INSTITUTO DE NEUROCIENCIAS

Consejo Superior de Investigaciones Científicas
Universidad Miguel Hernández
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CERTIFICA:

Que la Tesis Doctoral "*Analysis of the Drosophila asymmetric cell division regulator Canoe/Afadin in tumorigenesis*" ha sido realizada por D.ª Noemí Rives Quinto (DNI 15415854N) bajo la dirección de la Dra. Ana Carmena de la Cruz y da su conformidad para que sea presentada a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste a los efectos oportunos, firma el presente certificado en San Juan de Alicante a 14 de diciembre de 2015

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Dª Ana Carmena de la Cruz, científico titular del Consejo Superior de Investigaciones científicas,

AUTORIZA la presentación de la Tesis Doctoral titulada: "Analysis of the *Drosophila* asymmetric cell division regulator Canoe/Afadin in tumorigenesis", realizada por Dª Noemí Rives Quinto, bajo su inmediata dirección y supervisión en el Instituto de Neurociencias de Alicante, centro mixto CSIC-UMH, y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que así conste, y a los efectos oportunos , firma el presente Certificado en San Juan de Alicante, a veintiuno de enero de dos mil dieciséis.

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Analysis of the *Drosophila* asymmetric cell division regulator Canoe/Afadin in tumorigenesis

Memoria para optar al grado de Doctor presentada por

NOEMÍ RIVES QUINTO

Dirigida por la Dra. ANA CARMENA DE LA CRUZ

San Juan de Alicante, 2015

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Cuando prendemos la llama
siempre creemos que seremos capaces de controlar el fuego,
sin embargo, suele ser el propio fuego el que, si miramos con ojos humildes,
nos muestra el desarrollo natural de los acontecimientos.

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INDEX

ABBREVIATIONS

ACD – Asymmetric cell division

AEL – After Egg Laying

ALH – After Larval Hatching

Ase – Asense

Aur A – Aurora A

Brat – Brain tumor

Cas – Castor

Cno – Canoe

CNS – Central Nervous System

CSC – Cancer Stem Cell

D – Dichaete

Dlg – Discs Large

Dpn – Deadpan

Ds – Dachsous

Dsh – Dishevelled

Ex – Expanded

Ey – Eyeless

FLP – Flippase

FRT – FLP Recognition Target

Ft – Fat

Grh – Grainyhead

GMC - Ganglion Mother Cell

Hb – Hunchback

Hpo – Hippo

INP – Intermediate Progenitor

Insc – Inscuteable

IPC – Inner Proliferation Center

IPC – Intermediate Progenitor Cell (in mammalian context)

JNK- Jun N-terminal Kinase

Klu – Klumpfuss

Kr – Krüppel

LIII – Larvae III

L'sc – Lethal of Scute

L(2)gl – Lethal (2) Giant Larvae

MAPK- Mitogen-Activated Protein Kinase

Mats – Mob-as-Tumor-Suppressor

Mira – Miranda

NB – Neuroblast

NBI – Neuroblast type I

NBII – Neuroblast type II

NSC – Neural Stem Cell

OPC – Outer Proliferation Center

PH3 – Phosphohistone 3

PMC – Pericentriolar Material

PntP1 – PointedP1

Pon – Partner of Numb

PP2A – Protein Phosphatase 2A

PP4 – Protein Phosphatase 4

Pros – Prospero

RG cells – Radial Glial cells

Sav – Salvador

SC – Stem Cell

Scrib- Scribble

Sd – Scalloped

Su(H)- Suppressor of Hairless

SVZ – Subventricular Zone

TS genes – Tumor Suppressor genes

VNC – Ventral Nerve Cord

VZ – Ventricular Zone

Wg- Wingless

Wts – Warts

Yki – Yorkie

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ABSTRACT

Stem cells divide asymmetrically to give rise to one daughter cell that inherits the self-renewal potential and another daughter cell that is committed to differentiate. Hence, an impairment of the asymmetric cell division (ACD) process could render daughter cells with altered cell fates, unable to respond to cell growth control mechanisms. Indeed, over the past years an intriguing connection between ACD, stem cells and cancer biology has emerged. We previously showed that the PDZ protein Canoe (Cno) regulates ACD in embryonic neuroblasts (NBs), the neural stem cells of *Drosophila* central nervous system (CNS). In this work, we wanted to investigate whether the loss of *cno* in asymmetrically dividing NBs could lead to tumor-like overproliferation. We show that Cno is expressed in NBs and Intermediate Progenitors (IPs) of *Drosophila* larval brain type II NB (NBII) lineages. In *cno* mutant clones the ACD regulators aPKC, Pins and Dlg fail to form crescents and they accumulate all around the cortex or are mislocalized. Other defects such as size-reduced NB and abnormal morphology of the *cno* mutant clone are observed. Despite these failures, *cno* mutant clones do not show overgrowth. In fact, fewer IPs and Ganglion Mother Cells (GMCs) are detected. In addition, we have found that type II NB mutant clones of *scribble* (*scrib*), a well-known tumor suppressor gene, do not overgrow either. Moreover, most *scrib* mutant clones die. Intriguingly, the simultaneous loss of function of *cno and scrib* synergistically interacts displaying an overproliferation of progenitor cells that show disrupted cell polarity and give rise to a tumor-like overgrowth. Removing Ras signaling in the *cno, scrib* double mutant background rescues the overgrowth phenotype. In addition, the ectopic activation of Ras in *cno, scrib* double mutant clones does not seem to signal through the Raf-MAPK cascade in *Drosophila* brain NBs. Our data reveal a novel synergistic interaction between *cno* and *scrib* loss of function in NBII lineages that lead to tumor formation by, at least in part, an up-regulation of Ras signaling.

RESUMEN

Las células madre pueden dividirse de forma asimétrica para producir una célula descendiente que hereda su potencial para continuar dividiéndose y otra célula hija que iniciará un proceso de diferenciación. Por lo tanto, errores en el proceso de división asimétrica pueden conducir a que la producción de células hijas con identidades alteradas que no sean capaces de responder a los mecanismos de control de crecimiento. De hecho, a lo largo de los últimos años ha cobrado fuerza la relación existente entre la biología de la células madre y el cáncer. En trabajos anteriores en nuestro laboratorio mostramos que la proteína PDZ Canoe (Cno) es un regulador de división asimétrica en los neuroblastos embrionarios, las células madre nerviosas del sistema nervioso central del embrión de *Drosophila*. En este trabajo, quisimos investigar si la falta de función del gen *cno* en los neuroblastos larvarios que se dividen de manera asimétrica, puede conducir a la formación de tumores. En primer lugar, mostramos que Cno se expresa en los NBs y los Progenitores Intermedios (IP) de los NB de tipo II del cerebro de *Drosophila*. En los clones mutantes de *cno*, otros reguladores de división asimétrica tales como aPKC, Pins o Dlg fallan en formar acumulaciones apicales, encontrándose repartidos a lo largo de todo el córtex celular o simplemente desplazados de su posición correcta. Otros defectos como un NB de menor tamaño o una morfología anormal, también se hacen evidentes en los mutantes de *cno*. A pesar de estos fallos, los clones mutantes de *cno* no muestran sobrecrecimiento tumoral. De hecho, en estos clones se detecta un menor número de INPs y Ganglion Mother Cells (GMCs). Por otro lado, encontramos que clones mutantes para otro regulador de división asimétrica que ha sido descrito como un gen supresor de tumores, *scribbled* (*scrib*), tampoco son capaces de sobrecrecer. De hecho, la mayoría de los clones mutantes de *scrib* son eliminados mediante apoptosis. Sin embargo, la falta de función conjunta de *cno* y *scrib* interacciona de forma sinergísitca produciendo la sobreproliferación de células progenitoras que muestran una división asimétrica gravemente alterada produciendo finalmente formaciones tumorales. Pudimos observar que en este contexto de clones doble mutante para ambos genes, la eliminación de la señal del gen Ras es capaz de rescatar la sobreproliferación producida por ambas mutaciones, a pesar de que los clones

resultantes constinúan sin ser WT. Además la activación ectópica de Ras en los clones mutantes *cno scrib* parece no estar mediada por el efector dipMAPK. Todos estos datos revelan una nueva interacción sinergística entre la falta de función de los genes *cno* y *scrib* en los NBs larvarios de *Drosophila* que conduce a la formación de tumores, estando mediada esta interacción, al menos en parte, por la sobreactivación de Ras.

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INTRODUCTION

1. *DROSOPHILA* SP. AS A MODEL SYSTEM

Drosophila melanogaster, also known as the fruit fly, is an invertebrate that has been recognized as a one of the most powerful models to study development. The reasons behind this are several, from simply being the most studied genome (with only 4 chromosomes) to being the model organism with the vastest variety of genetic tools and rapid life cycle.

The life cycle of *Drosophila* lasts 10 days (Figure 1) at 25ºC. The process goes through a series of stages that start when the fertilized egg develops into an embryo, which after 24 hours hatches as a first instar larva. These larvae start feeding and growing along for about 4 more days until arriving to the most mature larval stage, the third instar larva (LIII). At the end of larval life, a pupal case is formed within which the metamorphosis takes place and from where a new adult fly will emerge in 5 more days.

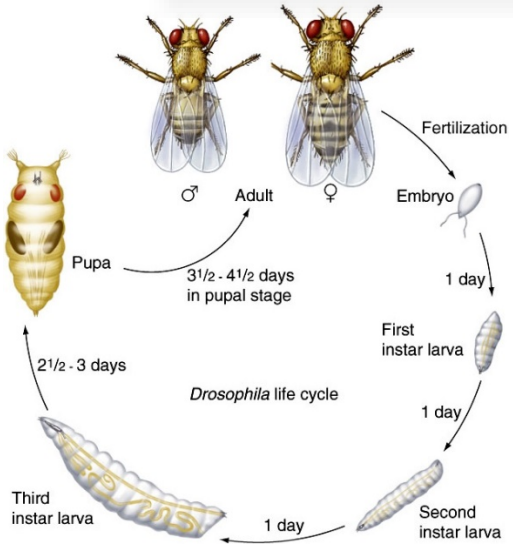

Figure 1. *Drosophila* life cycle: the stages from fertilization until new adults. After fertilization, it takes place the embryogenesis plus three larval stages and the pupal stage to get new adults after around 10 days *(Taken from Mc-Graw Hills companies)*.

Thus, easy manipulation and short periods of time to get different developmental stages permit to obtain results rapidly. Taking all of this into account, it is not surprising

that many important screens have been done in *Drosophila* and they have contributed to unveil major signaling and cellular processes in development that are conserved from flies to mammals. Actually, although the *Drosophila* central nervous system (CNS) has been used for a long time as a model due to its simplicity and the conservation of the molecules, recent discoveries along the last decades have renewed the interest on it as a hot model. For example, the discovery in *Drosophila* of the type II neuroblasts (NBsII), a new type of neural stem cells (SCs) in the larval brain that have many similarities with the mammalian ones, has opened a new field of extreme value to study the mechanisms controlling stem cell division in development and also in cancer.

2. THE MECHANISM OF ASYMMETRIC CELL DIVISION

One of the hugest steps in evolution was the multicellular organism appearance. The distinctive feature of these multicellular organisms is to have different kind of cells that are able to perform different functions. Therefore, cellular diversity generation is a fundamental process in evolution and developmental biology. A key mechanism to achieve such a process is asymmetric cell division (ACD). In developmental biology and stem cell biology, an ACD is defined as any division that gives rise to two sister cells that have different fates, a feature that can be recognized by differences in size, morphology, gene expression pattern, or the number of subsequent cell divisions undergone by the two daughter cells (Horvitz and Herskowitz, 1992). Of particular importance is the asymmetric nature of SC divisions. SCs must divide asymmetrically to simultaneously generate identical copies of themselves (self-renew) and progeny that are committed to differentiate. Most of our mechanistic insights into this process come from invertebrate model systems, especially *Drosophila melanogaster* and *Caenorhabditis elegans*.

Both *C. elegans* and *Drosophila* development rely heavily on ACD. In *C. elegans*, early development is essentially a series of ACDs, and especially the first division of the zygote has been intensely studied. In *Drosophila*, ACDs have been described in developing muscle, gut, malphighian tubules and nervous system. The asymmetric division of the neural SCs called neuroblasts (NBs)(Yasugi et al., 2008) in the CNS and of sensory organ

precursors in the peripheral nervous system have been particularly well studied (Betschinger and Knoblich, 2004).

Essentially, to divide asymmetrically cells have to establish an axis of polarity and orientate their mitotic spindle along this axis. Furthermore, the so-called cell fate determinants have to localize asymmetrically to one side of the cell. Thus, when the division occurs the determinants are going to be segregated into only one daughter cell generating, after cytokinesis, descendants with diverse identity. The ACD process can occur in two ways: by an intrinsic or an extrinsic mechanism (Figure 2). In the first case, cells usually follow a predefined developmental program where already in interphase, cells use the apical-basal or planar polarity of the surrounding tissue to set up the axis of polarity. On the other hand, in extrinsic or niche-controlled mechanism, cells orientate the mitotic spindle perpendicularity to the niche surface ensuring that only one cell can keep contact with the stem cell niche and retain the ability of self-renew. In contrast to intrinsic mechanism, the niche-controlled one offers more flexibility allowing sometimes the generation of two SCs for increasing the number of SC population or to compensate occasional loss. This has been proposed to be the reason why niche mechanisms are more common in adult SCs, whereas intrinsic ones predominate during development (Knoblich, 2008).

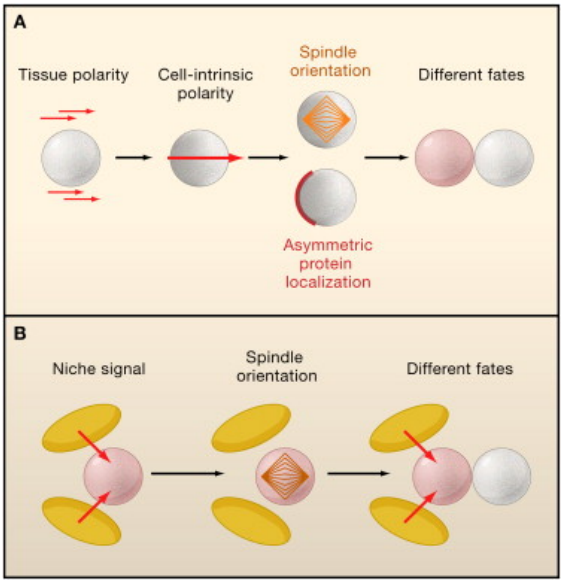

Figure 2. Intrinsic and extrinsic mechanisms of stem cell asymmetric division and self-renewal. A) Intrinsic mechanism: SCs set up its axis of polarity during interphase that will use during the mitosis to segregate asymmetrically different determinants into different cells. **B) Extrinsic mechanism:** SCs depend on the niche. They orientate the mitotic spindle perpendicularly to the niche surface ensuring that only one of two daughter cells maintains the ability to self-renew. *(Extracted and modified from Knoblich, 2008).*

Nowadays, the significance of ACD has been recognized to study not only the development of multicellular organisms, including humans, but also in context of cancer and biology of stem cells.

2.1 Cell biology of *Drosophila* NBs

The diversity of neural cell types that characterize the complex circuits of the nervous system is produced by the division of the CNS neural stem cells or NBs (Kang and Reichert, 2015). *Drosophila* CNS is formed by the central brain and the optic lobes in the head, and the ventral nerve cord (VNC) along the body. With further detail, we can distinguish thoracic and abdominal NBs in VNC, and NBs type I (NBsI), NBsII, mushroom body NBs and optic lobe NBs in the brain lobes (Figure 3 A).

The NBs of VNC and central brain have an embryonic origin; these NBs arise by delamination from the neuroectoderm during embryonic stages 9 to 11 (Figure 3 B). In the embryonic neuroectoderm, groups of cells, called proneural clusters, become manifest by the expression of proneural genes. NBs are singled out from these clusters by a Notch-dependent lateral inhibition established within the proneural clusters, process in which proneural gene activity is restricted to the future NB (Artavanis-Tsakonas and Simpson, 1991; Hartenstein and Wodarz, 2013). Following their specification, delaminated NBs enlarge and begin to proliferate producing a small subset of neurons, called primary neurons that will start to form the larval brain (but only the 10% of the final adult CNS). Later on, most of abdominal NBs are eliminated through a programmed cell death event (White et al., 1994), but NBs in the central brain and in the thoracic regions enter in quiescence, arresting their cell cycle, exiting from G1 into G0 (Figure 3 C). Exceptionally, the four mushroom body NBs do not undergo quiescence and continue dividing. At late first instar larval stage, about 8-10 hours after larva hatching, these NBs exit quiescence and re-enter mitosis again. Re-entrance of the NBs into the cell cycle is triggered by extrinsic signals, including nutritional or hormonal signals such as ecdysone (Colombani et al., 2012) where fat body and glial cell niche are involved. Finally, termed secondary or adult neurons, which are the majority of the neurons that make up the adult brain and VNC, are generated during an intense proliferative period that lasts from the end of the

first instar/early second instar larval stage until the end of the larval period/early pupal stage (Truman and Bate, 1988).

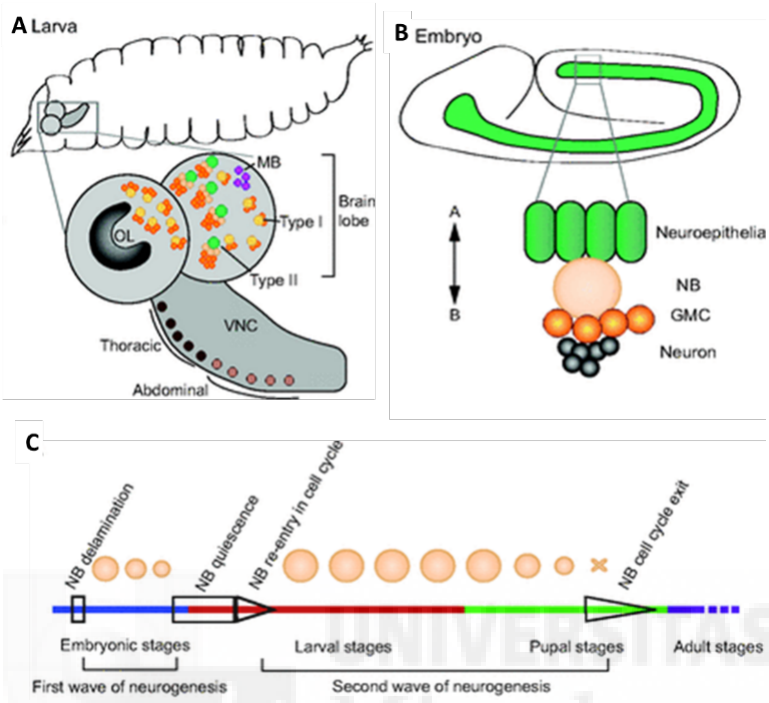

Figure 3. Neuroblast neurogenesis and development in *Drosophila*. A) CNS can be divided into the central brain and the optic lobes in the head and the ventral nerve cord (VNC), where we can distinguish abdominal and thoracic NBs in VNC and NBsI, NBsII, mushroom body and optic lobe NBs in the brain lobes. **B)** In the VNC and central brain, NBs first arise by delamination from the neuroectoderm during embryonic stages 9 to 11. **C)** Delaminated NBs enter in quiescence at the end of embryonic stage to re-enter in proliferative mode at the end of first instar larval stage. They continue dividing until pupal stages generating in that period the 90% of adult brain neurons *(Extracted and modified from Knoblich, 2012).*

NBs of the optic lobe do not follow the same developmental program. They are generated from the neuroepithelial cells of the optic anlagen in larval stages. During early larval development, the embryonic optic placode expands dramatically in size through symmetric divisions and becomes segregated into two separate epithelia called inner proliferation center (IPC) and outer proliferation center (OPC). At the medial edge of the OPC, the neuroepithelial cells are sequentially converted into NBs (Egger et al., 2007), and this dynamic transition is triggered by a synchronized medial to lateral wave of the proneural gene *lethal of scute* (*l'sc*) (Yasugi et al., 2008). Neurogenesis in the adult central brain and VNC has not been reported; however, a recent work indicates that adult

neurogenesis can occur in the optic lobe after acute damage (Fernandez-Hernandez et al., 2013).

In this doctoral work we are going to focus our attention on the NBs of the central brain, in concrete, NBsI and NBsII. NBsI constitute the majority of central brain NBs and are located in anterior and posterior sides of the brain and the VNC as well (Figure 3 A). In each brain lobe we can find around 100 NBsI, which divide asymmetrically to self-renew and to generate a daughter cell called ganglion mother cell (GMC) that is committed to differentiate and will divide just once more to produce postmitotic cells: neurons or glial cells (Figure 4 A). In contrast, only 8 NBsII are present in each brain lobe. These NBsII can be distinguished from NBsI by the absence of the proneural transcription factor Asense (Ase) (Figure 4 B) (Bello et al., 2008). Misexpression of *ase* in NBII has been shown to abolish type II characteristics and so appears sufficient to induce a type I identity; however, downregulation of *ase* in NBI is not sufficient for their conversion into NBsII (Morrison and Kimble, 2006). A remarkable difference of NBsII is that they generate their lineages through transit amplifying self-renewing secondary progenitors, which have been termed intermediate progenitors (INPs). Each INP divides around 5-7 times to self-renew and to give rise to a GMC that will divide once more. Since each NBII generates numerous INPs and each INPs generates several GMCs, a marked amplification of proliferation ensues, and lineages that are 4-5 folds bigger than NBsI are created (i.e. each NBII produces around 500 neurons) (Bello et al., 2008). The INPs follow a concrete program to be mature and able to divide. When they are generated they are immature and only express, very transiently, the transcription factor Pointed (PntP1) and later only Ase, whose expression will persist in the mature INP (Figure 5). During the following 4-5 hours after their generation they are arrested in G2, but later they mature and acquire the developmental potential to divide. When this happens, the mature INPs express Ase and the transcription factors Deadpan (Dpn) and Klumpfuss (Klu) (Figure 5) (Bayraktar et al., 2010). It has been reported that ectopic expression of PntP1 in NBsI is sufficient to induce INP-like progeny cells (Zhu et al., 2011). In addition, misexpression of Klu at early stages of INPs maturation is sufficient to revert them back into NBII (Xiao et al., 2012).

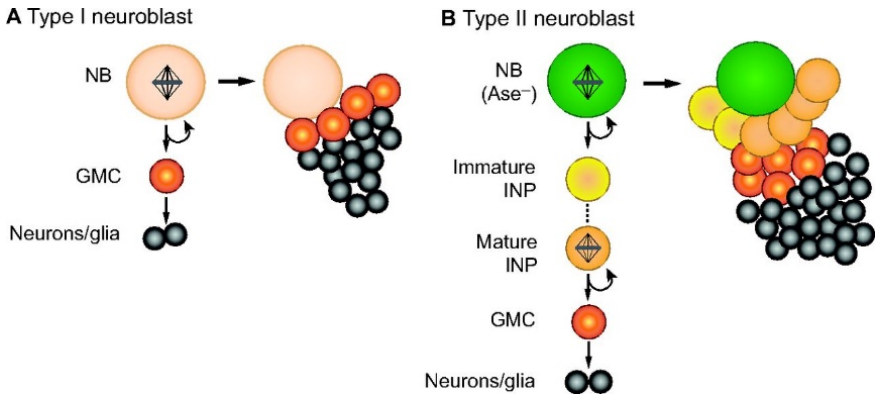

Figure 4. Central brain type I and type II NBs: ACD and progeny. A) NBsI divide asymmetrically to give rise to a cell committed to differentiate, the GMC, which will divide once more generating neurons or glial cells. **B)** NBsII also divide asymmetrically but generating in this case an intermediate progenitor cell (INP) that is able to divide asymmetrically several rounds of division generating another INP and a GMC that will produce neurons. *(Extracted from Knoblich 2008)*.

It is crucial a tight regulation of the precise time at which NBs stop dividing to get a correct number and balance of different neurons and glial cells. In the VNC, NBs terminate proliferation by undergoing Hox-gene mediated cell death (White et al., 1994). Central brain NBsI stop proliferating and undergo apoptosis or terminally differentiate. Differentiation includes changes such as reduction in cell size, lengthening of cell cycle and expression of nuclear Prospero (Pros) (Reichert, 2011). It has been shown that central brain NBsII terminate proliferation via apoptosis (Maurange et al., 2008) and in optic lobe NBs are currently poorly known.

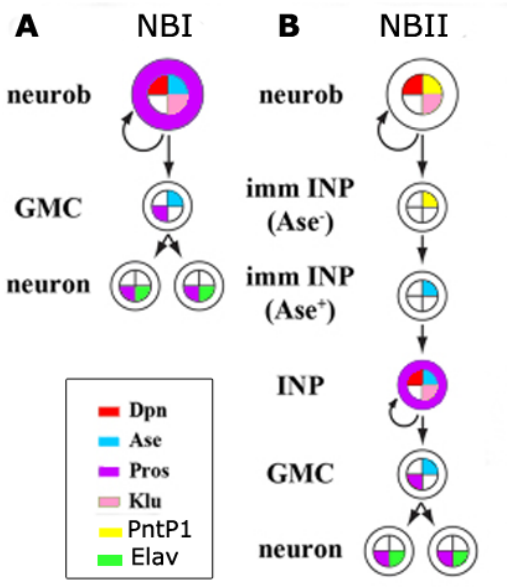

Figure 5. NBs lineages in the central brain and their transcription factor patterns of expression. A) Diagram summarizing the expression profile of molecular markers in the NBsI and **B)** NBsII.

Divided small circle represents the nucleus and the big one not divided, the cytoplasm. Each color represents a different marker.

Traditionally, ACD has been studied in NBsI but in the last past years, a huge amount of work has appeared around the NBsII and its lineages. This is due to their similarities with vertebrate neural SCs. Actually, comparable intermediate progenitors have been found in the developing brain of mammals (Gotz and Huttner, 2005). These similarities and the big capacity to proliferate are also reasons why NBsII have emerged as a great model to study overgrowth and tumor formation (see below).

To conclude with the description of NBs biology, it is necessary to mention a field that has become relevant with several good studies and descriptions in the last years, the temporal patterning of gene expression that codify NBs proliferation and progeny specification (Figure 5). To generate the diversity of neurons in the brain, proliferating NBs require positional and temporal information. Positional information is provided to each NB by the early embryonic expression of anteroposterior and dorsoventral patterning genes (Schmidt et al., 1997). In addition, temporal information is going to be provided by the moment of birth; different progeny is generated by the parental NB depending of the birth moment. The molecular basis that links birth order to neural fate, involve a cascade of transcription factors expressed in the parental NB. This stereotyped cascade includes: Hunchback (Hb), Krüppel (Kr), Pmd, Castor (Cas) and Grainyhead (Grh) (Pearson and Doe, 2003). Temporal specification is not limited to embryogenesis, also occurs during postembryonic stages. Loss of one of the transcription factors does not result in a block of the series but only in the skipping of one temporal identity (Tran and Doe, 2008). GMCs maintain expression of the temporal factor inherited from the sibling NB, and neurons of different identity are generated according to which temporal factor is being expressed. The NBII themselves serially express the transcription factors, Diachaete (D), Cas, and Seven up (Svp) and likely more that have not been described yet. In addition, each INP also expresses its own series, which includes D, Grh an Eyeless (Ey). This type of combinatorial temporal patterning composed by two axes leads to a large diversity of neurons (Bayraktar and Doe, 2013). Thus, the temporal patterning of gene expression is not only implicated in the diversity of neurons generated, also controls the number of cells that have to be produce in any time and the moment when proliferation has to end and NB has to disappear.

2.2 *Drosophila* NBs as a model system to study ACD

A defining feature of SCs is their ability to self-renew and to generate daughter cells that are committed to further differentiation, within the same cell cycle. This feature is usually linked to the ability of SCs to undergo ACD (Kang and Reichert, 2015). *Drosophila* embryonic NBs have been used for a long time as a valuable system that has permitted the discovery of many regulators and basic principles of ACD, but their restricted self-renewal capacity limit their usefulness as a true stem cell model. Larval NBIIs instead have extensive proliferative capacity and this allows the study of ACD in such a context, which is extremely relevant for cancer related studies.

Asymmetric NB division involves four major steps: (1) setting up an axis of polarity, (2) a proper orientation of the mitotic spindle along that axis of cell polarity, (3) the asymmetric localization of cell fate determinants in the dividing NBs and (4) the differential segregation of cell fate determinants between the two daughter cells (Homem and Knoblich, 2012). In the embryonic stage, when the NBs start delaminating, their apical-basal polarity is inherited from the epithelial cells of the neuroectoderm. Subsequent embryonic and larval NB divisions are aligned relatively to the axis of the previous ones. For this reason, it is thought that the NB apical centrosome serves as a reference point for apical accumulation of the Par complex during interphase to establish the apical-basal axis of polarity in the cell (Rebollo et al., 2009). NB polarity is set up in interphase, but cell fate determinants only localize asymmetrically during mitosis.

