope.

Results

Three different *C. elegans* strains were stained for cyclin E expression in muscle cells. Worms overexpressing cyclin D in muscle cells did not show cyclin E expression in muscle cells. This is shown in Figure 1A. The positive control overexpressing cyclin E did show expression of cyclin E in muscle cells. This is shown in Figure 1B. The negative control which had no overexpression showed no expression of cyclin E in muscle cells either. This is shown in Figure 1C.

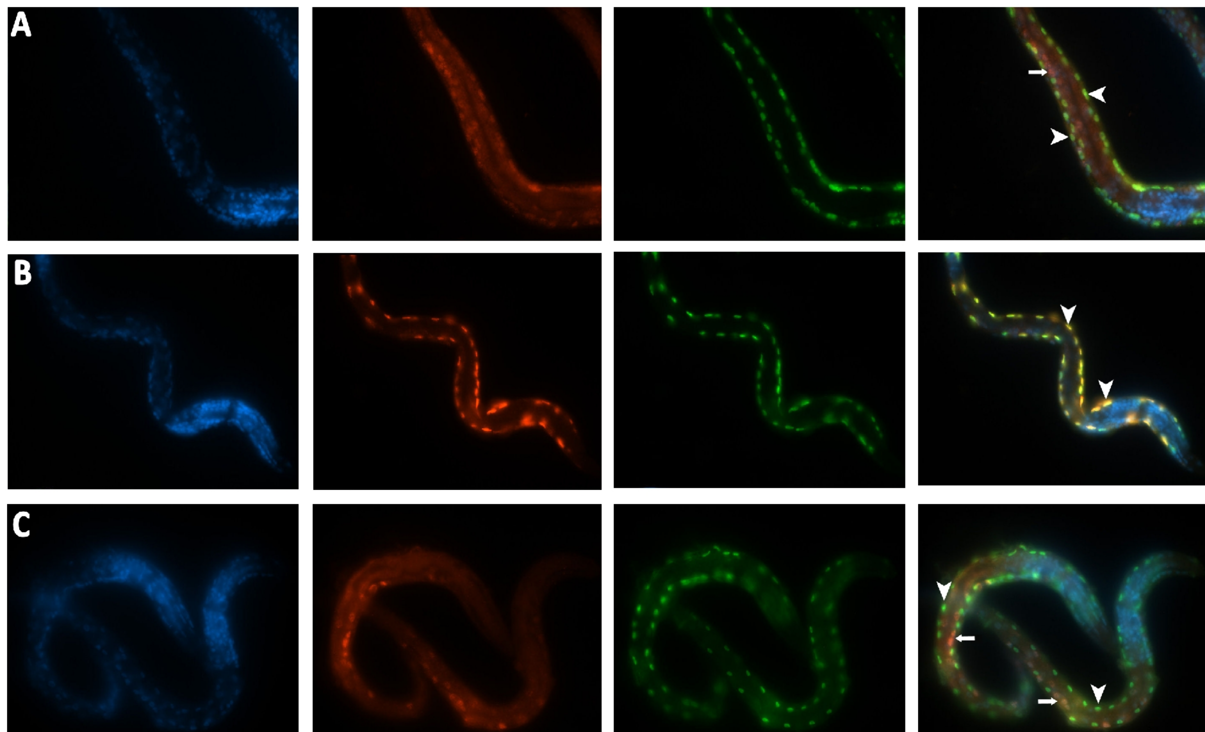


Figure 1. Expression of Cyclin E and cyclin D
A. staining of *myo-3::H2B::GFP*, *myo-3::cyd -1/Cdk4::venus* worms. Arrowheads indicate body wall muscle cells (green) not expressing cyclin E (red). The arrow indicates non-muscle cells expressing cyclin E. B. Staining of *myo-3::H2B::GFP*, *myo-3::cye -1/Cdk -2AF* worms used as a positive control. Arrowheads indicate body wall muscle cells expressing cyclin E. C. Staining of *myo-3::H2B::GFP* worms as a negative control. Arrowheads indicate muscle cells not expressing cyclin E. Arrows indicate non-muscle cells expressing cyclin E. Red staining indicates Cyclin E expression, blue staining indicates the nucleus stained by DAPI and green staining indicates muscle cells expressing GFP.

In Figure 2 the same results are shown, obtained by a confocal microscope. However, in Figure 2A a muscle cell with a larger than expression than the background is seen. Figure 2B and Figure 2C show results similar to Figure 1.

