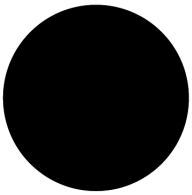


CSIC

UNIVERSITAS Miguel Hernández

INSTITUTO DE NEUROCIENCIAS

INSTITUTO DE NEUROCIENCIAS

CONSEJO SUPERIOR DE INVESTGACIONES CIENTÍFICAS (CSIC)

UNIVERSIDAD MIGUEL HERNÁNDEZ (UMH)

A NOVEL ROLE OF THE HIPPO SIGNALLING PATHWAY DURING ASYMMETRIC CELL DIVISION

Memoria para optar al grado de Doctor presentada por

ALYONA KEDER

Dirigida por Dra. ANA CARMENA

Presentada para la obtención del título de Doctor por la

Universidad Miguel Hernández

San Juan de Alicante, 2014

UNIVERSITAS
Miguel
Hernández

ACKNOWLEDGEMENTS

First of all, I would like to thank my thesis supervisor Dr. Ana Carmena for the guiding me through the difficulties that I met on my way of scientific career, and for the smart advises and for the constructive critics. I want to thank my dear colleagues that became my closer friends: Raquel Gómez, Jana Slováková, Noemí Ríves, Stephan Speicher, Maribel Franco, Fabiola Manfra, and Ania Pitas. I very appreciate your friendship and I hope that we can meet up more often independently of how far we are from each other. Ania and Noemí, thank you girls for being my closest friends and helping me in the most difficult times, and sharing happiness with me during the best moments. I hope to see you for a "coffee break" at any time anywhere we go, I really hope to not to lose this special connection with you girls! You are awesome!

Muchísimas gracias Raquel Pérez Gómez, la que me ha enseñado todo el castellano que manejo hoy en día, sin tu arte en explicarme los verbos reflexivos la vida en el laboratorio no sería tan divertida. Gracias a tí, Stephan, por cuidar de nosotras en el laboratorio, por introducirnos al mundo de la mosca, y por tu apollo a todas horas. Gracias a tí Janka, por el tiempo que pasémos juntas en el laboratorio y fuera de el. A Fabi, que ha estado poquito tiempo en nuestro laboratorio pero desde entonces es mi mejor amiga. Piccolina, ti voglio bene!

Хочу поблагодарить за огромную поддержку, за понимание и за мотивацию Лёшку, спасибо тебе за все твои "Ты справишся Лиса, ты сможешь, все у тебя получится!" Спасибо за твою в меня веру. Безусловно, огромное спасибо моей дорогой мамочке, которая всегда за меня переживала, поддерживала и подстегивала, когда надо. Я очень рада, что в трудные моменты ты всегда оказывалась рядом и придавала мне уверенности и решимости! Спасибо моему папе, моим дорогим бабушке и дедушке, моей тете Оле за поддержку, заботу и за дельные советы. Спасибо моей лучшей подруге Кате Гасилиной за ежедневное общение, за поддержку и за хорошие советы. Я дорожу нашей с тобой дружбой и верю, что где бы мы ни были, мы всегда найдем темы для разговора и возможность встретиться. Спасибо Катюше Кардаш, за наши с тобой понедельники и за моральную поддержку. Надо чаще созваниватся. Спасибо Вике за веселый досуг и за долгие телфонные разговоры по душам. Также спасибо всем тем кого я забыла перечислить, кто был со мной все эти 5 лет, переживал со мной все мои взлеты и падения, и разнообразил мою жизнь. А так же отдельное спасибо моему любимому животному, моему позитивному комочку счастья, который всегда заставляет меня улыбнутся и дарит необыкновенный заряд энергии, моей Еве.

Suurtänu ka sulle, Sven, ilma sinuta ma ei hakkaks postdokki otsima. Teretulemast Pariisi!

UNIVERSITAS
Miguel
Hernández

INDEX

ABBREVIATIONS 9

ABSTRACT 13

CHAPTER 1 15

INTRODUCTION 15

1. 1 ASYMMETRIC CELL DIVISION 15

1.1.1 ACD investigation models: Caulobacter crescentus 17

1.1.2 ACD investigation models: *Saccharomyces cerevisiae* 17

1.1.3 ACD investigation models: *Caenorhabditis elegans* 18

1.1.3.1 The PAR complex 19

1.1.3.2 Cell-fate determinants 19

1.1.3.3 Mitotic spindle position 20

1.1.4 ACD investigation models: *Drosophila melanogaster* 21

1.1.4.1 *Drosophila* Stem Cells 22

1.1.4.1.1 *Drosophila* Germline Stem Cells (GSCs) 22

1.1.4.2 *Drosophila* Sensory Organ Precursors (SOPs) 24

1.1.4.2.1 Establishment of planar polarity 24

1.1.4.2.2 Notch signalling in cell fate determination 26

1.1.4.3 *Drosophila* Neuroblasts 26

1.1.4.3.1 The apical protein complex 27

1.1.4.3.2 The cell-fate determinants 28

1.1.4.3.3 Daughter cell size differences and telophase rescue 29

1.1.4.3.4 Neoplastic tumour suppressor genes 29

1.1.4.3.5 Cell-cycle regulators 30

1.1.4.3.6 Phosphatases 31

1.1.5 ACD in Vertebrates 31

1.1.5.1 Meiotic ACD in Mammalian Oocytes 31

1.1.5.1.1 Conserved polarity proteins in mouse oocytes 32

1.1.5.2 ACD in mammalian neural stem cells (NSCs) and oligodendrocyte precursor cells (OPCs) 33

1.1.5.2.1 Apical polarity complex and cell-fate determination in mammalian NSCs 34

1.1.5.3 ACD in the epidermis 36

1.1.5.4 ACD in mammalian cell lines 37

1.2 PDZ DOMAIN-CONTAINING PROTEINS AND PROTEIN NETWORKS 38

1.2.1 Canoe/AF-6/Afadin 38

1.3. HIPPO SIGNALLING PATHWAY 41

1.3.1 The core kinase cassette 41

1.3.2 Upstream regulators of the Hippo core cassette 42

1.3.2.1 The Mer/Ex/Kibra complex 44

1.3.2.2 The Crb-Par apico-basal cell polarity complex 44

1.3.2.3 The Fat planar cell polarity complex 45

1.3.2.4 GPCR signalling 45

1.3.2.5 Extracellular matrix and cytoskeleton 46

1.3.3 Yki transcriptional targets 46

1.3.4 Mammalian Warts: noncanonical LATS regulation and function 47

CHAPTER 2 49

MATERIALS AND METHODS 49
2.1 CLONING 49
2.1.1 Strains of yeast and bacteria and growth medias 49
2.1.2 Plasmids 49
2.1.3 PCR, oligonucleotides and cloning 50
2.1.3.1 *wts* cloning 50
2.1.2.1.1 PCR and cloning 50
2.1.2.1.2 Heat Shock Transformation of DH5α competent cells 52
2.1.2.1.3 Plasmid DNA extraction 52
2.1.3.2 *cno*, *numb* and *baz* cloning 53
2.1.2.2.1 PCR and cloning 53
2.1.2.2.2 the Directional TOPO cloning 54
2.1.2.2.3 LR cloning 54
2.1.4 Quickchange multi site-directed mutagenesis 55
2.2 YEAST TWO-HYBRID 55
2.2.1 Yeast competent cells preparation 55
2.2.2 Transformation of yeast competent cells 56
2.2.3 Protein extraction from yeast colonies and probe preparation 56
2.2.4 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) 57
2.2.5 Western blot 57
2.2.6 β-galactosidase activity determination 58
2.2.7 β galactosidase reaction 58
2.2.8 Protein concentration determination 58
2.3 CELL CULTURE 59
2.3.1 S2 cell culture transfection and recombinant protein expression 59
2.3.2 Cell lysis and immunoprecipitation 59
2.3.3 Recombinant protein elution 59
2.4 *IN VITRO* KINASE ASSAY 60
2.5 CO-IMMUNOPRECIPITATIONS IN VIVO 60
2.5.1 Embryo collection 60
2.5.2 Immunoprecipitation 60
2.6 DROSOPHILA EMBRYO FIXATION 61
2.6.1 Embryo collection and dechorionation 61
2.6.2 Formaldehyde fixation 61
2.6.3 Heat fixation 61
2.7 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE 62
2.7.1 Antibody staining 62
2.7.2 DAB histochemistry 63
2.7.3 Immunofluorscence 63
2.8 DROSOPHILA STRAINS AND GENETICS 64
2.8.1 Stocks 64
2.8.2 GAL4-UAS system 64
2.8.3 Generation of the germline clones 64
CHAPTER 3 65
OBJECTIVES 65
CHAPTER 4 67
RESULTS 67
4.1 BACKGROUND 67

4.1.1 Warts is a potential Canoe partner 67
4.2 RESULTS 68
4.2.1 Wts functionally interacts with Cno during ACD 68
4.2.2 The Hippo signalling pathway functions in ACD 70
4.2.3 Wts modulates key aspects of asymmetric NB division 72
4.2.3.1 Apical and basal determinant localization is imapired in *wts*^{x1} mutant NBs 73
4.2.3.2 Cell fate determinants fail to segregate correctly in *wts*^{x1} mutant NBs during the telophase 78
4.2.3.3 Mitotic spindle orientation fails in *wts*^{x1} mutant NBs 79
4.2.3.4 Wts is expressed in embryonic NBs 79
4.2.3.5 Wts forms a complex in vivo with Baz and Insc 84
4.2.3.6 WTS directly phosphorylates Cno and Baz 85
4.2.4 The function of Wts and Hippo signalling in ACD is conserved in different tissues 87
4.2.4.1 Muscle/heart progenitor asymmetric division is impaired in HIPPO pathway mutants 87
4.2.5 Muscle and heart progenitor specification is altered in Hippo pathway mutant components 90
CHAPTER 5 93
DISCUSSION 93
CHAPTER 6 97
CONCLUSIONS 97
BIBLIOGRAPHY 99
ANNEX I 119
ANNEX II 129

UNIVERSITAS
Miguel
Hernández

ABBREVIATIONS

ACD – Asymmetric Cell Division

AJ – Adherens Junction

APC/C – Anaphase Promoting Complex/ Cyclosome

aPKC – atypical Protein Kinase C

App – Approximated

Arp2/3 – Actin related protein 2 and 3

Asp – Abnormal spindle

AurA – Aurora A

bam – bag of marbles

Baz – Bazooka

BCS – Bloomington Stock Collection

BMP – Bone Morphogenic Protein

BP – Basal Progenitor

Brat – Brain tumor

BSA – Bovine Serum Albumin

Cnn – Centrosomine

Cno – Canoe

CNS – Central Nervous System

Crb – Crumbs

D – Dachs

DA1 – Dorsal Acute 1

Dco – Disc overgrown

Diap1 – Drosophila inhibitor of apoptosis 1

DIL – DILute domain

Dl – Delta

Dlg – Discs large

DO2 – Dorsal Oblique 2

Dpp – Decapentaplegic

Ds – Daschsous

EPC – Eve Pericardial Cell

Eve – Even-skipped

Ex – Expanded

F – Founder

FERM – 4.1, Ezrin, Radixin, Moesin

FHA – ForkHeAd

Fj – Four-jointed

Fmn2 – Formin 2

Fz – Frizzled

Gbb – Glassbottom boat

GMC – Ganglion Mother Cell

GPCR – G-Protein Coupled Receptor

GSC – Germline Stem Cell

Hpo – Hippo

Hth – Homothorax

JAK-STAT – JAnus Kinase and Signal Transducer and Activator of Transcription

L´sc – Lethal of scute

Lft – Lowfat

Lgl – Lethal giant larvae

Mats – Mob-as-tumor suppressor

Mer – Merlin

MEX – Muscle EXcess

Mira – Miranda

MTOC – MicroTubule Organizing Centre

Mts – Microtubule star

Mud – Mushroom body defect

N – Notch

NB – NeuroBlast

NE – NeuroEctoderm

Neur – Neuralized

Nrt – Neurotactine

NSC— Neural Stem Cell

NuMa – Nuclear Mitotic Apparatus

oRG –outer Radial Glia

PAGE – PolyAcrylamide Gel Electrophoresis

PAR – PARtitioning defective

PDZ – PSD-95, Dlg, ZO-1

PH3 – PhosphoHistone 3

PIE – Pharynx and Intestine in Excess

Pins – Partner of Inscuteable

PNS – Peripheral Nervous System

Pon – Partner of Numb

POS – POsterior Segregation

PP2A – Protein Phosphatase 2 A

Pros – Prospero

RA – Ras-Associated

RASSF – Ras ASSociation Family

RG –Radial Glia

SARA – SMAD Anchor for Receptor Activation

Sav – Salvador

SCD – Symmetric Cell Division

Scrib – Scribble

Sd – Scalloped

SDS – Sodium Dodecyl Sulfate

Ser/Thr – Serine/ Threonine

SOP – Sensory Organ Precursor

Spdo – Sanpodo

Stau – Staufen

Stg – stage

STRIPAK – STRiatin-Interacting Phosphatase And Kinase

SVZ – SubvVentricular Zone

TAZ – Transcriptional co-Activator with a PDZ-biding motif

Tsh – Teashirt

Upd – Unpaired

VZ – Ventricular Zone

Wg – Wingless

Wts – Warts

YAP – Yes-Associated Protein

Yki – Yorkie

Zyx – Zyxin

αAda – αAdaptin

ABSTRACT

Asymmetric cell division is a universal mechanism to generate cellular diversity during development and a crucial process in cancer and stem cell biology. *Drosophila* neuroblasts, the neural stem cells of the central nervous system, divide asymmetrically to give rise to another neuroblast that keeps on proliferating and a ganglion mother cell that is committed to initiate a process of differentiation. Some years ago, we isolated Warts (Lats1/2 in mammals) in a yeast two-hybrid screening as a potential partner of the PDZ protein Canoe, which we showed is a key player in modulating asymmetric cell division. Warts is a serine/threonine kinase that function at the core kinase cascade of the Hippo tumor suppressor signalling pathway involved in regulating cell growth, apoptosis and organ size. Hence, we decided to analyze whether the Hippo pathway functions in the process of asymmetric cell division. In this work, we have found specific genetic interactions between Canoe and all components of the Hippo pathway. Additionally, *warts* genetically interacts with multiple components specifically involved in modulating asymmetric cell division, such as aPKC, Inscuteable and Numb. Moreover, *warts* loss-of-function mutant embryos display clear defects in the localization of the apical proteins aPKC, Bazooka/Par3 and Canoe, as well as in the basal cell-fate determinants Numb and Prospero. Failures in the mitotic spindle orientation in metaphase neuroblasts and in the generation of unequeal-sized daughter cells are also detected. Finally, co-immunoprecipitation experiments have revealed that Warts is forming a complex *in vivo* with Inscuteable and Bazooka, and *in vitro* kinase assay experiments have shown that Warts is phosphorylating both Canoe and Bazooka. Not only neuroblasts but also muscle/heart progenitors require Wasrts and other components of the Hippo pathway for their asymmetric division. Altogether, these data unveil a novel function for the Hippo signalling pathway in asymmetric cell division.

RESUMEN

El proceso de división asimétrica es un mecanismo universal para generar diversidad celular a lo largo del desarrollo y es un proceso importante en la biología de las células madre y del cáncer. Los neuroblastos de *Drosophila melanogaster*, las células madre neurales del sistema nervioso central, se dividen de forma asimétrica para dar lugar a un neuroblasto, que mantiene la capacidad de proliferación, y a una célula ganglionar madre, que va a iniciar un proceso de diferenciación. Hace unos años, en nuestro laboratorio se identificó la proteína Warts (Lats 1/2 en mamíferos) como un potencial interactor de la proteína PDZ Canoe, utilizando la técnica del doble híbrido en levadura. Warts es una serina/treonina proteína kinasa que funciona en la cascada de kinasas dentro de la vía de señalización supresora de tumores de Hippo. Dicha vía está involucrada en el control del crecimiento de órganos, de la apoptosis y de la proliferación celular. Con todo, decidimos analizar la posible función de la vía de Hippo en el proceso de división asimétrica. En el presente trabajo de tesis doctoral, hemos descubierto una interacción genética totalmente específica entre Canoe y todos los componentes de la vía de Hippo. Además, Warts interacciona genéticamente con múltiples proteínas reguladoras de la división asimétrica, tales como aPKC, Inscuteable y Numb. Hemos determinado que la falta de la función de *warts* en embriones muestra defectos claros en la localización de proteínas apicales, como aPKC, Bazooka/Par3 y Canoe, en neuroblastos metafásicos, así como defectos en la localización de determinantes de identidad basales, como Numb y Prospero. También hemos observado fallos en la orientación del huso mitótico en neuroblastos metafásicos y defectos en la generación de células hijas de diferente tamaño, ambos aspectos críticos en la división asimétrica del neuroblasto. Finalmente, experimentos de co-inmunoprecipitación han demostrado que Warts forma un complejo *in vivo* con las proteínas apicales Bazooka e Inscuteable, y experimentos de fosforilación *in vitro* han mostrado que Warts fosforila a Bazooka y a Canoe. No sólo los neuroblastos sino también los progenitores musculares y cardiales requieren Warts y otros componentes de la vía de Hippo para su división asimétrica. Todos esos resultados ponen de manifiesto una nueva función de la vía de señalización de Hippo en el proceso de división asimétrica.