2.2.1 The apical and basal complexes in dividing NBs

As we already mentioned, NBs are able to produce different cells after division segregating different neural precursor factors into each cell. Most of these factors are evolutionary conserved and we can find mammalian homologues with similar function and properties in ACD (Table 1). To be segregated into different daughter cells, proteins have to be placed in the apical or basal part of the cell (Figure 6). In wild type NBs, these protein complexes containing cell-fate determinants asymmetrically localize to the cell cortex. Complexes localized to the apical part of the NB sit at the top of a hierarchy responsible

for orchestrating a number of asymmetries in the dividing NBs, including restricting cell-fate determinants to the opposite (basal) cortex, orientating the mitotic spindle along the apical-basal axis and regulating daughter cell size asymmetry (Sousa-Nunes and Somers, 2013). There are two classical apical subcomplexes: the Par3/Par6/aPKC/Inscuteable (Insc) complex and the Pins/Gαi/Dlg/Mud complex (Kuchinke et al 98; Shober et al., 99; Wodarz et al., 99; Petroncki and Knoblich 2001; Wodarz et al 2000;Izumi et al., JCB2004; Schaefer et al 2001; Parmentier 2000; Schaefer et al., 2000; Yu et al., 2000) and the more recently described Canoe (Cno)/Rap1 complex (Speicher, 2008; Carmena, 2011). Basically, the first complex is involved in setting up and maintaining the apical-basal axis of polarity in the NB and it is also responsible for the basal localization of cell fate determinants through sequential phosphorylation events that occur in the apical region (Knoblich, 2008). The Cno/Rap1 complex also contributes to the basal localization of cell fate determinants and, along with the Pins/Gαi/Dlg/Mud complex, to the proper spindle orientation (Speicher et al, 2008; Carmena et al 2011; Wee et al., 2011).

Basally localized factors are going to be segregated into the GMC where they will promote the differentiation program. There are two independent protein complexes in the basal cortex: one containing the adaptor protein Miranda (Mira) (Schuldt et al., 1998) and its two linked cell fate determinants Prospero (Pros) (Doe et al., 1991) and Brain tumor (Brat) (Lee et al., 2006c), and the other is composed by the determinant Numb (Uemura, 1989) and its adaptor Partner of Numb (Pon) (Lu, 1998). Disruption of one complex does not affect the other. The function of Miranda is to localize basal complex cargo to the basal cortex from metaphase onwards, cargo (Pros and Brat) that will be segregated specifically into the GMC. Miranda persists for a while in the GMC cortex and later is degraded. At this moment, Pros is able to enter into the nucleus of the GMC (Ikeshima-Kataoka et al., 1997) where it represses expression of genes required for proliferation and self-renewal (such as *cyclin E, cdc25/string*, E2F and the transcription factors Dpn, Ase, Achaete, Scute, Snail, Hb and Kr) and it activates genes involved in the differentiation program (Choksi et al., 2006). Brat, the other protein that is located basically by Mira, inhibits self-renewal and promotes differentiation as well, but by unknown post-transcriptional mechanism involving the downregulation of the

transcription factor dMyc (Betschinger et al., 2006). The member of the other basal complex, the determinant Numb, is a PTB-domain protein that negatively regulates Notch signaling by promoting endocytosis of the Notch receptor in the differentiating daughter cell (Couturier et al., 2012).

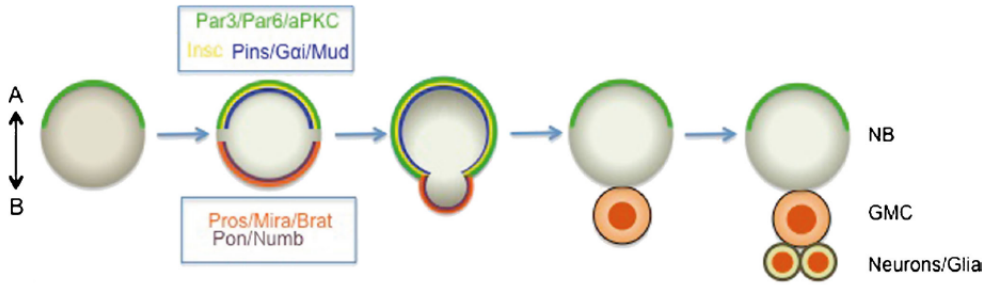

Figure 6. ACD in NBs. During ACD, in metaphase, proteins that compound the apical complexes including Par-3/Par-6/aPKC and Pins/Mud/Gαi, are localized to the apical cortex. A cascade of phosphorylation events localizes the cell fate determinats Pros, Brat and Numb and its adaptor proteins Mira and Pon to the basal cortex. Therefore, this asymmetric localization will permit that daughter cells inherit different proteins after division. The basal determinants are going to be segregated only into the GMC where they will activate a differentiation program *(Extrated from Kang and Reichert, 2015).*

2.2.2 Coordinating asymmetry and mitosis: asymmetric segregation of cell fate determinants and spindle orientation

The Par complex component aPKC is itself determinant for NB self-renewal (Lee et al., 2006b). A reason why aPKC is such a pivotal player in NB division is that its own activity is regulated by mitotic kinases and phosphatases, providing a mechanistic link between the NB cell-cycle and its asymmetry. Thus, mitotic kinase Aurora (Aur) turns active during mitosis and phosphorilates Par-6. Then, activated Par-6 activates aPKC to which it is bound and, in this way, restricts aPKC activity to the apical pole (Wirtz-Peitz et al., 2008). Apically activated aPKC can control now the position of different basal determinants. For example, aPKC phosphorylates the mediator cytoskeleton-binding protein called Lethal (2) Giant Larvae (L(2)gl) and it provokes a conformational change in L(2)gl that inactivates it. Active L(2)gl is localized around all cortex but after phosphorylation is unable to interact

with the cytoskeleton and it detaches from Par-6/aPKC complex being restricted to the basal pole. Furthermore, L(2)gl is able to inhibit aPKC basal localization as well. As a result of this mutual inhibition between aPKC and L(2)gl, the first one is restricted to the apical pole, and the second one is basally stable. All of this ensures the partitioning of aPKC to the future NB, where it will promote self-renewal, and of L(2)gl to the other daughter cell (Lee et al., 2006b) (Figure 7).

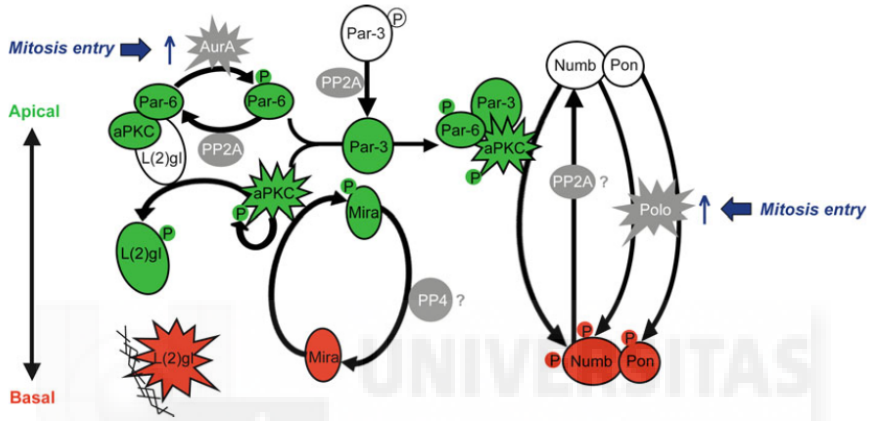

Figure 7. Schematic representation of the events that occur to regulate the NB asymmetry. In green, apical complex members; in red, basal complex members; in grey, uniformly cytoplasmic kinases and phosphatases (upon nuclear envelope breakdown); in white, specific post-translational modifications of asymmetric components that are not necessarily asymmetrically localised; black mesh, actin microfilaments; star-like shapes, activated forms of proteins; black arrows, (de)phosphorylation events; blue upward arrows, upregulation of protein activity by mitosis entry; encircled P, phosphorylation event(s) *(Taken from Sousa-Nunes et al., 2009).*

The exit of L(2)gl of the apical complex allows the association of Bazooka (Baz; Par-3 in vertebrates) to aPKC. Baz recruits Numb, which is then phosphorylated by aPKC and, consequently, Numb dissociates from the apical complex to be stabilized to the basal pole of the NB (Wirtz-Peitz et al., 2008). The mitotic kinase Polo also regulates the asymmetric segregation of the Numb/Pon complex via direct phosphorylation of both proteins (Wang et al., 2007). Phosphorylation of Pon restrics Numb to the basal cortex, so it can be segregated to the GMC to inhibit Notch signaling-promoted self-renewal in this cell. In the case of Mira, it is aPKC again the responsible of its phosphorylation to localize it to the basal cortex. Some works have shown the possibility that a dephosphorylation

event(s) involving the Protein Phosphatase 4 (PP4) could be required for the localization of Miranda (Sousa-Nunes et al., 2009). Finally, to restore the initial situation, some proteins require proper activation and inactivation as well. The catalytic subunit of Protein Phosphatase 2A (PP2A) has been shown to form a complex with Par-6 resulting in a suppression of aPKC signaling.

Drosophila gene/protein	Vertebrate orthologue(s)	Function	Defects associated with mutations in the nervous system	References
Asymmetric cell division				
Par complex: aPKC (Atypical Protein Kinase C), Bazooka/Par3, Par-6 (Partitioning defective 6)	PKCζ, PKCλ; Par3/Par-6	Establishes a polarity axis in NBs. Localizes and determines the apical side of the NB cell cortex. Inheritance by the undifferentiated NB after mitosis.	aPKC gain of function: NB-like cells and overproliferation Par complex loss of function: loss of apicobasal polarity; NBs prematurely enter cell cycle arrest	Reviewed by Knoblich, 2008
Insc (Inscuteable) and Gαi/Pins (Partner of Inscuteable)/ Mud (Mushroom Body Defective)	Mouse Insc, Gαi1-3, AGS3, LGN, NuMA	Insc: adaptor protein that links the Par complex to a second protein complex containing the proteins Gαi, Pins and Mud Gαi/Pins/Mud: apical complex; links apical cortex and astral microtubules to orient the mitotic spindle	Loss of function: misorientation of spindle during NB divisions	Reviewed by Knoblich, 2008
Lgl [Lethal (2) Giant Larvae]	Mgl	Lethal (2) giant larvae (Lgl) is a cytoskeletal protein that defines the basolateral domain and restricts the Par complex to the apical domain	Loss of function: overproliferation of NB-like cells	Reviewed by Knoblich, 2008
Numb	Numb, Numblike	Notch signaling inhibitor. Asymmetrically segregated to the basal daughter cell, where it lowers Notch levels and promotes cell differentiation.	Loss of function: overproliferation of NB-like cells	Wang et al., 2007
Pon (Partner of Numb)	–	Adaptor protein that facilitates the basal localization of Numb	Loss of function: overproliferation of NB-like cells	Wang et al., 2007
Brat (Brain Tumor)	Trim2, Trim3, Trim32	Translation inhibitor. Localizes basally in the dividing NBs. Is inherited by the basal differentiating daughter cell. Inhibits growth and self-renewal, and induces differentiation.	Loss of function: overproliferation of NB-like cells; reduction in number of differentiated cells	Bello et al., 2006; Betschinger et al., 2006
Mira (Miranda)	–	Adaptor protein that accumulates asymmetrically in the basal side of the dividing NB. Mira binds Brat and Pros, localizing these proteins to the basal cortex of NBs.	Loss of function: loss of Pros asymmetric localization in NBs; overproliferation of NB-like cells; reduction in the number of differentiated cells	Shen et al., 1997
Pros (Prospero)	Prox1	Homeodomain transcription factor. Represses expression of cell cycle genes and activates genes that specify cell fate and are required for terminal differentiation.	Gain of function: premature differentiation of NBs Loss of function: differentiating daughter cells revert back to Nb-like fate	Doe et al., 1991
Notch	Notch 1-4	Notch high levels are determinant of Nb fate	Gain of function: INPs revert back to NB-like cells Loss of function: loss of larval NBs	Bowman et al., 2008
Polo	Plk1 (Polo-like kinase1)	Cell cycle regulator, mitotic Ser/Thr protein kinase	Loss of function: overproliferation of NBs, and defective asymmetric localization of aPKC, Numb and Pon	Reichert, 2011
Aurora-A	Aurora	Cell cycle regulator, mitotic Ser/Thr protein kinase	Loss of function: overproliferation of NBs, and defective asymmetric localization of aPKC, Numb and Pon	Reichert, 2011

Tabla 1. Regulators of asymmetric NB division *(Obtained from Homen and Knoblich, 2012).*

Despite of all control mechanisms during all cell cycle steps, determinants can still mislocalize during methaphase. To further prevent errors, it exists another control mechanism able to act during anaphase/telophase to correct the localization of misplaced

cell determinants called “telophase rescue”. This is a phenomenon poorly understood that requires the activity of the Snail family of transcriptional repressors Snail, Escargot and Worniu (Cai et al., 2001) and it is the responsible that mutants that show defects in NB asymmetry during metaphase do not present big failures at the end of mitosis.

Coupling spindle orientation with cortical polarity is essential for an accurate segregation of cell-fate determinants and the maintenance of an appropriate balance between self-renewal and differentiation. Although some apical complex members have roles in both cortical polarity and spindle-orientation, the phenotype of certain mutants demonstrated that the mechanism responsible for each of these processes are separate and it is possible to alter one without disrupting the other one (Izumi, 2004). Alignment of the mitotic spindle along the apical-basal axis entails a two-step mechanism: an initial step involving centrosomes that initiate the assembly of the mitotic spindle in alignment with cortical polarity, and a later step involving mitotic spindle cortex-interaction that refines the alignment (Rebollo et al., 2007).

Centrosomes function as major microtubule-organizing centers of cells and are recognized as critical regulators of spindle-orientation (Gonzalez, 2007). One centrosome is larger and acts as the major microtubule-organazing center; it remains fairly stationary beneath the apical cortex, maintains its pericentrosomal material (PMC) and nucleates many astral microtubules. The other centrosome is smaller and moves extensively throughout the cytoplasm before positioning itself at the opposite part of the cell and is inherited by the GMC. The big centrosome is associated with the apical cortex and plays a role in maintaining a apical-basal axis along successive divisions, determining spindle axis prior to spindle formation and also specifying the cortical site where apical complexes should reassemble (Rebollo et al., 2009). As mentioned above, in addition to the apical centrosome, further events implying the apical complex, link cortical polarity to spindle orientation. One of the most relevant apical components for that function is the Gαi/Pins pathway (Izumi et al., 2004). It has been proposed that it orientate the spindle by maintaining a pulling force between the apical cortex and the apically localized

centrosome (Siller and Doe, 2009). Indeed, Pins interacts first with the tumor suppressor protein Dlg that is in turn associated with Khc-73, a kinesine linked to aster microtubules. Then Pins binds Mud that is associated with the Dynein-Dynactin complex, which also bound aster microtubules, further contributing to spindle orientation. The other important apical complex that links cortical polarity and spindle orientation is Cno/Rap1; Cno directly interacts with Pins contributing to spindle alignment by recruiting Mud to the apical cortex (Speicher et al., 2008) (Carmena et al., 2011) (Wee et al.,2011).

Another striking feature of NB division is the generation of asymmetrically sized daughter cells. The "new" NB has to be bigger than the committed cell. This asymmetry is the result of mechanism regulating spindle geometry and size. Until anaphase, the mitotic spindle is symmetric but along anaphase the apical microtubules enlarge while the basal ones shrink. Mitotic spindle asymmetry and unequal daughter size is controlled by two parallel pathways involving aPKC/Par and Pins/Gαi pathways (Cai et al., 2003).

2.3 Neural SCs and ACD in vertebrates

Despite the mammalian brain is much larger and more complex than any invertebrate brain, many basic aspects behind *Drosophila* brain development are conserved in mammals. As in flies, all neurons in the mouse cortex arise from ACD of a small set of progenitors that generate several neural subtypes in a spatially and temporal control manner (Homem and Knoblich, 2012) (Paridaen, 2014). The neocortex of adult mice is conformed by six layers. At embryonic day 9, the cortex consists of neuroepithelial progenitors, which extend from the apical ventricular surface to the basal surface of the neural tube. Before these neuroepithelial cells divide, their nuclei undergo interkinetic nuclear migration and move apically to undergo mitosis at the apical-most-position. Early divisions are symmetric and result in expansion of the progenitor pool. Neurogenesis starts around day 11, when progenitor cells start expressing characteristic features of glial cells (Mori et al., 2005) and turn into the so-called radial glial (RG) cells. RG cells extend an apical and basal process and are restricted to the most apical area of the cortex: the ventricular zone (VZ). They continue dividing asymmetrically into one self-regenerating

daughter cell and one cell that migrates into the more basally located cortical plate to differentiate into a neuron in a process called direct neurogenesis (Figure 8). Alternatively, RG cells can generate another RG cell and an intermediate progenitor cell (IPC) via a process termed indirect neurogenesis (Figure 8). IPCs reside in the cortical area between the VZ and the intermediate zone (IZ) where they form the layer called subventricular zone (SVZ). They undergo at least one more symmetric division to generate two differentiated neurons. Indirect neurogenesis is thought to be the predominant mode that occurs in the mouse cortex, at least during later stages and resembles the mode of division used by *Drosophila* type I NBs (Homem and Knoblich, 2012). Recent studies have revealed another type of progenitor called outer radial glial (oRG) (Wang et al., 2011b). These cells are located in the outer regions of the SVZ and arise from asymmetric divisions or RG cells (Figure 8). Although they lack the connection to the apical surface and no longer express apical plasma membrane markers, they contain a basal process and continue expressing the RG markers Pax6 and Sox2, and continue to self-renew. In humans, most cortical neurons are actually thought to arise from oRG cells, whereas in mice they contribute only to a small fraction. These oRG lineages closely resemble type II NB lineages in *Drosophila* and, therefore, both NBI and NBII lineages resemble to be recapitulated in the mammalian brain. As in *Drosophila* neural SCs, the polarity and spindle orientation machinery are conserved in mammalian brain. Mammalian Par-3, Par-6 and aPKC (Table 1) are necessary for both apical-basal polarity and for spindle orientation. Pins has two mammalian homologs, Ags3 and Lgn. The mammalian Mud homolog NuMa has a role in the establishment and maintenance of spindle poles. Finally the single vertebrate homolog for *Drosophila* Inscutable is required and sufficient for inducing non-planar spindle orientation (Postiglione et al., 2011). Thus, conserved molecular machinery regulates the orientation of progenitor divisions. How this influences cell fate in the daughter cells, however, is much less clear. Although the segregating determinants that were found in flies are conserved in mice, it is poorly understood the way that they act. Depletion of the mammalian homologs of *prospero* and *brat*, *Prospero- related homeobox 1* (*Prox1*) and *Tripartite-motif con-taining 32* (*Trim32*), respectively results in premature differentia- tion of RG cells into neurons, indicating that the complex is essential for progenitor maintenance (Gomez-Lopez et al., 2014).

Mammalian neural precursor divisions are morphologically highly asymmetric. Subcellular structures are asymmetrically inherited and they could contribute to asymmetric fate specification (Figure 8). These structures include the apical adherents junctions and the apical membrane domain, as well as the apical and basal processes that are characteristics features of RG cells. The apical membrane domain is very narrow and, as a consequence, the apical domain could be asymmetrically inherited not only in horizontal division but also in oblique ones. Although the apical process disappears during mitosis, recent studies in slice culture have shown that the basal process is actually maintained throughout division and is inherited by one of the two daughter cells even in division with a vertical cleavage plane.

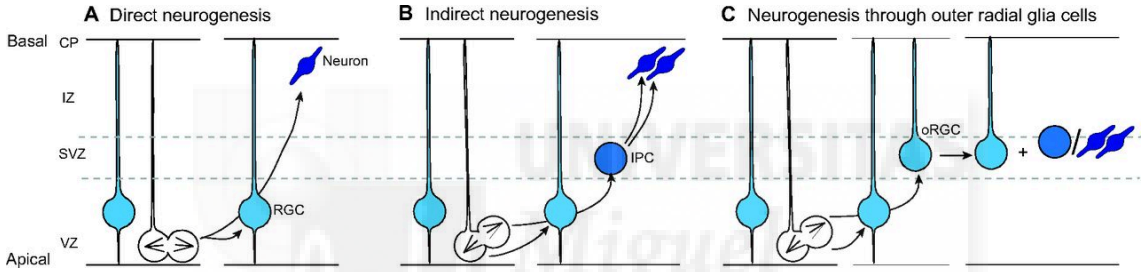

Figure 8. Neurogenesis in the mice neocortex. **A)** In the **direct neurogenesis**, the RG (light blue) cell divides asymmetrically to self-renew and to generate another cell that will differentiate in a neuron (dark blue) in the basal part of the cortex. **B) Indirect neurogenesis** consists in another type of asymmetric cell division of the RG cell where an IPC is generated in an oblique division. Then the IPC will divide once more to generate two neurons. **C) Neurogenesis** can also occur **through generation of the oRG cells**. In this case the RG cell divides obliquely to the ventricular surface and generates another RG cell and one oRG that localizes to the most basal region of the SVZ. oRG then divides to self-renew and to generate an IPC or two neurons. *(Extracted and modified from (Homem and Knoblich, 2012)*.

Given the remarkable conservation of molecular mechanism involved in nervous system in *Drosophila* and vertebrates, the investigation of all of these features of neural stem cell biology in the fly model is likely to help in understanding the roles of neural SCs in generating the highly complex human brain. From this point of view, the use of *Drosophila* NBs as a model for unraveling the mechanisms underlying not only brain development, but also stem cell derived brain tumors, could be really useful to get insight into the aberrant molecular mechanisms that cause brain tumors in human patients.

3. CONSEQUENCES OF DISRUPTING ACD: NEOPLASTIC GROWTH AND TUMORIGENESIS

3.1. NBs as a model for cancer stem cells

The majority of the cells that are present in adult animal tissues are short lived. The stability of the tissue in a living animal is actually a steady state in which permanent loss of injured or aged cells is supplied by specialized tissue SCs. Through this mechanism, a single SC can cover the lifespan of the individuals and generates new differentiated tissue when is demanded. Adult tissues such as colon, lung, prostate, peripheral blood and brain are maintained by the proliferation of tissue SCs that constitute a small fraction of the total cell mass (Reya et al., 2001). The same fact seems to occur in tumors that originate in these tissues, where only a small fraction of "tumor cells" are able to sustain the overgrowth along the time. This observation has put the focus on the research of which changes can occur in these adult SCs to become tumorigenic and the real contribution of them to the initiation of the cancer.

For years is has been assumed that any somatic cell could initiate tumor development. However, some characteristics of SCs such as being immortal, mitotically competent and localized in tissues where tumors often appear, made them good candidates to be the cells of origin of the tumor. According to this model, the neoplastic transformation of an SC in an adult tissue will lead to a "cancer stem cell" (CSC). Some observations provide further support to the idea that some types of cancer might originate from STs transformed in CSCs. For example, CSCs isolated from different tumors have been found to bear surface markers that are unique to the normal SCs of the tissues in which the original tumors arose (Piccirillo et al., 2006). Furthermore, these CSCs present some distinctive characteristics such as avoiding ageing, unrestrained cell-division potential, unlimited tissue-generating activity and refractory behavior to the signals that control tissue homeostasis (Gonzalez, 2007). Despite the CSC hypothesis is still controversial, some studies in the past decade have provided increasing evidence that CSCs exist in various cancers. The first CSCs were identified in acute myeloid leukemia (AML) but similar xenograft assays have made it possible the identification of CSCs in

many solid tumors, including breast, brain, ovary, colon, head and neck squamous cell carcinoma, melanomas, liver, lung, pancreas and prostate cancer (Reviewed in (Xie, 2009). An important implication of this model is that treatments that cause a major reduction of bulk tumor mass might not necessarily have significant long-term consequences for tumor progression if the small population of CSCs, which might actually be more resistant to the treatment, remains unaffected (Al-Hajj et al., 2004).

Drosophila NBs have emerged as a model system that recapitulates the transition from a normal SC to a tumor SC upon mutation of genes involved in ACD (Caussinus and Gonzalez, 2005). Identifying how mutant *Drosophila* NBs escape those controls may teach us something about the transition from normal to tumor-initiating stem cells that may occur in human tumors. Thus, several lines of evidence suggest that the link between increased neural SC self-renewal and brain tumorigenesis may be conserved from *Drosophila* to humans (Xie, 2009). Modeling cancer in *Drosophila* is not new. The pioneer works of E. Gateff and colleagues laid the foundations for future research by elaborating a classification of fly tumors (Table 2), providing protocols to work with them and identifying a considerable number of genes that are involved as regulators of SC divisions.

Tumor type	Implanted in adults		Implanted in larvae
	Invasive	Lethal	Capable of differentiation
Malignant	Yes	Yes	No
Benign	No	No	No
Hyperplactic	No	No	No

Table 2. Classification of tumor behavior in flies *(Extracted and modified from (Gonzalez, 2007)).*

The possible functional link between failed NB asymmetry and tumor growth was first suggested by the identification of well-known tumor suppressor (TS) genes, such as *l(2)gl* or *dlg* as key regulators of NB asymmetry (Wodarz, 2005). In fact, some of the key

regulators of the ACD were first identified in genetic screens for TS genes (Gateff, 1978, 1994). Thus, *Drosophila* larval tissues with mutations in *dlg1, l(2)gl or scrib* have impaired apical-basal polarity and neoplastic growth in imaginal discs and in the nervous system (Bilder, 2004; Gateff, 1978; Humbert et al., 2003; Wodarz, 2000). In human carcinomas, loss of cell polarity and malignant transformation have been also correlated (Liu et al., 2005). Several hypotheses have emerged to explain the possible link between these two events. Some of them relate the loss of cell polarity with malignant transformation throughout changes in cell architecture that impinge directly on the cell cycle by inhibiting signals that control and restrict proliferation or by enhancing mitogenic pathways. An alternative hypothesis suggests that the loss of cell polarity results in an impairment of the mechanism that controls the correct segregation of the cell fate determinants that will determine the identity of daughter cells. Thus, these daughter cells are unable to follow their normal developmental program and they do not respond to the mechanisms that control proliferation in the wild type condition (Bilder, 2004). This leads to a two possible scenarios where cells either do not stop proliferating causing overgrow or they prematurely stop proliferating via anticipated differentiation.

The first direct demonstration of a link between ACD failures and tumorigenesis came when pieces of larval brain tissue mutants for genes encoding asymmetric NB division regulators, including known cell-fate determinants, developed malignant tumors when they were transplanted to the abdomen of adult host (Caussinus and Gonzalez, 2005). Since then, many more of these proteins have been shown to be TSs both in allograft culture and *in situ*, in the larval brain (Table 3).

Mutant[a]	Overgrowth				References
	In situ		Allograft culture		
	Clonal analysis	NB duplication[b]	Immortal, invasive metastatic growth	Limited growth potential	
aPKC	–		–	–	Lee *et al.*, 2006c; Caussinus and Gonzalez, 2005
aPKC GOF	+				Lee *et al.*, 2006c
pins	–	+	+		Rebollo *et al.*, 2007; Lee *et al.*, 2006c; Caussinus and Gonzalez, 2005
mira	+		+		Betschinger *et al.*, 2006; Caussinus and Gonzalez, 2005
brat	+		+		Lee *et al.*, 2006b; Betschinger *et al.*, 2006; Bello *et al.*, 2006; Caussinus and Gonzalez, 2005; Gateff, 1978
pros	+		+		Lee *et al.*, 2006b; Choksi *et al.*, 2006; Betschinger *et al.*, 2006; Caussinus and Gonzalez, 2005
numb	+		+		Lee *et al.*, 2006a; Bowman *et al.*, 2006; Caussinus and Gonzalez, 2005
lgl	+		+		Lee *et al.*, 2006c; Betschinger *et al.*, 2006; Gateff, 1978
dlg	+		+		Gateff, 1978
mud	+	+			Siller *et al.*, 2006; Bowman *et al.*, 2006; Izumi *et al.*, 2006
sas6				+	Castellanos *et al.*, 2008
sas4		+	+		Castellanos *et al.*, 2008; Basto *et al.*, 2006
sak				+	Castellanos *et al.*, 2008
sak GOF		+	+		Basto *et al.*, 2008
asl		+		+	Castellanos *et al.*, 2008; Rusan and Peifer, 2007
cnn		+			Lucas and Raff, 2007
aurA	+	+	+		Castellanos *et al.*, 2008; Lee *et al.*, 2006a; Wang *et al.*, 2006
polo	+		+		Castellanos *et al.*, 2008; Wang *et al.*, 2007

[a]GOF: gain of function, all other situations refer to loss of function.
[b]Symmetric NB divisions reported by live imaging.

Table 3. Test of tumorigenic activity of genes implicated in ACD. (*Taken from (Januschke and Gonzalez, 2008).*

As it has been described previously in this work, the basal proteins are cell fate determinants that must be segregated only to one daughter cell to initiate the differentiation process. Pros is a transcription factor that it has to be segregated to the GMC where it enters in the nucleus and regulates genes that are crucial to initiate differentiation and repression of cell cycle. In the same way acts Brat that is sorted in the GMC as well where acts as a (post-transcriptional) repressor of cell growth. Thus, larval brain tissue mutant for *pros* or *brat* develops massive malignant tumors in allograft culture and displays significant overgrowth *in situ*. Miranda (Mira) is the adaptor protein responsible to ensure the localization of Pros and Brat to the basal pole of the NB to be properly segregated. Consistently, *mira* mutants lead to overgrowth *in situ* and malignant tumor growth in allograft cultures. Numb is another cell fate determinant that has to be segregated into the GMC where represses Notch activity and avoid self-renewal. Exactly in the same way that the previous ones, allograft culture of *numb* mutant clones, cause malignant tumors. Some components of the apical complex are also able to produce overgrowth when they are disrupted. Thus, ectopic cortical localization of aPKC affects to the localization of the basal proteins, as Numb, and leads to a dramatic increase in NBs in

detriment of differentiated cells *in situ*. On the other way around, loss of aPKC function provokes NB cell cycle arrest and premature loss of NBs, and this mutant tissue does not overgrow in allografts culture (reviewed in (Januschke and Gonzalez, 2008). Mutations in these three genes that previously we described as a TS genes: *dlg*, *l(2)gl* and *scrib*, also result in the inability of cell fate determinants to localize asymmetrically in NBs, being these mutations responsible for inducing the formation of neoplastic tumors in imaginal discs and nervous system (Betschinger and Knoblich, 2004; Lee et al., 2006b). The behavior of the *pins* mutant tissue is intriguing. *In situ*, loss of Pins function causes a reduction in the number of NBs in late larval stages. However, *pins* mutant NBs have been observed to divide symmetrically by live imaging and malignant tumors develop from *pins* mutant tissue in allograft culture. Interestingly, all cases of these mutants in which symmetric NB division have been observed, cause tumor growth, benign or malignant, in allograft culture (Lee et al., 2006a). Spindle misorientation without disruption of cortical polarity has been reported as another way to produce overgrowth, as it has been reported to occur in *mud* mutant NBs. Some of the main kinases that regulate the asymmetric NB division are also TSs, as AurA and Polo. Both are necessary to constrain aPKC to the apical cortex, to localize Numb basally and for spindle alignment. Thus, larval brain mutants for *polo* or *aurA* present supernumerary NBs and develop malignant tumors when they are implanted in adult host. Finally, different types of genome instability, including chromosome instability and centrosomal alteration are common traits in human and *Drosophila* tumors. Several types of centrosome dysfunctions, as duplications, have been found to be potent tumorigenic conditions in larval brains kept in allograft culture. Different hypothesis have emerged to explain how disruption in centrosome function can lead to tumor formation. It seems that the tumorigenic activity of centrosome function in *Drosophila* is directly related to failures in the segregation of cell fate determinants, rather than being linked to chromosome segregation (reviewed in (Januschke and Gonzalez, 2008).