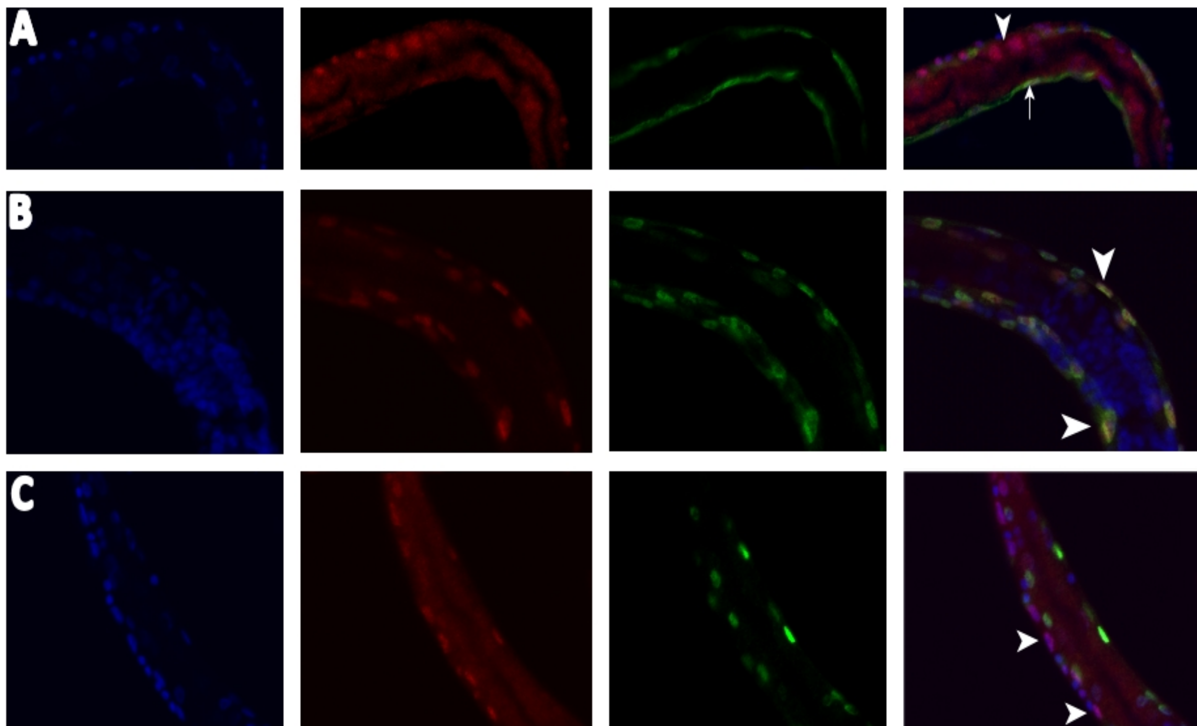


Figure 2. Confocal images of cyclin E and cyclin D expression
A. staining of *myo-3::H2B::GFP*, *myo-3::cyd -1/Cdk4::venus* worms. Arrowhead indicates non-muscle cells expressing cyclin E. The arrow indicates a muscle cell that seems to express cyclin E. B. Staining of *myo-3::H2B::GFP*, *myo-3::cye -1/Cdk -2AF* worms used as a positive control. Arrowheads indicate body wall muscle cells expressing cyclin E. C. Staining of *myo-3::H2B::GFP* worms as a negative control. Arrowheads indicate non-muscle cells expressing cyclin E. Red staining indicates Cyclin E expression, blue staining indicates the nucleus stained by DAPI and green staining indicates muscle cells expressing GFP. Images made with a confocal microscope.

Discussion

Based on the pictures obtained from the experiment, it seems that cells overexpressing cyclin D in the body wall muscle cells do not express cyclin E. This is similar to the expression pattern seen in the negative control. However, the muscle cell that is seemingly expressing cyclin E in figure 2A could prove otherwise. This result could have been caused by poor staining since the staining examined under the confocal microscope was weak in some worms. Perhaps too many worms were mounted on the slides, which resulted in poorly visible staining. To rule this possibility out the experiment should be repeated.
Worms overexpressing cyclin E do show cyclin E expression in muscle cells. This result suggests that cyclin D expression does not indirectly stimulates cyclin E. If cyclin D was stimulating cyclin E expression it would have been visible in the muscle cells of the cyclin D overexpressing strain. However, this result was only shown in L1 stage larvae and should be examined in other stages as well. In addition, the influence of other regulators of the cell cycle could be examined such as E2F, RB and CIP/KIP proteins. The finding described here could prove to be important in understanding the regulation of the cell cycle not only in *C. elegans* but perhaps in mammals as well.

Creating a cyclin E reporter line

Introduction

An important factor in regulation of the cell cycle is cyclin E. It is also a good marker for detecting cells that are actively dividing since cyclin E expression promotes the expression of *mcm-4* a known marker for the S phase of the cell cycle. By creating a GFP cyclin E fusion protein it is possible to visualise the expression of cyclin E and thus the activity of dividing cells in real time. Here, we tried to create a worm line which incorporates GFP into the cyclin E gene. For this purpose two fusion constructs where used previously described by Brodigan et al.: PKM 1110 and PKM 1191. Both promoters were shown to be expressed in gonads, vulval precursor cells, P cells, seam cells, embryo's, neurons and intestinal cells (Brodigan et al., 2003). By creating two lines expressing these promoters, the same expression pattern which Brodigan et al. found will hopefully be visualised.