CHAPTER 1

INTRODUCTION

1. 1 ASYMMETRIC CELL DIVISION

Cellular diversity generation is a crucial and fundamental process in developmental biology. During embryogenesis many different tissues are formed. Cells in these tissues differ by their shape, size, morphology and gene expression. Asymmetric cell division (ACD) is a universal mechanism to generate cellular diversity, and so it is an important process during development (Betschinger and Knoblich 2004). Moreover, over the past decades ACD has become very relevant in the context of cancer- and stem cell biology (Knoblich 2010). In an ACD, the mother cell divides giving rise to two distinct daughter cells with different fates; one daughter cell maintains the proliferative/self-renewal capacity of the mother cell, whereas the other daughter cell is committed to initiate a process of differentiation. ACD balances stem cell self-renewal and differentiation by regulating the ratio between stem cell and differentiated cell populations. The establishment of an axis of the cell polarity is a fundamental prerequisite for the ACD. The mitotic spindle must be orientated along this polarity axis. Likewise, the so-called cell fate determinants locate asymmetrically respect to this axis of the cell polarity and they will be segregated into only one daughter cell (Knoblich 2008, Siller and Doe 2009).

The process of ACD has been intensively studied in simple model organisms such, as the bacteria *Caulobacter crescentus*, the budding yeast *Sacharromyces cerevisiae*, and especially in the nematode *Caenorhabditis elegans* and in the fruit fly *Drosophila melanogaster* (Figure 1).

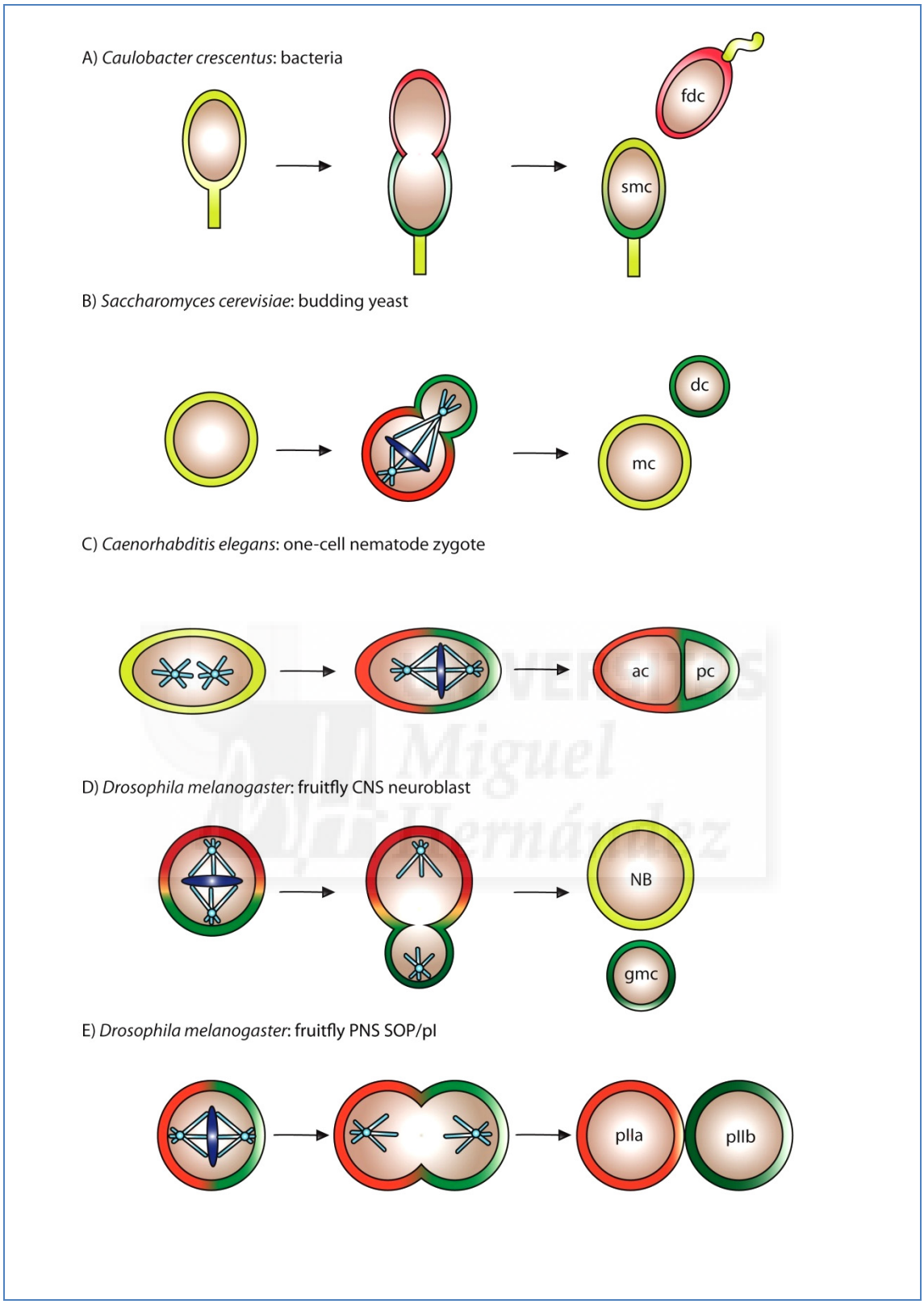


Figure 1. Asymmetric cell division (ACD) in different model organisms:

A) *Caulobacter crescentus* ACD. Bacteria *C. crescentus* divides asymmetrically giving rise to the stalked mother cell (smc) and to the flagellar daughter cell (fdc). **B) *Saccharomyces cerevisiae* ACD**. After asymmetric division the budding yeast *S. cerevisiae* gives rise to smaller daughter cell (dc) and to the larger mother cell (mc). **C) *Caenorabdithis elegans* ACD.** As a result of asymmetric division of one-cell

embryo two different daughter cells are generated: the anterior cell (ac) and the posterior cell (pc). **D) *Drosophila melanogaster* neuroblast ACD**. Neuroblast (NB) in the central nervous system (CNS) divides asymmetrically to give rise to a self-renewing NB and a differentiation initiating smaller ganglion mother cell (gmc). **E) *Drosophila melanogaster* SOP ACD.** Sensory organ precursor (SOP/pI) in the peripheral nervous system (PNS) is dividing to the pIIa and pIIb cells. Cell polarity proteins are shown in red and they are localized in the opposite side to the cell-fate determinants that are shown in green and that exclusively will be segregated to one daughter cell after the division; DNA at the methapase is shown in blue.

1.1.1 ACD INVESTIGATION MODELS: CAULOBACTER CRESCENTUS

The bacteria *Caulobacter crescentus* has been a good model in which to analyze the process of ACD because it is a single-cell organism that coordinates cell divisions and multiple differentiation events (Skerker and Laub 2004). *Caulobacter* divides asymmetrically generating two daughter cells with differences in cell morphology, replicative potential and motility. *Caulobacter* develops a stalk to attach to the surface of a plant prior to DNA replication. After division, the mother cell retains the stalk remaining fixed in the place, while the daughter cell develops the flagella to swim out from the fixed mother cell. This asymmetric division is triggered by the transcriptional factor CtrA that binds to the origin of replication, silencing it in the motile daughter cell. In addition, the phosphorylated form of CrtA governs the activation of genes essential for the morphogenesis and cell division. CtrA is phosphorylated in the flagellar daughter cell and it is degradated in the stalked cell, which initiate DNA replication. Many different kinases and phosphatases are involved in the regulation of CrtA during ACD (Tsokos and Laub 2012). This simple model has been intensively used to identify new kinases and phosphatases involved in the process of the asymmetric division. Recently two bi-functional histidine kinases, PleC and CckA, have been identified in *C. crescentus* (Subramanian et al. 2013).

Another unicellular model organism that shed light on the ACD process is budding yeast *Sacharomyces cerevisiae*.

1.1.2 ACD INVESTIGATION MODELS: *SACCHAROMYCES CEREVISIAE*

Saccharomyces cerevisiae is a eukaryote that divides asymmetrically developing a larger aging mother cell that is able to switch mating type, and a smaller young daughter cell (the bud) that maintains its original mating type. Many different proteins, such as

the small GTPase Cdc42, localize to the bud cortex and orient actin cables toward the polarity axis (Slaughter et al. 2009). Also, the protein Ash1 is asymmetrically localized only in the nucleus of the daughter cell, repressing in this cell the switching of the mating type. The asymmetric localization of Ash1 is achieved by polarized Ash1 mRNA trafficking using the actin and myosin transport system (Chartrand et al. 2001, Keder and Carmena 2013). The mother cell has a limited number of divisions and so produces only a limited number of daughter cells. Senescence or aging-factors, such as extrachromosomal rDNA circles (as a result of the recombination), damaged proteins and protein aggregates are asymmetrically segregated into the mother cell, which "protects" the daughter cell from receiving these deleterious products (Henderson and Gottschling 2008). In budding yeast, a mitotic spindle orients towards the axis of the mother-to-daughter cell polarity and distinct pathways tightly regulate this process (Pereira and Yamashita 2011).

1.1.3 ACD INVESTIGATION MODELS: *CAENORHABDITIS ELEGANS*

ACD is a mechanism that is conserved from unicellular to multicellular organisms. In the multicellular organisms the significance of ACD is even greater, because of its importance in the proper development of the organism and in the cellular diversity generation. One of the best-studied processes to understand ACD is the zygotic development of the nematode *Caenorhabditis elegans*.

The *Caenorhabditis elegans* (*C. elegans*) one-cell zygote starts with the first round of asymmetric divisions (there are four rounds of ACD in total). This first division generates two daughter cells, with different sizes and cell-fates: a larger anterior AB cell and a smaller posterior P_1 cell. Later on, P_1 cell also divides asymmetrically generating founder cells for germline and some somatic lineages, whereas the AB cell is the stem cell for the primary ectoderm (Munro and Bowerman 2009). Prior to the asymmetric division, the one-cell zygote is polarized as a consequence of the entire actin cortical cytoskeleton movement to the anterior pole of the embryo. These cytoskeleton movements and subsequent embryo polarity maintenance is dependent on small GTPases, such as RHO-1 (Motegi and Sugimoto 2006, Schonegg and Hyman 2006), and CDC-42 (Kumfer et al. 2010, Keder and Carmena 2013). Hence, the first important feature for a proper asymmetric division is the polarization of the embryo. Two cortical

domains are established as a result of the embryo polarization: an anterior and a posterior domains, which differ in protein composition.

1.1.3.1 THE PAR COMPLEX

Partitioning defective or *par* genes were identified in a screening looking for embryonic lethal mutations in *C. elegans*. These mutant embryos shared the same phenotype: they divided symmetrically instead of asymmetrically. Prior to the polarization, all PAR proteins are uniformly distributed at the cell cortex, later on, upon polarization the distribution of the PAR proteins changes. Atypical protein kinase 3 PKC-3 (Tabuse et al. 1998) with the PDZ domain-containing adaptor proteins PAR-6 (Watts et al. 1996, Hung and Kemphues 1999) and PAR-3 (Etemad-Moghadam et al. 1995) localize in the anterior side of the embryo, whereas the serine/threonine (Ser/Thr) kinase PAR-1 (Guo and Kemphues 1995) and the RING domain-containing protein PAR-2 (Boyd et al. 1996) localize in the posterior side of the embryo (Figure 2 A). Inhibitory interactions between the anterior and the posterior complexes, mainly by phosphorylation, ensure their localization and stabilization at the opposite cortical domains. In addition to the PAR protein complex, many other proteins are involved in the polarization of the one-cell embryo prior to its division (Munro and Bowerman 2009).

1.1.3.2 CELL-FATE DETERMINANTS

PAR proteins regulate the asymmetric segregation of cell-fate determinants, such as CCCH-Zn finger proteins Muscle Excess 5 (MEX-5) and MEX-6 (Schubert et al. 2000), which localize at the anterior part of the zygotic cytoplasm, and MEX-1; (Guedes and Priess 1997), Posterior segregation protein 1 (POS-1; (Tabara et al. 1999) and pharynx and intestine in excess protein 1 (PIE-1; (Mello et al. 1996, Seydoux et al. 1996), which localize to the posterior half of the zygote (Figure 2 B). Also, ribonucleoproteic P-granules (Strome and Wood 1983) localize in the cytoplasm of the posterior P_1 cell and a specific concentration of these granules is required to confer the germline cell-fate (Hubbard and Greenstein 2005)

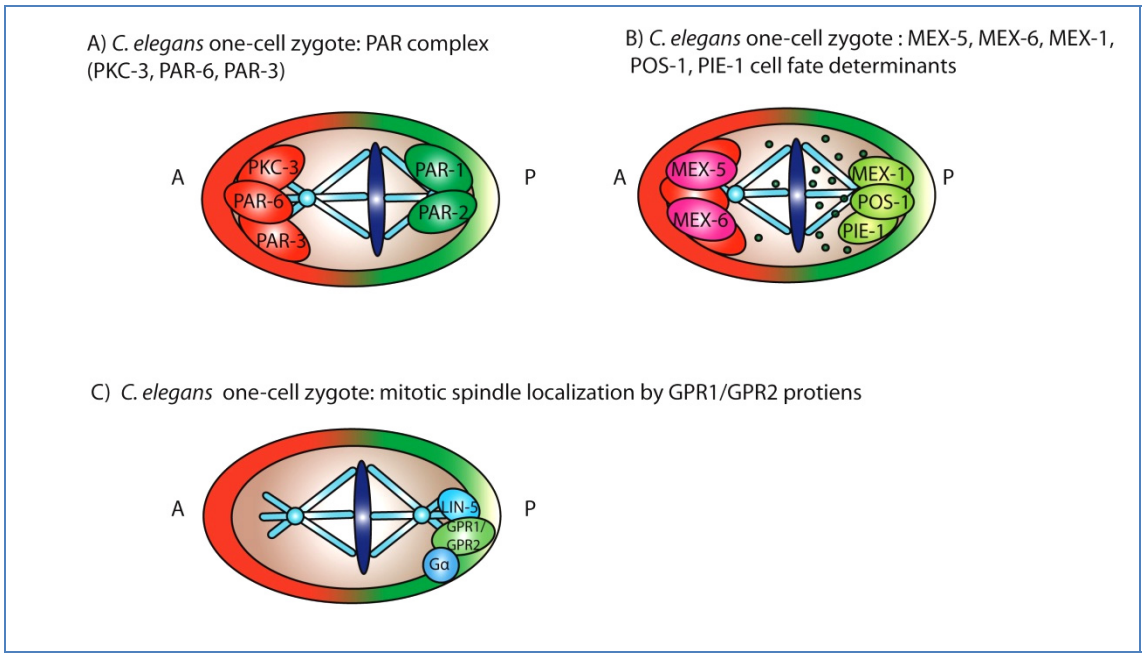


Figure 2. ACD in one-cell embryo of nematode *C.elegans*: A) PAR protein complex distribution during mitosis. PKC-3, PAR-6 and PAR-3 localize to the anterior (A) pole of the polarized embryo, whereas PAR-1 and PAR-2 localize to the opposite or to the posterior (P) side of the embryo prior division. **B) Cell-fate determinants localization in a polarized fashion.** MEX-5 and MEX-6 localize at the anterior (A) membrane, and MEX-1, POS-1 and PIE-1 and P-granules (showed as green bubbles) localize at the posterior (P) membrane of the embryo during mitosis. **C) Mitotic spindle localization**. Mitotic spindle localization closer to the posterior (P) side is regulated by proteins LIN-5, GPR1/GPR2 and Gα.

1.1.3.3 MITOTIC SPINDLE POSITION

The orientation of the mitotic spindle along the anterior-posterior polarity axis is the second crucial event for a proper asymmetric division. To ensure a difference in daughter cell sizes, the mitotic spindle has to orientate asymmetrically on the posterior side of the embryo before cleavage (Hyenne et al. 2010). Initially, the spindle localizes in the centre of the zygote but later, during anaphase, the spindle elongates toward the posterior pole. The same evolutionally conserved PAR protein complex coordinates the forces applied on the mitotic spindle, through the regulation of the heterotrimeric protein subunits Gα called COA-1 and GPA-16 (Colombo et al. 2003, Srinivasan et al. 2003). The asymmetric regulation of these Gα proteins occurs via the guanine-nucleotide dissociation inhibitors (GDI) called GPR-1/GPR-2, which are enriched at the posterior pole of the embryo during anaphase (Gotta et al. 2003). GPR-1/GPR-2

complex links microtubules of the mitotic spindle to the Gα subunits via coiled-coiled protein LIN-5 (Srinivasan et al. 2003) (Figure 2 C).