Trying to understand how this transformation occurs in SCs to become CSCs two main models have emerged. Both models have the starting point in failures during the ACD and, consequently, in the identity of the daughter cells generated. The simplest

working model suggests the direct expansion of NBs by symmetric divisions (Lee et al., 2006a; Lee et al., 2006b; Siller et al., 2006; Wang et al., 2011a), while the second model call on reversion of GMCs or INPs to NBs state (Betschinger et al., 2006; Bowman et al., 2008; Choksi et al., 2006; Lee et al., 2006c). In the first model, direct expansion of NBs might apply to cases in which uncoupling of spindle orientation from the apical-basal axis of the cell results in cleavage planes that bisect the NB in two equal halves, in terms of size and determinants. This situation occurs in *mud* (Siller et al., 2006)*, Aurora A (Lee et al., 2006a)* or *Ana2* (Wang et al., 2011a) mutants. This model implies that the presence of the apical components as aPKC is dominant over the presence of basal determinants that promote differentiation; thus, cells that inherit both retain SC identity. Something similar has been reported in the combination of loss of function of *pins* and *lgl,* where aPKC is ectopically localized over most of the NB cortex. It has been also reported the same situation in the cases where is expressed aPKC-CCAX (constitutive active form of aPKC) (Lee et al., 2006b). On the other hand, reversion of the GMC to the NB state has been proposed as a model to explain tumor growth in *pros* (Choksi et al., 2006) and *brat* (Betschinger et al., 2006; Lee et al., 2006c) mutants. In those cases, *pros* and *brat* loss of function act as a dominant situation over the presence of other cell differentiation determinants, such as Numb. Thus, the basal cell is not able to start the differentiation program and revert to a NB state. Finally, the overgrowth has also been proposed to occur as a result of reversion of INPs, in the case of NBII, in mutant conditions as a loss of *brat* (Lee et al., 2006c) or *Numb* (Bowman et al., 2008). In these conditions, the Ase-negative INP immature is unable to progress in the maturation program and reverts to the NB state.

In summary, the neoplastic transformation of *Drosophila* NBs can be triggered by the perturbation of several molecular mechanisms that control the segregation of cell fate determinants during ACD, resulting in hyperproliferation of SCs and contributing to tumor development. Similarly, these defects have been proposed to affect tissue SCs in other species. Thus, in mouse models, defects associated to the segregation of Numb or L(2)gl homologs lead to hyperproliferation of neural progenitors and brain dysplasia (Klezovitch et al., 2004; Li et al., 2003).

3.2. Loss of cell polarity, tumor growth and cell competition

As it was mentioned before, mutations in TS genes are found in most human tumors and are believed to be a prerequisite for the accumulation of additional mutations that conclude in tumor formation. A lot of these TS genes are critical players controlling polarity in ACD. This is the case of three well-known TS genes, *scrib, dlg* and *l(2)gl* that were initially studied in epithelial tissues. *dlg* and *l(2)gl* were first identified as TS genes (Jacob et al., 1987; Woods and Bryant, 1991) and later *scrib* joined the group being also characterized as a TS gene with very similar properties (Bilder et al., 2000; Bilder and Perrimon, 2000). Thank to these great works, now we know that *Drosophila* TS genes *scrib, dlg, l(2)gl* appear to act in a common pathway and mutation in any of these genes lead to loss of apical-basal polarity and overproliferation in epithelia (Wodarz, 2000). Thus, these works revealed a clear link between cell polarity and cell proliferation. Almost at the same time, studies of these genes were performed in NBs, where it was shown that *l(2)gl* and *dlg* regulate basal protein targeting, but not apical complex formation or spindle orientation in both, embryonic and larval NBs. Dlg protein is enriched in the apical pole and is required for maintaining cortical localization of Lgl protein and then Lgl mediates the localization of basal components during ACD (Ohshiro et al., 2000; Peng et al., 2000). Some years later, more studies in NBs incorporated the role of Scrib protein. Scrib and Dlg are apically enriched in early mitosis. Lgl can be detected as well in the apical pole of the NB but due to the phosphorylation event by aPKC, Lgl is excluded from the apical pole, finding the active form of the protein only in the basal pole (Lee et al., 2006b). Dlg/Scrib/Lgl are interdependent for localization in epithelia but in NBs, Dlg is required for localization of Scrib and Lgl, however the localization of Dlg is not affected in *scrib* and *lgl* mutants. It has also been reported that all *scrib, dlg and l(2)gl* mutants show an apical domain smaller than in control conditions, resulting in symmetric or inverted cell divisions (Albertson and Doe, 2003). Hence, these three proteins are important not only regulating cortical polarity but also controlling cell size asymmetry and mitotic spindle asymmetry in NBs.

These works opened the door to continue studying the mechanism underlying tumor formation and since then, a lot of studies have come out relating how loss of cell

polarity drives tumor growth and invasion, mainly in epithelial tissues. In this sense and taking into account that cancer is a multistep process involving cooperation between oncogenic or TS mutations and interactions with the surrounding normal tissue, it was designed a model to reproduce the development of tumors in mammals. The system implied the use of clonal analysis that permits to produce a piece of mutant tissue surrounded of wild type cells. This approximation has been really useful because it has made possible to investigate how mutant tissue interacts with normal one. In addition, it has also allowed to describe the loss of function phenotype of genes that opposite to the case of *dlg, l(2)gl* and *scrib*, are embryonic lethal, or dye before reaching the larval stage development to study the consequences of its mutation, when they are in homozygosis. This is, for instance, the case of our gene of interest, *cno*. *cno* null alleles are embryonic lethal and then we cannot study the effect of *cno* loss of function in larvae (LIII), but using clonal analysis we can skip this problem. Interestingly, when *scrib, dlg* or *l(2)gl* mutant clones were produced in imaginal discs, none of them were able to produce tumor growth alone (Igaki et al., 2006). First descriptions show that *scrib* mutant clones in the eye disc exhibit ectopic expression of cyclin E and ectopic cell cycles but clones do not overgrow due to increased cell death mediated by the Jun N-terminal Kinase (JNK) pathway and the surrounding wild type tissue. Thus, even though *scrib* mutant clones show in this context a loss of cell polarity and start to proliferate, the surrounding wild type tissue prevents this overgrowth by the JNK pathway that mediate the apoptosis of the mutant cells. In contrast, when oncogenic (constitutively activated) forms of Ras (Ras^{v12}) or Notch (N^{intra}) are overexpressed within the *scrib* mutant clone, cell death is prevented and neoplasms develop (Brumby and Richardson, 2003). The metastatic behavior has been another aspect investigated in these tumors. These cell polarity genes (*scrib, dlg* or *l(2)gl)* cannot drive metastatic behavior alone or in combination with other tumor-initiating alterations. But, the cooperation between oncogenic Ras^{v12} overexpression and the inactivation of any of these polarity genes leads to metastatic behavior, including basement membrane degradation, loss of E-cadherin expression, migration, invasion and secondary tumor formation (Pagliarini and Xu, 2003). Few years later these researchers also demonstrated that the metastatic behavior reported in those tumors was due to JNK pathway. Thus, mutation of different apical-basal polarity genes activates JNK signaling and then activated JNK cooperate with Ras^{v12} in promoting tumor

growth cell autonomously. Additionally, in this cooperative context, JNK signaling switches its proapoptotic role to a progrowth effect, being essential for tumor invasion and metastasis (Igaki et al., 2006). It is important to mention that Ras^{v12} alone only induces non-invasive moderate overgrowths in epithelia. Therefore, only the combination of these two conditions, loss of cell polarity and Ras^{v12}, promotes both tumor growth and invasion. Going further in the analysis, it has been proved that this cooperation between mutations is not only a cell autonomous effect. Clones of cells bearing different mutations can cooperate to promote tumor growth and invasion in *Drosophila*. Ras^{v12} and *scrib* mutations can also cause tumors when they affect different adjacent epithelial cells (Wu et al., 2010), demonstrating an interclonal oncogenic cooperation. The same work shows that $scrib^{-}/Ras^{v12}$ clones upregulates the *unpaired* genes (*upd, upd2* and *upd3),* which encode JAK/STAT-activating cytokines related to interleukin 6. In fact, and elevated expression of JAK/STAT reporter STAT-GFP is detected, thus correlating high expression of Upd cytokines with increased JAK/STAT activity. They show that induction of Upd cytokines was due to JNK signaling in $scrib^{-}$ cells, placing JAK/STAT signaling downstream of JNK. $scrib^{-}$ clones cause JNK activation both autonomously and non-autonomously. In imaginal discs, wounding also induces JNK activation that is able to propagate away from injured zone. Thus, stress-induced JNK signaling can contribute to tumor development in flies. Notably, tissue damage caused by conditions such as chronic inflammation has been linked to tumorigenesis in humans (Wu et al., 2010). In the model proposed, damaged tissue activates the JKN pathway to eliminate the injured/mutants cells and a compensatory growth, mediated by JAK/STAT-activating cytokines, to ensure normal tissue homeostasis. But, if this lesion appears in a Ras^{v12} background, JNK signaling switches its pro-apoptotic role to a pro-growth role, which leads to tumor and metastatic behavior (Figure 9).

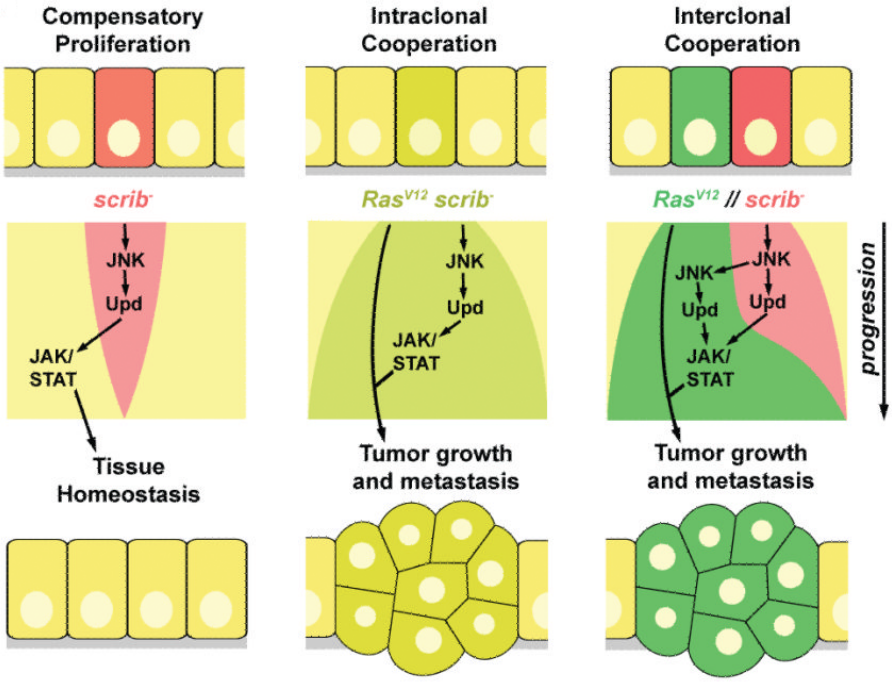

Figure 9. Model for the involvement of JNK and JAK/STAT signalling in intraclonal and interclonal cooperation between *Ras*^{v12} and *scrib*$^{-}$. Compensatory proliferation. *scrib*$^{-}$ mutant siuation provokes the activation of JNK pathway that mediates apoptosis in this cells. Then the upregulation of the diffusible Upds are able to activate JAK/STAT signalling in surrounding normal tissue to compensate the effect. **Intraclonal cooperation**. *scrib*$^{-}$ triggers JNK activation, that in *Ras*^{v12} background mediates JAK/STAT activation trough Upds mediating tumor growth and metastasis. **Interclonal cooperation**. JNK and Upd signaling are diffusible between cell that provokes that cell carrying different mutations (*Ras*^{v12} and *scrib*$^{-}$) cooperate to produce tumor growth and metastasis *(Taken from (Wu et al., 2010)*.

Another pathway that is implicated in tumor growth is the Hippo pathway (Doggett et al., 2011). It is the dual role of the Hippo pathway in negatively regulating both cell proliferation and survival that makes its loss such a potent driver of tissue overgrowth. The pathway is regulated through input from upstream components including Merlin and Expanded (Exp), and the transmembrane proteins Fat (Ft) and Dachsous (Ds). It is proposed that the primary function of the Hippo pathway is to incorporate positional cues within an epithelial field to dictate the ultimate size of organ development. The pathway is highly conserved and also functions to restrain organ size in mammals. Components of the Hippo pathway have been reported as a hyperplastic TSs, such as Hippo (Hpo) and Warts (Wts) kinases, and their adaptors proteins, Salvador (Sav) and Mob-As-Tumor-Suppressor (Mats). Hpo phosphorilates and activates Wts, and activated Wts phosphorylates and thereby inactivates the transcriptional co-factor Yorkie

(Yki) that, in this way, stays in the cytoplasm. Loss of Hippo components leads to reduced phosphorylation of Yki and, as a consequence, Yki translocates to the nucleus where it binds to its DNA binding partner, Scalloped (Sd) and promotes expression of proteins involved in cell proliferation as Cyclin E (CycE), cell growth as Myc and cell survival like *Drosophila* Inhibitor of Apoptosis 1 (DIAP1). The neoplastic TSs genes *scrib, dlg* and *l(2)gl* also interact with the Hippo pathway. Loss of *scrib* promotes eye and disc epithelial tissue overgrowth, as well as the cooperative neoplastic overgrowth with oncogenic Ras-Raf signalig mentioned above, through impaired Hippo pathway signaling and the consequent upregulation of Yki. Failure in the Hippo pathway is independent of the JNK signaling activation in *scrib* mutants, as it remains impaired even when the JNK signaling is blocked. Hippo pathway deregulation in *scrib* mutants do depend upon aPKC signaling, which is ectopically activated in these mutants. In fact, aPKC is sufficient to downregulate the Hippo pathway independently of the JNK signaling (Doggett et al., 2011). In sum, the impairment of the Hippo pathway, and therefore of Yki activation, contributes to the neoplastic transformation when *scrib* is mutated in an oncogenic Ras-Raf background. However and despite of such an important contribution of Hippo, knockdown of *yki* fails to rescue the loss of apical-basal cell polarity in *scrib*$^{-}$ mutants, the capacity of *scrib*$^{-}$*/Ras*^{v12} to invade, and to fully rescue tumor overgrowth in these double *scrib*$^{-}$*/Ras*^{v12} mutants. These evidences suggest that other deregulated pathways in *scrib*$^{-}$ mutants are likely important for promoting tumor overgrowth (Doggett et al., 2011). Although all these studies have been performed in epithelial developmental tissues, there are works that demonstrate *scrib*/Ras cooperation also in adult SCs, for instance in *Drosophila* malphigian tubules (Zeng et al., 2010).

Despite most studies have used the combination between *scrib* and *Ras*^{v12} to report oncogenic cooperation, the same effects have been described for the combination between *l(2)gl* and *Ras*^{v12} (Menendez et al., 2010) or Dlg and Ras^{v12} (Willecke et al., 2011) indicating that other polarity mutation can cooperate with *Ras*^{v12} to produce tumor growth and metastasis.

A conventional view of development is that cells cooperate to build an organism. In the same sense, cancer was considered for a long time as a strictly cell-autonomous process in which oncogenes and TS genes drive clonal cell expansion. However, it is currently widely accepted the existence, during normal development and homeostasis, of a process called cell competition, in which less fit cells are eliminated by surrounding normal cells. These less fit cells, named "loser cells" are viable and able to grow, but only when they are surrounded by other "loser cells". However, when they are mixed with "winner cells", they are at growth disadvantage and undergo apoptosis. Loser cells are considered slower-growing cells or structurally defective cells (Tamori and Deng, 2011). Tumors, far from being homogenous lumps of cells, consist of different cell types that function together and are communicated to each other. Interestingly, several studies have revealed that cells bearing mutations in TS genes, which show overgrowth and tumorigenesis in an homotypic situation, are frequently eliminated through cell competition from tissues in which they are surrounded by wild type cells (Tamori and Deng, 2011). Furthermore, new studies in mammals have revealed that this process is universal and that many factors and mechanisms are conserved (Amoyel and Bach, 2014). This implies a new level of complexity in which a research into this social cell biology behaviour is critical for understanding development of normal but also tumoral tissues, being ultimately this, a possible novel target for treatments.

4. THE PDZ DOMAIN-CONTAINING PROTEIN CANOE/AFADIN AND ITS ROLE IN ACD AND CANCER

PDZ domains are globular structures of about 90 aminoacids that are involved in protein-protein interactions. These domains recognize specific short peptide sequences, normally at the C-terminus of their interacting partners, but can also interact with internal protein motifs, other PDZ domains and even with lipids. Most PDZ proteins are cytoplasmic and closely associated to the plasmatic membrane at specific locations such as cellular junctions or synapses. PDZ proteins are considered scaffold-proteins because

multiprotein complexes can be formed around them. Furthermore, these PDZ domain-containing proteins can contribute to anchor cytosolic proteins to the plasmatic membrane and to cluster receptors and channels at the membrane in specific domains, controlling the rate and fidelity of signal transduction. In addition, these proteins normally contain additional protein-protein interaction domains involved in regulating signaling events. Thus, all these characteristic make PDZ proteins good candidates to modulate signaling networks (reviewed (Carmena, 2008). Indeed, it has been shown that PDZ proteins can display direct and more dynamic functions regulating signaling events in addition to their established role as static scaffolds (Carmena et al., 2006).

The PDZ protein Cno is a cytoplasmic protein associated to adherens junctions (AJs) in epithelial tissues where interacts with different proteins, such as Polychaetoid (the *Drosophila* ZO-1), Rap1, Echinoid (nectin),DE-Cad and F-actin (O'Keefe et al., 2009) (Takahashi Mech Dev 98; Sawyer JCB 2009; Wei et al., Dev Cell 2005) (Figure 10).

Cno participates in multiple developmental and morphogenetic processes, such as muscle, eye, bristle and wing development and epidermal dorsal closure, physically or genetically interacting with different signaling pathways, including Ras-Mitogen Activated Protein Kinase (MAPK), JNK, Notch or Wingless (Wg) pathways (Carmena et al., 2006; Matsuo et al., 1997; Matsuo et al., 1999; Miyamoto et al., 1995; Takahashi et al., 1998) Gaengel and Mlodzik, Development 2003; O´Keefe et al 2009, Sawyer. Et al, JCB 2009).

Cno is an example of PDZ protein that has been shown to mediate signaling pathway cross-communication, as mentioned above. Specifically, Cno modulates Wg/Wnt, Ras-MAPK and Notch signaling pathways cross-communication. Cno has a repressive effect on all these three signaling pathways by physically interacting with Ras, Notch and the cytoplasmic protein Dishevelled (Dsh) (a key effector of Wg/Wnt), coordinating at the membrane level these interactions during muscle/heart progenitor specification in the *Drosophila* embryo (Carmena et al., 2006). Other functions of Cno that have been shown are its implication in neuron-glia interaction (Slovakova and Carmena,

2011), axon guidance at midline (Slovakova et al., 2012) and glial-neuroephitelial cell interactions during optic lobe development (Perez-Gomez et al., 2013).

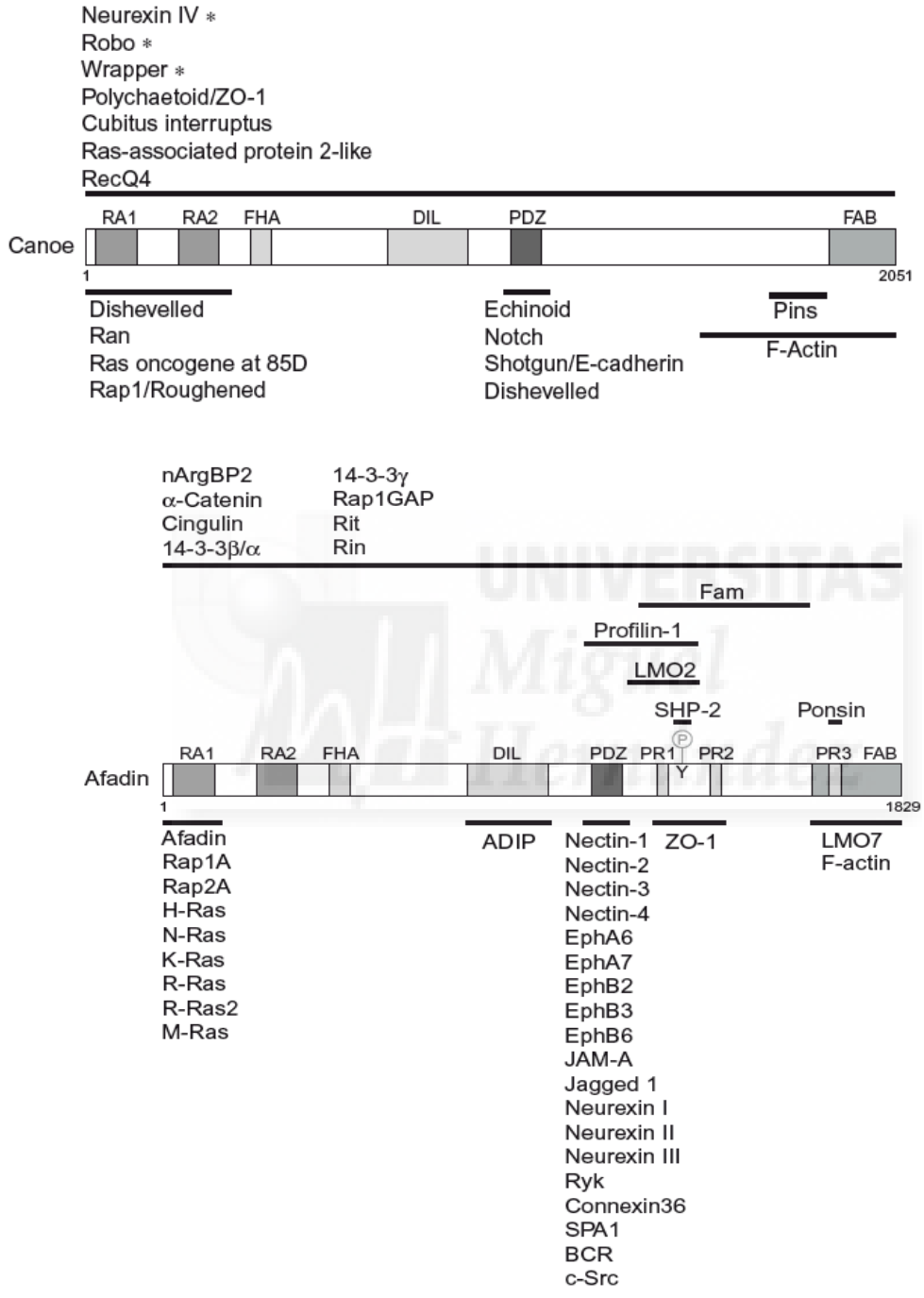

Figure 10. Domains and binding partners of Canoe (top) and Afadin (bottom). First and last amino acids are numbered. The following binding domains are shown: RA1, RA2, Ras-associated domain-1 and -2; FHA, forkhead-associated domain; DIL, dilute domain; PDZ, PDZ domain; PR1, PR2, PR3, proline-rich domain-1 to -3; FAB, F-actin-binding domain. *It is not known whether the interaction is direct or indirect. *(Extracted from (Mandai et al., 2013).*

The first evidence of Cno as a key regulator of ACD was reported in 2008 (Speicher 2008). Cno localizes apically in metaphase embryonic NBs and functions as a new component of the apical complex during asymmetric NB division forming a complex *in vivo* with Pins and functionally interacting with Insc, Gαi and Mud. Cno contributes to regulate key processes in the asymmetrically dividing NB, such as the localization of cell-fate determinants, the orientation of the mitotic spindle and the generation of unequal-sized daughter cells (Speicher et al., 2008). Later, it has been demonstrated that Cno directly binds to Pins tretratricopeptide repeats (TRPs) domain. This interaction is essential for recruiting Mud to Pins, which binds Mud through the same domain ($Pins^{TRP}$-Mud interaction), and thus for the spindle orientation (Wee et al., 2011). The Ras-like small GTPase Rap1, which binds Ras–associated domains (RA) (Figure 10) also contributes to regulate asymmetric NB division through the Ral guanine nucleotide exchange factor Rgl, Ral and Cno. This Rap1–Rgl–Ral signaling network cooperates with other apical proteins to regulate cortical polarity and spindle orientation in NBs (Carmena et al., 2011). Cno^{RA} domains have also been shown in S2 cells to bind directly RanGTP and to promote Mud recruitment to the cortical Pins domain required for the mitotic spindle orientation pathway (Wee et al., 2011). The apical protein Insc also binds the PinsTPR domain. How all these interactions (Insc-PinsTPR, Cno-PinsTPR and Mud-PinsTPR) are temporallay coordinated remains to be explored (Figure 11). Altogether this data strongly demonstrates that Cno is an important regulator of ACD in *Drosophila* embryonic NBs.

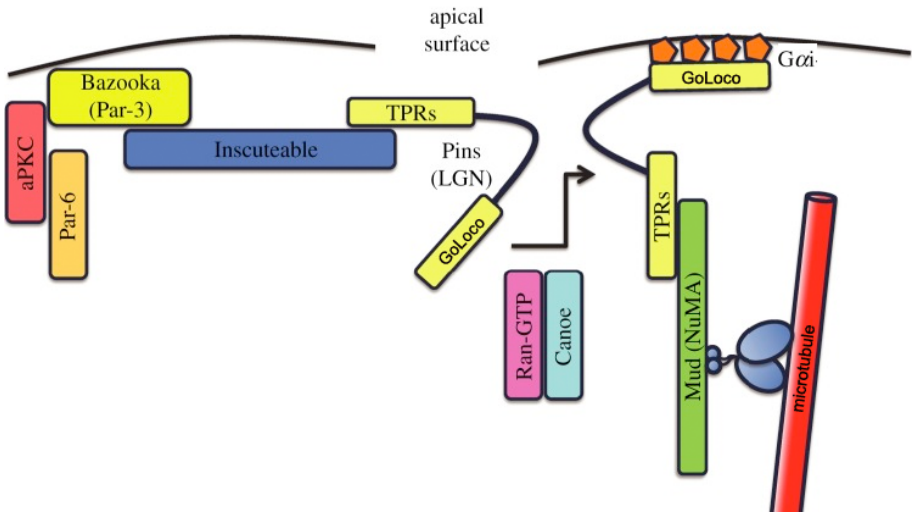

Figure 11. Spindle orientation pathway needs the cooperation between several proteins. Spindle orientation in NBs relies on Pins-Gαi recruitment of Mud. Inscuteable apical localization

requires Bazooka (Par-3), a component of the apical Par-6/aPKC/Bazooka complex. First, Inscuteable recruits Pins apically by binding to $Pins^{TPR}$ domain, to be replaced subsequently by Mud, which also binds to the Pins N-terminal TPR motif. Cno, in cooperation with Ran-GTP, also binds to the $Pins^{TPR}$ domain to help recruit Mud. This specific interaction, as the timing, is unclear. Pins C-terminal domain (GoLoco) binding to Gαi facilitates membrane anchoring of the Pins/Mud/Gαi tripartite complex and spindle orientation (Taken and modified from (Bergstralh et al., 2013).

Recent work performed in our lab has contributed to clarify more details about this dynamic process. We have found a novel role of Hippo pathway in asymmetric cell division in *Drosophila* embryonic NBs. We have demonstrated that Wts, a kinase member of the Hippo pathway, phosphorylates Cno and this phosphorylation event contributes somehow to mediate the exchange between Pins/Insc to Pins/Mud and to recruit other proteins to the latter complex required for spindle orientation (Keder et al., 2015).