Materials and methods

In order to create a cyclin E reporter line a wildtype N2 strain was injected with a fusion construct. DH5a cells were transformed with the constructs PKM1110 and PKM 1191 and grown for about a day. For more information about the constructs consult the introduction section. These constructs were then injected in young adult stage worms. Together with the constructs λDNA was injected to stabilise the injected DNA, lin-48 mCherry was injected as co-injection marker and H2O as solvent. Concentrations for the injection are in the table below:

	Concentration
Lin-48 mCherry	15 ng /µl
PKM 1110/PKM1191	30 ng /µl

Worms containing the injected construct were selected based on the expression of the co-injection marker and picked under a fluorescence microscope. Worms were selected for red fluorescence in neurons caused by mCherry. Worms were grown to generate F2 progeny. F2 progeny was picked and examined under the fluorescence microscope.

Results

Worms from two N2 strains were injected with either of two different constructs and examined under the fluorescence microscope. The construct containing the promotor PKM 1191 yielded a poorly visible result and is therefore not suitable to create a cyclin E reporter line with. On the other hand, the PKM 1110 promotor containing construct did show expression in expected structures such as the seam cells, the intestine and ventral cord cells. This is shown in Figure 4, 5 and 6.

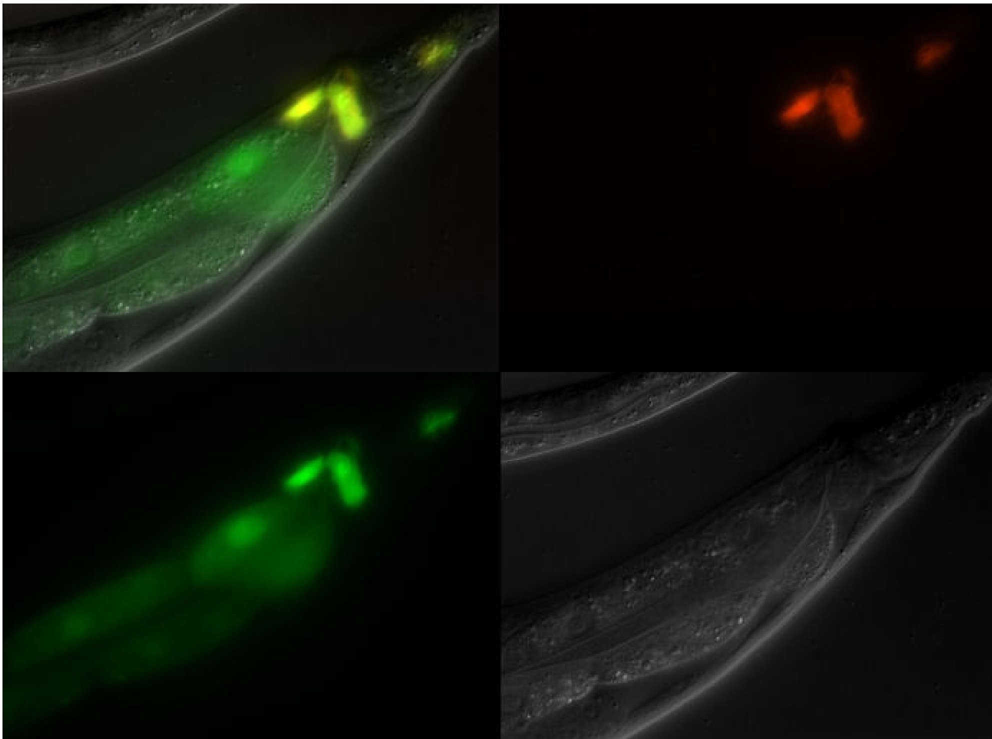


Figure 3. Intestine showing expression of cyclin E
Intestine of worms injected with the PKM1110 promotor containing cells. Green indicates Cyclin E GFP expression, red indicates the co-expression marker lin-48.