1.1.4 ACD INVESTIGATION MODELS: *DROSOPHILA MELANOGASTER*

The most extensively studied model organism for describing the mechanisms underlying the process of ACD is the fruit fly *Drosophila melanogaster*. Drosophila per se is an excellent model system with many advantages. For example, it has only 4 chromosomes, a 10-day life cycle and a fully sequenced genome that offers us quite easy and fast way to analyze molecular functions of unknown genes *in vivo.* Drosophila life cycle consists of 4 differentiated stages: 1) embryo development that takes about 22 hours, 2) larva stage, where 1st instar larva is feeding and growing until its 3rd instar stage (it takes 4 days approximately), 3) pupa stage during which larva becomes encapsulated and all adult fly organs are developed during 4-day long metamorphosis, and finally, 4) adult fly stage where adult fly emerges from pupa (Figure 3). The lifespan of *D.melanogaster* is 30 days approximately.

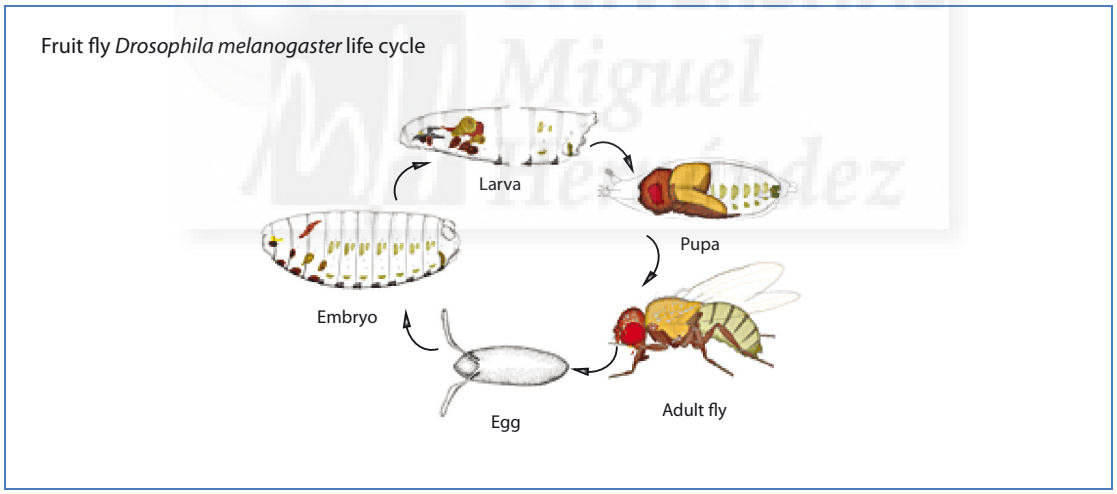


Figure 3. Fruit fly *Drosophila melanogaster* life cycle. An adult female lays fertilized egg that later develops into the embryo, which hatches as a larva, which in turn develops into the pupa, where metamorphosis occurs. Different organs are represented in different colors and correspond to the same colors in the embryo, larva and pupa stages. (Adapted from www.flybase.org and originates from (Hartenstein 1993).

For the ACD analysis our investigation model was the Drosophila neural stem cells of the embryonic central nervous system (CNS).

1.1.4.1 *DROSOPHILA* STEM CELLS

The interest in the process of ACD has been increased during past decade in a context of stem cell biology (Knoblich 2010). Stem cells can divide asymmetrically maintaining a tissue homeostasis and regulating a ratio between stem cells self-renewal and differentiating population of cells. There are two different types of mechanisms to coordinate an ACD: extrinsic, or niche-controlled regulatory mechanisms, and intrinsic or autonomous regulatory mechanisms. In the extrinsic mechanisms, distinct environmental signals surround daughter cells generating asymmetries within them. In the intrinsic mechanisms, cell-fate determinants segregate asymmetrically into only one daughter cell conferring to this daughter cell a specific cell fate (Knoblich 2008, Neumuller and Knoblich 2009).

1.1.4.1.1 *DROSOPHILA* GERMLINE STEM CELLS (GSCS)

The best understood example of extrinsic regulation of ACD is the female and male germline stem cells (GSCs) of *Drosophila* (Inaba and Yamashita 2012). GSCs receive diffusible signals from a stem cell niche, which is the hub cells in testis and the cap cells and terminal filament cells in the ovary, to control self-renewal. Both GSCs in the testis and in the ovary are in a tight contact with the niche through adherens junctions enriched in E-cadherin and β-catenin, which in male GSCs (mGSCs) mediate a key polarity cue that orients the mitotic spindle perpendicularly to the hub cell niche (Fuller and Spradling 2007).

Oogenic cap cells synthesize the ligands called Decapentaplegic (Dpp) and Glass bottom boat (Gbb) that activate Bone morphogenetic protein (BMP) signalling pathway in GSCs, therefore repressing differentiation promoting genes (Chen and McKearin 2003, Song et al. 2004). GSC align their spindle perpendicularly to the niche, thus placing one daughter cell in contact with the stem cell niche while the other daughter cell, called cystoblast, is positioned away from the niche (Figure 4 A).

The cystoblast loses direct contact with the stem cell niche, no longer receives the BMP signal provided by the niche, and initiates *bag of marbles* (*bam*) transcription that will promote a differentiation process (Chen and McKearin 2003).

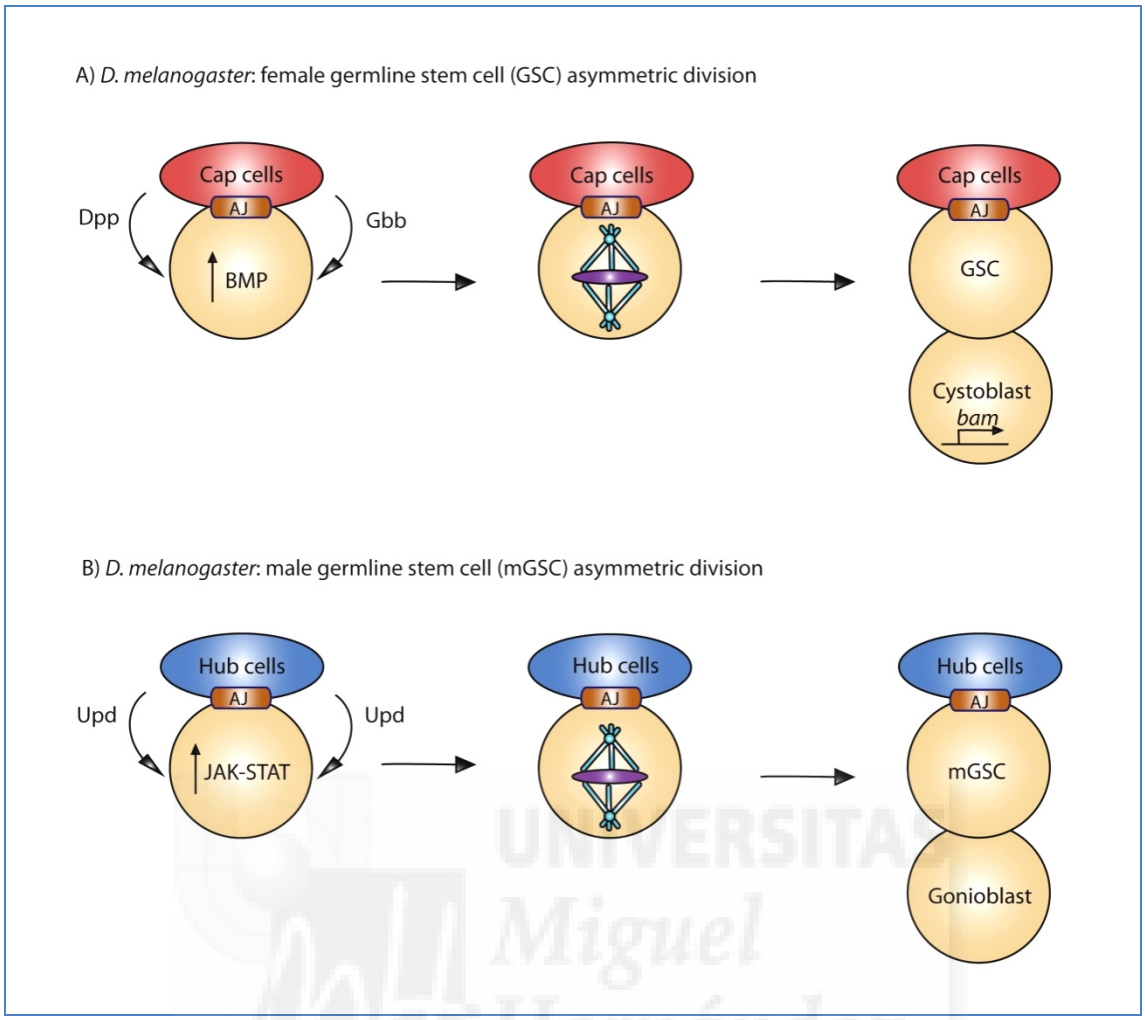


Figure 4. Germline stem cells (GSCs) of fruitfly *D.melanogaster* undergo ACD: A) Female GCS ACD. Cap cells are forming a signal-sending niche for the female GCS prior division. Decapentaplegic (Dpp) and Glass bottom boat (Gbb) signals sent from the cap cells activate Bone morphogenetic protein (BMP) signalling pathway in GCS. Cap cell niche is linked to the GCS through adherens junctions (AJs). As a result of female GCS division another GCS and a cystoblast are generated. *bag of marbles* (*bam*) transcription induces a differentiation process in the cystoblast. **B) Male mGCS ACD.** The niche for the mGCSs is formed by Hub cells that are in a tight contact with mGCS throug thr AJs. Hub cells secret Unpaired (Upd) ligand to activate JAK-STAT signalling in the mGCS. After the division, a Gonioblast and another mGCS are generated.

mGSCs divide asymmetrically to generate two daughter cells; one daughter cell stays attached to the hub cells (niche) and retains mGSC identity, whereas the other daughter cell, called gonioblast, is displaced away from the niche and initiates a process of differentiation to become a cyst of spermatocytes (Figure 4 B). In testis, hub cells secret the cytokine-like ligand called Unpaired (Upd) that activates the Janus kinase and signal transducer and activator of transcription (JAK-STAT) signalling in mGCS. This signal

prevents differentiation in mGCS by regulating some target genes that remain to be identified (Kiger et al. 2001, Tulina and Matunis 2001).

1.1.4.2 *DROSOPHILA* SENSORY ORGAN PRECURSORS (SOPS)

Progenitors of the *Drosophila* peripheral nervous system (PNS), the sensory organ precursor cells (SOPs), and of the central nervous system (CNS), the neural stem cells called neuroblasts (NBs), have been extensively studied. They are very good examples of cells that undergo asymmetric division mainly by intrinsic regulatory mechanisms, though extrinsic mechanisms also operate. SOP/pI cell divides asymmetrically giving rise to pIIa and pIIb daughter cells, which are going to divide once more to generate the four cells that form the external sensory organ (Figure 5 A). pIIb gives rise to a neuron and a sheath cell, while pIIa division originates a socket cell and a hair cell.

1.1.4.2.1 ESTABLISHMENT OF PLANAR POLARITY

During mitosis, SOP/pI cell polarizes along the anterior-posterior axis in response to a planar polarity signalling provided by the Wingless (Wg)/Wnt receptor Frizzled (Fz), which forms an anterior crescent with Discs large 1 (Dlg 1), Partner of Inscuteable (Pins) and the G protein subunit Gαi (Gho and Schweisguth 1998, Bellaiche et al. 2001a, Schaefer et al. 2001). The mitotic spindle is orientated along this antero-posterior polarity axis. The Dlg 1/Pins/Gαi complex controls the positioning of Bazooka (Baz)/PAR-3, Par-6/PAR-6 and aPKC/PKC-3 at the posterior cortex (Bellaiche et al. 2001b).

The cell-fate determinant Numb and its effector Partner of Numb (Pon) are asymmetrically localized as a crescent in the anterior cortex during SOP division, and are segregated only into one daughter cell (Guo et al. 1996, Bellaiche et al. 2001b) Bellaiche Y 2001 Cell). Pins colocalizes with Numb in the anterior pole of the SOP/pI, and this Pins localization is required, along with Baz at the posterior side, to regulate the asymmetric localization of Numb (Bellaiche et al. 2001b). Cell cycle regulators, such as cyclin-dependent kinase Cdc2 and Aurora A, have also been implicated in the asymmetric localization of Numb and in centrosome maturation (Berdnik and Knoblich 2002, Hutterer et al. 2006) (Figure 5 B).

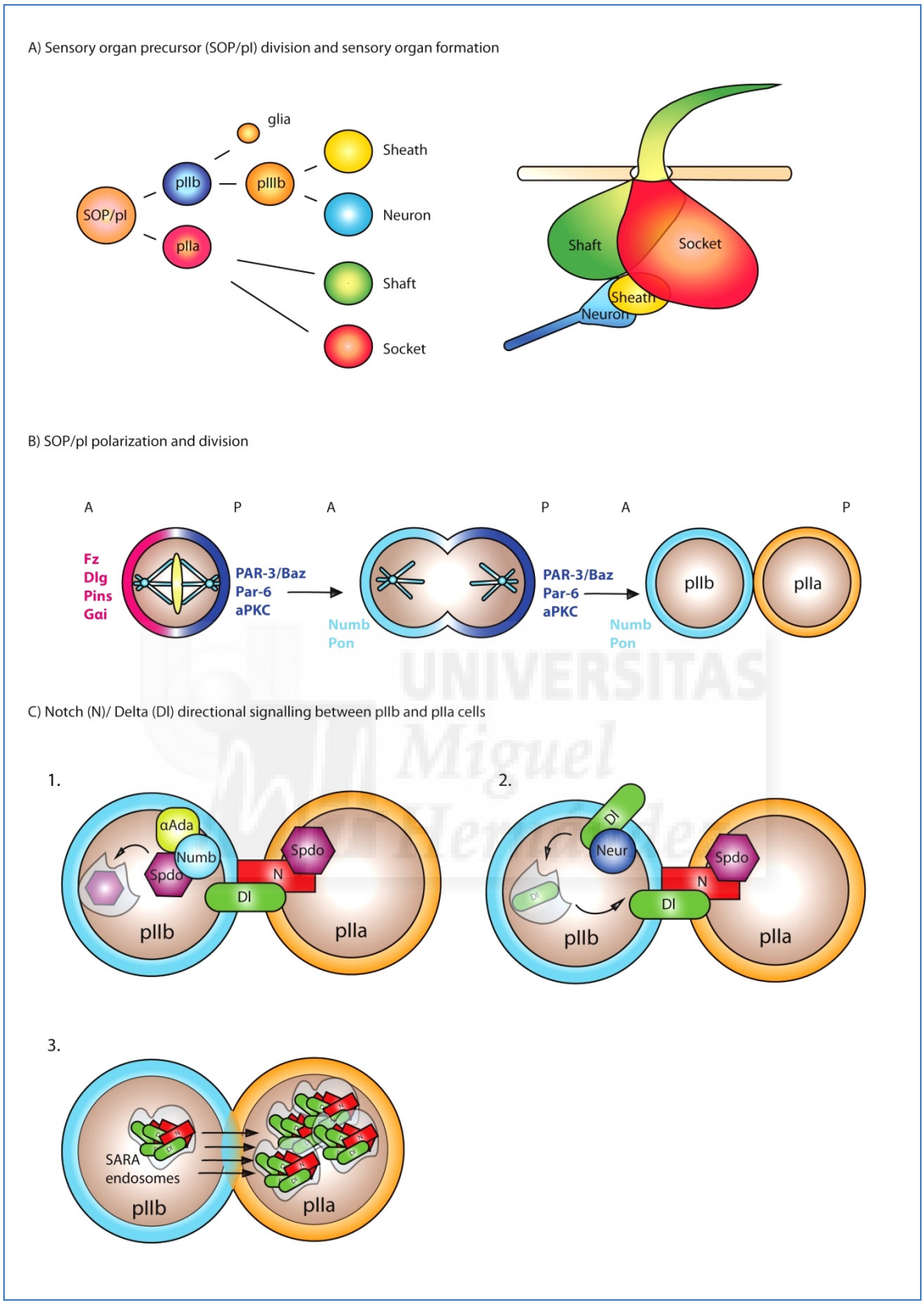


Figure 5. Sensory organ precursor (SOP/pI) ACD: A) Sensory organ formation. *D. melanogaster* SOPs divide asymmetrically to give rise to two daughter cells, pIIa and pIIb, which in turn divide asymmetrically to generate the four different cells that will form the sensory organ: a neuron, a sheath, a shaft, and a socket. **B) SOP is polarized prior ACD.** Frizzled (Fz), Discs large (Dlg), Pins and Gαi localize to the anterior (A) side of the dividing SOP, and PAR-3/Bazooka, Par-6 and aPKC localize to the

posterior (P) side, while Numb and Pon localize at the anterior (A) membrane of the SOP and then segregates to the anterior (A) pIIb daughter cell **C) Notch-Delta signalling in pIIb and pIIa cells.** 1. Numb and α Adaptin (αAda) promote the endocytosis of Notch (N) activator Sanpodo (Spdo) in pIIb cell 2. Neuralized (Neur) promotes the recycling of the N ligand Delta (Dl) in the pIIb cell 3. SARA endosomes transport Dl and N to the pIIa daughter cell. All these features make the pIIb a signal-sending cell and pIIa a signal-receiving cell

1.1.4.2.2 NOTCH SIGNALLING IN CELL FATE DETERMINATION

Directional Notch signalling between pIIa and pIIb contributes to define the different cell fates of these cells. Numb acts as a selective Notch signaling inhibitor and segregates only to the anterior pIIb cell (Bellaiche et al. 2001b). Numb in a complex with α-Adaptin (Berdnik et al. 2002) antagonizes Notch signalling in the pIIb cell by promoting the endocytosis of the Notch regulator Sanpodo (Spdo; (Hutterer and Knoblich 2005). Another protein, the E3 ubiquitin ligase Neuralized (Neur), similarly to Numb, localizes at the anterior cortex of the SOP/pI cell during mitosis, and it is exclusively inherited by the pIIb cell. Neur promotes endocytosis of Delta (Dl), which is one of the ligands of the Notch receptor (Le Borgne and Schweisguth 2003). All these mechanisms provide the directionality of the Dl/Notch signalling, with pIIb becoming the ligand-presenting cell and pIIa the signal-receiving cell. SMAD Anchor for Receptor Activation (SARA) endosomes play also an important role in asymmetric endocytosis during asymmetric SOP/pI cell division (Le Borgne and Schweisguth 2003). SARA endosomes transport and asymmetrically segregate Dl/Notch to the pIIa cell, where Notch signalling will be activated (Coumailleau et al. 2009). (Figure 5 C)

1.1.4.3 *DROSOPHILA* NEUROBLASTS

The greatest progress in understanding the process of ACD has been made in the *Drosophila* neural stem cells called neuroblasts (NBs) (Homem and Knoblich 2012). NBs generate different neurons and glial cells in the embryo and in the larva. Embryonic NBs delaminate from the ventral neuroectoderm and undergo up to 20 rounds of asymmetric divisions to generate different neural types; then, at the end of embryogenesis NBs enter to the first phase of quiescence (Truman and Bate 1988). In each division, a NB gives rise to a large daughter cell that retains the self-renewal properties of the NB, and a smaller ganglion mother cell (GMC) that it is committed to initiate a process of differentiation. The GMC divides only once more thus producing two neurons or glial cells.