The human ortholog of Cno, Afadin, encoded by the *MLLT4* gene (alias *AF-6*) was initially identified as a fusion partner of the Mixed-Lineage Leukemia (*MLL)* gene (alias *ALL-1*), resulting the fusion product MLL-AF6 (Figure 12), which is involved in human leukemias (Prasad et al., 1993). *MLL* gene encodes a transcriptional regulator and is required for hematopoiesis (Ernst et al., 2004). *MLL* is a frequent target of chromosomal translocations associated with particularly aggressive acute leukemias, including acute lymphoid (ALL), myeloid (AML), bi-phenotypic (ABL) and chemotherapy-related secondary leukemias that affect children and adults. These genetic events result in the consistent production of novel, dominant-acting oncogenic proteins in which MLL is fused with one of at least 50 distinct novel partner proteins dependent on the loci that participate in the translocation. The translocation forms fusion genes in which the truncated form of MLL and the partner gene are fused in frame, leading to a gain of function of MLL-fusion gene complexes. The fusion partners can be broadly classified into cytoplasmic proteins and nuclear proteins. Afadin, in normal conditions, is a cytoplasmic protein abundant at Cadherin-based AJ´s in epithelial cells (like Cno), endothelial cells and fibroblasts. It contains multiple domains and interacts with many proteins, including cell adhesion molecules and their associated molecules and signaling molecules. Cno and Afadin share a similar structure (Figure 10): one PDZ motif (Ponting et al., 1997); a Forkhead (FHA) or

Kinesin-like domain and a Dilute (DIL) or Myosin-V-Like domain, both characteristic motifs present in proteins that interact with cytoskeleton components (Ponting, 1995); and two Ras-associating domains (Ponting and Benjamin, 1996), through which Cno/Afadin binds to the activated form of Ras (Kuriyama et al., 1996) an other small GTPases. Afadin has been reported to participate in the formation of cell junctions, cell polarization, migration, survival, proliferation and differentiation. In addition, as we mentioned before, it is also involved in oncogenesis and metastasis (review in (Mandai et al., 2013). The ability to activate MLL oncogenesis relies on RA1 Afadin domain, which it is conserved in *Drosophila* (Liedtke et al., 2010). The RA1 domain of Afadin mediates its self-association, which activates the oncogenic potential of MLL-AF6 fusion protein in myeloid progenitor cells. (Liedtke et al., 2010). However, it is unknown if Afadin by itself plays a role in normal hematopoiesis and leukemogenesis.

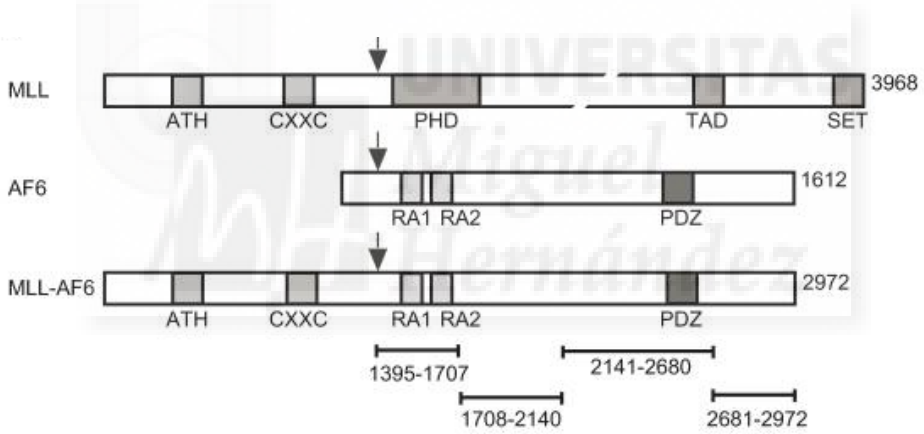

Figure 12. Schematic diagram depicting MLL, AF6, and MLL-AF6 fusion protein. ATH indicates AT hook motifs; PHD, plant homeo-domain related; TAD, transcriptional activation domain; SET, Suvar3-9/enhancer-of-zeste/trithorax motif. Total number of amino acids comprising each protein is indicated on the right. Black arrows above each protein indicate the typical position of protein fusion after chromosomal translocations. Specific AF6 protein segments fused with MLL are indicated by brackets below the schematic *(Taken from (Liedtke et al., 2010).*

A recent work published in 2014 has demonstrated the negative regulation of Ras by Afadin in myeloid leukemia (Manara et al., 2014). They show that Afadin is normally expressed in the cytoplasm of healthy bone marrow cells and controls Ras-GTP levels. By

contrast, in MLL-AF6 rearranged cells, MLL induces the localization of Afadin in the nucleus, leading to aberrant activation of RAS in the cytoplasm and of its downstream targets. After silencing MLL-AF6, Afadin localization becomes again cytoplasmic and there is a significant reduction of RAS-GTP levels. Hence, MLL-AF6 oncoprotein potentiates the activity of the RAS pathway through retention of AF-6/Afadin within the nucleus (Manara et al., 2014). All these data together confirm the high conservation of the relationship between Ras and Cno/Afadin from flies until humans and support the use of RAS a target for novel potential therapeutic strategy in patients carrying the translocation t(6;11).

Afadin also plays a role in oncogenesis in solid tumors. Loss of Afadin has been reported in 15% of breast cancer cases and is associated with adverse prognosis, suggesting that *MLL4* might be a tumor suppressor gene (Letessier et al., 2007). In fact, downregulation of Afadin increases tumorigenecity and metastasis in mice (Fournier et al., 2011) and increases invasive behavior in cultured breast cancer cells (Chatterjee et al., 2012). The 300kb segment of human chromosome 6q27 in which *MLL4* gene locus is located is commonly deleted in ovarian cancer (Saito et al., 1996). However, the role of *MLL4* gene in oncogenesis of ovarian cancers is not clear.

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OBJECTIVES

MAIN OBJECTIVE

To analyze a potential function of the asymmetric cell division regulator *cno* in tumorigenesis.

To investigate this hypothesis, we divided the work in the following parts:

1. To study in detail the expression pattern and localization of Cno in larval brain NBs.
2. To characterize the *cno* loss of function in larval NBIIs lineages.
3. To analyze functional relationships between *cno* and known tumor suppressor genes.

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RESULTS

1. *cno* IS EXPRESSED IN LARVAL BRAIN NBII LINEAGES

Previous works in our lab showed that *cno* is expressed in embryonic NBs where it plays an important role in ACD (Speicher et al). In this thesis work, we wanted to assess whether *cno* behaves, as other ACD regulators, as a TS gene. Hence, we first analyzed Cno expression in larval NBII lineages, the ones that are susceptible of overgrowth when ACD is compromised. To have a precise view of Cno localization, we generated transgenic fly lines with the endogenous *cno* gene tagged with GFP that allow us to see its whole expression profile. We found that Cno is detectable in the cytoplasm of NBs and INPs of NBII lineages (Figure 13 A,A'). Citoplasmatic expression of Cno in NBI lineages was also observed. However, not clear expression was found out in GMCs or neurons (Figure 13 B,B'). Using phosphohistone 3 (PH3) as a marker of dividing cells we investigated the dynamics of the protein in fixed material. We found that during metaphase, Cno was apically enriched forming a crescent in NBs and INPs. Furthermore, Cno expression was detected in both centrosomes during progenitor division, expression that is not observed in embryonic NBs (Figure 13 C,C',D,D').

2. Cno FUNCTIONS AS A KEY REGULATOR OF ACD IN NBII LINEAGES

To study the role of Cno in NBII lineages we started with loss of function experiments. *cno* null mutants are not viable in homozygosis; larvae die before arriving to LIII stage. Hence, we took advantage to the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique that permits to observe the development of GFP labeled null mutant groups of cells (clones) in an unlabeled wild type surrounding environment.

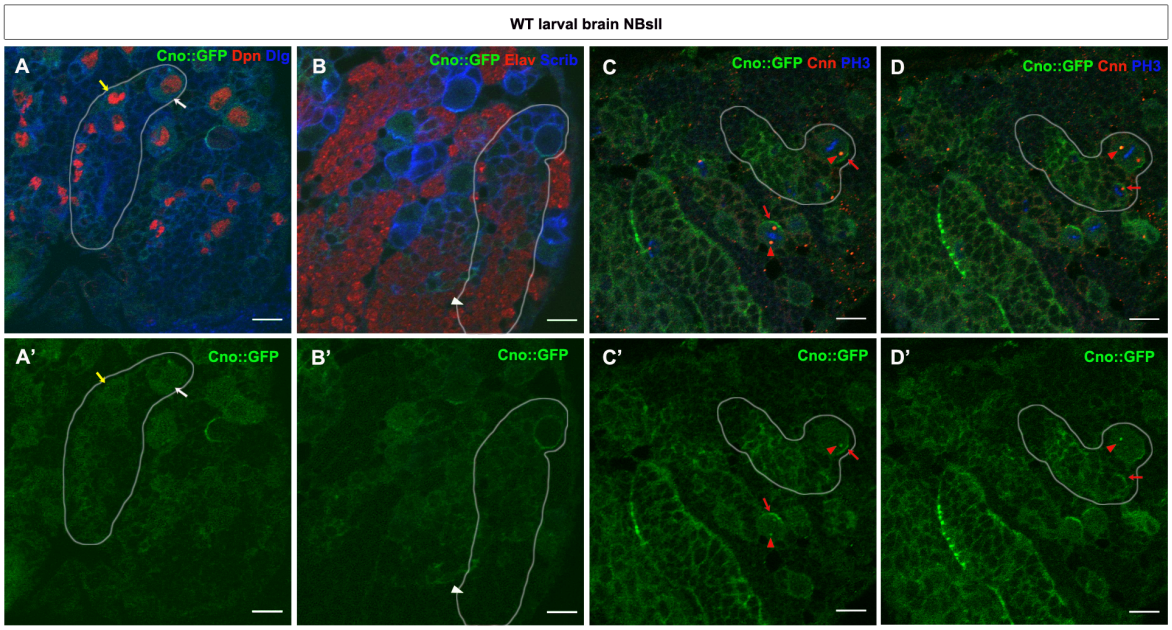
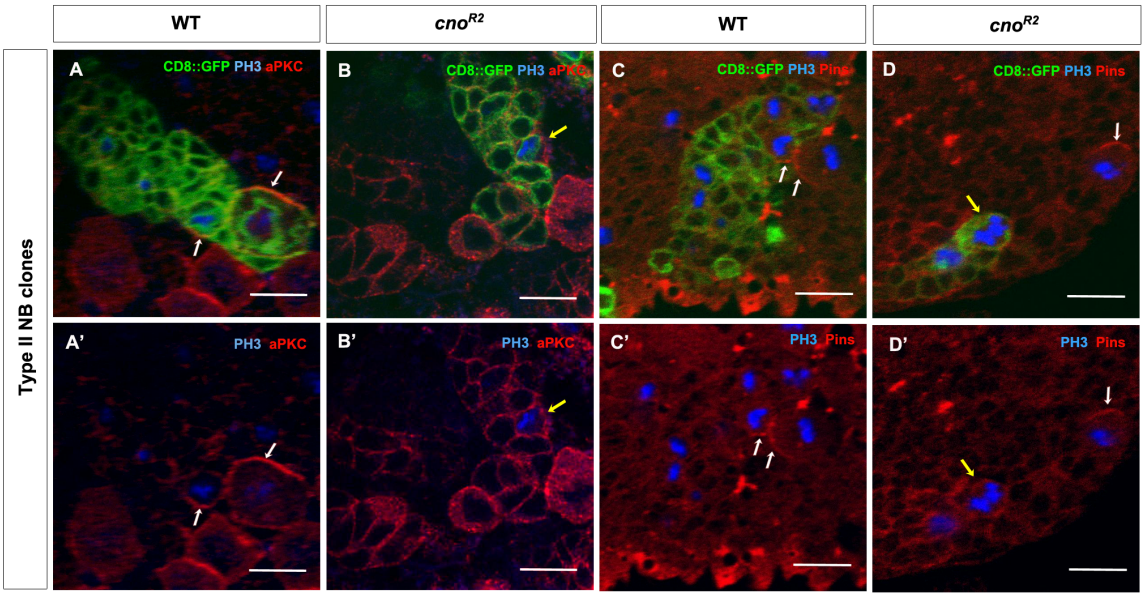


Figure 13. Cno is expressed in larval brain type II NB lineages. **A,A')** Cno is expressed in NBs (white arrows) and INPs (yellow arrows). **B,B')** Cno is not detected in differentiated cells (neurons; white arrowheads). **C,C',D,D')** Cno forms crescents in metaphase NBs and INPs (red arrows) and it is also detected in centrosomes (red arrowheads). Scale bar: 10µm.

2.1. Cortical localization of ACD regulators is altered in progenitor cells of *cno^{R2}* mutant clones

Due to the known role of Cno in ACD in embryonic NBs and its presence and dynamics during NBII division, we first wanted to analyze the effect of *cno* loss of function in NBII ACD. To test if this process was altered, we used as reporters, regulators of ACD with well-known dynamics. While in WT conditions aPKC (n=18) and Pins (n=28), two key ACD regulators, showed an apical enrichment (apical pole in cells is defined in WT conditions by the presence of apical components as these two) in most metaphase NBs and INPs analyzed, in *cno^{R2}* mutant clones the restricted apical localization of aPKC and Pins failed in 85% (n=20) and 50% (n=32), respectively, of the progenitors analyzed (Figure 14). Another apical component is Dlg, which whereas in WT conditions did not show alteration during ACD (n=18) in *cno^{R2}* mutant clones was mislocalized in 23.8% (n=42) of the progenitors analyzed. The apical protein Baz also showed, to a lesser extent, distribution defects (13.4%, n=15, compared with 0.1% defects, n=26, found in WT clones). The basal localization of the cell fate determinant

Numb was not affected (Figure 14 E). These results suggest that Cno also functions in larval NBII ACD.



E

	WT				cno^R2			
	n	Failures(%)		Total (%)	n	Failures (%)		Total (%)
		absent (%)	cortical mislocalization (%)			absent(%)	cortical mislocalization (%)	
aPKC	18	0	11.1	**11.1**	20	0	85	**85**
Baz	26	0	0.1	**0.1**	15	6.7	6.7	**13.4**
Pins	28	21.4	0	**21.4**	32	21.9	28.1	**50**
Dlg	18	0	0	**0**	42	0	23.8	**23.8**
Numb	13	0	0	**0**	10	0	0	**0**

Figure 14. Cortical localization of ACD regulators is altered in progenitor cells of *cno*^{R2} mutant clones. **A,A',C,C')** WT NBsII clones show apical crescents of aPKC and Pins at metaphase in progenitors (NBs and INPs; white arrows). **B,B',D,D')** In *cno*^{R2} mutant clones both aPKC and Pins are mislocalized all around the cellular cortex (yellow arrows). **E)** Quantification of the phenotypes analyzed in WT (control) and *cno*^{R2} mutant clones for the ACD regulators indicated; n=number of metaphase/anaphase progenitors analyzed. Scale bar: 10µm.

2.2. *cno*^{R2} mutant clones show altered cellular composition and lineage development

After showing that ACD was not functioning properly in *cno*^{R2} mutant clones we wanted to further investigate the implications of these failures in the clone lineage development. First of all, we observed that the NB of *cno*^{R2} NBII mutant clones displayed a significant reduced size compared with the NB of WT clones (Figure 15). In WT clones, the NB presented a diameter of 13.4 ± 1.91 µm, while in *cno*^{R2} clones the

NB was reduced until 8.2 ± 1.19 µm (n= 15 clones per genotype), almost the half part of the normal size of a WT NB. It is known that after every cytokinesis, the new NB has to recover its normal size to continue dividing. Therefore, this observation suggests that *cno* is also implicated in the maintenance of NB size along division period.

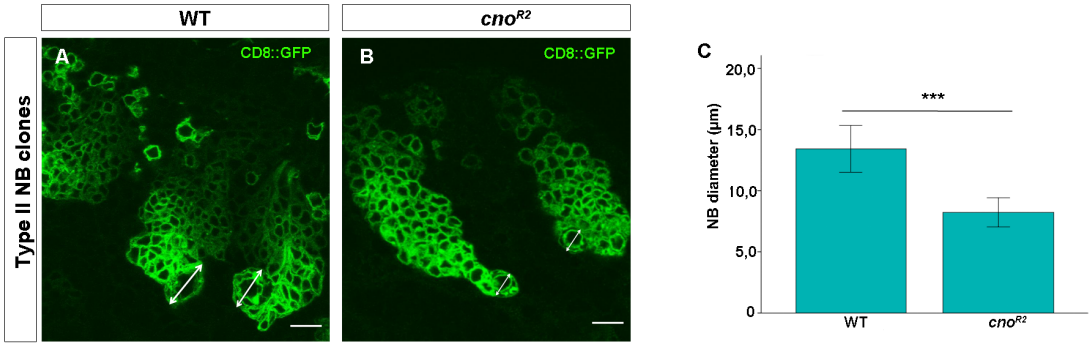


Figure 15. *cno*^{R2} mutant clones show a reduced NB size A-C) NB diameter is significantly reduced (P=0.000) in *cno*^{R2} mutant clones compared with WT NB diameter. Scale bar: 10µm.

Next, we studied in detail the cell population of *cno*^{R2} mutant clones, analysis that revealed more alterations in NBII lineage development. For example, an ectopic "NB-like" cell was occasionally detected in *cno*^{R2} mutants, appearing two or even three Dpn, PntP1 positive cells (n=15 clones). WT NBII clones always contained only one NB (that is the only cell that it is double marked by the transcription factors Dpn and PntP1) (n=13 clones) (Figure 16 A-C). In addition, WT NBII clones had a mean of 24 mature INPs (recognized by the simultaneous expression of the transcription factors Dpn and Ase) (n=14 clones), 19 GMCs (Ase and Pros positive cells) (n=13 clones) and hundreds of neurons (Elav positive cells). By contrast, the cell population of *cno*^{R2} NBII mutant clones was clearly reduced, presenting a mean of 17,5 mature INPs (n=15 clones) and 12.9 GMCs (n=19 clones) (Figure 16 D-H). Furthermore, the shape and the development of these mutant clones were altered, probably as a consequence of all these changes. For example, in WT NBII clones the most differentiated cells are located distal to the NB, in the final part of the clone called "clone tail". Only a small population of neurons generated in the first division event during LI, the primary neurons, is located in the medial part of the clone. Thus, along the division period, the

clone grows generating a loop where cells are placed far away from the NB as more differentiated they are. (Figure 17 C).

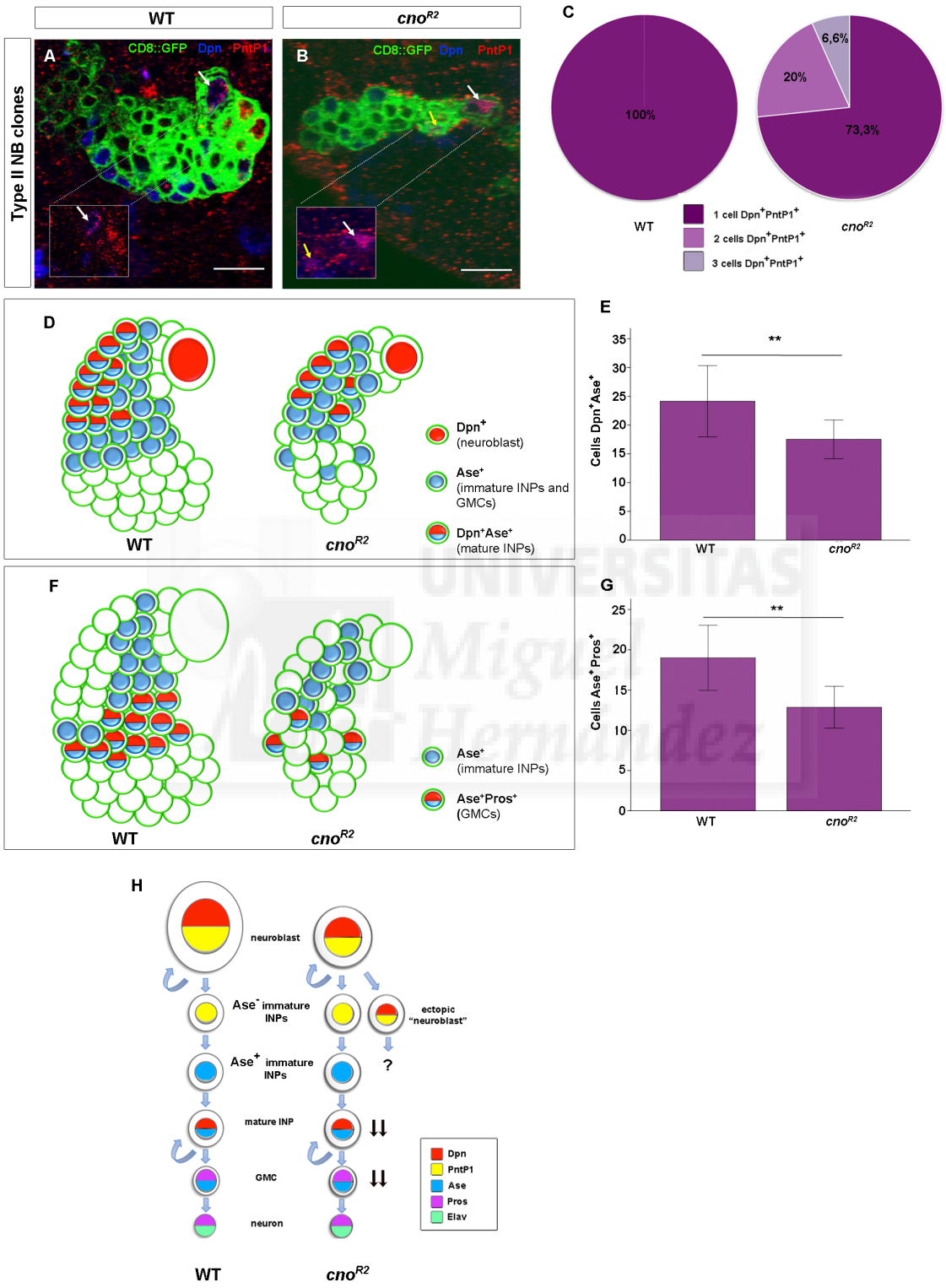


Figure 16. Cellular composition is modified in *cno*^{R2} mutant clones compared with WT clones A-C) In *cno*^{R2} mutant clones "ectopic NBs" (yellow arrow) are occasionally detected (P=0.049) (white arrows indicate the original NB of the clone). **D)** Diagram representing the relative number and distribution of mature INPs in WT and *cno*^{R2} mutant clones. **E)** The

number of mature INPs is significantly lower (P=0.002) in *cno*^{R2} clones compared with WT clones. **F)** Diagram illustrating the relative number and position of GMCs in WT and *cno*^{R2} clones. **G)** *cno*^{R2} mutant clones contain fewer GMCs than WT clones (P=0.002). **H)** Representation of the temporal expression profile of molecular markers in WT and *cno*^{R2} type II NB lineages; in *cno*^{R2} mutant lineage (black arrows represent the decreased number of INPs and GMCs found in these mutants). Scale bar: 10µm

By contrast, *cno*^{R2} mutant clones showed a different development where two main morphologies were detected: (1) a thin clone where the loop was almost lost (Figure 17 D) or (2) a clone where the NB ended up surrounded by the other cells (Figure 17 E). In both cases, *cno*^{R2} mutant clones seemed to be smaller than the WT ones. But the fact that made more evident the wrong development of these mutant clones was the presence of differentiated cells, neurons, in direct contact with the NB (Figure 17 A-E). All these data together strongly support that ACD is disrupted in *cno*^{R2} mutant clones, this leading to changes in cell identity, cellular composition and, consequently, to alterations in the morphology of mutant clones.

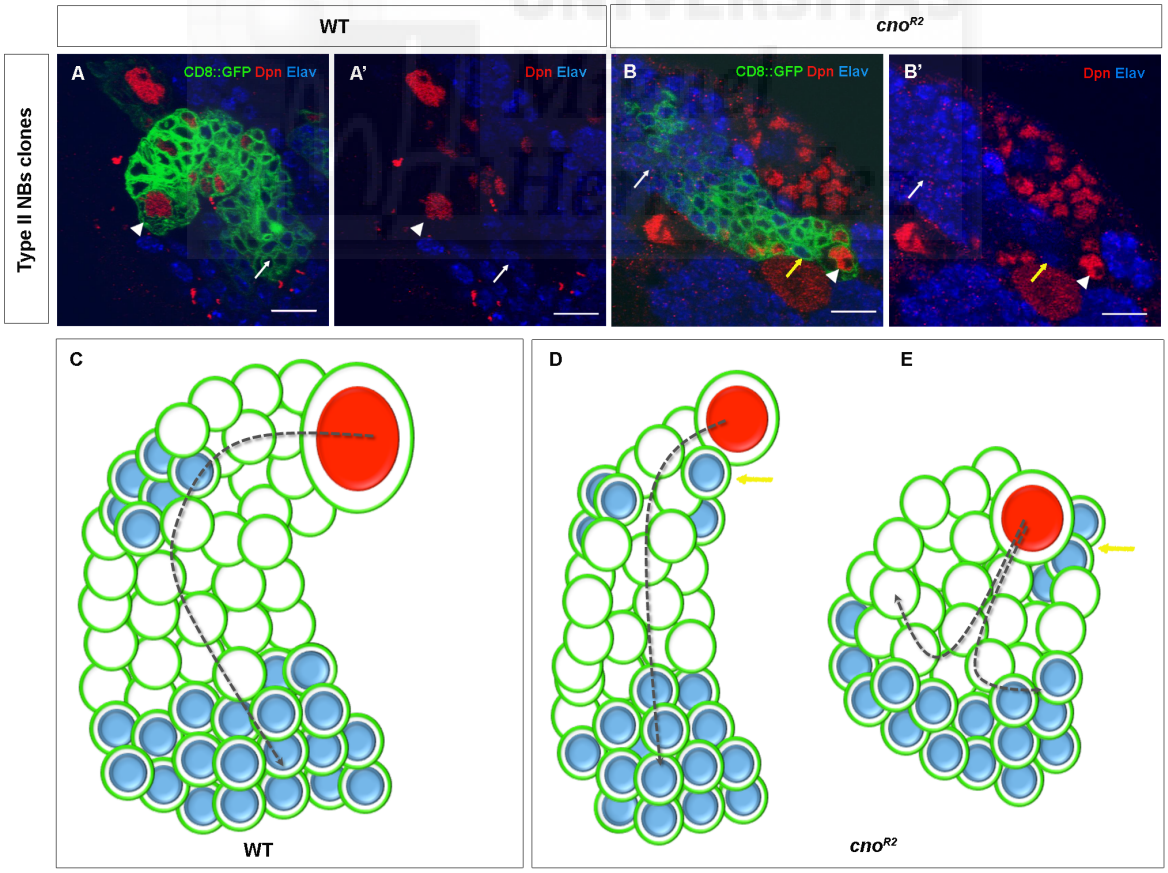


Figure 17. *cno*^{R2} mutant clones show aberrant shape and development. A,A') Differentiated cells (Elav^{+} neurons) are located in the distal part of the clone (white arrows), far from the NB (white arrowhead). Only primary neurons appear in the medial part on the

clone. **B,B')** In *cno*^{R2} mutant clones some neurons appear directly in contact with the NB (yellow arrows). **C-E)** Drawing representing the way the development (grey discontinuous line) of a WT (C) or *cno*^{R2} mutant clones (D,E) where neurons are found in unusual position (yellow arrows). Scale bar: 10µm.

2.3. Mitotic index of INPs is lower in *cno*^{R2} mutants than in WT clones

Trying to understand the reduction of the cell number in *cno*^{R2} NBII mutant clones, we investigated the cell cycle of mature INPs. INPs normally divide asymmetrically several times to continue producing more INPs and also GMCs that will give rise to neurons. Therefore, they produce the majority of cells that compound NBII clones. First of all we decided to investigate if INPs of *cno*^{R2} NBII mutant clones were normally dividing. To test this we first used CycE as a marker of the progression between G1 and S phase in the cell cycle. We counted the number of progenitor cells (Dpn$^+$, excluding the NB) that were CycE positive, finding not significant differences between the number of progenitors that were active in cell cycle between WT and *cno*^{R2} mutant clones (Figure 18 A). To know if these INPs were progressing until M phase (mitosis), we considered the number of Dpn$^+$ cells (excluding the NB again) that were actually dividing (PH3$^+$) respect to the total Dpn$^+$ cells, both in WT and *cno*^{R2} mutant conditions. In this case, whereas in WT clones 23% of INPs were dividing (n=12), only 17% of INPs (n=10) were found in mitosis in *cno*^{R2} mutant clones (Figure 18).

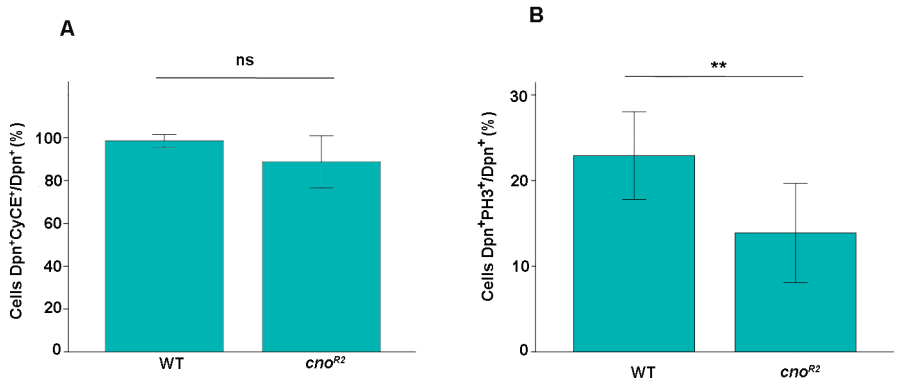


Figure 18. INPs of *cno*^{R2} mutant clones have lower mitotic index than WT clones. A) The percentage of INPs (Dpn$^+$) that are active in cell cycle (CyCE$^+$) is not significantly different in *cno*^{R2} mutant clones compared with WT clones. **B)** The mitotic index (number of cell in mitosis, PH3$^+$) of INPs in *cno*^{R2} mutant clones is significantly lower than in WT clones (P=0.001).

This result suggests that INPs in *cno*^{R2} mutant clones are dividing less than WT ones. Therefore, despite INPs in mutant clones are able to enter into the cell cycle, it may be taking place some block or delay to arrive to M phase, resulting in a mitotic index lower that in WT clones. This result contributes to explain the cell population reduction in *cno*^{R2} mutant clones.