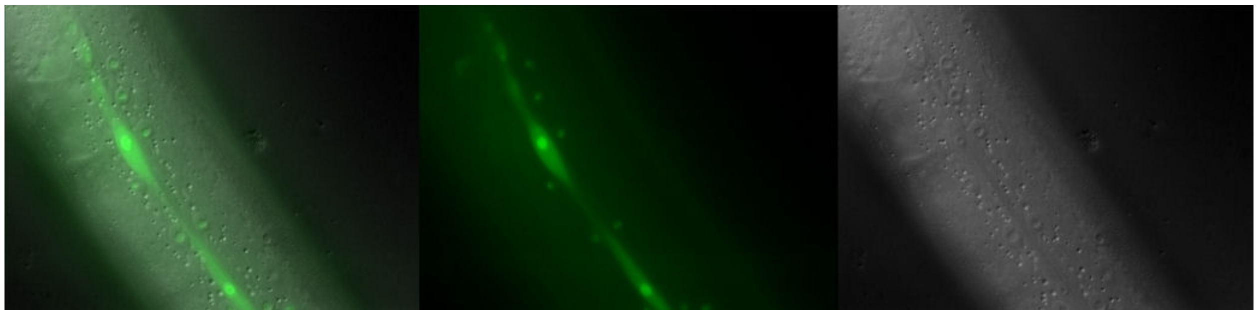


Figure 4. Seam cells showing expression of cyclin E
Seam cells of worms injected with the PKM1110 promotor containing cells. Green indicates Cyclin E GFP expression, red indicates the co-expression marker lin-48.

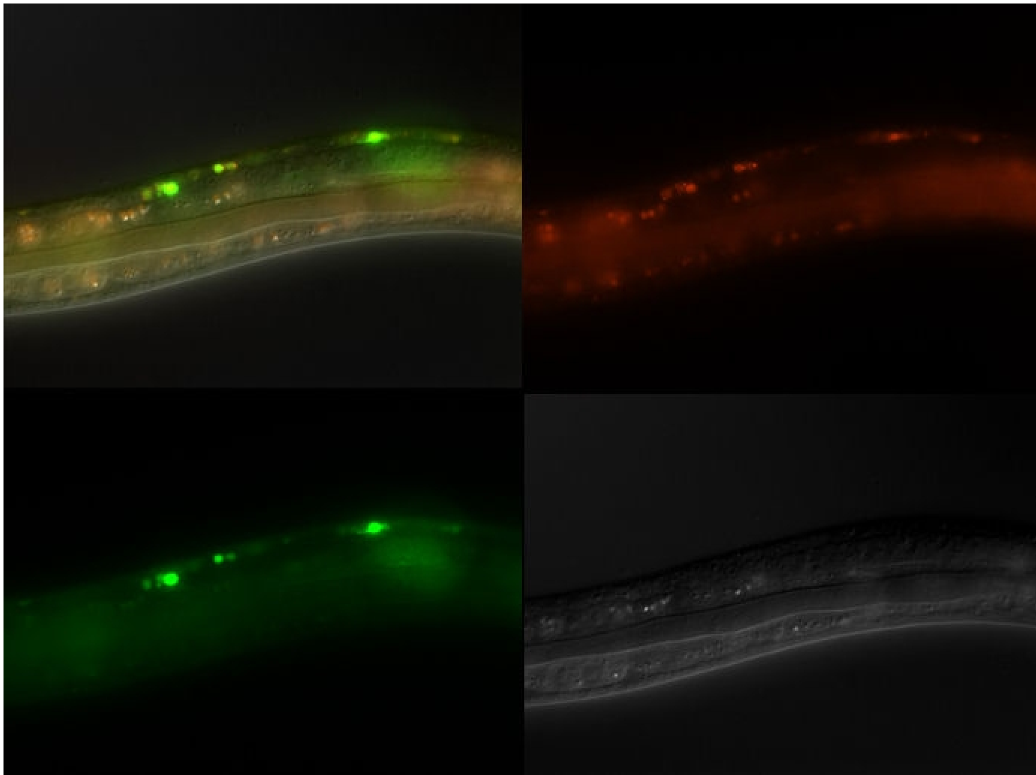


Figure 5. Ventral cord showing expression of cyclin E
Ventral cord (dorsal in the picture) of worms injected with the PKM1110 promotor containing cells. Green indicates Cyclin E GFP expression, red indicates the co-expression marker lin-48.

Discussion

The results indicate that the PKM 1110 line can be used to research the function of cyclin E *in vivo*. A likely experiment to perform now is knocking out stimulating factors of cyclin E and visualise the effect on the cells in the worm. A useful technique to perform this is RNAi either by injecting or feeding. A conditional knock-out can be made as well to determine the effects on separate structures. It is still unclear why the PKM 1191 line was not usable to visualize cyclin E expression since this expression was measured by Brodigan et al. in structures which were not visible in the worm line (Brodigan *et al*., 2003). Perhaps the injection was not successful which explains the faint GFP expression under the microscope. Even embryo's did not express GFP which should express cyclin E. Repeating this experiment would most likely yield better results, but perhaps the promoter is not as suitable as PKM 1110.

References

Brodigan, T. M., Liu, J., Park, M., Kipreos, E. T. and Krause, M. (2003) Cyclin E expression during development in Caenorhabditis elegans. *Dev. Biol.* **254,** 102-115.

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