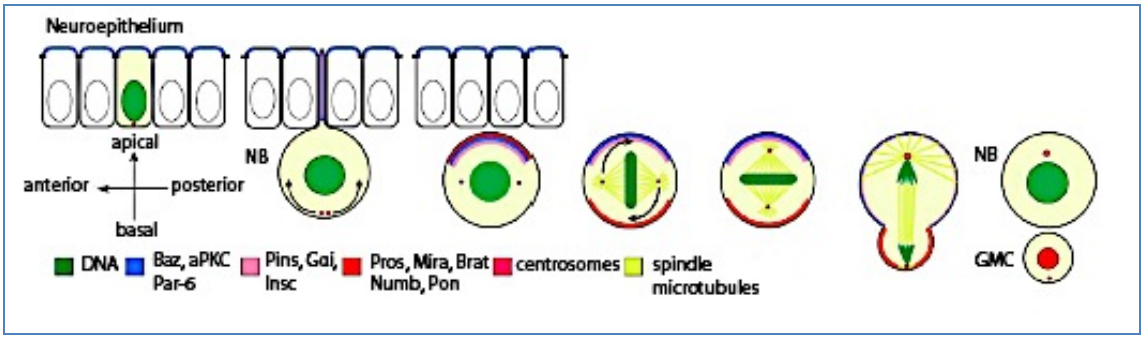


Figure 6. ***D.melanogaster asymmetric*** **NB division.** NBs delaminate and divide in the ventral neurogenic region of the Drosophila embryo. The subcellular localization of apical polarity regulators, cell-fate determinants and their adaptor proteins is indicated in different colors (see legend). The centrosomes movements are indicated with arrows (Adapted from (Wodarz and Huttner 2003).

During the delamination from the neuroectoderm and during asymmetric division, NBs retain an apico-basal polarity. The mitotic spindle, initially parallel to the neuroectoderm, rotates 90°orientating itself along the apico-basal polarity axis, and thus perpendicular to the plane of the neuroectoderm (Figure 6). At early stages of larval development, quiescent NBs receive signals to start dividing again asymmetrically and to produce the neurons and glial cells that will form part of the adult nervous system (Maurange and Gould 2005). Some important differences exist between larval and embryonic NB divisions (Reichert 2011). Here, I am going to describe the asymmetric division of the embryonic NBs.

1.1.4.3.1 THE APICAL PROTEIN COMPLEX

The establishment and the maintenance of the apico-basal cell polarity is regulated by proteins that are polarized at the apical side of the NB cortex. Three evolutionarily conserved polarity proteins that have already been mentioned above are forming the so-called PAR protein complex: Baz (Kuchinke et al. 1998, Schober et al. 1999, Wodarz et al. 1999), aPKC (Wodarz et al. 2000) and Par-(Petronczki and Knoblich 2001). This Baz/Par -6/aPKC complex is enriched at the apical cortex of the NB at metaphase and it is associated through the interaction between Baz and the adaptor-protein Inscuteable (Insc, (Kraut and Campos-Ortega 1996, Kraut et al. 1996) to the Partner of Inscuteable (Pins) complex. Pins (the homolog of *C.elegans* GPR-1/GPR-2 proteins, and LGN/AGS-3 in mammals) (Parmentier et al. 2000, Schaefer et al. 2000, Yu et al. 2000), in turn, binds the heterotrimeric G protein subunit Gαi (Schaefer et al. 2001) and the microtubule- and dynein-binding protein Mushroom body defect (Mud), (the

Drosophila homolog of the Nuclear Mitotic Apparatus (NuMA) (Bowman et al. 2006) in order to regulate the mitotic spindle orientation. Gαi together with the subunit Gβγ (Yu et al. 2000) recruits Pins complex to the apical membrane and contributes to the apico-basal mitotic spindle orientation. Pins and Gαi form a complex with the adaptor protein Discs large 1 (Dlg 1). The kinesin Khc-73 binds to Dlg/Pins complex and to the astral microtubules, and thus clusters the polarity complex in the direction of the mitotic spindle (Siegrist and Doe 2005). Abnormal spindle (Asp) is a microtubule-associated protein that has been shown to localize in the poles of the mitotic spindle where it regulates γ-tubulin to organize the microtubule asters (do Carmo Avides and Glover 1999). The Rho GTPase Cdc42 binds directly Par-6 and could be involved in its cortical recruitment (Peterson et al. 2004, Atwood et al. 2007). It has been shown that Cdc42 acts downstream of Baz regulating apical localization of Par-6 and aPKC (Atwood et al. 2007). All these proteins control the correct orientation of the mitotic spindle and the localization of cell-fate determinants at the opposite side of the NB, where they form a basal cortical crescent (Figure 6).

1.1.4.3.2 THE CELL-FATE DETERMINANTS

The cell-fate determinants segregate into only one daughter cell endowing it with specific properties. During metaphase, cell-fate determinants form a crescent at the basal domain of the NB cortex and then they segregate into the basal GMC inducing a process of differentiation. Almost two decades ago, Numb was the first cell-fate determinant identified in the SOP cells and then it has also been shown to segregate asymmetrically in the NBs (Uemura et al. 1989, Rhyu et al. 1994, Wai et al. 1999). Numb acts as a Notch pathway repressor (Guo et al. 1996). In mitotic NBs, aPKC phosphorylates Numb, which then dissociates from the apical cortex and localizes basally. This Numb phosphorylation by aPKC is conserved from flies to mammals (Smith et al. 2007). The Numb adaptor protein called Partner of Numb (Pon) also localizes basally in the NB during division (Lu et al. 1999). In *pon* mutants Numb basal crescent formation is delayed in NBs (Lu et al. 1998). The homeodomain transcription factor Prospero (Pros) is another cell-fate determinant that also segregates asymmetrically into the GMC (Figure 6), where it enters to the nucleus and activates the transcription of specific GMC genes (Knoblich et al. 1995, Spana and Doe 1995). In the NB, Pros is not active and is forming a basal cortical crescent. Staufen (Stau) is an RNA-binding protein that binds Pros mRNA and participates in its transport to the basal

membrane (Li et al. 1997). Recently characterized cell-fate determinant called Brain tumour (Brat) also plays a role in regulating the balance between NB self-renewal and differentiation (Betschinger et al. 2006). Brat was known as an inhibitor of cell growth and ribosome biogenesis (Sonoda and Wharton 2001). Brat cooperates with Pros to specify GMC fate (Betschinger et al. 2006). The coiled-coiled adaptor protein Miranda (Mira) is also required during asymmetric NB division to attach Pros and Brat to the basal membrane domain of the NB (Shen et al. 1997, Lee et al. 2006a). Mutations in *baz, insc, pins* or *mud* result in distribution defects of Mira with the consequent defects in Pros and Brat basal crescent formations (Schober et al. 1999, Wodarz et al. 1999, Bowman et al. 2006).

1.1.4.3.3 DAUGHTER CELL SIZE DIFFERENCES AND TELOPHASE RESCUE

During metaphase the mitotic spindle is symmetric, whereas during anaphase the spindle microtubules elongate at the apical side and shorten at the basal side, thus generating the spindle asymmetry. As a result two daughter cells of different size are generated: a larger NB and a smaller GMC (Kaltschmidt et al. 2000). Par and Pins complexes redundantly regulate this spindle asymmetry and cell size (Cai et al. 2003). The heterotrimeric G protein subunits Gβ13F and Gγ1 play a major role in cell size asymmetry (Fuse et al. 2003, Yu et al. 2003, Izumi et al. 2004). PDZ-domain protein Canoe (Cno) and its effector Rap1 GTPase also contribute to the regulation of the unequal daughter cell size generation (Speicher et al. 2008, Carmena et al. 2011).

1.1.4.3.4 NEOPLASTIC TUMOUR SUPPRESSOR GENES

Neoplastic tumours are distinguished from other tumours by disruption of apico-basal polarity and normal tissue architecture caused by mutations in neoplastic tumour suppressor genes. The incorrect segregation of apical and/or basal proteins in NBs is a major cause of neoplastic growth and tumourigenesis. Mutations in the genes *lethal giant larvae* (*lgl*), *discs large 1* (*dlg 1*), and *scribble* (*scrib*) induce the formation of neoplastic tumours and promote defects in cell-fate determinants localization (Peng et al. 2000, Betschinger et al. 2003, Albertson et al. 2004). The proteins that encode these tumour suppressor genes are present in the whole NB cortex and thus can be directly involved in cell-fate determinants targeting to the membrane. Lgl, Dlg1, and Scrib are involved in the regulation of apico-basal polarity by antagonizing the activity of the apical Par complex. Lgl is phosphorylated by aPKC and thus inactivated; as a

consequence, the cell-fate determinant Mira is released from the apical cortex and accumulates basally (Peng et al. 2000, Betschinger et al. 2003). Interactions between Lgl and non-muscle myosins, such as Myosin II (Zipper in *Drosophila*) and Myosin VI (Jaguar in *Drosophila*) allow the transport of cell-fate determinants to the basal pole of the NB (Barros et al. 2003, Petritsch et al. 2003). It has been proposed a mechanism of exclusion of Mira from the apical side of the NB that involves aPKC, Lgl, Myosin II and Myosin VI (Erben et al. 2008) (see Annex II, Keder and Carmena, 2013). Pon localizes basally independently of Myosin VI but depends on Myosin II (Erben et al. 2008). Recently, it has been reported that Mira can be directly phosphorylated by aPKC (Atwood and Prehoda 2009). Finally, it has been demonstrated that double mutations in *pins* and *lgl* as well as in *dlg1* and *Gβγ* also result in spindle misorientation and overproliferation phenotypes (Kelsom and Lu 2012).

1.1.4.3.5 CELL-CYCLE REGULATORS

During prophase NBs are already polarized but cell-fate determinants localize basally only during metaphase of mitosis. This localization has been shown to be coupled to cell-cycle regulation. The Cdc2 kinase is a crucial factor for mitotic entry, and, moreover, is an essential regulator for the correct localization of Insc and cell-fate determinants (Wodarz and Huttner 2003). Two other cell-cycle regulators, Aurora A (AurA) and Polo, are also involved in asymmetric NB division regulation. AurA is critical for centrosome maturation and mitotic spindle formation (Lee et al. 2006b). In addition, AurA phosphorylates Par-6, which activates aPKC and this, in turn, phosphorylates Lgl (Wirtz-Peitz et al. 2008). As a consequence, aPKC is released from the apical complex and Baz enters to the Par complex, changing the substrate specificity of aPKC, allowing it to phosphorylate Numb. Phosphorylated Numb is released form the apical cortex and localizes basally. Similarly to Numb, Mira location depends on direct phosphorylation by aPKC; however, redundant mechanisms of Mira basal localization must exist, as in *AurA* mutants, in which aPKC is mislocated, Mira is unaltered (Lee et al. 2006b, Wang et al. 2006). Mira transport involving non-muscle myosins Myosin II and Myosin VI has been proposed (Barros et al. 2003, Petritsch et al. 2003). Polo kinase phosphorylates centrosomal microtubule-associated protein Asp; and it also phosphorylates Pon allowing its correct localization at the basal domain of the NB (do Carmo Avides et al. 2001, Wang et al. 2007). The mitotic regulator Anaphase-Promoting Complex/Cyclosome (APC/C) is required for the adaptor protein Mira and

its cargoes Pros and Brat to localize basally in mitotic NBs (Slack et al. 2007). APC/C consists of many protein subunits and functions as an E-ubiquitin ligase, targeting proteins for degradation. APC/C may facilitate ubiquitination of Mira essential for Mira basal recruitment to the membrane during metaphase (Slack et al. 2007).

1.1.4.3.6 PHOSPHATASES

Protein Phosphatase 2A (PP2A) regulates the localization of aPKC, Numb and Pon. PP2A is a Ser/Thr-specific phosphatase. PP2A dephosphorylates Par-6 (phosphorylated by Aur-A), thus inactivating aPKC (Ogawa et al. 2009). In mutants for the PP2A catalytic subunit *microtubule star* (*mts*) the correct localization of Mira is affected (Ogawa et al. 2009). In addition, phosphorylation of Numb and Pon are reduced in *mts* larval brain mutant NBs and seem to be Polo-dependent (Wang et al. 2009). PP2A also dephosphorylates Baz at the PAR-1 specific phosphorylation site (Krahn et al. 2009). Finally, protein phosphatase 4R3 (PP4R3) is responsible for cortical Mira localization and has been shown to interact directly with Mira (Sousa-Nunes et al. 2009).

Many novel ACD regulators have been identified by using simple model organisms. Most of the proteins critical for the ACD in *Drosophila* and *C.elegans* have their counterparts in vertebrates with a conserved function (see below). Thus, and in order to understand the complexity of this crucial process during stem cell and cancer biology, further investigation has to be made in higher organisms.

1.1.5 ACD IN VERTEBRATES

1.1.5.1 MEIOTIC ACD IN MAMMALIAN OOCYTES

Immature mammalian oocytes undergo two rounds of asymmetric divisions to produce haploid gametes during meiosis. As a result, four daughter cells are produced: the bigger egg and 3 small polar bodies destined to apoptosis. Similarly to *Drosophila* NBs, the asymmetry between daughter cells correlates with asymmetric positioning of the spindle and the plane of cell division. In contrast to mitotic ACD, the site of polar body extrusion is determined by the chromatin, which induces the cortical polarity. The meiotic spindle in mouse oocytes has atypical centrosomes without centrioles (review of (Schatten and Sun 2011). As a result, the so-called cap-like domain, enriched of F-actin surrounded by a ring of myosin II, defines the site of cell division. It has been shown that chromatin breaks the mouse oocyte symmetry by the meiotic spindle displacing

from the centre to a subcortical area. The migration of the meiotic spindle is dependent on the remodelling of Formin 2 (Fmn2)-nucleated F-actin meshwork (Azoury et al. 2011). This meshwork remodelling correlates with Fmn2 degradation and reaccumulation. Recently, it has been shown that the meiotic spindle migration occurs in two phases: during the first phase, the spindle migrates to the periphery in an Fmn2 dependent manner; later, in the second phase, Actin Related proteins 2 and 3 (Arp2/3) complex nucleates the dynamic actin network producing a pushing force for the spindle, which localizes toward the Arp2/3 concentrated cortex domain (Yi et al. 2013). In contrast to the *Drosophila* NBs, the meiotic spindle and the chromatin itself regulate cell polarity and the plane of division during mouse oocyte meiosis (Figure 7).