3. *cno* AND *scrib* LOSS SYNERGISTICALLY INTERACT TO PROMOTE TUMOR-LIKE OVERGROWTH IN LARVAL BRAIN

Our analysis of *cno*^{R2} NBII mutant clones revealed that they not only do not overgrow but also have even fewer cells than WT clones. This result would suggest that *cno* is not acting as a TS gene. However, mutant clones of different genes that have been categorized as TS genes, do not overgrow either. There are several reasons that contribute to explain this fact. The environment (i.e. the WT cells that surround the mutant clone) can avoid the appearance of a tumor through a process known as "cell competition" (see Introduction). Furthermore, cancer is a multistep process that normally is produced by an accumulation of several mutations, which on the whole permit to ignore the control growth signals of the system. Taking all of this into account, we decided to start investigating whether *cno* could be cooperating with other TS genes whose mutations by themselves do not produce an overgrowth either.

3.1. *scrib* NBII mutant clones do not overgrow

scrib is a well-studied TS gene. Despite the fact that null *scrib* mutations produce overgrowth in homozygous *scrib* mutant tissues, *scrib* mutant clones are not able to overgrow and finally die through apoptosis mediated by the JNK-pathway. Different groups have showed these results in epithelial tissues (imaginal discs)(Bilder and Perrimon, 2000; Brumby and Richardson, 2003). Thus, first we wanted to investigate the behavior of *scrib* mutant clones in our system. For this clonal analysis we also used the MARCM technique. While WT clones were detected in 33% of the brains analyzed (n=39), *scrib*^{1} NBII mutant clones were present only in 6% of brains analyzed (n=36). This fact already suggested that *scrib*^{1} mutant clones were dying. Indeed, in the small

number of *scrib*[1] NBsII mutant clones still detected, the JNK-pathway was up-regulated (Figure 19).

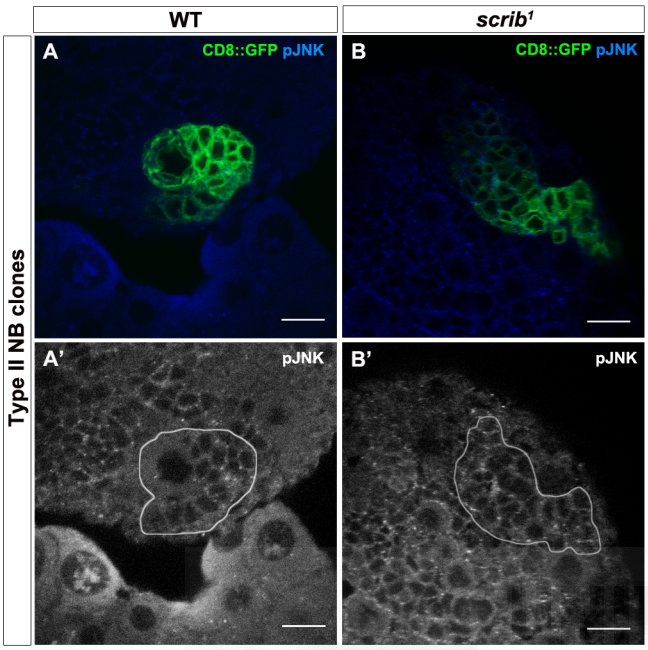


Figure 19. *scrib*[1] NBII clones do not overgrow and are eliminated through JNK pathway-mediated apoptosis. A-B') *scrib*[1] mutant clones (B,B') present un up-regulation of pJNK (white arrows) compared with WT clones (A,A'). Scale bar: 10µm.

These data support the idea that *scrib*[1] NBII clones are dying by the action of the JNK-pathway in the brain as it happens in epithelial tissues.

3.2. *cno*[R2] *scrib*[1] NBII clones overgrow

As we mentioned before, normally cancer appears as a summation of failures in a system. According to this, it has been demonstrated that the combination of mutations in *two TS genes* can lead to hyperplasia (Pagliarini and Xu, 2003) or the mutation in a TS gene and the up-regulation of an oncogene can produce a malignant tumor (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). In addition, a big number of proteins involved in ACD have been characterized as TSGs. In fact, both *scrib* and *cno* are ACD regulators. Taking all of these into account, we wanted to analyze whether the combination of the loss of function of this two genes, *cno* and *scrib,* could lead to tumor formation.

We also performed MARCM clone analysis in LIII larval stage for this study. First of all, we observed that *cno*^{R2}*scrib*^{1} double mutant clones did not die, as *scrib*^{1} single mutant clones did, neither were as small as *cno*^{R2} clones. Conversely, *cno*^{R2}*scrib*^{1} highly overgrew. To analyze this in detail, we started comparing the area that WT, *cno*^{R2} and *cno*^{R2}*scrib*^{1} NBII mutant clones occupied respect to the hemisphere area. While a WT NBII clone (n=23) occupied normally around 2,9% of the brain lobe plane analyzed, *cno*^{R2}*scrib*^{1} NBII clones (n=28) colonized the 4,2% of the brain hemisphere. Moreover, some *cno*^{R2}*scrib*^{1} double mutant clones massively overgrew, taking up the 10% (n=7) of the brain lobe. In addition, we verified that *cno*^{R2} mutant clones (n=20) were smaller than WT, occupying the 2,3% of one brain lobe (Figure 20 A-D). Thus, this study reveal that double mutant *cno*^{R2}*scrib*^{1} NBII clones are able to overgrow producing tumor-like masses in late LIII larval stage.

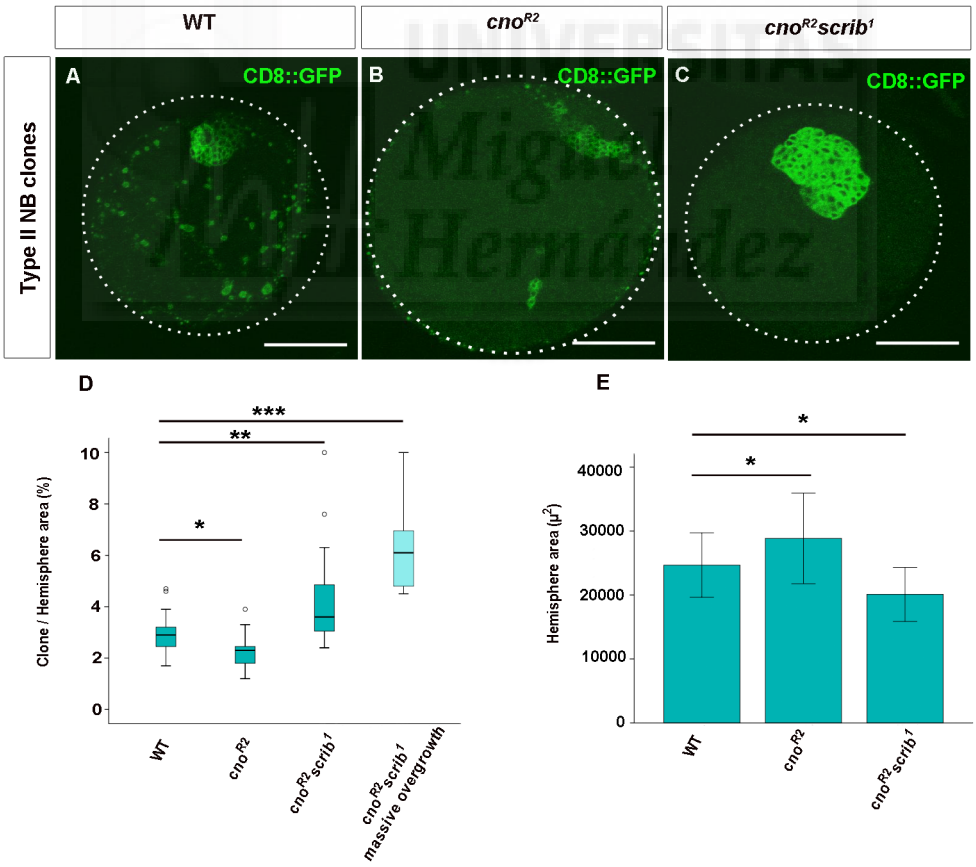


Figure 20. Simultaneous loss of function of *cno* and *scrib* promotes overgrowth in NBII clones. A-C) Overview of clones in a brain hemisphere. **D)** The percent of space occupied by *cno*^{R2} clones is smaller than in WT clones (P=0,013), whereas *cno*^{R2}*scrib*^{1} double mutant clones significantly overgrow compared with WT clones (P=0,002 and P=0,000, in whole population of *cno*^{R2}*scrib*^{1}

and the percentage that massive overgrow respectively). **E)** Brain lobes carrying *cno^{R2}* or *cno^{R2}scrib^{1}* mutant clones are bigger (P=0,037) or smaller (P=0,01), respectively, than brain lobes with WT clones. Scale bar: 50µm

Furthermore, we observed that not only the clone size was altered; the hemisphere area was reduced in *cno^{R2}scrib^{1}* mutants (20.099,2µ^2; n=28) in comparison with WT (24.675,1µ^2; n=23). In the case of *cno^{R2},* the hemispheres were bigger (28.753,8µ^2; n=20) than WT ones (Figure 20 E). These data evidences the influence of the surrounding environment in tumor development.

Hence, despite *scrib^{1}* or *cno^{R2}* single mutant clones do not lead to NBII overgrowth, the combination of both mutations synergistically interact and it is enough to avoid control signals leading to tumor formation. In addition, these experiments suggest that the WT surrounding tissue responds to changes that happen within NBII lineages.

3.3. *cno^{R2}scrib^{1}* tumors are composed by a large amount of progenitor cells in detriment of neurons

After demonstrating that double *cno^{R2}scrib^{1}* NBII mutant clones were able to overgrow, we wanted to study in detail the cellular composition of them. To do that, we employed Dpn as a nuclear marker of progenitor cells to explore differences between WT and *cno^{R2}scrib^{1}* NBII mutant clones. We observed that even not all *cno^{R2}scrib^{1}* clones formed enormous tumors; in all cases the population consisted almost entirely of progenitor cells (Dpn$^+$) with very few neurons detected (characterized by the expression of the transcription factor Elav) (Figure 21). WT clones presented an average of 21 Dpn$^+$ cells (n=23) and we did not report cases with more than 30 Dpn$^+$ cells. Taking this number as a superior limit, we classified *cno^{R2}scrib^{1}* NBII mutant clones (n=37) in two groups. One group did not present more than 30 Dpn$^+$ cells (49%; n=18) showing an average of 20 Dpn$^+$ cells. The second group contained more than 30 Dpn$^+$ cells per clone and showed a patent tumor overgrowth (51%; n=19) with an average of 62 Dpn$^+$ cells per clone (Figure 21 C). In

addition, in this last group, we identified a population (27%; n=5) that showed a massive overgrowth with more than 100 Dpn⁺ cells per clone. (Figure 21 C). Despite the number of Dpn⁺ cells, all *cno*^{R2}*scrib*^{1} NBII mutant clones, as we mentioned before, were almost exclusively composed by Dpn⁺ cells.

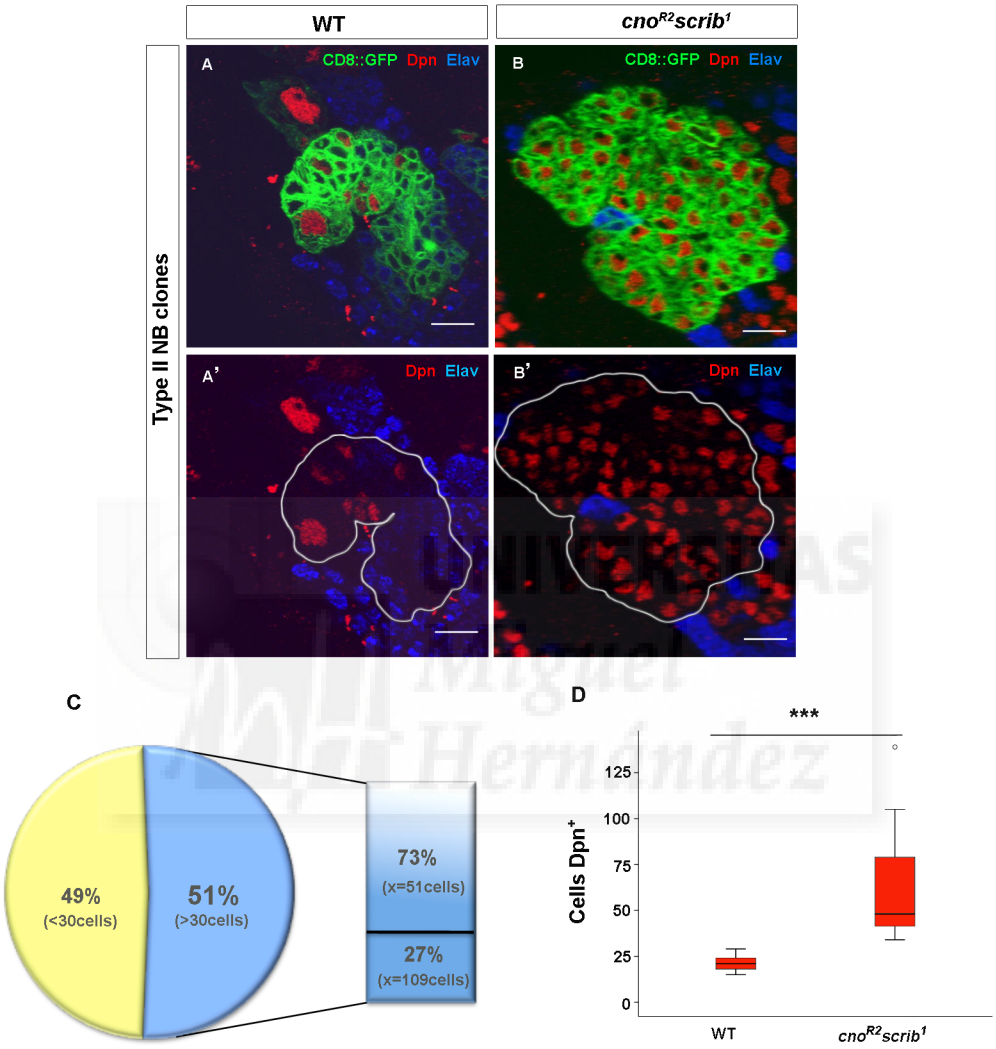


Figure 21. ***cno*^{R2}*scrib*^{1} tumors present a big amount of undifferentiated cells and low number of neurons. A-B')** Confocal images showing a WT clone (A,A') and a *cno*^{R2}*scrib*^{1} mutant clone (B,B'), which is mostly composed by progenitor cells (Dpn⁺ in red) and only few neurons (Elav⁺ in blue). **C)** Diagram showing the distribution of *cno*^{R2}*scrib*^{1} mutant clones depending on the number of Dpn⁺ cells present in the whole clone. **D)** *cno*^{R2}*scrib*^{1} clones that overgrow (51%) present a significantly higher number of Dpn⁺ cells than WT clones (P=0,000). Scale bar: 10µm.

A highly conserved component of the type II NB self-renewal machinery is Notch, which encodes a transmembrane protein that upon proteolytic activation, the

intracellular domain translocates into the nucleus where it complexes with the DNA-binding protein Suppressor of Hairless (Su(H)) to directly activate the expression of different targets, including genes of the E(split) complex and genes related with self-renewal such as Dpn. Then trying to further confirm the identity of the cells in NBII mutant clones, we used Su(H)-lacZ as a reporter of Notch activity. Compared with WT clones in which only the NB and INPs are β-gal positive, we found an increased number of cells with Notch pathway active in *cno^{R2}scrib^{1}* NBII mutant clones (Figure 22). This was consistent with the increase in Dpn^{+} cells present in these mutant clones, as mentioned before. All together, these data support the increment of self-renewing progenitors in *cno^{R2}scrib^{1}* tumors.

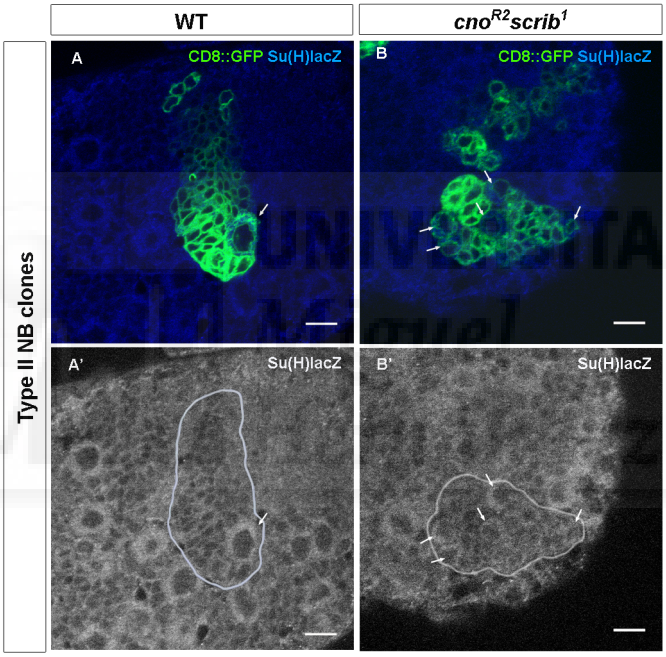


Figure 22. *cno^{R2}scrib^{1}* NBII mutant clones present a big number of self-renewing cells. A,A') A WT NBII clone presents a reduced number of Su(H)-lacZ^{+} cells (white arrows), whereas a **B,B')** *cno^{R2}scrib^{1}* tumors **(B,B')** show an increased number of Su(H)-lacZ^{+} marked cells. Scale bar:10µm.

Both progenitor cells, the NB and INPs express Dpn in a WT clone. Thus, to know how many of Dpn^{+} cells present in *cno^{R2}scrib^{1}* mutant clones were actually NBs we performed a double staining with antibodies against the transcription factors Dpn and PntP1, combination that is only present in NBs. We verified that in WT clones there was always only one NB (Dpn^{+}PntP1^{+})(n=13) whereas in *cno^{R2}scrib^{1}* NBII mutant clones the majority of cells labeled with Dpn were PntP1^{+} as well (Figure 23 A-B''). When we considered the whole population of *cno^{R2}scrib^{1}* mutant clones analyzed,

the mean value of Dpn$^+$PntP1$^+$ positive cells was 6,1 (n=18). This number increased until 10 Dpn$^+$PntP1$^+$ cells (n=7) when we considered only the population that overgrew. Even the population of *cno^{R2}scrib^1* that did not overgrow presented more NBs than WT clones (mean= 3,5; n=11) (Figure 23 C).

Thus, *cno^{R2}scrib^1* NBII mutant clones have a significant higher number of NBs than WT clones, even in the *cno^{R2}scrib^1* mutant clones that do not overgrow. Same results were found when we examined the number of immature INP cells that are labeled only with PntP1. In WT clones, the number of PntP1$^+$ cells is 4,7 (n=13) whereas *cno^{R2}scrib^1* clones always contained a significant bigger number of these PntP1$^+$ cells. The whole population of *cno^{R2}scrib^1* mutant clones had an average of 7,7 PntP1$^+$ cells (n=18), number that increased until 9,7 in the case of the mutant clone population that overgrew (n=7). The population of *cno^{R2}scrib^1* that did not overgrow also showed a bigger number of immature INPs (mean=6; n=11) than WT clones, demonstrating again that even this population of mutant clones that did not visible overgrow were abnormal clones mostly composed by progenitor cells (Figura 23 D). These experiments evidence that the loss of *cno* synergistically interact with the loss of *scrib* in NBsII producing tumors that are composed basically by undifferentiated, self-renewing cells and very few neurons.

3.4. Tumors show a progressive reduction of mature INP population

Trying to understand the progression of *cno^{R2}scrib^1* tumors we followed them through the third larval stage. We used antibodies against the transcription factors Dpn and Ase to investigate the composition and evolution of the progenitor cell population, both NBs (Dpn$^+$) and mature INPs (Dpn$^+$Ase$^+$), along tumor development. As we showed before, at late LIII the number of NBs (Dpn$^+$) and immature INPs (PntP1$^+$) was pretty elevated in *cno^{R2}scrib^1* clones. Then, we wanted to follow the number of mature INP cells in WT and *cno^{R2}scrib^1* at this stage. Despite *cno^{R2}scrib^1* mutant clones were bigger than WT NBII clones, we found that the percent of mature INPs was lower in these double mutants than in WT clones at late LIII. Whereas mature INP population (Dpn$^+$Ase$^+$) represented the 53% of the whole Ase$^+$ cells in WT clones (n=14), in

cno^{R2}*scrib*^{1} NBII mutant clones this population was the 44% (n=12), decreasing until 38% in the population that showed a massive overgrowth (Figure 24 C).

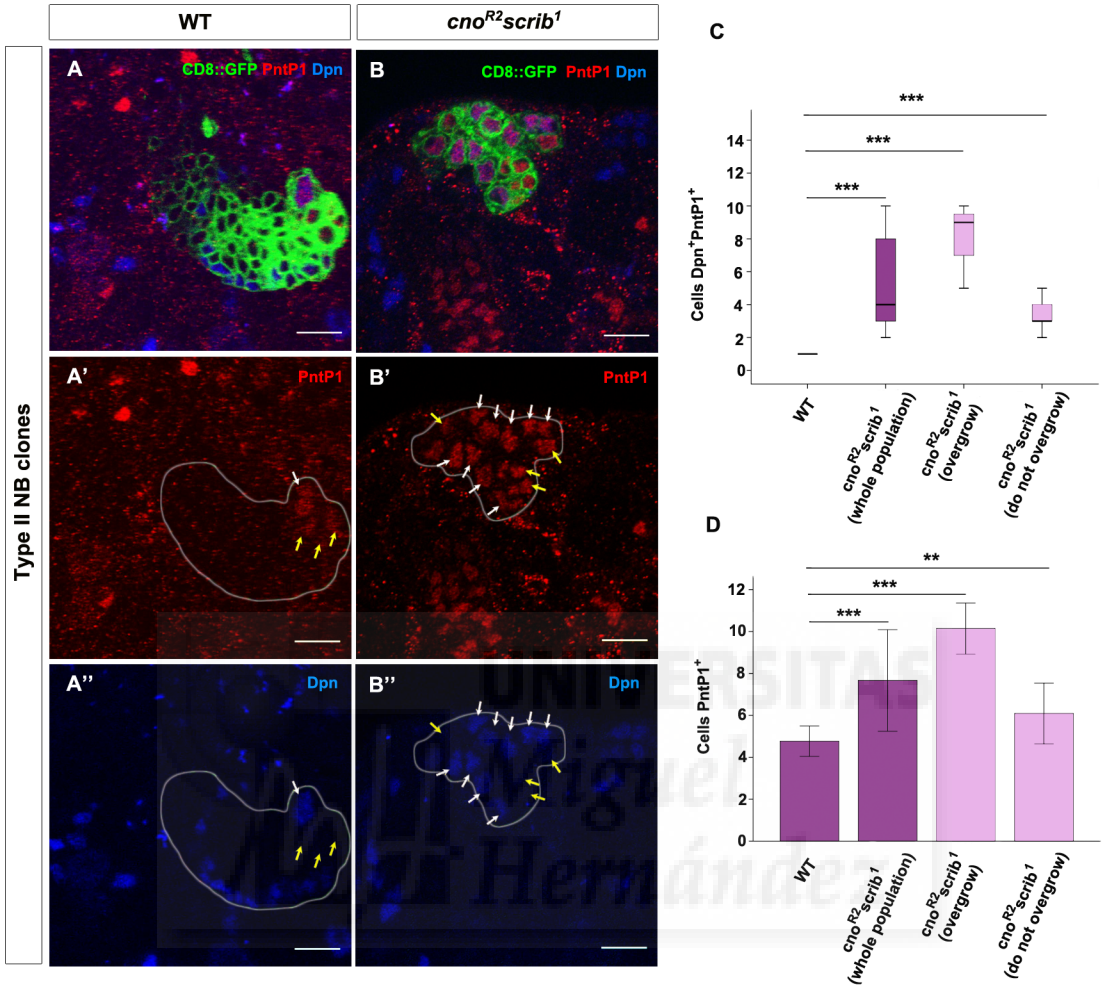


Figure 23. *cno*^{R2}*scrib*^{1} mutant clones comprise an increased number of NBs and immature INPs. A-B'') ** WT clones (A-A'') only contain 1 NB (Dpn^{+}PntP1^{+}; white arrow) and a small number of immature INPs (PntP1^{+}; yellow arrows) whereas *cno*^{R2}*scrib*^{1} mutant clones (B-B'') are composed by many more NBs, as well as more immature INPs. **C) WT clones always present 1 NB while *cno*^{R2}*scrib*^{1} mutant clones show always more than 1 (whole population purple and separated populations in pink) (P=0,000). **D)** WT clones have a mean of 4,7 INPs, whereas *cno*^{R2}*scrib*^{1} mutant clones (whole population in purpple) show 7,7 as an average (P=0,000). This number increases until 9,7 in the population that overgrows (pink bar) (P=0,008) and 6 in the rest of *cno*^{R2}*scrib*^{1} mutant clones (pink bar) (P= 0,000). Scale bar=10µm.

To further understand this, we followed the mature INP population along third larval stage in *cno*^{R2}*scrib*^{1} NBII mutant clones. At early LIII stage the population of mature INPs represented the 51% of the whole Ase^{+} cells, percentage that decreased until 44% in late LIII stage as we mentioned above. This reduction was even more

evident in the mutant clones in which the tumor massively overgrew, where the population of mature INPs decreased until 38% of the whole Ase+ cells at late LIII stage (Figure 24 D). This data evidence a progressive reduction of mature INP population through the third larval stage in *cno^{R2}scrib^{1}* mutant clones that may suggest a reversion of INPs to a more undifferentiated stage.