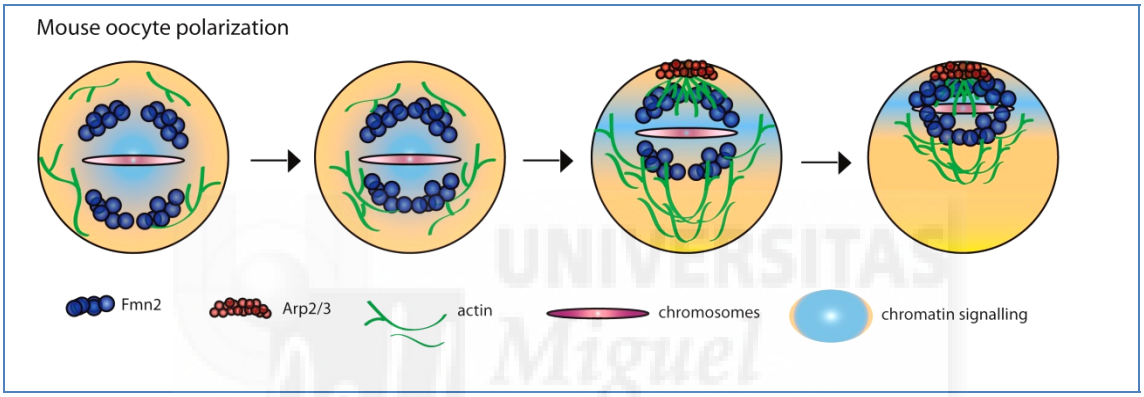


Figure 7. Mouse oocyte polarization. During mouse oocyte meiosis, the oocyte polarization is driven by choromatin signalling. The chromosome movement is driven by pushing forces induced by Formin2 (Fmn2), the Arp2/3 complex and an actin meshwork remodelling. Fmn2 nucleates the F-actin meshwork and chromosomes start to migrate to one pole of the oocyte, where the F-actin cap domain is formed of actin filaments and the Arp2/3 complex.

1.1.5.1.1 CONSERVED POLARITY PROTEINS IN MOUSE OOCYTES

The homolog in mammals of *Drosophila* Pins, the protein LGN (named this way because of the presence of Leucine-Glycine-Asparagine tripeptides at the N-terminus), plays a pivotal role in the meiotic spindle organization and in the cortical domain polarization during oocyte division (Guo and Gao 2009). The small GTPases Ran and Cdc42 localize at the cap-like domain and they are required for spindle stabilization and positioning (Na and Zernicka-Goetz 2006, Halet and Carroll 2007). In addition, the conserved proteins Par3 (the mammalian homolog of *Drosophila* Baz) and Par6 also concentrate at this cap region where the polar body extrusion takes place, thus defining the site of oocyte division (Vinot et al. 2004, Duncan et al. 2005). In mouse oocytes,

NuMA (the mammalian homolog of *Drosophila* Mud) localizes along spindle microtubules, similarly to LGN, regulating the spindle position during the asymmetric oocyte division (Lee et al. 2000).

1.1.5.2 ACD IN MAMMALIAN NEURAL STEM CELLS (NSCS) AND OLIGODENDROCYTE PRECURSOR CELLS (OPCS)

Mammalian embryonic neural stem cells (NSCs), called radial glia (RG) cells, arise from a polarized neuroepithelium. RG cells have an apical process and a long basal fiber to contact the ventricular and pial surfaces, respectively. Thus, RG cells attached to the neural tube divide asymmetrically in the ventricular zone (VZ) producing neurons or basal progenitors (BPs) that lose both ventricular and pial attachments and migrate basally. In the subventricular zone (SVZ) BPs divide symmetrically to produce two neurons that would migrate further basally (Gomez-Lopez et al. 2013). Recently, different groups have reported another type of neurogenic progenitors called outer RG (oRG) cells that are present in human, ferret and mouse embryonic brain (Fietz et al. 2010, Hansen et al. 2010, Wang et al. 2011). oRG cells arise from asymmetrically dividing RG cells and inherit their basal fiber; however, they lose the apical process and migrate basally to the outer SVZ, where they undergo ACDs giving rise to neurons and more oRG (Figure 8).

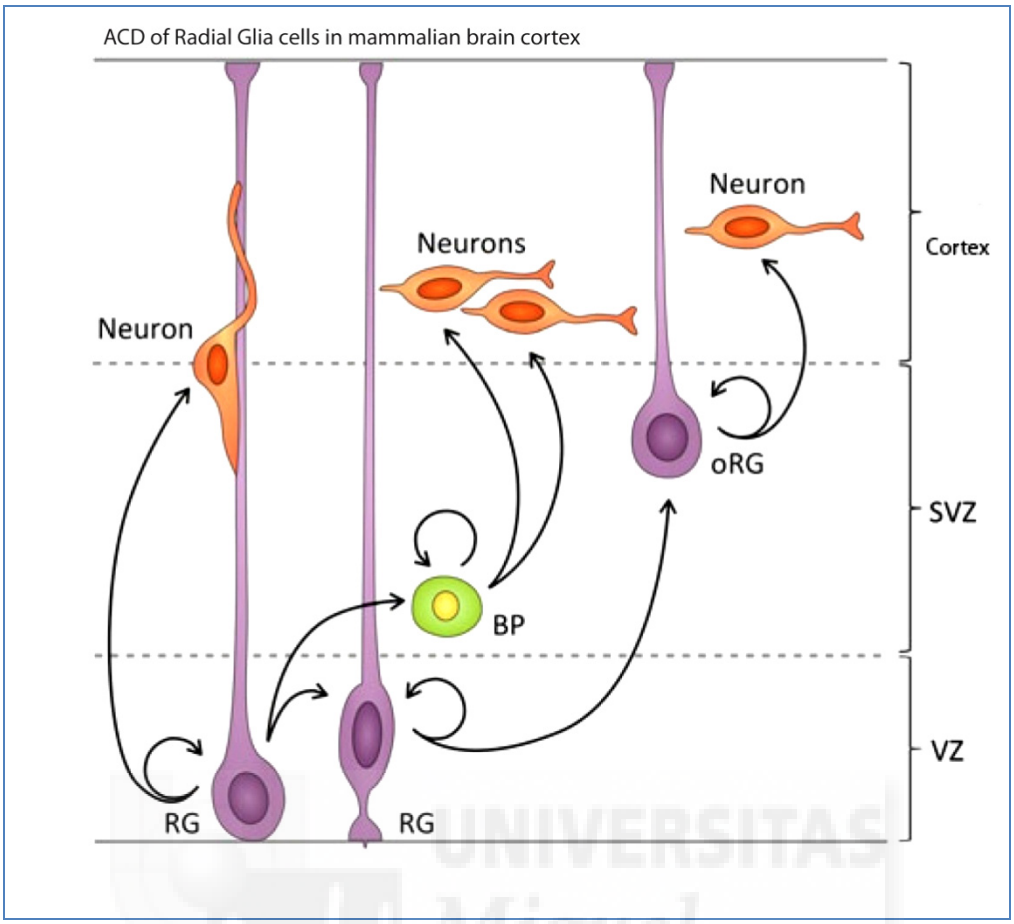


Figure 8. ACD of Radial Glia: Radial glia (RG) cells undergo asymmetric division in the ventricular zone (VZ) of the cortex generating one RG cell and one neuron, or, one RG cell and one basal progenitor (BP). BP migrates then to the subventricular zone (SVZ) and divides to produce neurons. Asymmetric division of RG cells also produce outer radial glia (oRG) cells that lose their ventricular attachment and migrate to the SVZ to divide asymmetrically and to produce neurons (Adapted form (Gomez-Lopez et al. 2013)

1.1.5.2.1 APICAL POLARITY COMPLEX AND CELL-FATE DETERMINATION IN MAMMALIAN NSCS

Similarly to *Drosophila* NBs, mammalian NSCs are apico-basally polarized. Pins and Insc homologs in mammalians, LGN and mInsc respectively, bind to each other and regulate the correct mitotic spindle orientation (Du et al. 2001). The plane of the RG cell division is mostly vertical. However, a small fraction of RG cells have an oblique

plane of division where mitotic spindle orients between 30° and 60° (Figure 9).

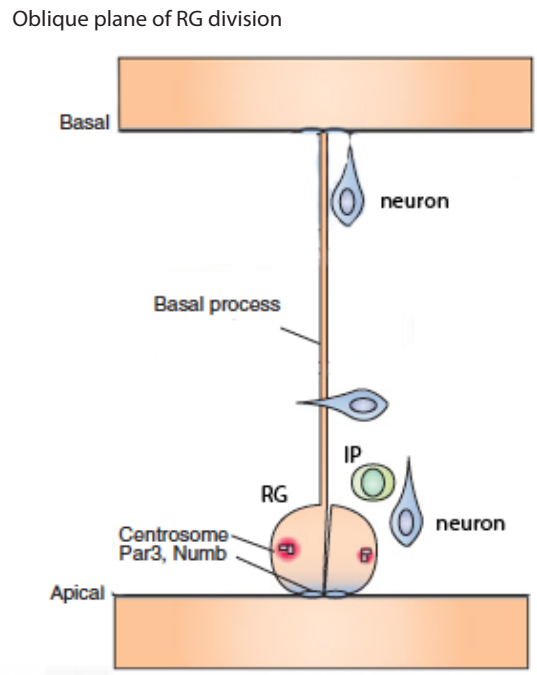


Figure 9. Oblique plane of the RG division. The mitotic spindle orients between 30 ° and 60 ° in the asymmetrically dividing RG cells. The identical RG cell with both basal process and the oRG cell are generated. The oRG then divides asymmetrically giving rise to the intermediate progenitors (IP) and the migrating neuron. Par3 binds Numb in the apical domain of dividing RG cell (Adapted from (Shitamukai and Matsuzaki 2012).

Oblique RG cell divisions create a cleavage furrow that bypasses the apical junctions, splitting the apical and basal epithelial structures into two daughter cells. The cell that inherits the basal process maintains the ability to self-renew, whereas the apical daughter is committed to differentiate. The Par polarity complex that consists of Par3/ aPKC localizes at the apical domain of RG cell and is inherited only by one daughter. Par3 directly binds Numb; as a consequence aPKC phosphorylates Numb and activates it. Hence, in the RG cell that does not inherit Par3, Numb is inactive leading to the acquisition of a different fate in this RG cell (Nishimura and Kaibuchi 2007, Bultje et al. 2009). mInsc localizes in an apical crescent in dividing RG cells and it seems to control the plane of division. An excess of mInsc increases the amount of oblique divisions, whereas the absence of mInsc renders only vertical (or symmetric) divisions (Postiglione et al. 2011).

Some mammalian orthologs of the *Drosophila* basal cell-fate determinants have been shown to participate in the asymmetric RG cell divisions. Recently reported by two different groups, the RNA-binding protein Staufen2 (Stau2), the mouse homolog of *Drosophila* Stau, is enriched apically in RG cells and it preferentially segregates into the BP, endowing it with differentiation properties (Kusek et al. 2012, Vessey et al. 2012). Stau2 cargoes are Pros and Brat homologs in mammals, Prospero related homeobox 1 (Prox1) and Tripartite-motif containing 32 (Trim32), respectively (Kusek et al. 2012, Vessey et al. 2012). It has been proposed that Stau2, similarly to its function in *Drosophila* NBs, localize Prox and Trim32 mRNAs asymmetrically, so that they can confer the differentiation fate to the daughter cell.

1.1.5.3 ACD IN THE EPIDERMIS

It has been recently reported that the innermost layer cells of epidermis undergo asymmetric divisions in 13.5 mouse embryos (Poulson and Lechler 2010). The mitotic spindle orients along the apico-basal polarity axis and cells switch from a symmetric to an asymmetric mode of cell division.

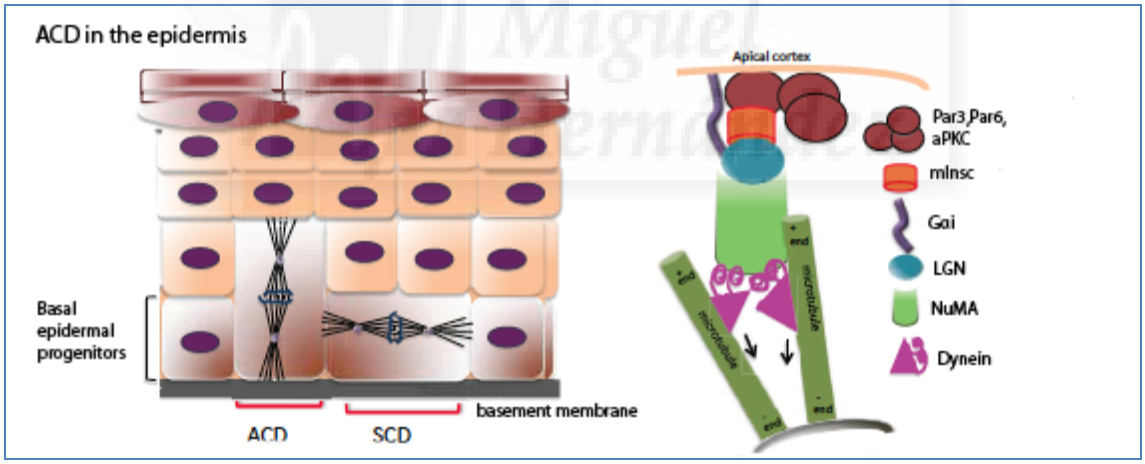


Figure 10. ACD in the epidermis. Basal epidermal progenitors are localized in a tight connection to the basement membrane that acts as a niche in multi-layered epidermis. Basal epidermal progenitors can orient their mitotic spindle parallel or perpendicular to the basement membrane to undergo symmetric (SCD) or asymmetric cell division (ACD), respectively. Par protein complex, linked to mInsc, LGN and NuMA, orients mitotic spindle along the apico-basal axis of cell polarity to drive ACD in the epidermal cells (Figure adapted from (Ray and Lechler 2011).

The difference between the two daughter cells produced as a result of the asymmetric division is the expression of different Keratins: the basal daughter cell expresses Keratin14, whereas the other suprabasal daughter cell expresses Keratin10 (Poulson and

Lechler 2010). There is an extrinsic regulation of ACD provided by a tight contact with the "niche" or basement membrane. The integrin family of proteins attaches epidermal basal cells to the basement membrane and it maintains the basal epidermal progenitors in a proliferative state (Watt 2002). Similarly to *Drosophila* GSCs, during asymmetric epidermal progenitor division one daughter cell is displaced from the 'niche" and adopts a differentiation cell-fate. The majority of basal progenitors can undergo both symmetric and asymmetric divisions (Figure 10). The regulation of the mitotic spindle orientation during ACD is key to understand the switch between symmetric versus asymmetric progenitor divisions. Similarly to what happens in *Drosophila* NBs, in the epidermal basal progenitors the mitotic spindle is also coupled with a group of polarity proteins, such as Par3 and aPKC, which form a crescent at the apical side of the progenitor. In addition, LGN, Gαi, and NuMA are linked by Dyneins to the microtubules of the mitotic spindle and are required for its perpendicular orientation (Lechler and Fuchs 2005).

Transient overexpression of mInsc leads to increased amounts of ACD versus symmetric cell divisions (SCD) of basal epidermal progenitors (Figure 10). Ectopic mInsc co-localized apically with both NuMA and LGN. It has been demonstrated that the overexpression of mInsc during a long time leads to failures in apical NuMA localization, and it results in a decrease of ACD, indicating that mInsc levels may be regulating NuMa apical recruitment and acting as a baseline for the ACD vs SCD rate (Poulson and Lechler 2010).

1.1.5.4 ACD IN MAMMALIAN CELL LINES

Experiments *in vitro* in human embryonic stem cells (hESC), and in mammalian somatic cell line Cos7, as well as *in vivo* in *Drosophila* blastoderm revealed an asymmetric segregation of the proteins targeted for proteosomal degradation. Proteins such as Smad1 phosphorylated by MAPK and glycogen synthase kinase 3 (GSK3), phosho-β-catenin, phospho-Mad, and the proteins that are targeted by polyubiquitination signal, are inherited preferentially by one daughter cell after the division (Fuentealba et al. 2008). During the asymmetric division of T cells certain proteins like Leucocyte Function-Associated antigen 1 (LFA-1) and Co-receptors for the T cell receptors protein cluster of Differentiation 8 (CD8) and CD3 localize at the Microtubule Organizing Centre (MTOC). During mitosis, these proteins remain

associated with nonmigrating centrosome and are inherited by only one daughter cell (Chang et al. 2007). PKCζ, a mammalian counterpart of aPKC localizes at the opposite site of MTOC, whereas Scrib colocalizes with the MTOC during division. Additionally, C-X-C chemokine receptor type 4 (CXCR4) is highly enriched in one pole, where it colocalizes with the small Rho GTPase Rap1, with Par3, PKCζ and Cdc42; antigen CD44 and intercellular adhesion molecule 1 and 3 (ICAM1/3) are however redistributed to the opposite pole (Gerard et al. 2007).

As it has been described before many different polarity proteins, tumour-suppressors, kinase and phosphatase signalling networks are involved in the process of ACD. To describe the whole protein-protein interaction and signalling networks interconnection map, many scaffolding-proteins, so-called signalling-nodes and signalling-junctions, have been described (Jordan et al. 2000). Signalling-junctions function as signal-integrators, whereas signalling-nodes split the signals and route them to multiple outputs. An important group of proteins for the assembly of large signalling complexes are the anchoring scaffolding PDZ domain-containing proteins (Ziff 1997, Garner et al. 2000)

1.2 PDZ DOMAIN-CONTAINING PROTEINS AND PROTEIN NETWORKS

PDZ (PSD-95, Dlg, and ZO-1) domains are traditionally involved in protein-protein interactions. PDZ domains bind short peptide sequences normally located at the C-terminus of their interacting partners, although sometimes they are internal protein motifs, and also can interact with some lipids. Multiprotein complexes can be formed around PDZ-based scaffolds. PDZ domain-containing proteins are usually associated to the cellular membrane (Sheng and Sala 2001). Different PDZ-domain-containing proteins are involved in the process of ACD, such as Par-6, Baz, Dlg1 and Scrib (Figure 12 from (Carmena et al. 2011).