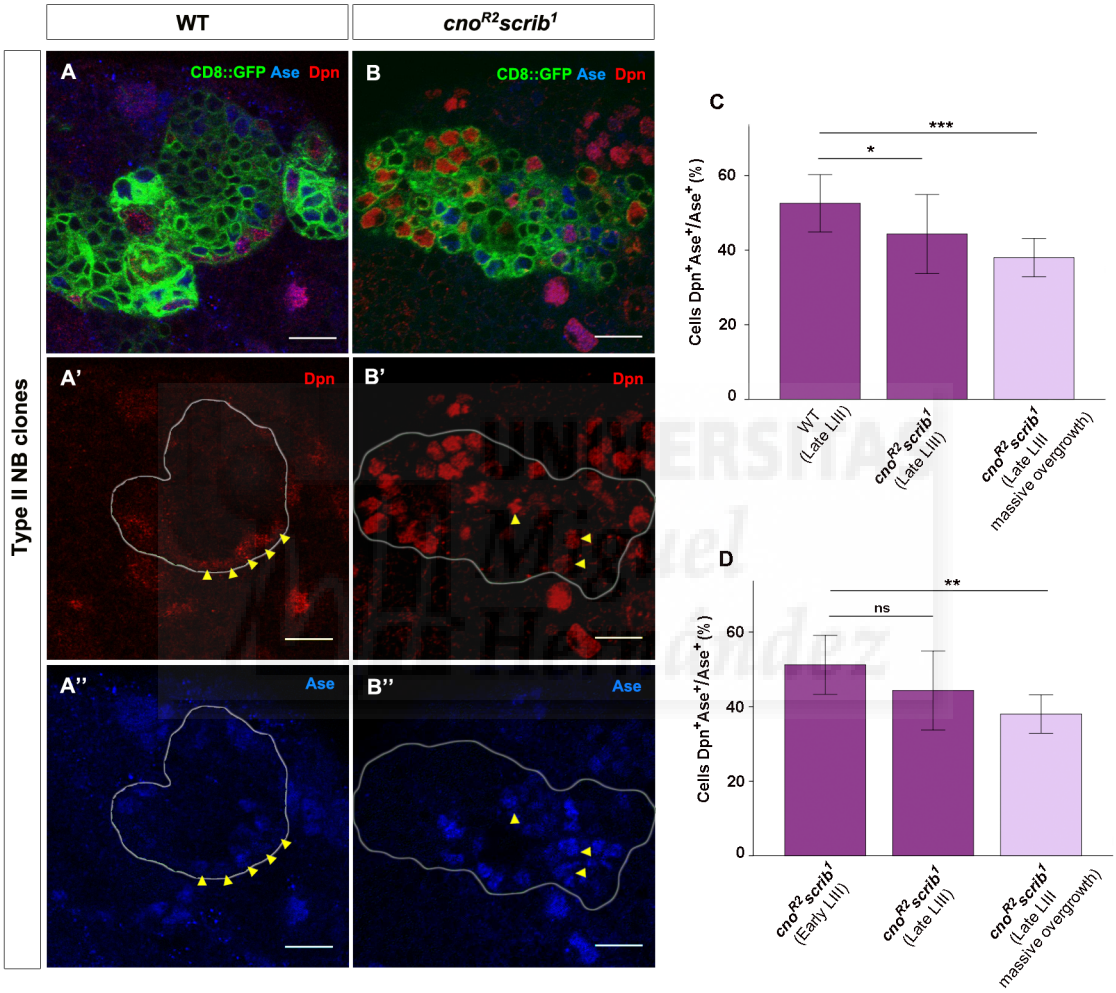
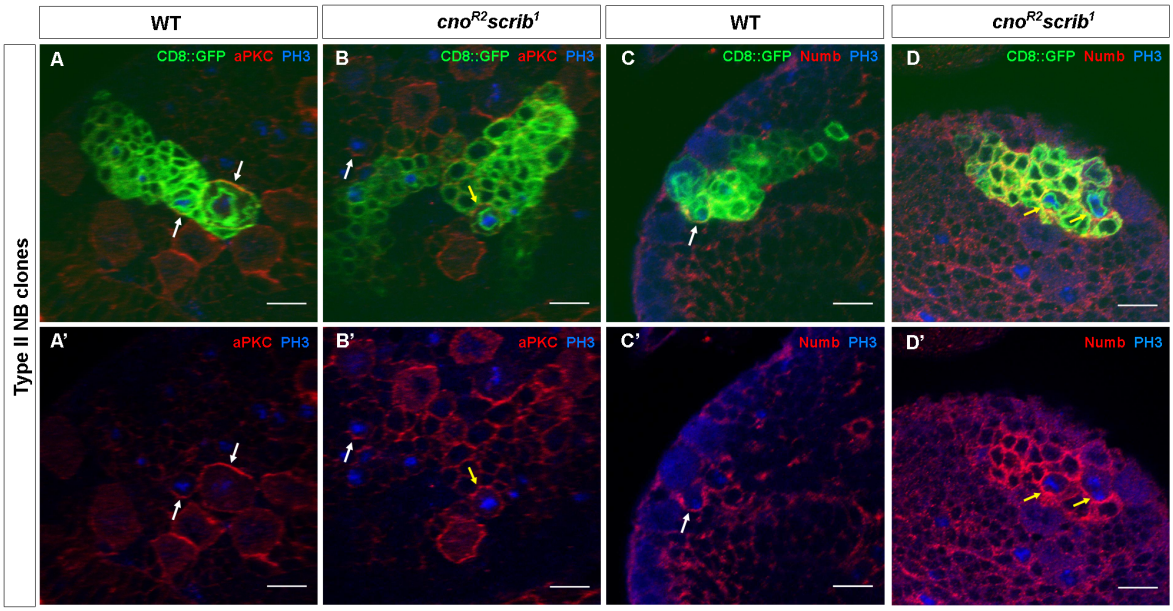


FIGURE 24. Tumors show a progressive reduction in the mature INP population. A-B'') Compared with WT clones (A-A'''), there are few mature INPs (Ase^{+}Dpn^{+}; yellow arrowheads) in *cno^{R2}scrib^{1}* clones (B-B''). **C)** *cno^{R2}scrib^{1}* clones present fewer mature INPs (% of Dpn^{+}Ase^{+} cells relative to the total Ase^{+} cells) (mean= 44% and 38%, respectively; considering whole population, purple bar, and the part of population that overgrows, light purple bar) than WT (mean=53%) (P=0.031 and P=0,000) at third larval stage (LIII). **D)** The number of mature INPs decreases in *cno^{R2}scrib^{1}* clones throughout LIII stage; early LIII=51% (purple bar), late LIII=44% (purple bar), late LIII clones with massive overgrowth=38% (light purple bar) (P=0,004). Scale bar:10µm

4. ACD is severely altered in *cno^{R2}scrib^{1}* tumors

After the clear alterations in cell composition/identity found in of *cno^{R2}scrib^{1}* mutant clones we observed, and given that both *cno* and *scrib* function in this process, we wanted to know until which extent ACD was disrupted in these double mutant clones. To test that, we investigated the segregation of two crucial proteins in ACD: aPKC, which is part of the apical complex and the cell-fate determinant Numb. aPKC forms an apical crescent at metaphase NBs/INPs that permits its segregation into the daughter cell that will keep on dividing. On the other hand, Numb accumulates in the basal part of the metaphase progentior and will be segregated into the daughter cell that will start a differentiation program (Figure 25 F). Using PH3 as a marker of mitotic cells, we identified the progenitor cells that were in metaphase or anaphase and analyzed the localization of these proteins. In *cno^{R2}scrib^{1}* mutant clones, the position and segregation of both aPKC and Numb was seriously altered during division. While in WT clones aPKC apical crescents were well formed in almost 90% of the progenitor cells analyzed (n= 18), in *cno^{R2}scrib^{1}* NBII mutant clones aPKC localization was never apical (Figure 25 A-B'). In 28,6 % of the cases, aPKC was forming a non-apical crescent and in the rest 71,4% of the cases, aPKC was detected all around the cellular cortex (n=14). The situation that we found in Numb was pretty similar. In WT clones no defects were detected, in 100% of analyzed progenitor cells (n=13) Numb was basally located. Conversely, in *cno^{R2}scrib^{1}* tumors, Numb was never properly located in progenitor cells during metaphase/anaphase (n=7): in 57,1% of the cases, Numb formed a non-basal crescent, and in the other 42,9% Numb was detected all around the cellular cortex. These data show that aPKC and Numb localization and segregation are strongly altered and, consequently, the ACD machinery is severely disrupted in *cno^{R2}scrib^{1}* tumors. The defects found in these double mutant clones were stronger than those we observed in *cno* and *scrib* single mutant clones, despite the defects identified in the few *scrib* clones that survived and we could analyze where stronger than in *cno* single mutants. Hence, it may occur something else to explain the strong growth synergism between *cno* and *scrib* that finally lead to tumor formation.



E

	Meta/Anaphase progenitors (n)	aPKC mislocalized (%) no present	aPKC mislocalized (%) crescent mislocalization	aPKC mislocalized (%) cortical	Total (%)	Meta/Anaphase progenitors (n)	Numb mislocalized (%) no present	Numb mislocalized (%) crescent mislocalization	Numb mislocalized (%) cortical	Total (%)
WT	18	0	11.1	0	**11.1**	13	0	0	0	**0**
cno^{R2}scrib^{1}	14	0	71.4	28.6	**100**	7	0	57.1	42.9	**100**

F

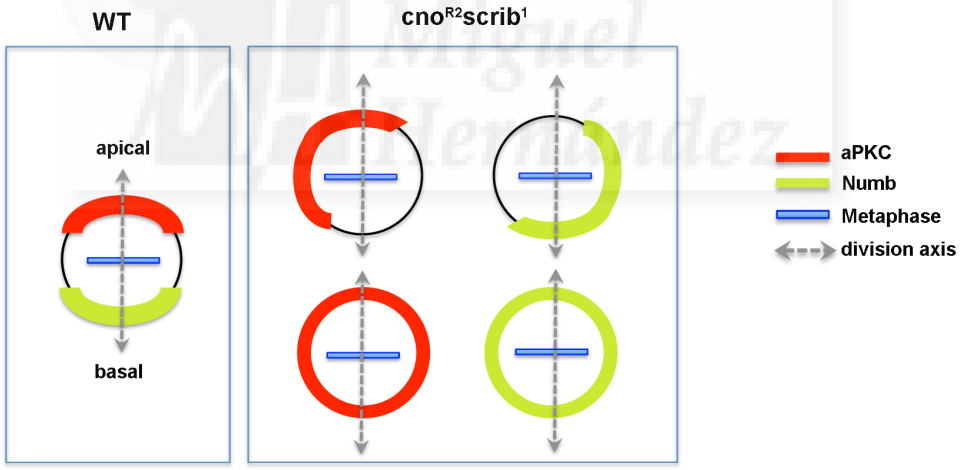


FIGURE 25. *cno^{R2}scrib^{1}* clones show defective aPKC and Numb cortical localization. A,A',C,C') WT NBII clones show apical aPKC and basal Numb enrichment in metaphase progenitors (NBs and INPs), (white arrows). **B,B',C,C')** In *cno^{R2}scrib^{1}*, both aPKC and Numb display cortical mislocalization (yellow arrows). **E)** Quantification of the phenotypes analyzed in WT (control) and *cno^{R2}scrib^{1}* mutant clones for aPKC and Numb; n=number of metaphase/anaphase progenitors analyzed. **F)** Drawing representing aPKC (in red) and Numb (in green) localization during metaphase (blue line) in dividing cells. In WT aPKC is apically enriched while Numb is restricted to the basal pole. It permits its segregation to the mother cell in the case of aPKC or to the daughter cell in the case of Numb, after every division. In *cno^{R2}scrib^{1}* clones apical aPKC and basal Numb localization is clearly disrupted. Both can be found forming a crescent but mislocalized respect to the division axis (grey line) or around all the cortex of the dividing cell. Scale bar: 10µm.

5. *cno* acts via Ras to promote tumor growth in cooperation with *scrib*

At this point, the main question was why *cno*^{R2}*scrib*^{1} mutant clones do not die as *scrib* clones do. Given that *scrib* clones die through the activation of the JNK pathway that in turn promotes apoptosis, one possibility was that in absence of *cno*, the JNK pathway could no longer be activated. To test this possibility we used the antibody against pJNK, as we did with *scrib*^{1} clones, finding that in *cno*^{R2}*scrib*^{1} NBII tumors (n=6) the JNK was still active (Figure 26). Thus, the loss of *cno* does not seem to directly affect the JNK activation.

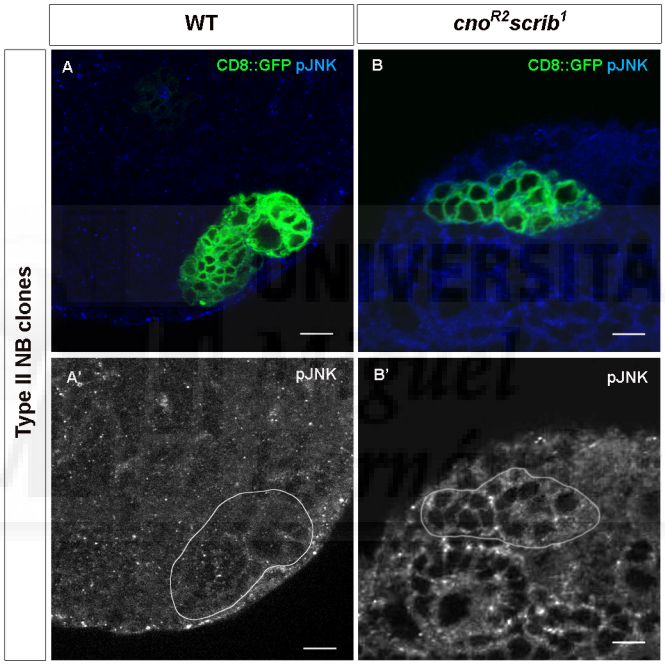


Figure 26. The JNK signaling pathway is active in *cno*^{R2}*scrib*^{1} NBII clones. A,A',B,B') *scrib*^{1}*cno*^{R2} mutant clones present an activation of the JNK pathway, as *scrib*^{1} single mutant clones. Scale bar: 10µm.

Intriguingly, it has been shown that the JNK pathway changes its pro-apoptotic function for a pro-growth role in particular contexts. For example, in epithelial tissues (imaginal discs) the constitutive activation of the oncogen Ras (Ras^{V12}) has this effect when it is combined with *scrib* mutants. Whereas *scrib* clones die through JNK-mediated apoptosis, *Ras*^{V12}*scrib*^{1} mutant clones massively overgrow, as many works have described (Brumby and Richardson, 2003; Pagliarini and Xu, 2003; Wu et al., 2010). Our lab showed before that in other systems Cno is repressing Ras pathway

(Carmena et al., 2006). Thus, we wanted to investigate if the suppression of *cno* function in *cno^{R2}scrib^{1}* NBII mutant clones provoked up-regulation of Ras, which then could be cooperating with *scrib* loss of function to produce the tumors observed. To test that we eliminated Ras signal in *cno^{R2}scrib^{1}* background using a dominant negative forms of Ras (DNRas). First of all, we analyzed *Ras^{V12}* NBII mutant clones and we found that *Ras^{v12}* alone is not enough to produce a big overgrowth, as it happens in epithelia (data not shown); and we also tested the DNRas in WT background where we did not found alteration in NB either (data not shown). After verifying this, we proceeded to assay dominant negative forms of Ras, in *cno^{R2}scrib^{1}* background. The effect of removing Ras signal in *cno^{R2}scrib^{1}* NBII mutant clones resulted in a suppression of the overgrowth phenotype (Figure 27). Again, using the number of Dpn^{+} cells as a measure of growth, we observed that the amount of these cells decreased significantly from 48,6 in *cno^{R2}scrib^{1}* NBII mutant clones (n=20) until 24,4 (n=14) when a dominant negative form of Ras was expressed in the same mutant background. It is important to notice that even when the overgrowth is suppressed by the use of DNRas in the double mutant background, a WT phenotype was not restored. Despite the clones did no longer overgrow, the clone cellular composition and shape were still disrupted. This data strongly evidence that *cno* acts through Ras activation to promote overgrowth in *cno^{R2}scrib^{1}* NBII mutant clones.

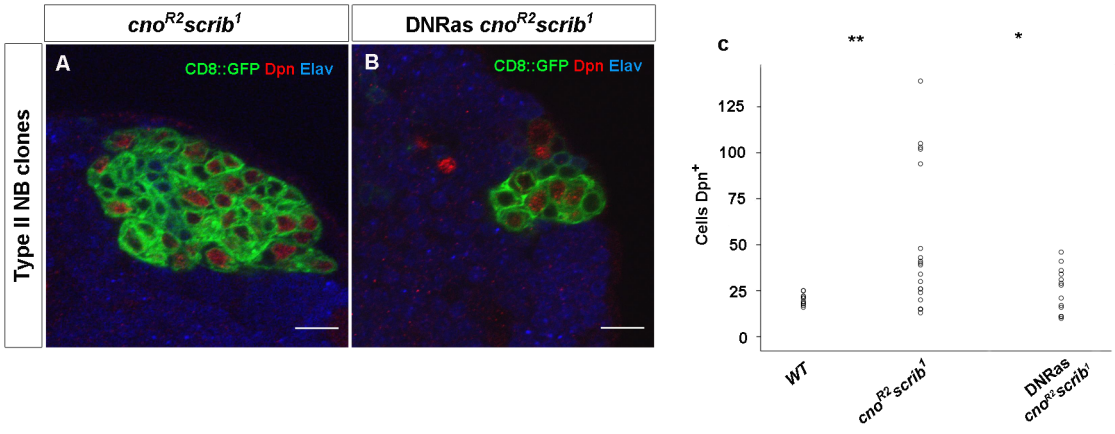


Figure 27. Downregulation of Ras rescues *cno^{R2}scrib^{1}* overgrowth. A-C) A *cno^{R2}scrib^{1}* mutant clone showing the overgrowth phenotype (A). Removing Ras through a dominant negative form in a *cno^{R2}scrib^{1}* mutant background (B) suppresses the overgrowth phenotype. **C)** Graphic comparing the number of Dpn^{+} cells as a measure of growth, between WT, *cno^{R2}scrib^{1}* mutant clones and a Ras dominant negative form in *cno^{R2}scrib^{1}* background. As we reported before, *cno^{R2}scrib^{1}* mutant clones present a bigger number of Dpn^{+} cell than WT clones (p=0,009) but the suppression of Ras signal by the use of the dominant negative form

in the double mutant background, produced a significant decrease in the number of Dpn+ (P=0,034). Scale bar: 10µm

6. UAS-Ras^{V12}*scrib*^{2} NBII mutant clones survive but do not overgrow

As mentioned above, it has been shown that the overexpression of the oncogenic Ras^{v12} in combination with the loss of function of *scrib* cooperates to lead to overgrowth in epithelial tissues. Given that in *cno*^{R2}*scrib*^{1} mutant clones Ras was upregulated, we wondered whether the former combination (UAS-Ras^{v12} *scrib*^{2}) would produce the same effect in larval brain NBII. To assay that, we performed MARCM clones with that combination in NBII. First of all, we observed that UAS-UAS-Ras^{V12}*scrib*^{2} double mutants did not die as *scrib*^{1} clones did. That effect avoiding the death of *scrib*^{1} clones was similar that the one we already observed when we combined *cno*^{R2} with *scrib*^{1}. However, even though UAS-Ras^{V12}*scrib*^{2} double mutant clones showed alterations in their cellular composition, they did not overgrow (Figure 28 A-B). Using the transcription factors Dpn and Ase we identified cells with NB identity (Dpn^{+}Ase^{-}) in both WT and UAS-Ras^{V12}*scrib*^{2} NBII mutant clones, finding that while in WT clones never appeared more that one NB (n=12), in UAS-Ras^{V12}*scrib*^{2} double mutants we observed two or even three "NB-like" (Dpn^{+}Ase^{-}) in 18,2% and 36,3% of the cases analyzed respectively (n=11) (Figure 28 C).

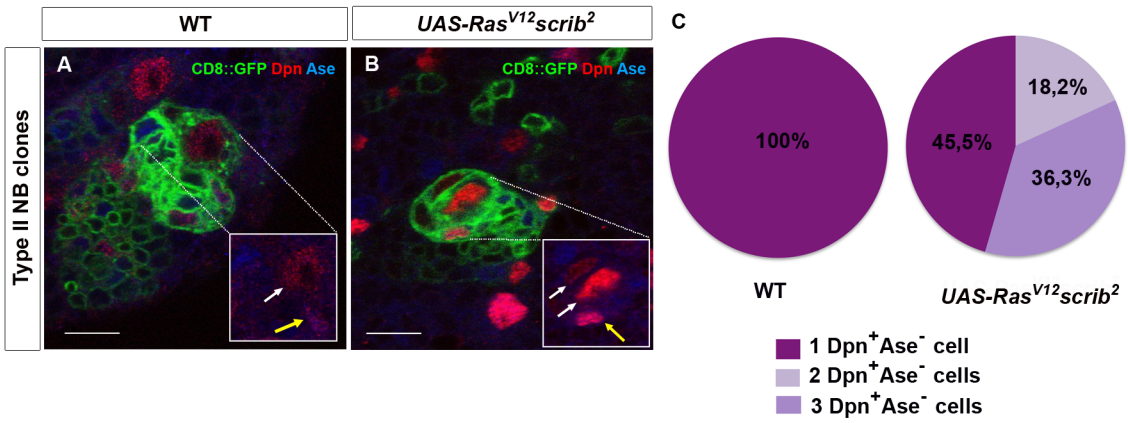


Figure 28. UAS-Ras^{V12}*scrib*^{2} NBII mutants clones show ectopic NBs but they do not overgrow. A-C) WT NBII always present only one NB (Dpn^{+}Ase^{-}) (white arrow) and some INPs (Dpn^{+}Ase^{+})(yellow

arrow), **B-C)** while UAS-Ras^{V12}*scrib*^{2} NBII mutants clones presented in many case more than one NB per clone (white arrows). **C)** Diagram showing the percentages of NBs found in both genotypes. Scale bar:10µm

These results suggest that the effect of Ras overexpression produces the same effect than removing *cno* in a *scrib* mutant background, both being able to rescue the death of *scrib*^{1} NBII clones. This supports a repressive effect of Cno on Ras signaling in WT larval brain NBs. However, these results also evidence that the combination of UAS-Ras^{V12} with *scrib*^{2} is not enough to produce overgrowth in NBII, suggesting that, in this context, the loss of function of *cno* in a *scrib* mutant background have additional consequences than de-repressing Ras signaling that finally lead to a tumor-like overgrowth.

7. dipMAPK is not expressed in larval NBIIs.

After all the results that strongly suggested the role of Ras signaling in *cno*^{R2}*scrib*^{1} NBII mutant clones we wanted to investigate if Ras was acting via the Raf/MAPK cascade, as it has been reported in UAS-Ras^{V12}*scrib*^{2} clones in epithelial tissues (Dow et al., 2008). To test this possibility we used an antibody that recognizes the active (di-phosphorylated) form of MAPK. We found that in WT NBII clones dipMAPK was not detected (Figure 19 A,A'). When we removed *cno* function from the NBII, dipMAPK was still undetectable (Figure 29 C,C'). Even in the *cno*^{R2}*scrib*^{1}double mutant clones dipMAPK was not expressed (Figure 29 D,D'). Only when we overexpressed Ras^{v12} alone in NBII clones we were able to detect some dipMAPK inside of the clones (Figure 29 B,B'). In all of the studied cases we found dipMAPK signal outside of the clone.

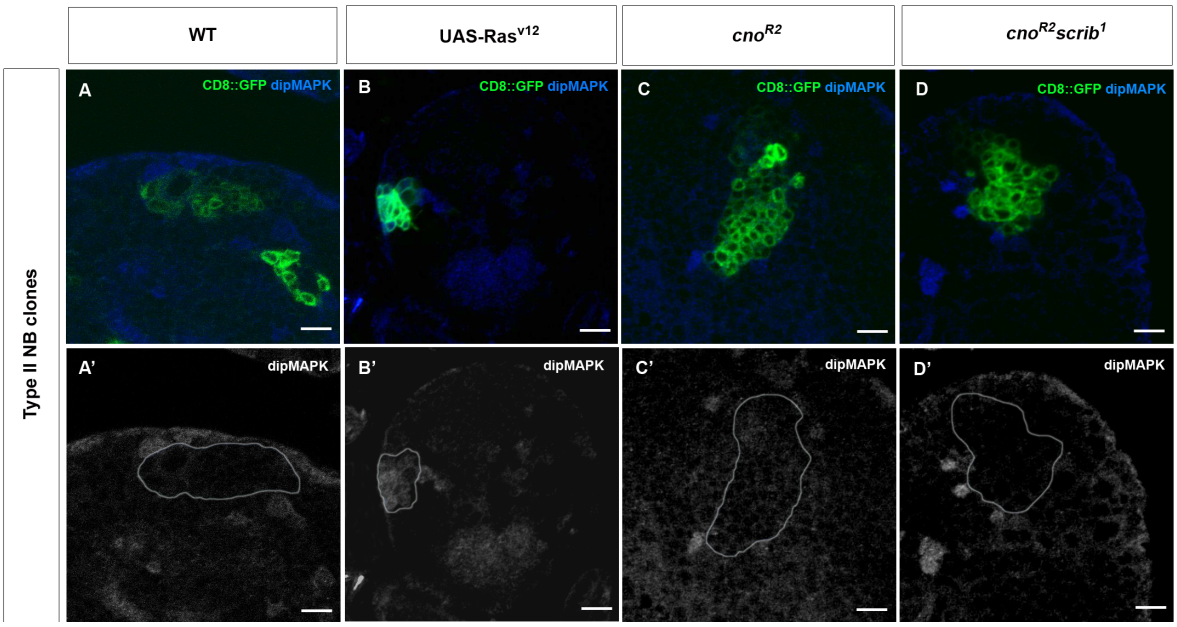


Figure 29. MAPK is not activated in larval NBIIs. A) We do not report dipMAPK signal inside of WT larval brain NBIIs. **C)** Either in *cno*^{R2} **D)** or *cno*^{R2}*scrib*^{1} mutant NBII clones. **B)** It expression is only detected when we overexpress Ras^{v12} inside of the clone. Scale bar:10 µm

These data suggest that Ras signaling is not functioning via dipMAPK in *cno*^{R2} or *cno*^{R2}*scrib*^{1} larval brain NBII mutant clones.

8. Cno and Scrib are expressed in epithelial cells of eye-antennal imaginal discs

Trying to understand better the mechanism behind of *cno*^{R2}*scrib*^{1} phenotype, we moved from the brain to epithelial cells where the mechanism that lead to tumor overgrowth in UAS-Ras^{V12}*scrib*^{2} clones has been widely studied (Bilder, 2004; Bilder et al., 2000; Brumby and Richardson, 2003; Pagliarini and Xu, 2003; Wu et al., 2010). First of all we analyzed the expression of both proteins in eye-antennal discs (Figure 30).

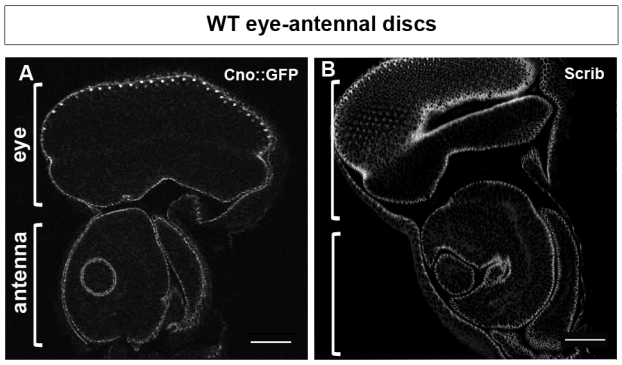


Figure 30. Scrib and Cno are both expressed in eye-antennal discs. A) Cno is expressed in epithelial cells of both antennal and eye discs. **B)** Scrib is also expressed in these areas. Scale bar: 50µm

These data revealed that both Cno and Scrib are expressed in these imaginal discs.

9. *cno^{R2}scrib^{1}* antennal disc clones also overgrow and dipMAPK expression is up-regulated

In this work we have shown how *cno* and *scrib* loss of function cooperate in larval brain NBII to produce tumor overgrowth, and how that cooperation is mediated, at least in part, by Ras. We have also demonstrated that Ras is not acting via MAPK activation in the larval brain. As we said before, it has been clearly demonstrated that Ras functions via Raf/MAPK cascade in the combination UAS-Ras^{V12}*scrib*^{2} that lead to tumor overgrowth in imaginal discs. Then we wanted to test the behavior of *cno^{R2}scrib^{1}* mutant clones in this epithelial tissue to analyze whether in this system there was also a synergistic cooperation between them mediated by Ras and, if so, whether MAPK was activated in this context. First, we observed that in antennal discs *cno* and *scrib* did interact synergistically too and the double mutant clone overgrew in 28% of the cases analyzed (n=14) (Figure 31).

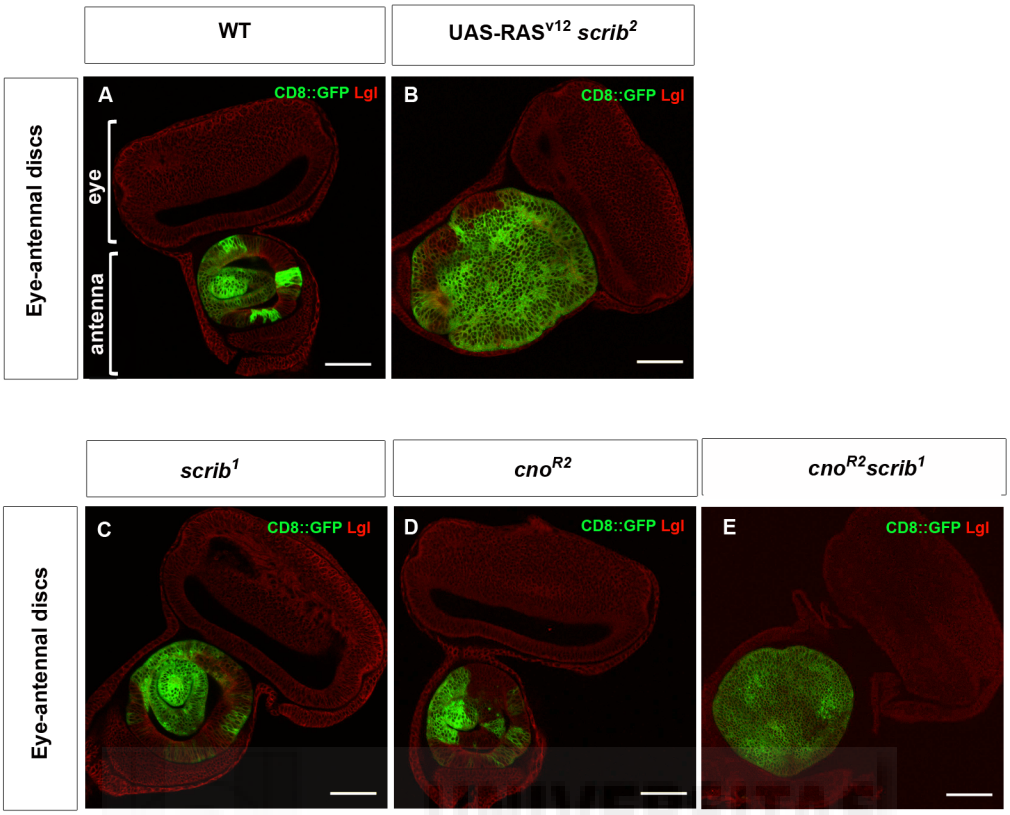


Figure 31. *cno* and *scrib* also interact in antennal disc leading to overgrow. WT **(A),** scrib^{1} **(C)** or cno^{R2} *(D)* clones do not show overgrow in antennal disc. We verified that Ras^{V12} in combination with *scrib* loss produce the overgrowth of the clone **(B).** The combination of *scrib* and *cno* loss cooperates synergistically to produce tumor growth in antennal discs as it happens in NBsII **(E).** Scale bar: 50μm

Next we wondered if in this case the overgrowth was due to an up-regulation of Ras signaling where the downstream player was the Raf/MAPK pathway. We found that in WT discs dipMAPK was not expressed at significant levels (Figure 32 A,A') whereas in UAS-Ras^{V12}*scrib*^{2} antennal clones dipMAPK was highly up-regulated inside of the overgrown clone (Figure 32 B,B'). In *cno*^{R2} or *scrib*^{1} antennal clones dipMAPK did not show levels significantly higher than in WT conditions (Figure 32 C,C'-D,D'). However, the expression of dipMAPK in *cno*^{R2}*scrib*^{1} antennal clones (Figure 32 E,E'), was up-regulated even though the levels were no so high and uniformily distributed as in UAS-Ras^{V12}*scrib*^{2}. Hence, in epithelial tissues Ras signals via MAPK activation in *cno*^{R2}*scrib*^{1} clones as it does in UAS-Ras^{V12}*scrib*^{2}. These results support that the loss of function of *cno* can collaborate with the loss of function of *scrib* by up-regulating Ras signaling.

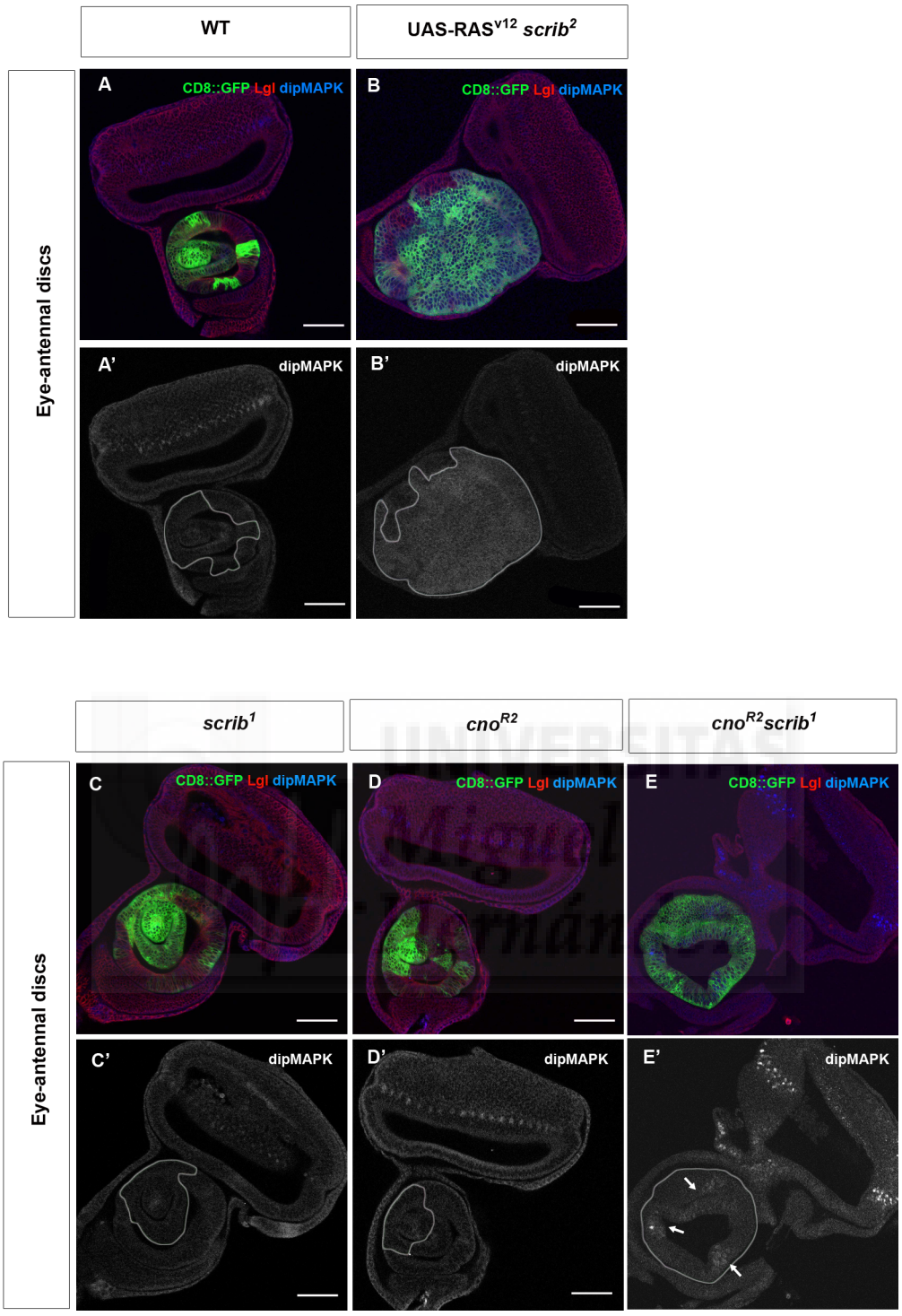


Figure 32. Ras functions via dipMAPK in the eye-anntennal disc. A,A') WT antennal clones do not show dipMAPK signal**. B-B')** UAS-Ras^{V12}*scrib*^{2} antennal clones overgrow and show an up-regulated dipMAPK signal**. C,C'-D,D')** Neither *cno*^{R2} nor *scrib*^{1} antennal clones show significant differences with the WT. **E,E')** In *cno*^{R2}*scrib*^{1} antennal clones the dipMAPK levels are higher than in WT or in the single mutants but lower that in UAS-Ras^{V12}*scrib*^{2} clones. Scale bar: 50µm

DISCUSSION

In this thesis research project we have shown that the asymmetric cell division regulator Cno is implicated in tumorigenesis. The loss of function of *cno* and the loss of the tumor suppresor gene *scrib* synergistically cooperates producing the up-regulation of Ras signaling that contribute to tumor formation in *Drosophila* larval brain.

First, using a fused protein Cno::GFP we were able to see the pattern of expression of Cno in NBsII lineages, the ones that have been related with tumor formation in *Drosophila* brain when ACD is disrupted . This tool resulted very useful to follow the endogeus protein pattern in the cell. In this regard, we reported for the first time Cno expression in both centrosomes of protenitors cells (Cno is not present in embryonic NBs in the centrosomes), so this observation might open a new research line in future. Centrosomes are recognised as critical regulators of spindle-orientation and also in tumorigenesis (Gonzalez, 2007). Indeed, different types of genome instability, including choromosome instability and centrosomal alteration are common traits in human cancers (Januschke and Gonzalez, 2008). It is necesary that proteins of the apical complex couple with the mitotic spindle directed by centrosomes to ensure a correct division, and alteration in that process have been extensevely reported in overgrowth phenotypes (review in (Januschke and Gonzalez, 2008). Hence, it will be interesting to investigate whether a potential function of Cno in centrosomes may contribute to its tumor-suppressor potential.

cno^{R2} mutant NBsII clones showed clear alterations in the asymmetric cell division process proving that *cno* also functions as an asymmetric cell regulator in larval brain NBs. The first clues about that the process could not be working properly appereared when we observed an abnormal clone development. In the central brain, after every division NBs have to recover their original size to continue dividing normally (Nishimura et al., 1992), but in cno^{R2} mutant clones the NBs revealed a reduced size compared with WT NBs. The reduction in NB size is a sign for

termination of the divisions and the initiation of either differentiation or death, and it occurs when the NB is in the end of the proliferating period (Maurange et al., 2008). The fact that *cno*^{R2} mutant NBsII clones displayed a sized-reduced NB suggests that the NBs may not be able to recover its size after every division and that these clones could stop proliferating prematurely. This fact in addition to the lower mitotic rate reported in the INPs of *cno*^{R2} NBsII mutant compared with the WT, could contribute to explain why those mutant clones presented a smaller number of cells and therefore smaller sizes than WT ones. In addition, we can not discard that cell death is acting in *cno*^{R2} mutants reducing the final number of cells by eliminating the cells bad especified or "weakers" than the WT environment and also remodeling the clone, what could explain the wrong positioning of the neurons. This last fact could be also explained by a premature differenciacion of INPs or GMCs. We did not explore the kind of neurons that were finally produced in both conditions WT and *cno*^{R2} mutants; it is known that in *Drosophlia* and vertebrates neural progenitors can generate various neuronal subtypes over the proliferating period due to different temporal patterning of gene expression (Bayraktar and Doe, 2013). Therefore, the fact that *cno*^{R2} mutant clones had less cells could not only affect at the final number of neurons but also the types of neurons generated, having diffent consecuences in adult flies, depending on the type of neural loss. More investigation will be necessary to study possible implications of this in adult brain formation and fly behaviour.

Focusing on the asymmetric cell division process, we showed that the positioning and segregation of different proteins of the apical complex was disrupted in *cno*^{R2} NBsII mutant clones. aPKC, Dlg, Pins and Baz were the apical proteins tested that showed errors, being placed around all cell cortex in metaphase and anaphase NBsII in *cno*^{R2} mutant instead of forming apical enrichment as in WT clones. Insc apical localization requires Bazooka (Par-3), a component of the apical Par-6/aPKC/Bazooka complex. Thus, first, Insc bound to the Par complex recruits Pins-GαI to the apical pole by binding to Pins^{TPR} domain In normal conditions, spindle orientation in NBs relies on Pins-Gαi recruitment of the tumor supressor Dlg and Mud. Pins C-terminal domain (GoLoco) binding to GαI (which is attached to the membrane) facilitates the membrane anchoring of the Pins/Gαi/Dlg/Mud complex and spindle

orientation. Dlg binds the Pins LINKER middle domain and the Kinesin heavy chain 73 (Khc-73), which is associated to aster microtubules, whereas Mud, like Insc, binds the $Pins^{TPR}$ domain. A recent work in our lab has helped to clarify more the contribution of Cno to this process. We demonstrated that Wts, a kinase member of the Hippo pathway, phosphorylates Cno and this phosphorylation event contributes somehow to mediate the exchange between $Pins^{TPR}$ /Insc to $Pins^{TPR}$ /Mud and to recruit other proteins to the latter complex required for spindle orientation such as the above mentioned Dlg (Keder et al., 2015) (see introduction Figure 11). The mutated form of Cno that affect the phosporilation sites by Wts showed alterations in Dlg position in dividing larval brain NBs but did not affect aPKC or Pins positioning (Keder et al., 2015). In this thesis work, we found that the loss of function of the protein Cno in these NBsII, not only affects the positioning of Dlg but also of aPKC and Pins. This suggests that the even though the phosphorilation of Cno by Wts is not necessary for the positioning and/or stability in dividing NB of aPKC or Pins, the presence of a functional Cno protein is required for that during the exchange of Pins/Ins to Pins/Mud complexes. In addition, a paper published by Chris Doe suggested that Pins could be important for anchoring aPKC in the apical pole of the NBs (Lee et al., 2006b). In *pins* single mutants aPKC showed a weak uniform cortical localization in metaphase. They also showed that it exists a mutual antagonism between Lgl and aPKC that restricts aPKC in the apical part of the cell, and in lgl mutans aPKC is also uniformly distributed in the cortex. Furthermore, uniform cortical localization of aPKC showed to be essential to generate ectopic NBs (Lee et al., 2006b).. Whereas *lgl* mutant NB clones were able to divide producing more than one NB, the opposite phenotype was seen in *pins* mutant brains, were NBs occasionally fail to self-renew, resulting in GMC/GMC siblings and termination of the lineage. They proposed that in *pins* mutants, aPKC is delocalized all around cell cortex but it is nonfunctional due to Lgl activity, thereby reducing self-renewal; however, in *pins lgl* double mutants, aPKC was both delocalized and fully active, leading to NBs overproliferation (Lee et al., 2006b). All this together could help us to explain the situation in cno^{R2} mutant NBs were we saw aPKC and Pins delocalized around all cell cortex during NBs division, but only in few cases appeared more than one NB, as Lgl localization was not altered in cno^{R2} mutant NBs. Despite of having aPKC all around of cellular cortex, the self-renewing situation was not the dominant situation, because Lgl may be inhibiting

aPKC avoiding extra self-renewing cells in *cno*^{R2} mutants, as it happens in *pins* mutant clones. To try to clarify more the mechanistic alterations that could be happening in *cno*^{R2} mutants clones, more proteins and cell fate determinants and spindle orientation should be analyzed in detail.

As I mentioned in the introduction, cell competition is a developmental mechanism that identifies and eliminates cells that are weaker than their neighbors or have features that make them different or not well adapted to their surroundings. This is an important homeostatic mechanism to contribute to the general fitness of tissues and also it is responsible for the removal of malignant or aberrant cells that may appear during development (Ballesteros-Arias et al., 2014). The so-called loser cells are the ones that are not well adapted to a particular developmental context, and this is manifested by having a lower proliferation rate than their neighbors or by not having the identity corresponding to the cell population to which they belong. Loser cells are commonly killed by the JNK-mediated apoptosis pathway. This is the case of for example the mutant clones for the genes *scrib*, *lgl, dlg* or *Rab*5 in epithelial tissues; despite the growth potential of these mutant clones, they are normally eliminated as a result of the interactions with their non-mutant neighbors (Ballesteros-Arias et al., 2014; Brumby and Richardson, 2003; Chen et al., 2012; Igaki et al., 2009; Menendez et al., 2010; Tamori and Deng, 2011). This is the mechanism that has been described for removing oncogenic cells defective in cell polarity as *scrib* clones, and this is what we observed also in our system. In the case of *cno*^{R2} mutant clones, they presented features of loser cells (such as low mitotic rate and cells with wrong identity) that could imply be subject of a cell competition process and their potential growth being avoided by the WT surrounding environment. Thus, the "ectopic-NBs" that sometimes we observed could had been detected as cells with wrong identities and eliminated by the neighbors. But sometimes, the WT cells can be the "losers" in the cell competition process, and the best-documented cases involve the overexpression of the Ras pathway in *lgl* or *scrib* mutant clones (Brumby and Richardson, 2003; Igaki et al., 2006; Menendez et al., 2010; Pagliarini and Xu, 2003). One critical feature of these clones and one reason of their growth is that they have a high proliferation rate. Since a classical feature of cell competition is the interaction of slow and fast dividing cells, a

simple interpretation of these results is that, having acquired a higher proliferation rate in a Ras activation background, *lgl* or *scrib* mutant cells become "supercompetitors" that can eliminate the non-tumor neighbors (review in (Ballesteros-Arias et al., 2014). All of these could help us to understand what is happening in our double *cno^{R2}scrib^{1}* NBsII mutant clones. Despite that *cno^{R2}* and *scrib^{1}* single mutant were not able to grow, the summation of both mutations in the cells permitted them to avoid grow control signals and to form a tumor–like cellular population. Furthermore, we reported an up-regulation of Ras signal in those *cno^{R2}scrib^{1}* NBsII double mutant clones that supports the idea that they were behaving as *lgl*Ras^{v12} or *scrib*Ras^{v12} in epithelial tissues, in the sense that they are able to avoid control signals and overgrow. Another interesting thing in the context of the environment responses and changes is the effect in the hemisphere size. The growth of a *cno^{R2}* single mutant clone subjected to the control of the WT neighbors resulted in a decrease of the clone size, and at the same time the WT environment compensated that cell loss and the hemisphere showed a moderate bigger size than WT hemispheres. On the contrary, when the clone was a supercompetitor, cells with a higher capacity to grow than the surrounding ones, as it was the case of *cno^{R2}scrib^{1}* double mutant clones, those mutant tumors were able to eliminate the non-tumor surrounding cells, as it was revealed by a decreased in the hemisphere size. In this case WT cells would be the loser cells, as mentioned above, and the tumor cells would grow at the expense of the WT surrounding tissue, feature that in fact occurs in many tumors (Gabay et al., 2014). Despite that *lgl*Ras^{v12} and *scrib*Ras^{v12} cells have shown to possess proliferation advantages with respect to surrounding non-tumoral cells, they are frequently recognized as aberrant and are eliminated. Menendez et al., proposed that in order to develop a tumor, *lgl*Ras^{v12} or *scrib*Ras^{v12} cells have to generate a protective microenvironment to evade the effect of cell competition (Menendez et al., 2010). This protection could result from the merging of several clones into a only one tumor mass (because it has been shown in epithelial tissues by different experimental designs, that when low density of mutant clones are generated they normally are eliminated, but on the contrary, when the density of the mutant clones is big, tumoral masses appear). It could be a clue to understand the differences in terms of growth inside of *cno^{R2}scrib^{1}* NBII mutant clones. As we explained in the results, not all of those double mutant clones grew in the same extend, being some of them really

massive and other no so big. It could be due to that, in some cases, the clonal assay produced only one mutant NBII. In those cases, even if the double mutant NB had the potential to overgrow, the WT environment better contained this capacity. But in other cases, we could obtained more than one mutant NB per hemisphere, which could merge during their proliferation period becoming more efficient avoiding the control restriction of the environment and getting the biggest sizes. Then, clone technique have proved its value as a tool to study tumor development because in this way we can follow how mutant cells interact and develop in a WT environment, situation that more similarly reproduce the tumor appearance in nature.

Regarding to *cno*^{R2}*scrib*^{1} tumor cell composition and development we found that independently of the final clone size all of them were almost totally composed by progenitor cells. When we studied in detail those population of progenitors we reported that a big amount of them were actually NBs and that the population of mature INPs was decreasing along the time. Thus, clone development could be explained by symmetric divisions of the NBs and the fate-reversion of INPs to previous stages (NB-like identity). In NBsII it was identified a regulatory cascade that promotes commitment to a progenitor cell identity by restricting their response to the self-renewal machinery; and many evidences suggest that aberrant activity of stem cell self-renewal pathways can transform progenitor cells into tumor initiating cells (Liu and Zong, 2012; Schwitalla et al., 2013; Visvader, 2011). Brat and Numb initiate this cascade by asymmetrically extinguishing the activity of the self-renewal factors in the cell in which segregate. Subsequently Erm and the SWI/SNF complex stably restrict the competence of the progenitor cell to respond to reactivation of self-renewal mechanism. Together, these cascades program the progenitor cell to undergo limited rounds of division, generating exclusive differentiated progeny (reviewed in (Janssens and Lee, 2014). A highly conserved component of the NBs self-renewal network is Notch, which encodes a transmembrane protein that upon proteolytic activation, the Notch intra-cellular domain translocates into the nucleus where it complexes with the DNA binding protein Su(H) to activate target gene expression (Liu et al., 2010). Notch is both, necessary and sufficient to promote NBsII self-renewal, and over expression of the Notch intracellular-domain in NBsII leads to

supernumerary NB formation (San-Juan and Baonza, 2011; Song and Lu, 2011). A direct target of Notch is Dpn, whose overexpression also induces supernumerary NBs (San-Juan and Baonza, 2011). All these data proposed that an increased function of self-renewal factors could cause tumorigenesis. Actually, it was proved that tumor-initiating cells arise from the reversion of newly born Ase- INPs, which aberrantly misexpressed self-renewal factors (Janssens et al., 2014; Xiao et al., 2012). Cell fate determinants Brat and Numb, which in normal conditions are uniquely segregated into the newly born INPs function to prevent tumorigenesis by preventing aberrant activation of the self-renewal machinery in these progenitors. Brat prevents newly born INPs fron reverting into supernumerary NBs by avoiding the aberrant expression of Klu and Dpn. Consistent with this, Dpn is aberrantly expressed in INPs in *brat* mutants (Janssens et al., 2014; Xiao et al., 2012). Numb functions as an evolutionary conserved negative regulator of Notch signaling (Giebel and Wodarz, 2012; Kandachar and Roegiers, 2012) and promotes the commitment to a functional INP identity by restricting Notch function in this cell. Therefore, Brat and Numb presence and levels are critical to INP restriction and identity configuration. *lgl (Haenfler et al., 2012)*, *aurora-A (Lee et al., 2006a)* and *polo (Wang et al., 2007)* mutants present supernumerary NBs, and in all the cases aPKC and Numb were found all around the cell cortex during progenitors division. The explanation for those phenotypes is that Numb failed to be segregated into only one of the daughter cells. Thus, the absence of an accumulation of Numb in the basal pole of the cell during the division caused that Numb levels in the basal daughter cell were not sufficient to efficiently suppress Notch and so, the self-renewing machinery, leading to reversion of the immature INPs to a NB identity. In $cno^{R2}scrib^{1}$ tumors, asymmetric cell division was strongly altered. aPKC and Numb were always found all around the cell of dividing cells instead of forming an apical and a basal accumulation respectively. These results strongly suggest that, as it happens in the above mentioned mutants, Numb segregation fails in $cno^{R2}scrib^{1}$ tumoral cells, leading to not enough Numb levels in the new born INPs to suppress Notch activity and therefore permitting them to revert to NB identity (Figure 1). Supporting this idea, we reported more cells with Notch activity in $cno^{R2}scrib^{1}$ tumors than in WT. But not only the newly born (immature) INP (Ase^{-}) could revert to a NB state. It has been shown that mature INPs also revert to a NB state in *erm* mutants (Janssens et al., 2014). Erm functions

temporally after Brat and Numb to direct the SWI/SNF (chromatin remodeling complex), thereby restricting the competence of INPs to respond to the self-renewal machinery, i.e. these mature INPs also self-renew to generate more INPs and GMCs, but they have to divide just only 5-7 rounds more. In *cno*^{R2}*scrib*^{1} tumors the percentage of mature INPs within the population of the clone was smaller than in WT clones; furthermore, that percentage was decreasing along tumor development, suggesting that in *cno*^{R2}*scrib*^{1} double mutants mature INPs also failed to stabilized their identity leading to a potential reversion to previous stages along time (Figure D1). We cannot discard that mature INPs die but it does not seem the most likely explanation as the tumors where the percentage of INPs was the smallest were the biggest tumors that massively overgrew.

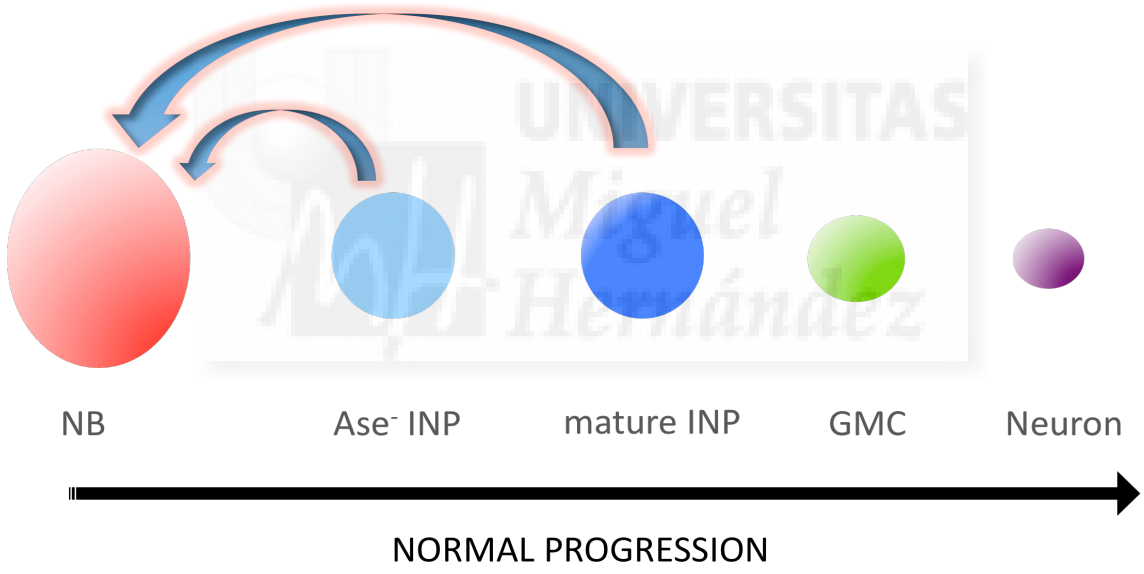


Figure 33. NBsII normally divide asymmetrically to produce an immature INP and to self-renew. The INP follows a maturation program to acquire a restricted proliferation capacity to divide between 5 to 7 times more producing more INPs and GMCs which will divide one more to produce neurons. We proposed that in *cno*^{R2}*scrib*^{1} double mutants both, immature Ase- and mature INPs are not able to efficiently restrict and stabilize the self-renewal machinery triggered by insufficient levels of Numb, and then revert to a NB stage along tumor progression (blue arrows).

To complete the description of *cno*^{R2}*scrib*^{1} tumoral overgrowths, it would be required to address if they behave as malignant tumors, i.e if they are inmortal, able to

continue dividing after larval stage and if they can cause metastasis. To investigate this behavior it would be necessary to study adult brains and also to perform allograft transplantation assays of mutant brain tissue into WT adult host, where we could follow the tumor development and its potential invasiveness properties.

In this study we have revealed that even though *cno* and *scrib* loss cooperate in NBsII to produce tumor formation at least in part by an up-regulation of Ras signaling, the loss of function of *scrib* with the up-regulation of Ras (Ras^{v12}) does not behave exactly in the same way in the brain. On one hand, Ras^{v12} was able to rescue cell death of $scrib^{1}$ mutant clones as cno^{R2} did in the same *scrib* mutant background. This reinforces the idea that the rescue of cell death by *cno* loss in *scrib* mutant background is due to an up-regulation of Ras signal. In addition, $scribRas^{V12}$ showed, like $cno^{R2}scrib^{1}$, supernumerary NBs. On the other hand, $Ras^{V12}scrib^{2}$ mutant clones did not overgrow to the same extent that $cno^{R2}scrib^{1}$ did. This could be due to several facts, such as the additional role of Cno during ACD. *scrib* NBsII mutant clones showed failures in the ACD process but these defects were not so severe as the ones observed in $cno^{R2}scrib^{1}$ tumors. Then, the simultaneous loss of function of two ACD regulators, *scrib* and *cno* get worse the process. In fact, we observed failures in ACD in 100% of $cno^{R2}scrib^{1}$ mutant clones analyzed. In addition, *cno* loss could affect other signaling pathways apart of Ras, as it has been shown in other contexts (Carmena et al., 2006), that could contribute to tumor growth and progression. More investigation will be needed to clarify the possible implication of other signaling pathways.

It has been extensively reported in epithelial tissues that, in the cooperation between cell polarity gene *scrib* and oncogenic Ras^{V12}, the downstream effector of Ras is the Ra/MAPK pathway (Brumby and Richardson, 2003). In this cooperative context, the JNK pathway, activated by *scrib* loss, changes its apoptotic role for a pro-proliferative effect, activating the JAK/STAT pathway and inducing tumor growth and invasion (Wu et al., 2010). In mammals, oncogenic Ras is thought to exert its effects through a number of different effectors including the Raf/MAPK pathway, the growth regulator phosphatidylinositol 3-kinase (PI3K) pathway and cell architecture regulators, such as Ral and Rho small GTPase family members (review in (Shields et

al., 2000). In the *Drosophila* eye disc, the ectopic expression of PI3K, Ral or Rho in a *scrib* mutant background does not phenocopy the effects of activated Ras. Only the Raf/MAPK pathway and its downstream targets seem to be responsible for the effect of activated Ras in the *scrib* mutant background. Indeed, Ras and the Raf/MAPK pathway have been implicated in a number of developmental processes in the Drosophila eye disc (Brumby and Richardson, 2003). However, in the *Drosophila* central brain, we did not observed Ras activation (by analyzing diPMAPK expression) in WT conditions, and removing Ras signaling using the DNRas form in WT clones did not have any effect in those clones (data not shown). This suggests that Ras is not normally active in *Drosophila* NB in the central brain during development. Conversely, Ras is signaling in in $cno^{R2}scrib^{1}$ mutant clones, as the expression of DNRas in $cno^{R2}scrib^{1}$ tumors effectively rescued the overgrowth phenotype. All these data together suggest that Ras is only activated under special mutant conditions in *Drosophila* larval central brain. Furthermore, we studied the posible activation of the Raf/MAPK pathway by Ras in the NB in the central brain. Raf-RNAi did not have effect on $cno^{R2}scrib^{1}$ tumors (data not shown). dipMAPK was not expressed in any of the studied conditions (WT, *scrib*, *cno* and $cno^{R2}scrib^{1}$ mutant clones), and only in low levels in clones after over-expressing UAS-Ras^{V12} alone. These data suggest that Ras pathway is not likely functioning via Raf/MAPK in the *Drosophila* central brain. Some studies using the developing *Drosophila* wing have shown that Ras, dMyc and PI3K control rates of cellular growth and progression through regulation of the G1/S transition during the cell cycle (Prober and Edgar, 2002). In this system, Ras is required to maintain normal dMyc but not dPI3K levels during development. However, in special conditions, when the oncogenic form of Ras (Ras^{v12}) is expressed, it can act activate PI3K signaling as it happens in mammals to promote growth (Prober and Edgar, 2002) (Figure 34).

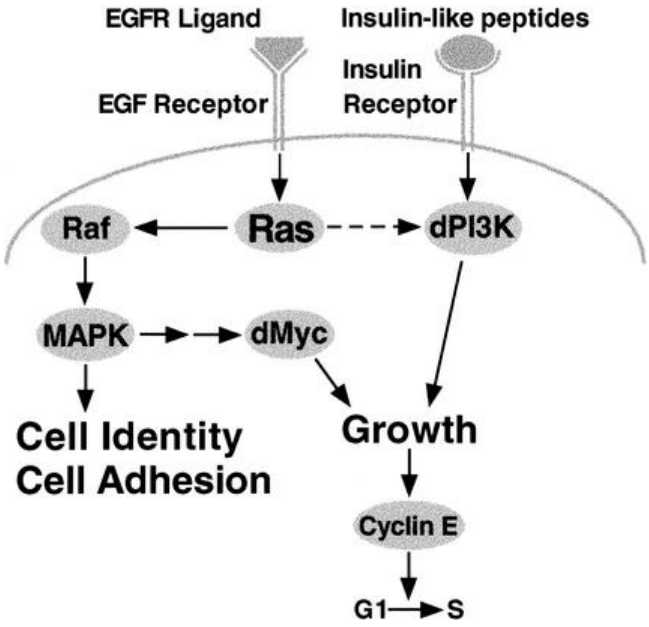


Figure 34. Model for interactions between Ras, dMyc, and dPI3K in the developing *Drosophila* wing. Ectopic expression of Ras^{V12} drives cell growth via at least two genetically separable pathways. Ras^{V12} activates Raf/MAPK signaling, which increases levels of dMyc protein. Ras^{V12} also independently activates dPI3K signaling. The resulting increased rate of cell growth increases cyclin E protein levels, thereby promoting G*1*/S progression (Prober and Edgar 2000). Ras is normally activated by the binding of ligands to the EGF receptor, and is required to maintain normal levels of dMyc protein but not dPI3K signaling (dashed arrow). dPI3K is likely normally regulated by the binding of insulin-like peptides to the Insulin receptor. Ras^{V12} also affects cell identity and adhesion via Raf/MAPK signaling. Arrows indicate genetic interactions and do not imply direct molecular interactions *(Extracted from (Prober and Edgar, 2000, 2002).*

Thus, these authors found that higher levels of Ras activity than can be normally generated in WT cells are required to activate PI3K, situation that actually is frequent also in human tumors (Barbacid, 1987). Furthermore, it has been proved in $Dlg^{RNAi}Ras^{V12}$ tumors in wing discs that they critically depend on PI3K signaling, i.e loss of PI3K/Akt signaling reduces $Dlg^{RNAi}Ras^{V12}$ tumor growth while it only slightly affects growth in WT tissue (Willecke et al., 2011). Also, expression of dAkt in *scrib- lasts-* mutant cells in imaginal disc increased tumor size (Pagliarini and Xu, 2003). These data support the idea that Ras can also act through PI3K when it is over-activated. Then, with all of our results, we suggest that in *Drosophila* central brain NBs, Ras is not being activated under normal conditions, but when it is up-regulated in $cno^{R2}scrib^{1}$ tumors, Ras could act through PI3K signal instead of the Raf/MAPK pathway. Future experiments in the lab will further clarify this point.