1.2.1 CANOE/AF-6/AFADIN

The PDZ protein Canoe (Cno) and its counterpart in vertebrates called AF-6 and Afadin is an actin binding protein enriched in cadherin-based adherens junctions (AJs) in the epithelial cells, endothelial cells, and fibroblasts (Mandai et al. 2013). Many Cno-interacting partners have been identified from different studies, unveiling its important role as an integration node between different signalling pathways. Indeed, it has been

shown that Cno acts as an integration and regulatory node between Ras-MAPK, JNK, Notch and Wnt signalling pathways (Carmena et al. 2006). Cno is also crucial in diverse biological processes, such as cell-cell adhesion, cell polarization, migration, survival, differentiation, and asymmetric division (Matsuo et al. 1997, Kanai-Azuma et al. 2000, Gaengel and Mlodzik 2003, Speicher et al. 2008, Sawyer et al. 2009). In vertebrates, Afadins bind the C-terminus of Nectins through their PDZ domain This interaction is the most important feature in cell-cell adhesion (Takai et al. 2008). In addition, Cno regulates cell shape changes during embryo dorsal closure (Boettner et al. 2003). In all these processes, Cno interacts physically with many proteins, such as the transmembrane proteins Echinoid and Shotgun (*Drosophila* E-cadherin, Slovakova et al. 2012), (Wei et al. 2005), the membrane protein Polychaetoid (*Drosophila* ZO-1) in the AJs of the polarized epithelia (Takahashi et al. 1998); the small GTPases Rap1 and Ran, and Pins in NBs (Kuriyama et al. 1996, Boettner et al. 2003, Wee et al. 2011). Cno also forms a complex with the immunoglobulin superfamily proteins Wrapper and Robo, and the transmembrane protien Neurexin IV to regulate neuron-glia interactions and axon guidance in the midline of the CNS (Slovakova and Carmena 2011, Slovakova et al. 2012).

Apart from a PDZ domain, Cno contains two Ras-Associated domains (RA1, RA2), a ForkHeAd domain (FHA), a DILute domain (DIL), and an F-actin binding region (Figure 11).

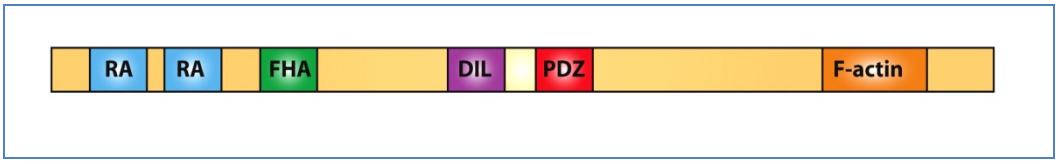


Figure 11. Canoe/ AF6/ Afadin structure: Cno contains two Ras-associated domains (RA), one Forkhead domain (FHA), one Dilute domain (DIL), one PDZ domain and an F-actin binding region.

Recently, in our lab we demonstrated a novel role of Cno in the regulation of asymmetric NB division. Cno localizes apically in NBs forming a complex with Pins in vivo (Speicher et al. 2008). In *cno* mutant embryos, the orientation of the mitotic spindle and the localization of cell-fate determinants such as Numb, Pros, Brat and Mira fails in dividing NBs. The function of Cno in the process of ACD is also conserved in

other tissues, such as the mesoderm, in which muscle/heart progenitors also divide asymmetrically (Speicher et al. 2008).

In the lab we were interested in identifying and in characterizing novel Cno-interacting partners to further understand the complex process of ACD. A yeast two-hybrid screening was performed with this aim and the small GTPase Rap1 was identified as a very strong Cno-interacting partner. Indeed, we demonstrated that Rap1, along with the guanine nucleotide exchange factor Rgl and the Ras-like small GTPase Ral forms a new signalling network that contributes to ACD through the correct mitotic spindle orientation and basal cell-fate determinants localization (see Annex I, (Carmena et al. 2011). Rap1 is forming a complex *in vivo* with aPKC and Baz apically and regulates Numb, Pros and Mira cell-fate determinants correct localization.

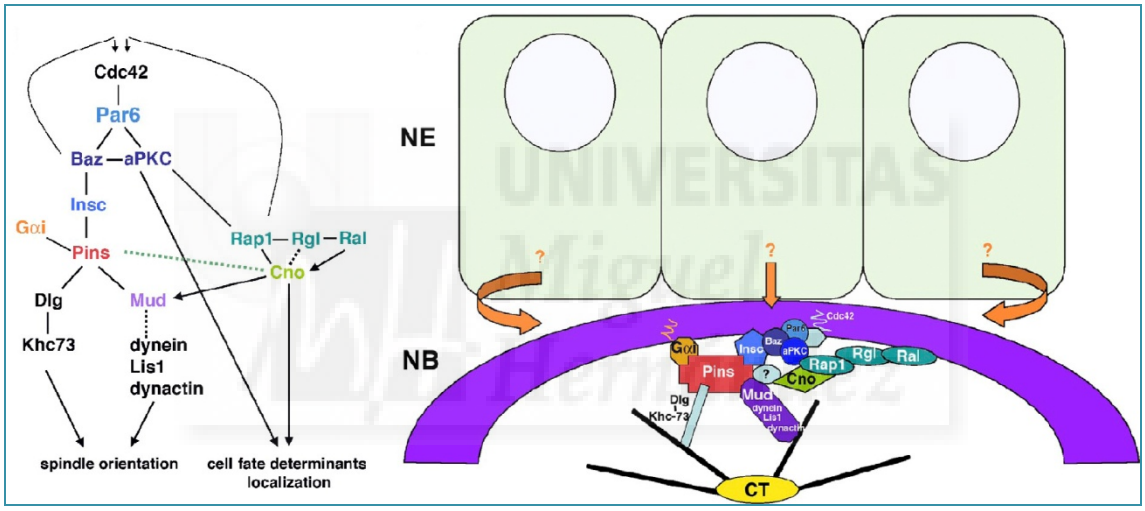


Figure 12. Apical protein complex In the left the diagram represents the apical proteins interaction map. Continuous line indicates a physical interaction. Dotted lines indicate potential physical interactions. Cno and Pins are forming a complex in vivo (green dotted line). Arrows indicate genetic interactions. In the right the location of ACD regulatory proteins at the apical mitotic NB cortex is represented. Par6, aPKC, Baz complex is linked through Insc to the Pins, hat links Gαi to the Mud, dynein and dynamin complex and to the Dlg and Khc-73 complex to the microtubules of the centrosome. Cno together with Rap1, Rgl and Ral network contributes to the mitotic spindle orientation. Orange arrows represent unknown extrinsic signals coming from the neuroectoderm (NE). Centrosome (CT) (Adapted from (Carmena et al. 2011).

Another positive though much weaker Cno-interacting partner that came out from the yeast two-hybrid screening was the Ser/Thr protein kinase called Warts (Wts). Wts is a

key kinase within the well-characterized tumour suppressor pathway known as the Hippo signalling pathway.

1.3. HIPPO SIGNALLING PATHWAY

Hippo signalling pathway controls tissue proliferation and organ growth and it is evolutionarily conserved between species. It was identified over the past decade in *D.melanogaster* by genetic mosaic screens for tumour suppressor genes (reviewed in (Oh and Irvine 2010, Yu and Guan 2013). Inactivation of genes, including *wts* (Justice et al. 1995, Xu et al. 1995), *hippo* (*hpo*) (Pantalacci et al. 2003, Udan et al. 2003, Wu et al. 2003), *salvador* (*sav*) (Tapon et al. 2002), and *mob-as-tumour suppressor* (*mats*) (Lai et al. 2005) resulted in similar tissue overgrowth phenotype due to cell overproliferation and reduced cell death. The transcriptional co-activator Yorkie (Yki), a major target of the Hippo signalling (Huang et al. 2005), in a complex with the transcription factor Scalloped (Sd) activates the transcriptional program of different proliferation and anti-apoptotic genes (Wu et al. 2008). Given that Yki has a growth-promoting activity and so acts as an oncogene, upstream tumour suppressor proteins have to regulate its activity. Recently, many new signalling components of the Hippo pathway have been identified in *D. melanogaster* and in mammals, becoming a large protein network (reviewed in (Harvey et al. 2013, Yu and Guan 2013).

1.3.1 THE CORE KINASE CASSETTE

The heart of the Hippo pathway is a core cassette that consists of a pair of Ser/Thr protein kinases, Wts and Hpo, together with the adaptor proteins Sav and Mats. Sav is a scaffolding protein that links Hpo and Wts (Tapon et al. 2002, Udan et al. 2003). Mats is an essential Wts co-factor that permits Wts kinase to autophosphorylate itself and to increase its activity (Lai et al. 2005; Wei et al. 2007). The signal transduction starts from Hpo autophosphorylation, then Hpo phosphorylates Sav, Wts and Mats; once it has been phosphorylated, Wts associates with Mats and autophosphorylates to achieve the maximum kinase activity (Wu et al. 2003, Dong et al. 2007). Then active Wts kinase phosphorylates Yki in three Ser residues inactivating it and preventing the Yki entrance to the nucleus (Huang et al. 2005, Dong et al. 2007, Oh and Irvine 2009). Yki phosphorylated by Wts in Ser168 is retained in the cytoplasm by interaction with 14-3-3 proteins (Dong et al. 2007, Zhao et al. 2007, Oh and Irvine 2008). Hence, by avoiding Yki translocation into the nucleus and by preventing the transcriptional activation of the

proliferation and the anti-apoptotic genes, the upstream kinase cascade controls organ growth. (Figure 13). Recently, Ribeiro and colleagues showed that the Ser/Thr phosphatase complex STRiatin-interacting Phosphatase And Kinase (STRIPAK) is recruited by the scaffolding protein Ras ASSociation Family (RASSF) and modulates the activity of the Hpo kinase by inhibiting its autophosphorylation (Ribeiro et al. 2010).

The Hippo signalling is highly conserved in mammals: MST1/2 are Hippo kinase orthologs that belong to the STE20 family protein kinase; Sav1 (WW45) is the Sav ortholog and Lats1/2 are the Wts orthologs; MOB1A and MOB1B are orthologs of Mats; Yes-Associated Protein (YAP) and Transcriptional co-Activator with a PDZ-binding motif (TAZ) are Yki orthologs, and TEAD1-4 are Sd orthologs (Dong et al. 2007, Wu et al. 2008). The mammalian Hippo signalling occurs similarly to the *Drosophila* Hippo pathway, in which MST1/2 kinase prosphorylates Sav1, MOB1A and MOB1B, and Lats1/2 kinase (Wu et al. 2003), (Chan et al. 2005, Callus et al. 2006, Praskova et al. 2008). Lats1/2, in turn, phosphorylates YAP and TAZ promoting their cytoplasmic sequestration by 14-3-3 proteins, thus avoiding YAP and TAZ translocation to the nucleus and their association to the DNA-binding protein TEAD1-4 (Huang et al. 2005, Dong et al. 2007, Zhao et al. 2007, Lei et al. 2008, Oh and Irvine 2008). It has been shown that after phosphorylation by Lats1/2, YAP/TAZ is subsequently phosphorylated by Casein Kinase 1 (CK1δ/ε) and it is recruited by a subunit of SCF ubiquitin ligase E3 (that belongs to the β-transducin repeat-containing protein family) and this leads to the YAP/TAZ degradation (Liu et al. 2010, Zhao et al. 2010).

1.3.2 UPSTREAM REGULATORS OF THE HIPPO CORE CASSETTE

Hippo pathway is mainly regulated by proteins involved in cell-cell contacts and cell polarity. Five upstream regulatory branches of the Hippo signalling have been identified: the Merlin (Mer)/ Expanded (Ex)/ Kibra complex, the apico-basal cell polarity complex, the planar cell polarity complex (Staley and Irvine 2012, Yu and Guan 2013), a G-protein-coupled receptor signalling, and the extracellular matrix and cytoskeleton (Codelia and Irvine 2012).

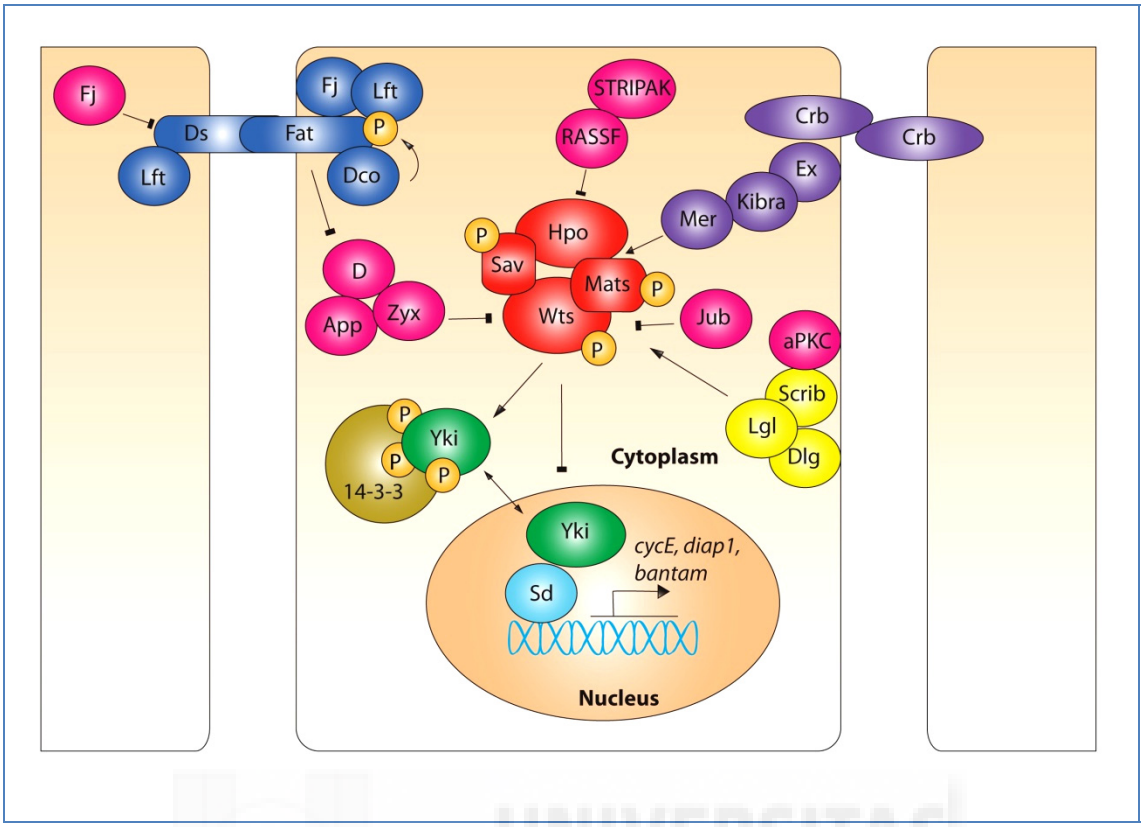


Figure 13. Hippo signalling pathway Core kinase cassette (showed in red) is formed by two kinases Hippo (Hpo) and Warts (Wts) and two adaptor proteins Salvador (Sav) and Mats. Sav, Mats and Wts are phosphorylated by Hpo that leads to Wts kinase activation. Active Wts phosphorylates co-transcriptional activator Yki (showed in green) in three serine residues thus preventing its translocation to the nucleus. Phophorylated Yki is retained in the cytoplasm by the interaction with 14-3-3 proteins. When the Hippo pathway is down-regulated, unphosphorylated Yki enters to the nucleus where it forms a complex with the DNA-binding protein Scalloped (Sd, blue) and activates the transcription of proliferation and anti-apoptotic genes, such as *cyclinE*, *diap1*, and microRNA *bantam*. Three main regulatory branches are represented: 1. Crumbs (Crb), Merlin (Mer), Expanded (Ex) and Kibra protein complex (showed in violet) activates the Hippo signalling 2. Fat and Dachsous (Ds) complex (showed in dark blue), including Four jointed (Fj), Lowfat (Lft) and Discs overgrown (Dco) kinases, by inhibiting Dachs (D), activates Hippo signalling. 3. Polarity proteins Scribble (Scrib), Discs large (Dlg), Lethal giant larvae (Lgl) (showed in yellow) also positively modulate the Hippo pathway activity. Hippo pathway inhibitors (showed in pink) Dachs (D), Approximated (App), Zyxin (Zyx), Ajuba (Jub) and aPKC alter Wts levels or/and activity thus activating Yki. Phosphatase complex Stripak together with Ras associatied family protein (Rassf) (showed in pink) dephosphorylate and thus represses Hpo kinase activity.

1.3.2.1 THE MER/EX/KIBRA COMPLEX

Ex and Mer are 4.1, Ezrin, Radixin, Moesin (FERM) domain-containing proteins. Both Mer and Ex have tumour suppressor functions to regulate cell proliferation and differentiation (McCartney et al. 2000). Mutations in any of these genes result in Yki activation and in an increased tissue growth, similar to that observed in Hpo mutations. Mer and Ex can independently regulate Hpo activity; however, mutations in both genes lead to stronger phenotypes (Hamaratoglu et al. 2006).

Kibra (WW and C2 domains-containing protein) has been recently shown to physically interact with Mer and Ex and to cooperate with them in Wts activation (Baumgartner et al. 2010, Genevet et al. 2010, Yu et al. 2010). It has been shown that Sav and Hpo physically interact with Mer and Ex, and Wts interacts with Kibra (Genevet et al. 2010, Yu et al. 2010). Mer/Ex/Kibra complex localizes at the apical domain in the polarized epithelial cells (Boedigheimer et al. 1997). The C2 domain of Kibra interacts with phospholipids and recruits the Hpo protein complex to the apical membrane (Kremerskothen et al. 2003).

1.3.2.2 THE CRB-PAR APICO-BASAL CELL POLARITY COMPLEX

Epithelial cells are polarized. Two main domains can be distinguished: an apical domain and a basolateral membrane domain, which differ in protein composition (Royer and Lu 2011). Par and Crb complex localizes apically, whereas the Scrib-Lgl-Dlg1 protein complex localizes at basolateral membranes. Multiple studies have shown the regulation of the Hippo signalling pathway and activity by these polarity complexes. Crb regulates Hippo pathway activity through its direct association with Ex by its intracellular FERM-binding motif. Overexpression of Crb leads to Ex mislocalization, Hpo inactivation and, consequently, to Yki activity (Chen et al. 2010, Parsons et al. 2010, Robinson et al. 2010). Overexpression of aPKC, similarly to the Crb, induces Yki activity through the Hpo and the RASSF basolateral mislocalization and inactivation (Grzeschik et al. 2010). Scrib regulates Wts levels and stability by interacting with the proteins Ex and D (Verghese et al. 2012). In mammals, the down-regulation of the Crb homolog Crb3 and Scrib leads to YAP translocation to the nucleus and target gene expression (Varelas et al. 2010, Cordenonsi et al. 2011). Lgl in *Drosophila* eye discs acts antagonistically to aPKC, and can influence Yki activity by regulating Hpo and the apical localization of the Ras association family member (Rassf) (Grzeschik et al. 2010). Also, the LIM

proteins Ajuba in mammals and Jub in *Drosophila* have been shown to inhibit Wts ((Das Thakur et al. 2010).

1.3.2.3 THE FAT PLANAR CELL POLARITY COMPLEX

Additionally to the apico-basal polarization, cells are also polarized along the planar cell polarity axis (Simons and Mlodzik 2008). Two different planar polarity complexes are critical in the epithelial cells: a Frizzled/Flamingo (Fz/Fmi) complex and a Fat/Dachsous (Fat/Ds) complex. The Fat/Ds regulatory complex has been shown to regulate the Hippo pathway in *Drosophila.* Loss of Fat leads to Yki activation by inactivation of Ex and Wts (Bennett and Harvey 2006, Cho et al. 2006, Silva et al. 2006, Willecke et al. 2006, Feng and Irvine 2007). Ds is an atypical cadherin and a ligand for another large cadherin called Fat that acts as a transmembrane receptor. Fat and Ds form intracellular heterodimers, and this interaction is regulated by phosphorylation mediated by Four-jointed (Fj) (Simon et al. 2010). Ds and Fj are expressed in complementary gradients, and this expression is critical for Fat activity in many tissues (Willecke et al. 2006, Rogulja et al. 2008). The Ser/Thr kinase Discs overgrown (Dco) and protein Lowfat (Lft) regulate Fat activity by phosphorylating it and by stabilizing its interaction with Ds, respectively (Mao et al. 2009, Sopko et al. 2009). Atypical myosin Dachs (D) can regulate Yki activity by regulating the levels of Wts and Ex (Bennett and Harvey 2006, Cho et al. 2006, Silva et al. 2006, Willecke et al. 2006, Feng and Irvine 2007). And Palmytoiltransferase Approximated (App) regulates the localization and activity of the atypical myosin D (Matakatsu and Blair 2008). Wts degradation is promoted by its interaction with a novel Hippo pathway component called Zyxin (Zyx) in a D polarization-dependent manner (Rauskolb et al. 2011). Very recently, Degoutin and colleagues demonstrated that the WD40 repeat protein Riquiqui (Riq) and the DYRK-family kinase Minibrain (Mnb) promote Wts-phosphorylation-dependent inhibition and the activation of Yki. This is a new Hippo signalling regulation branch, in which Fat activates Ds and through Riq and Mnb promotes Yki activity (Degoutin et al. 2013).

1.3.2.4 GPCR SIGNALLING

Recently two groups have demonstrated that two ligands for GPCRs, lysophosphatidic acid (LPA) and sphingosine-1-phospate (S1P), activate Yap, the mammalian ortholog of Yki, and the coactivator TAZ (Miller et al. 2012), (Yu et al. 2012). Both LPA and S1P

associate to GPCR and activate Rho GTPases to activate Yap/TAZ. GPCRs send signals via heterotrimeric G proteins. $G\alpha_{12/13}$, $G\alpha_{q/11}$, and $G\alpha_{i/o}$ induce YAP/TAZ activity by inhibiting the Wts orthologs in mammals Lats1/2, whereas $G\alpha_s$-coupled signals repress YAP/TAZ activity by promoting the activation of Lats1/2 (Yu et al. 2012). Another group has shown that Thrombin, an activator for Protease-Activated Receptors (PARs), also stimulates YAP/TAZ activity via Rho GTPases (Mo et al. 2012). The actin cytoskeleton seems to be involved in this YAP/TAZ regulation; however, the exact mechanism of Hippo signalling regulation by these receptors is not fully understood.

1.3.2.5 EXTRACELLULAR MATRIX AND CYTOSKELETON

Cell-cell communication is important to appropriately respond to the environmental stimuli by promoting proliferation, differentiation, migration or apoptosis. Two subunits of the actin-capping protein, Cpa and Cpb, have been reported to be involved in Hippo pathway signalling. Capping proteins limit F-actin disassembly, thus regulating the levels of F-actin. Mutations in actin-capping proteins lead to the apical accumulation of the F-actin and the subsequent Yki activation (Fernandez et al. 2011, Sansores-Garcia et al. 2011). Moreover, the F-actin and Hippo pathway interaction is evolutionarily conserved in mammals, indicating that F-actin regulators are essential for growth control by modulating the Hippo pathway (Sansores-Garcia et al. 2011, Zhao et al. 2012). Studies from mammals showed that the cytoskeleton reorganization and cell detachment from the extracellular matrix leads to Hippo pathway activation, and, particularly to Lats1/2 kinase activation. As a result, YAP becomes phosphorylated, and cells undergo apoptosis (Zhao et al. 2012). However, other cytoskeleton modulators and their implication in the Hippo signalling should be further investigated.

1.3.3 YKI TRANSCRIPTIONAL TARGETS

The main role of the Hippo signalling cascade is to inhibit Yki activity by retaining it within the cytoplasm. When the Hippo pathway is down-regulated, Yki enters into the nucleus where it binds to a different subset of DNA-binding proteins. One of the first described Yki partners was the transcriptional activator Scalloped (Sd). Recently, new Yki-binding partners have been identified, such as Mothers against Dpp (Mad) (Alarcon et al. 2009, Oh and Irvine 2011), Homothorax (Hth) and Teashirt (Tsh) (Peng H 2009 Genes Dev). Hth and Mad promote the transcription of microRNA gene *bantam,*

whereas Sd promotes the transcription of the *Drosophila inhibitor of apoptosis* (*Diap1*), *cyclin E* and *myc* genes (Oh and Irvine 2011). The identification of new Yki interacting partners has shed light into the tissue specificity response of the pathway. For example, Hth is a key partner of Yki in the eye disc (Peng et al. 2009), whereas Sd is a key partner for Yki in the wing disc (Goulev et al. 2008, Wu et al. 2008).

1.3.4 MAMMALIAN WARTS: NONCANONICAL LATS REGULATION AND FUNCTION

Drosophila Warts and mammalian Lats1/2 are members of the Ser/Thr nuclear Dbf2 related kinases 1/2 (NDR1/2). Lats kinases are highly conserved between species (Figure 14).

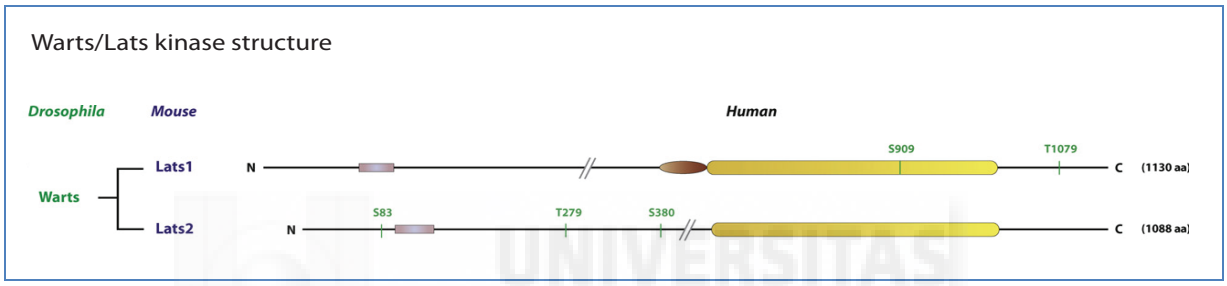


Figure 14. Warts/Lats kinase structure. Lats kinases are highly conserved in their structural composition. In yellow the kinase domain is represented, the ubiquitinilation domain is shown in pink and MOB-binding domain is represented in brown. Lats kinases are phosphorylated and actiated by upstream kinases at different serine (S) and threonine (T) positions and this phosphorylation leads to their kinase activation (Adapted from (Avruch et al. 2012).

In *S. cerevisiae* Lats kinase counterparts Cbk1 maintains the polarized growth during budding and mating (Racki et al. 2000, Bidlingmaier et al. 2001), and Dbf2 is forming a part of the mitotic exit network (MEN) and governs the inactivation of the CDKs (Toyn and Johnston 1994, Visintin and Amon 2001), while in the *Schizosaccharomyces pombe*, Lats kinase homologs: the kinase Sid2 forms part of the septation initiation network (SIN) that promotes the cytokinesis checkpoint (Balasubramanian et al. 1998, Sparks et al. 1999) and ser/thr protein Orb6 is necessary for the maintenance of the polarized growth and for the morphogenesis regulation (Verde et al. 1998). *Drosophila* Wts and Tricornered (Trc) kinases are homologs of Lats and NDR kinases, and they are key players in Hippo signalling and dendrite tilling, respectively (Emoto et al. 2006). Human LATS is localized to the mitotic apparatus where it is phosphorylated by the kinase Cdc2 (Nishiyama et al. 1999). Human LATS2, along with the LIM protein Ajuba, regulates the accumulation of γ-tubulin at the centrosomes during mitosis and

thus organizes the spindle apparatus during mitosis (Abe et al. 2006). Human LATS1, along with Mats ortholog MOB1A, promotes G2 arrest (Bothos et al. 2005). The regulation of Lats1/2 by the Hippo pathway has been previously discussed. Lats kinases are regulated by phosphorylation in the activation motif (T-loop) and in the hydrophobic motif (HM) located at the C-terminus. Both sites are required for Lats1/2 kinase activity. Additionally to these two phosphorylation events Lats2 is phosphorylated by Aurora-A and it plays a role in the mitotic progression. LATS1 phosphorylates YAP/TAZ at different ser positions in the phosphorylation consensus Hx(R/H/K)xx(S/T) in the context of the Hippo signaling pathway (Hao et al. 2008).

Lats1/2 have additional substrates in mammalian cells, such as Snail1, the Adaptor Associated protein Kinase 1 (AAK1), the Cyclin-dependent Kinase inhibitor 1 p21/WAF1, 14-3-3 proteins, the Dual-specificity tYrosine-phosphorylation-Regulated Kinase 1A (DYRK1A), Rabin8 and Myosin Phosphatase-Targeting subunit 1 (MYPT1) (Reviewed in (Hergovich 2013). Thompson group has recently demonstrated that Wts kinase, apart of Yki, has other phosphorylation substrates in *Drosophila*, such as the protein Enabled (Ena) (Lucas et al. 2013). Further investigation in the field of Wts/Lats kinase substrates has to be done to shed light into the noncanonical function of the Lats kinases.

Here I have described the model systems and the mechanisms underlying ACD, a fundamental process in development, stem cell and cancer biology. It has been highlighted how many different proteins tightly regulate different aspects of ACD, such as the establishment of apico-basal cell polarity, the correct orientation of the mitotic spindle and the localization of cell-fate determinants. However, recent studies from our lab about the function of Cno and Rap1 in ACD have revealed how it still remains unknown all the signals involved in regulating this crucial process, and how those signals are integrated within the complex protein network that very precisely must modulate ACD. Both ACD and the Hippo signalling pathway are evolutionarily highly conserved. Given that we identified Wts in our yeast two-hybrid screening as a positive and novel Cno-interacting partner, and that Wts, within the Hippo pathway, has an important function in the control of growth and proliferation, as well as in the regulation of the cell cycle, we decided to investigate whether the Hippo signalling pathway was modulating, along with Cno, the process of ACD. This hypothesis constituted the starting point of this PhD thesis research work.

CHAPTER 2

MATERIALS AND METHODS

2.1 CLONING

2.1.1 STRAINS OF YEAST AND BACTERIA AND GROWTH MEDIAS

Bacteria *Escherichia coli* (*E. coli*) strain DH5α was used for cDNA plasmids amplification. One Shot® Top10 *E. coli* cells (*Invitrogen*) were used for the Gateway Entry-cloning protocol (*Life Technologies*). Cells were grown in LB media (*Luria Bertani,* 10g/l bacto-tryptone, 5 g/l of bacto-yeast extract, 5 g/l NaCl; *Sigma*), where 100 μg/ml of ampicillin or 50 μg/ml of kanamycin were added. Yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) strains PJ69-4A (James, P. et al 1996) and L40 (Bai, C. and Elledge, S. 1997) were used for yeast-two hybrid analysis. Cells were grown in YPDA (rich media Yeast extract (*Fluka*), Peptone, Dextrose and Adenine, (*Qbiogene*)) and drop out media (DOB, 1xBSM UHLT and mixture of amino acids, *Qbiogene*).

2.1.2 PLASMIDS

cDNA (5771 bp) sequence of a gene *wts* form plasmid pAc5.1/V5-HisB (Wu et al, 2003 Cell) was used for PCR amplification. *wts* gene coding sequence (3318 bp) and its C-terminal part (1802 bp) were amplified and then were cloned to the plasmids pACT2 AD (*Clontech*) and pGBKT7 BD (*Clontech*) using SmaI and NcoI cloning sites. Both pACT2 AD and pGBKT7 BD contained MCS and ampicillin or kanamycin resistance genes. Additionally, these plasmids had yeast selectable markers TRP1 or LEU2, respectively. pLexA and pAs2-1 plasmids, containing *cno* NH sequence (Carmena A, 2006 PlosOne.) were used for yeast two-hybrid screening. Both pLexA BD and pAs2-1 BD plasmids contained MCS, ampicillin resistance gene and yeast selectable markers HIS3 and TRP1, respectively.

cDNA (6976 bp) sequence of the gene *cno* from the plasmid pOT2 (LD24616, *Drosophila Genomics Resource Center*) was used for the PCR amplification. *cno* C-terminal part (2853 bp) was amplified and then cloned into the pENTRY vector (*Life Technologies*) using Gateway Topo-cloning protocol (*Life Technologies*).

2 C-terminal truncations of 1762 bp and 2188 bp were created for the *baz* gene from the pDONR/Zeo vector (N.Tapon´s lab) and cloned to the pENTRY vector (*Life Technologies*) using Gateway Topo-cloning protocol (*Life Technologies*).

Full-length *numb* gene was cloned to the same pENTRY vector from the cDNA clone (RE15808, *Drosophila Genomics Resource Center*).

2.1.3 PCR, OLIGONUCLEOTIDES AND CLONING

2.1.3.1 *WTS* CLONING

2.1.2.1.1 PCR AND CLONING

wts gene coding sequence (3318 bp) and its C-terminus part (1802 bp, designed from nucleotide position 1516 - 3318) were amplified from pAc5.1/V5-HisB vector (Wu et al, 2003 Cell). PCR conditions are shown in the table 1. Used oligonucleotides are shown in the table 2. PCR reaction is shown in the table 3.

Table 1. PCR thermal cycling using Expand High Fidelity PCR System (*Roche*)

Process	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1x
Denaturation	94°C	15 sec	10x
Annealing	65 °C	30 sec	10x
Elongation	72 °C	2 min 6 sec	10x
Elongation	72 °C	5 min	1x
Denaturation	94°C	15 sec	20x
Annealing	72 °C	30 sec	20x
Elongation	72 °C	2 min 6 sec	20x
Final Elongation	72 °C	7 min	1x
Cooling	4 °C		1x

Table 2. Oligonucleotides for *warts* and *wts* C terminus part amplification from pAc5.1/V5-His vector. Restriction sites for the cloning *NcoI* (ccatgg) and *SmaI* (cccggg) are underlined. F (forward), R (reversed), wts (warts), stop (stop-codon).