As I already mentioned, we have observed some differences between *Drosophila* epithelial tissues (antennal discs) and central brain NBs regarding to downstream effetors of Ras signaling. It was described that Ras^{V12} cooperates whith *scrib* loss of function in imaginal discs via MAPK pathway, and according with this we observed an up-regulation of MAPK signals in $Ras^{V12}scrib^{2}$ mutant clones in antenal discs compared with the WT clones in the same system. In cno^{R2} mutant clones or $scrib^{1}$ mutat clones in disc we did not observed a clear up-regulation of MAPK. We propose that *cno* loss of function is able to up-regulate Ras signaling (because our previous results in NBs and data already published in other systems where it was proved this relationship between cno and Ras). Furthermore, it has been reported that mammalian Scrib, direcly modulates MAPK signaling, being able to downregulate it signal. In human epthelial cells expresing oncogenic Ras or Raf, loss of *scrib* promotes invasion of cells, and the mechanism by which this occurs is the regulation of MAPK signaling by Scrib (Dow et al., 2008). The supression of MAPK signaling is higly conserved function of *scrib* as it also prevents Ras-mediated defects in Drosophila wing disc. All these together, we propose that *cno* and *scrib* loss of function clones in antenal discs are actually up-regulating Ras/MAPK levels, but it may be possible that this increase does not overtake the threshold of detection by the antibody. But when we observed clones with both genes mutated, $cno^{R2}scrib^{1}$, then we were able to observe MAPK signal, suggesting likely both mutation together get a higher up-regulation of MAPK that now it is detectable. This result support previous results regarding that $cno^{R2}scrib^{1}$ synergistically interact to up-regulate Ras signal.

The development of tumors requires a number of genetic and epigenetic alterations including normally both the loss of tumor suppressive mechanisms and the acquisition of tumor promoting/oncogenic changes. In this thesis project we show a novel synergistic interaction between the loss of function of two ACD regulators, *cno* and *scrib* that lead to tumor formation, at least in part, by up-regulating Ras signaling. The down-regulation of the mammalian homolog of *cno*, Afadin has been associated with a bad prognosis in breast cancer (Letessier et al., 2007) and a recent work demonstrated the negative regulation of Ras by Afadin in myeloid leukemia (Manara

et al., 2014). In addition, even still contradictory roles reported for the mammalian Scrib, either as an oncogene or as a tumor suppressor gene, at least some of these studies have demonstrated that Scrib expression is decreased in specific tumors, such as those associated with HPV infection (Massimi et al., 2004). Therefore, similar cooperative mechanism, as the one that we present in this thesis project between *cno* and *scrib* loss, could exist in the development of some human cancers. *Drosophila* has long been recognized as a valuable tool for understanding the mechanisms by which tumors are formed and regulated, given the conservation of many gene functions. Thus, studies in this model system can help us to understand better the mechanisms behind cancer formation and eventually contribute to the development of more effective drug therapies.

CONCLUSIONS

I. Cno is expressed in the cytoplasm and centrosomes of progenitor cells in *Drosophila* central brain NBs, showing an apical enrichment at metaphase.

II. *cno* also functions as ACD regulator in NBsII. cno^{R2} mutant clones are smaller than WT clones, presenting a reduced-sized NB and an altered clone development and cellular composition (less INPs and GMCs).

III. $scrib^{1}$ NB mutant clones are eliminated by the action of the JNK pathway in the central brain as it happens in epithelial tissues.

IV. *cno* and *scrib loss* synergistically cooperate to lead to tumor growth in *Drosophila* central brain.

V. The cooperation between *cno* and *scrib* loss is mediated, at least in part, by an up-regulation of Ras signaling.

VI. Ras is not active in WT conditions in NBsII in *Drosophila* larval brain.

VII. The role of Ras in $cno^{R2}scrib^{1}$ mutant clones is not mediated by Raf/MAPK pathway in NBsII of *Drosophila* larval brain.

VIII. Ras^{V12} rescues the cell death in *scrib* mutants. $Ras^{V12}scrib^{2}$ clones display supernumerary NBs but they do not overgrow to the same extent that $cno^{R2}scrib^{1}$ mutant clones.

IX. In antennal discs *cno* and *scrib* loss also cooperate to promote overgrowth and an up-regulation of Ras signaling, which in this system functions via the Raf/MAPK pathway.

CONCLUSIONES

I. Cno se expresa en el citoplasma y en ambos centrosomas de los progenitores de los NBsII del cerebro central de *Drosophila,* mostrando un enriquecimiento apical durante la metafase celular.

II. *cno* también funciona como regulador del proceso de división asimétrica en NBs larvarios. Los clones mutantes cno^{R2} muestran un NB de tamaño reducido y el desarrollo y la composición del clon están alterados, siendo los clones de menor tamaño que los WT (menos INPs y GMCs).

III. Los NBs mutantes $scrib^{1}$ NB son eliminados mediante la acción de la vía de la JNK en el cerebro tal y como sucede en tejidos epiteliales.

IV. La falta de función de *cno y scrib* interaccionan de forma sinergística produciendo el desarrollo de crecimientos tumorales en el cerebro central de *Drosophila*.

V. La cooperación producida por la falta de función de los genes *cno* y *scrib* está mediada, al menos en parte, por el aumento de señalización de la vía de Ras.

VI. Ras no está activo en condiciones WT en los NBsII del cerebro de *Drosophila*.

VII. El papel de Ras en los mutates $cno^{R2}scrib^{1}$ no está mediado por la vía Raf/MAPK en los NBsII de *Drosophila*.

VIII. La sobreexpresión de Ras (Ras^{V12}) rescata la muerte celular que sufren los clones mutantes de *scrib*. $Ras^{V12}scrib^{2}$ presenta NBs ectópicos pero estos mutantes no sobrecrecen de la misma manera que los dobles mutantes $cno^{R2}scrib^{1}$ mutant.

IX. En los discos de antena la falta de función de *cno* y *scrib* loss también coopera para promover el aumento de la señalización de Ras, pero en este sistema Ras funciona a través de la vía de MAPK.

MATERIALS AND METHODS

1. *DROSOPHILA* STRAINS AND GENETICS

1.1. Stocks

Fly stocks were obtained from Bloomington Stock Center (BSC) or alternative published sources. The following mutant stocks were used for the current work: *cno*::GFP (generated in our lab), UAS-*cno* (Carmena et al., 2006), *hsFlp* (a gift from María Domínguez), cno^{R2} *(Sawyer et al., 2009),* $scrib^{1}$ (BSC), UAS-Ras^{v12}, UAS-$Ras^{v12}scrib^{2}$ (both provided by G. Halder Leuven), iRNA-Ras (VRDC), DNRas (Lee et al., 1996), UAS-mCD8::GFP on the second and the third chromosome (Lee et al., 1999), Dll-Gal4 (BSC), Su(H)-lacZ (our lab).

1.2. GAL4-UAS system

GAL4-UAS system (Figure 36 A) was used to get ectopic expression of genes or constructs of interest in different tissues or systems. The following fly construct was used to drive the expression of GFP in larval brain NBII membranes: DllGal4-UASCD8::GFP.

All crossed GAL4-UAS were carried out at 29ºC. *yellow white* (*yw*) strains were used as the reference control wild-type strain. Balancer chromosomes containing different GFP or RFP transgenes were used for identification of homozygous mutant larval brains.

1.3. Minos Mediated Integration Cassette (MIMIC)

Transposable elements (TEs), also known as "jumping genes," are DNA sequences that move from one location on the genome to another. These elements were first identified more than 50 years ago by geneticist Barbara McClintock. Since decades

geneticists have been taking advantages from them to manipulate *Drosophila* genome and to assess the function of the genes. The most commonly used transposons are the *P*-elements, *piggyBac* and *Minos* (Ryder and Russell, 2003; Venken and Bellen, 2005, 2007). *P*-elements mobilize efficiently and often excise imprecisely but they show a preference insertion position in the 5' end of the genes. *piggyBac* elements show much less restriction regarding to the insertion sites but mobilize less efficiently than *P*-elements and only excise precisely. *Minos* elements insert almost randomly along genome, transpose stably and efficiently in numerous organisms and excise imprecisely at a useful frequency (Venken et al., 2011).

One of the most popular applications of transposons is to create mutation directly by insertion or by imprecise excision, being *Minos* and *P*-elements especially useful in this last application. If P-elements have been being widely used along last decades, *Minos* elements are nowadays very used as well due to their demonstrated versatility.

Minos Mediated Integration Cassette (MiMIC) contains a gene-trap cassette (consisting of a splice acceptor (SA) followed by stop codons in all three reading frames, the coding sequence of the fluorencent protein GFP and an SV40 polyadenylation signal sequence), a *yellow*$^{+}$ marker flanked by two inverted bacteriophage φC31 *att*P sites (Figure 35 A). The *att*P sites allow the replacement of the intervening sequence of the transposon with any other sequence through recombinase mediates cassette exchange (RMCE). This replaces the *yellow*$^{+}$ marker, so RMCE events can be identified by loss of body pigmentation. Since donor cassettes can contain any DNA fragment, MiMIC provides enormous flexibility. Thus, MiMIC insertions near the 5' and 3' ends of genes allow the integration of regulatory elements such as enhancers or insulators to direct or restrict expression respectively. Such insertion can also be used to integrate an *FRT* site for creating Flp-based chromosomal rearrangements. Insertions in 5' UTR introns allow the incorporation of binary expression components, such as GAL4/UAS and QF/QUAS and recombinases such as Flp. Insertions in coding introns allow integration of protein tags including an

ever-expanding repertoire of fluorescent markers and conditional protein destruction tags, and other gene-trap mutator cassettes (Figure 35 C). Finally, any insertion can be used as a generic docking sites for integration transgenes (Venken et al., 2011).

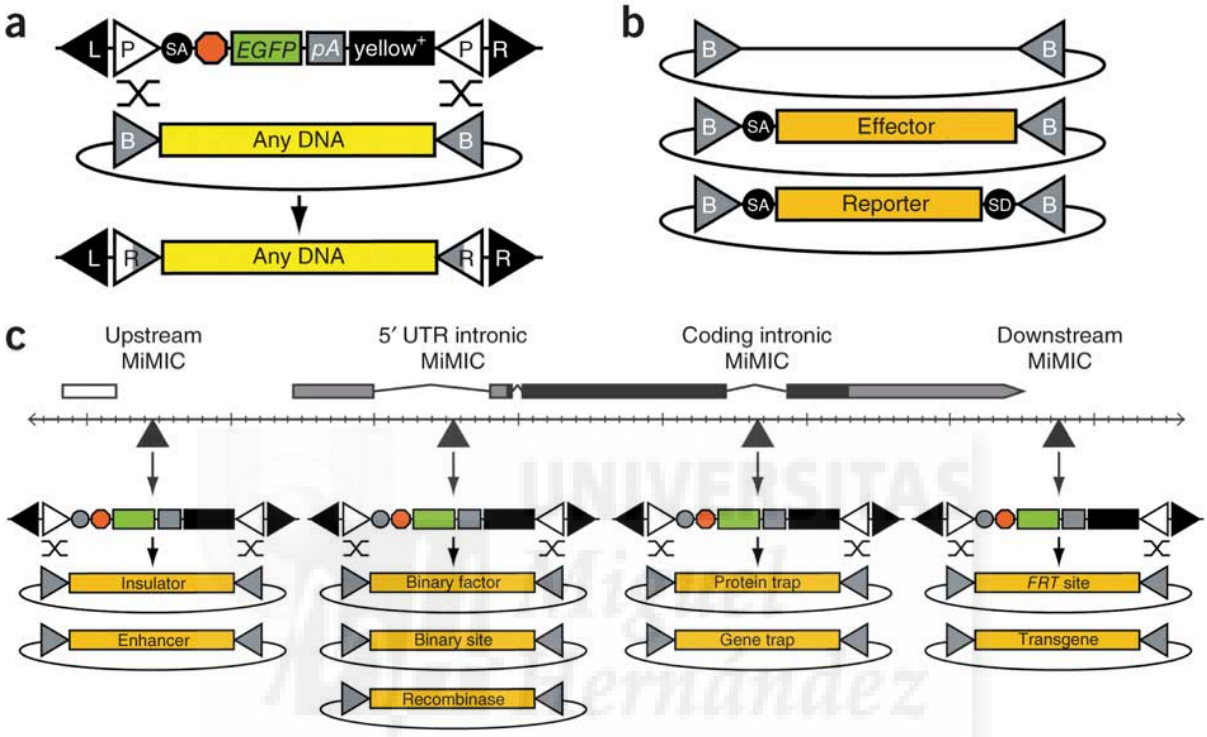


Figure 35. **A)** MiMIC consists of two *Minos* inverted repeats (L and R), two inverted ΦC31 integrase *attP* sites (P), a gene-trap cassette consisting of a splice acceptor site (SA) followed by stop codons in all three reading frames and the *EGFP* coding sequence with a polyadenylation signal (*pA*), and the yellow^{+} marker. The sequence between the *attP* sites can be replaced via RMCE with a plasmid containing two inverted *attB* sites (B), resulting in two *attR* sites (R). **B)** Three *attB* plasmids for RMCE: a correction plasmid consisting of a multiple cloning site, a gene-trap plasmid consisting of an SA fused to a downstream effector, and a protein-trap plasmid consisting of a reporter flanked by SA and splice donor site (SD). **C)** Various MiMIC insertions in a hypothetical gene with a regulatory element (white), 5′ and 3′ untranslated regions (gray), and coding regions (black) that can be used for several applications as indicated. *(Taken from (Venken et al., 2011)*

2. MARCM LARVAL BRAIN CLONES

To generate NBII mutant clones in a wild-type surrounding environment we took advantage of the Mosaic Analysis with a Repressible Cell Marker (MARCM) (Venken and Bellen, 2005) clones technique. The basic premise of MARCM is generation of positively label cells or group of cells related by lineages homozygous for a mutant chromosome using the heat shock-inducible flipase (FLP) recombinase/FLP recombination target (FRT) system (Golic and Lindquist, 1989). The principle of the MARCM system is schematically depicted in Figure 36.

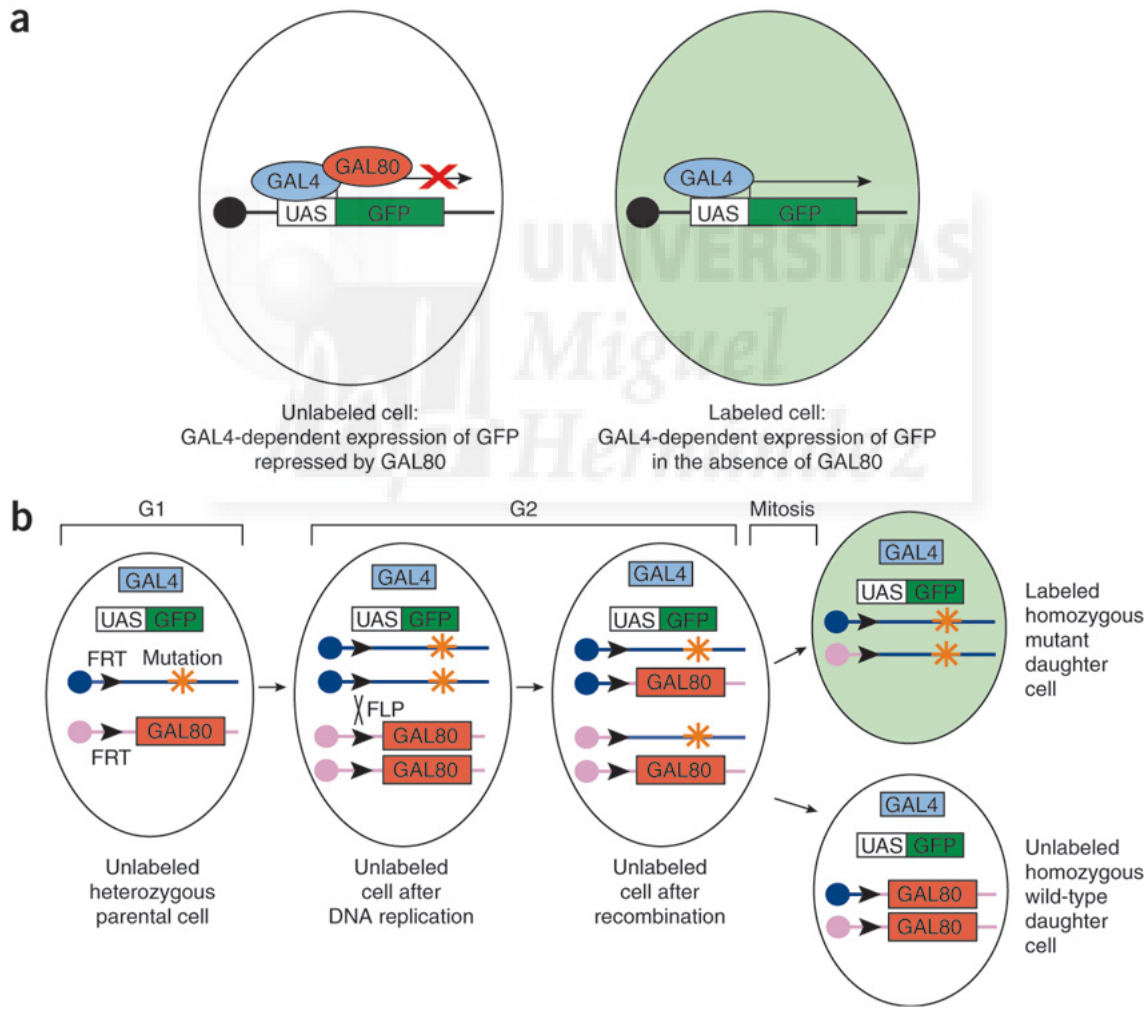


Figure 36. Schematic representation of the GAL4-UAS system with GAL80 and the MARCM genetic system. A) In cells containing the GAL80 protein, GAL4-dependent expression of a UAS-gene (GFP) is repressed. By contrast, cells containing GAL4 but lacking GAL80 will express the UAS-gene (GFP). In this schematic, genes are denoted by colored

boxes whereas proteins are denoted by colored ovals. **B)** MARCM requires two FRT sites located at the same position on homologous chromosomes, GAL80 located distal to one of FRT sites, FLP recombinase located anywhere in the genome, GAL4 located anywhere in the genome except distal to FRT site on the FRT, GAL80 recombinant chromosome arm, UAS-marker located anywhere in the genome except distal to FRT site on the FRT, GAL80 recombinant chromosome arm, and optionally a mutation distal to FRT in trans to but not on the FRT, GAL80 recombinant chromosome arm. Site-specific mitotic recombination at FRT sites (black arrowheads) gives rise to two daughter cells each of which is homozygous for the chromosomes arm distal to the FRT sites. Ubiquitous expression of GAL80 represses GAL4-dependent expression of a UAS-marker (GFP) gene. Loss of GAL80 expression in homozygous mutant cells result in specific expression of GFP. *Taken from (Wu and Luo, 2006)*

In the heterozygous and homozygous wild-type tissue, a GAL-80 transgene under the control of a ubiquitous promoter represses GAL4 activity and prevents expression of membrane associated reporter (UAS-CD::GFP). FRT sites are placed proximal to the mutation in one chromosome arm and proximal to GAL80 in the homologous chromosome arm. Heat shock-induced mitotic recombination generates homozygous mutant clones that have lost the repressive GAL80 and are thus labeled by the expression of GFP. GFP can be visualized in all mutant clones it is driven by ubiquitous GAL4 driver or in only a subset of the mutant cells when using a specific GAL4 driver. Furthermore, the ability to select the timing to induce the heat shock and therefore produce the mitotic recombination at a specific stage is useful to define patterns of neurogenesis or select cells that are dividing in this stage of development.

The easiest way is to generate "MARCM-ready" flies that contain FLP recombinase, a FRT site, GLA4, tubulin 1 promoter (tubP)-Gal80 and UAS-marker. These flies are ready to cross to a line containing the corresponding FRT and mutation of interest for MARCM analysis. In this work we generated a common stock containing the DllGal4-UASCD8::GFP (chromosome II) system to drive GFP expression in NBII and FRT82btubPGal80 (chromosome III) repressible system that we crossed we flies containing hsFLP (chromosome x) and FRT82b followed by the mutation of interest (chromosome III) (Figure 37). Crosses and egg lying were performed at 25º during 6 hours. After that, adult flies were taken out from the laying egg cage containing standard yeast food and eggs developed until arriving to 48 hours. Thus, 48 hours after egg lying (AEL), at late 1st/early 2nd instard larvae, we induced 2 hours

heat-shock at 37º. After heat-shock, larvae were placed at 25º (or 29º in the case that the resulting larvae have two UAS transgenes to activate) to develop 72 hours more. Larvae were collected at 120 AEL (or 96 after larvae hatching (ALH)) at 3rd instar larvae (Figure 38).

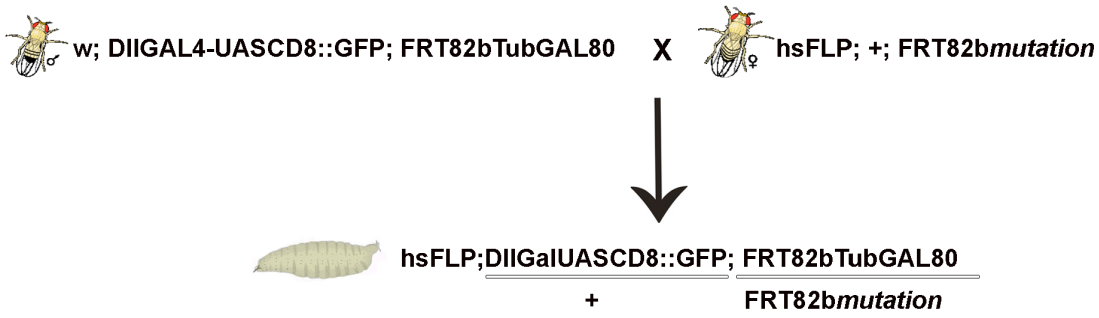


Figure 37. Schematic representation of the crosses made to get MARCM clones in larval brain NBsII system.

Especial clarification it is needed in the case of *cno*^{R2}*scrib*^{1} mutant clones. Larvae carrying mutant clones in the brain suffer a developmental delay of 24 hours approximately. So in this case, larvae that were collected 120 ALL (or 96 ALH) were at early LIII stage. We needed to wait 24h more (144 AEL or 120 ALH) to have late LIII larvae. They were recognized because they stop feeding and crawled out of the food to start pupation.

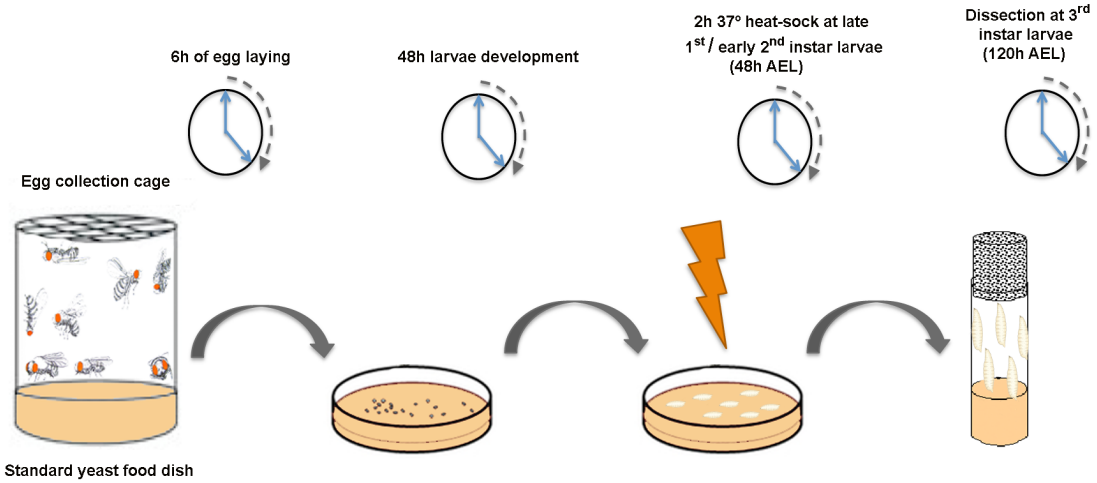


Figure 38. Drawing describing process to get WT and mutant NBII clones with MARCM technique. We put around 10 to 20 female virgins with 1 to 10 males in egg collection cages

with standard yeast food and they stayed together during 6h for egg laying. After that time, adult flies were removed and we put the dishes containing eggs at 25º to complete 48h of development. At 48h AEL, when larvae were between late 1st and 2nd instar stage we induced heat-shock during 2h at 37º. The last step consisted in let them grow until 3rd instar larvae along 72h more at 25º. Finally we dissected them when they stopped feeding and started to crawl out of the food at 120AEL.

3. DISSECTION AND FIXATION OF LARVAL BRAINS

3.1. Larval brain dissection and selection

To get larval brain for its examination we dissect Drosophila larval brain in PBS medium. All the brains that were dissected were individually placed in PBS drops to be inspected. Using fluorescence microscopy we could select brains carrying GFP positively label clones and discard brains that didn't present clones in the area of interest.

3.2. Formaldehyde fixation

After picking up the GFP positive brains they were placed in crystal dishes containing 1,5mL of 4% Formaldehyde dilution. We covered the dishes putting opaque lids on them and they were incubated for 40 minutes at room temperature in the shaker.

3.3. Heat methanol-method

Some types of proteins, like Cno, need an especial treatment to be efficiency label. In this case we used heat methanol-method to fix and prepare the tissue for Cno staining. Again after selecting GPF positive brains, we placed them in a 1,5mL eppendorf containing E-wash solution (70mM NaCl, 0,1% Triton X-100) and fixed them during 16 seconds at 80º. Brains were chilled with an excess volume of ice-cold E-wash and incubated on ice for 2 minutes (Tepass, 1996). To wash the brains we remove the E-wash media and added PBT buffer and maintained the brains 5 minutes more on ice. Later we transferred the brains to a crystal dish were we added methanol. We covered

the dishes putting opaque lids on them and they were incubated for 40 minutes at room temperature in the shaker.

4. IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

4.1. Antibody staining

Fixed tissue was washed for 1 hour in phosphate-buffered saline (PBS) containing 0,3% Triton X-100 (PBT), blocked for 1 hour at room temperature in PBT containing 0,1% bovine serum albumin (BSA), followed by an over-night (ON) incubation at 4ºC or 2 hours at room temperature (RT) with the first antibody. First antibody was always diluted in PBT-BSA following antibody concentration indicated (incubation and washing was done in the shaker). After washing three times for 15 minutes in PBT, tissue was incubated with the secondary antibody also diluted in PBT-BSA for 1 hour at RT, washed three time for 15 minutes in PBT and mounted. To this last step we used Vectashield mounting media for fluorescence (Vector labs), which was directly added to the tissue after washing. Preparations where set up in glass slides with brains or imaginal discs where placed in dorsal position following a line and covered with a covership. Fluorescence images were recorded by using Leica upright DM_SL Confocal miscroscope (Leica Spectral Confocal acquisition software). All images were taken with an HCX PL APO 63x or 40x oil CS objective. Images were assembled using Adobe Photoshop CS3.

4.2. Immunofluorescence

The following primary antibodies were used: rabbit anti-Cno 1:400 (Speicher et al., 2008) rat anti-Elav 1:400 (DSHB), rabbit anti-Baz 1:1000 (Wodarz et al., 1999), ginea pig anti-Numb 1:400 (Rhyu et al., 1994), rabbit anti-phospho-histone-H3 1:400 (Millipore), rabbit anti-Cnn 1:400 (gift from Thomas C. Kaufman), rabbit anti-aPKCς 1:100 (C-2 Santa Cruz), rabbit anti-Scrib 1:4000 (gift from Chris Doe), Mouse anti-Pros 1:50 (Hybridoma Bank), ginea pig anti-Dpn 1:200 (our lab), ginea pig anti-Dpn (1:1) (a gift from Cheng-Yu Lee lab), rabbit anti-Ase (our lab), Mouse anti-Dlg 1:100 (Parnas et

al., 2001) , rabbit anti-L(2)gl 1:100 (Santa Cruz), rabbit anti-PntP1 1:500 (a gift from Skeat lab), rabbit anti-Pins 1:200 (Parmentier et al., 2000), rabbit anti-Mud 1:100 (gift from Yasushi Izumi), rabbit anti-CyCE 1:100 (Santa Cruz), Rabbit anti-pAKT 1:100 (Promega).

Direct secondary antibody conjugated with fluorescents dyes were used: Alexa Flour 633 1:200 (Invitrogen), Alea Fluor 546 1:200 (invitrogen), Alexa Fluor 488 1:200 (Invitrogen).

4.3. Image acquisition, processing and data analysis

Fluorescence images were recorded by using Leica upright DM_SL Confocal miscroscope (Leica Spectral Confocal acquisition software). All images were taken with an HCX PL APO 63x or 40x oil CS objective. Images were analyzed using image processing package ImageJ and FIJI and assembled using Adobe Photoshop CS3. Statistic analyses were carried out with SPSS softwate using a t-test, Mann-Whitney rank sum test or ANOVA (Games-Howell test). Data graphic representation was done using simple bars in the case that data was analyzed by t-test. When population did not adjust to t-test criteria or variance was not equal, data was represented using box-plots or dispersion graphics.

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APPENDIX

- Perez-Gomez, R., Slovakova, J., **Rives-Quinto, N**., Krejci, A., and Carmena, A. (2013). A Serrate-Notch-Canoe complex mediates essential interactions between glia and neuroepithelial cells during Drosophila optic lobe development. J Cell Sci *126*, 4873-4884.

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