Oligonucleotide name	Sequence
F_wts	5` AT acc atg gaa atg cat cca gcg ggc gaa aaa 3`
F_wts_part	5` AT acc atg gaa gcc ttg agg gtg ctc cag gca 3`
R_wts	5` AT acc cgg gat gac gta aac cgg cgc ctg atc 3`
R_wts_stop	5` AT acc cgg gat tca gac gta aac cgg cgc ctg 3`

Table 3. PCR reaction mix for *wts* and *wts* C terminus part amplification using Expand High Fidelity PCR System (*Roche*)

PCR reaction mix (50 µl volume)	Final concentration
10x Expand High Fidelity buffer with $MgCl_2$	1x
Deoxynucleotide mix, 10 mM of each dNTP	200 µM
Forward primer 10 µM	300 nM
Reverse primer 10 µM	300 nM
warts cDNA	20 ng
Expand High fidelity enzyme mix 3.5 U/µl	2.6 U

DNA fragments were resolved by agarose electrophoresis in 1xTAE buffer (40 mM Tris-Acetate, 1mM EDTA, pH 8.3). Agarose concentration in the gel (0.8-1.2%) and voltage of electrophoresis depended on the size of DNA fragments. Ethidium Bromide (EtBr) was added to the agarose gel in final concentration 1 µg/ml. EtBr was used for nucleic acid visualization at 260 nm in UV-iluminator. PCR products were purified

from agarose gel according to Agarose Gel Extraction Kit (*Larova*) protocol. Restriction reaction of vectors pACT2 and pGBKT7 (*Clontech*) and PCR products of *warts* and *warts* C terminus part was performed. 10 U of SmaI restriction enzyme, 0.01% of Bovine Serum Albumine (BSA, *Takara*) and 1x T buffer (*Takara*) was used in final volume of 20 µl for 1 µg DNA digestion. Restriction reaction was performed at 30^{o}C for 1 hour, and then 10 U of NcoI, 0.01% of BSA and 1x K buffer (*Takara*) were added to the 50 µl of the final volume. DNA digestion was performed at 37 ^{o}C for 2 hours. 2U of Alkaline Phosphotase (AP, *Roche*) were added to the vectors for 2 hours at 37 ^{o}C to avoid the auto ligation of the vector. Restriction products were ligated in the final volume of 20 µl. 1 U of T4 DNA ligase in 1x T4 Ligation buffer (*Roche*) was used to perform ligation reaction between vectors and PCR products. Reaction was performed at 18 ^{o}C overnight. Only vector without insert was used as a control for ligation reaction.

2.1.2.1.2 HEAT SHOCK TRANSFORMATION OF DH5A COMPETENT CELLS.

80 µl of *E.coli* strain DH5α competent cells were incubated on ice for 20 min. 10 µl of ligation mix were added to the cells and then incubated on the ice for 30 min. Transformation of the bacteria was performed for 2 min at 42 ^{o}C and then direwctly placed to the ice for another 3 min. Then 1 ml of the preheated (at 37 ^{o}C) LB media was added to the cells. Bacteria cells were incubated at 37^{o}C for one hour in the shaker (220 rpm). Then cells were harvested by the centrifugation at 1200×g for 3 min. Cell pellet was resuspended in 100 µl of media and plated to LB agar plates containing 100 µg/ml of ampicillin for pACT2 or 50 µg/ml of kanamycin for pGBKT7. Bacteria colonies were grown at 37 ^{o}C overnight.

2.1.2.1.3 PLASMID DNA EXTRACTION

6 colonies were picked up from each transformation LB plate and were grown in 2 ml of LB media containing ampicillin or kanamycin at 37^{o}C on the shaker (220 rpm) overnight.

Then cells were harvested by centrifugation at 1200×g for 3 min. Plasmid DNA extraction was performed according to the protocol of Minipreps: High Pure Plasmid Isolation Kit (*Roche*).

Then restriction analysis of DNA was performed in final volume of 10 µl. 2 U of SmaI and 1 U of NcoI were added to 1×T buffer (*Takara*) and reaction lasted for 1 hour at 37 ^{o}C. DNA fragments were resolved in 0.8% TAE agarose gel. Two positive clones of each construct were chosen for nucleotide sequencing. After sequencing one positive clone of each construct was chosen for midi preparation. Extraction of DNA using midi preparation was done according to the manufacturer protocol of Qiagen Plasmid Midi Kit (*Qiagen*). DNA concentration was measured in spectrophotometer NanoDrop 2000.

2.1.3.2 *CNO*, *NUMB* AND *BAZ* CLONING

2.1.2.2.1 PCR AND CLONING

C-terminal parts of *cno* and *baz*, and *numb* coding sequences were amplified using next oligonucleotide primers (Table 4). PCR *Pwo Master Mix* (*Roche*) protocol was used to amplify all these genes. All 5´forward primers contained CACC nucleotides for the directional Gateway cloning (*Life Technologies*).

Table 4. Oligonucleotides for the amplification of *numb* full-length, *cno* and *baz* C terminus part from corresponding vectors. Forward primers contained 5´CACC nucleotides for the directional Gateway cloning are underlined. fw (forward), rev (reversed), cno (canoe), baz (bazooka), trun (truncation), the number (1100, 738, 880) corresponds to the amino acid number of the trucation, stop (stop-codon) .

Oligonucleotide name	Sequence
1100trun-cno-fw	5` <u>cacc</u>tatcacgggttggctacactactt 3`
cno-stop-rev	5` ttagtgcaccgcgtctatatctcgtt 3`
738trun-baz-fw	5` <u>cacc</u>cgatcggccagttccagtgacatt 3`
880trun-baz-fw	5` <u>cacc</u>tggtcgccggcgcagatgcactta 3`
baz-stop-rev	5`tcacaccttggaggcgtgtggctgggaa 3`
numb-fw	5`<u>cacc</u>atgggaaactcctcgtcacaca 3`
numb-rev	5`gagctgcacctggaatgactgc 3`

2.1.2.2.2 THE DIRECTIONAL TOPO CLONING

The pENTR Directional TOPO cloning was performed according to the protocol from the Invitrogen. 2 µl of the PCR mix was directly added to the mixture of the TOPO vector, salt solution and water. Topo reaction was performed at room temperature for 30 mins and 2 µl of the reaction was added to the chemically competent One Shot *E. Coli* cells on the ice. After 30 min-incubation heat shock was performed at 42 ^{o}C for 45 seconds and placed directly to the ice. Cells were grown in SOC media for an hour and half of the mixture was plated to the LB agar plate containing 50 µg/ml of the kanamycin. Bacteria colonies were grown at 37 ^{o}C overnight.

Six bacteria clones were picked up from the plate and DNA was extracted according to the previous protocol (see 2.1.2.1.3 plasmid DNA extraction). The plasmids were sequenced for the presence of the insert and appropriate clones were then cloned to the destination vector using Gateway LR cloning protocol (*Invitrogen*).

2.1.2.2.3 LR CLONING

Gateway LR cloning protocol (*Invitrogen*). LR cloning technology, based on the site-specific recombination properties of the lambda bacteriophage, is effectively used to clone DNA sequences to the destination vectors.

pENTRY clone contains the gene of the interest between two recombination sites attL1 and attL2, whereas the destination vector contains corresponding recombination sites attR1 and attR2. pENTRY clone containing *baz*, *cno* and *numb* was recombined with pAFW destination vector, that contains the actin promoter and 3xFLAG-tag in the N-terminus (for *baz* and *cno* cloning) or pAWF (in the case of *numb*), containing the actin promoter and 3xFLAG-tag in the C-terminus. The recombination reaction contained pENTRY clone, destination vector, and LR Clonase enzyme mix. The reaction was performed at room temperature for one hour. Then 1 µl of Proteinase K was added to terminate the reaction and incubated at 37 ^{o}C for 10 mins. 1 µl of the LR reaction was transformed to the One Shot cells according to the previously describing protocol.

2.1.4 QUICKCHANGE MULTI SITE-DIRECTED MUTAGENESIS

To introduce multiple mutations in the same vector simultaneously Quickchange multi site-directed mutagenesis kit (*Agilent Technologies*) was used. To introduce mutations from serine to alanine one to two nucleotides had to be changed per one mutagenesis site. Primers were designed according to the protocol, where 15 nucleotides from each side from the mutation were identical to the template. Primer sequences are shown in the table 5. Mutated forms of *baz*, *cno* and *numb* were then cloned to the FLAG-tagged destination vector using LR cloning kit (see 2.1.2.2.3).

Table 5. Oligonucleotides used to mutate different sites in the *numb, cno* and *baz* genes. Changed nucleotides are underlined. fw (forward), cno (canoe), baz (bazooka), trun (truncation), the number (757, 1196, 1366 etc) corresponds to the amino acid where serine is mutated to alanine.

Oligonucleotide name	Sequence
757,759baz-fw	5′cacagtcatagccatgccaatgctagcggtggcagcaat 3′
1049baz-fw	5′ catgccaagactgcacagtatcgagcaaaga 3′
1308baz-fw	5′ catcagcattaccatgcgcagcgcagtgcccgc 3′
1366,67,68baz-fw	5′atcaagcatagccacgccgccgccgccacgtcgtcctcc 3′
1196cno-fw	5′ catcaccacacgggagcgggtaccatatccttg 3′
161numb-fw	5′ cacgaacgcggcttcgcctacatatgccgggat 3′

2.2 YEAST TWO-HYBRID

2.2.1 YEAST COMPETENT CELLS PREPARATION

Cells were grown in 5 ml of YPDA media (containing 2% of glycose) at 30^{o}C overnight in the shaker. Then cell culture was transferred to 50 ml of YPDA (containing 2% of glycose) and was incubated at 30 ^{o}C for 3 hours in the shaker (200 rpm). Cells were harvested by centrifugation at 970×g for 5 min. Pellet was washed in 5 ml of sterile 1x TE buffer pH 7.5 (10 mM Tris-HCl, 1 mM EDTA) and then was centrifuged again at

the same conditions. Cell pellet was resuspended in 250 µl of freshly prepared sterile 1x LiOAc/TE buffer (100 mM LioAc pH 7.5, TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA).

2.2.2 TRANSFORMATION OF YEAST COMPETENT CELLS

plexA-*cnoNH* construct was transformed to L40 yeast strain. Salmon sperm DNA (10 mg/ml, *Fluka*) was boiled for 10 min and then was placed directly to the ice. 10 µl of boiled salmon sperm DNA was added to 100 ng of the construct DNA. Then 100 µl of yeast competent cells and 600 µl of a sterile freshly prepared LiOAc/TE/PEG solution (100 mM LiOAc pH 7.5, TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA) in 50% polyethylene glycol PEG 3350) were added. Transformation mix was incubated at 30^{o}C for 30 min in the shaker (200 rpm). 70 µl of dimethyl sulfoxide (DMSO) were added to the transformation mix and were mixed by gentle inversion of a tube. Heat shock of the cells was performed at 42^{o}C for 15 min and then cells were chilled on the ice for 2 min. Cells were harvested at maximum speed for 5 sec and then 200 µl of a sterile 1xTE buffer pH 7.5 were added. Cells were plated using Roll & Grow^{TM} Plating Beads (*Q-biogene*) to the DOB plates (DOB media containing 1x BSM UHLT 10% of agar and 1mg/ml of URA, 10 mg/ml of LEU, 10 mg/ml of HIS and 10 mg/ml of TRP) absent for His (pLexA-*cnoNH* construct containing HIS gene). 6 positive colonies were picked up from the His absent DOB plate and plated to another His absent DOB plate at 30^{o}C for 3 days to increase the amount of yeast material. The presence of pLexaA-*cnoNH* in the yeast cells was checked by Western blot technique. Rabbit anti–lexA Binding DNA region antibody (*Millipore*) in a concentration 1:2000 was used. Positive clone that contained LexA-*cnoNH* construct was grown in 10 ml of His absent DOB media at 30 ^{o}C overnight. Then the same clone was transformed again by the empty vector pACT2.1 as a negative control, by pACT2.1-*Yd4 (dishevelled* that interacts directly with *canoe)* as a positive control and pACT2.1-*wts* and pACT2.1-*wts_part.* Transformation was performed as described before. Cells were plated using Roll & Grow^{TM} Plating Beads (*Q-biogene*) to the DOB plates absent for His and Leu (pACT2.1 constructs containing LEU gene). Colonies were grown on the plates at 30 ^{o}C for three days.

2.2.3 PROTEIN EXTRACTION FROM YEAST COLONIES AND PROBE PREPARATION

Transformed colonies were grown in 10 ml of His absent DOB media at 30 ^{o}C overnight. Celles were harvested by centrifugation at 970×g for 5 min and were washed once with PBS (Phosphate Buffered Saline pH 7.4) and then were transferred to a new

tube. Cells were centrifuged at 10000×g for 30 sec. PBS was removed and a cell pellet was frozen at -80°C for 30 min and then cells were thawed on ice. 200 µl of lysis buffer (50 mM TrisCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% SDS, 1 mM PMSF) were added. 100 µl of Silica beads (*OPS diagnostics*) were added and then cells were destroyed by vortex at 4 °C for 15 min. Cell lysate was centrifuged at 1000×g for 30 sec. Supernatant was transferred to a new tube and equal volume of 2×Laemmli (50 mM TrisHCl pH 6.8, 100 mM DTT, 0.1% bromophenol blue, 10% glycerol, *Fluka*) was added. Proteins were denaturized at 95°C for 5 min before SDS-PAGE gel loading.

2.2.4 SDS-PAGE (SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS)

Proteins were resolved in 10% of SDS polyacrylamide gel by gel electrophoresis in 1×SDS buffer (0.025 M Tris, 0.19 M glycine, 0.1% SDS). Vertical SDS-PAGE phoresis was used (Mini- PROTEAN 3, *Bio-Rad*). Page Ruler™ Prestained Protein ladder marker (*Fermentas*) was used for protein molecular mass detection.

2.2.5 WESTERN BLOT

The gel with resolved proteins was incubated in a transfer buffer Semi-dry (25 mM Tris, 190 mM glycine, 20% methanol). Proteins were transferred to Hybond™ ECL™ nitrocellulose filter (*Amersham Biosciences*) that was also incubated in the same Semi-dry buffer for 10 min. Proteins were transferred to the nitrocellulose filter at 100 mV for 1-2 hours at 4 °C (*Bio-Rad*). The transfer time depended on the protein molecular mass. The nitrocellulose filter then was incubated in 5% of Western blocking solution (5% non-fat milk in TBT buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween20) for 1 hour in the shaker (100 rpm) at room temperature. Primary antibody Rabbit anti-lexA Binding DNA region (*Millipore*) was used in concentration1:2000 in 2% of Western Blocking solution at 4°C overnight in the shaker (100 rpm). The filter then was washed with Western washing TBT buffer three times for 15 min in thee shaker (100 rpm) at room temperature. Goat anti- rabbit-HRP in concentration 1:10000 was used as secondary antibody in 2% of Western blocking solution for one hour in the shaker at room temperature. The filter was washed three times again with the same Western Washing TBT buffer. For signal detection ECL detection reagents (*Amersham Biocsiences)* were used. For image acquirement the luminescent image analyzer with electronically cooled camera system LAS-1000 was used (*Fujifilm*).

2.2.6 B-GALACTOSIDASE ACTIVITY DETERMINATION

6 positives colonies were picked up from His and Leu absent DOB plates and were grown in 10 ml of His Leu absent DOB media at 30 ^{o}C in the shaker overnight. Cell density then was measured at OD_{600}, cells were resuspended at a final concentration 0.3 OD_{600}/ml and then were grown at 30 ^{o}C for 4 hours in the shaker to reach the final cell density at 0.5 OD_{600}/ml. Cells were centrifuged at 970×g at 4 ^{o}C for 5 min. Then the cells were transferred to a new tube and centrifuged at maximum speed for 15 sec. Supernatant was completely removed and 100 µl of Silica beads (*OPS diagnostics*) were added. Then 300 µl of Z buffer (16.1 mg/ml of $Na_2HPO_4 \cdot 7H_2O$, 4.7 mg/ml NaH_2PO_4, 0.75 mg/ml KCl, 0.246 mg/ml $MgSO_4$ pH 7.0, 3.2 mM β mercaptoethanol freshly added prior to use) were added and cells were destroyed by vortex at 4 ^{o}C for 10 min. Then protein lysates were collected by centrifugation at maximum speed for 15 sec. 200 µl of protein extracts were used for β galactosidase reaction and 40 µl were used for protein concentration measurement.

2.2.7 B-GALACTOSIDASE REACTION

The reaction was performed at 28 ^{o}C in a water bath. 200 µl of yeast protein extracts and 600 µl of Z buffer were added to plastic 5-ml vials. 800 µl of Z buffer were used as a blank for measurement at OD_{420}. The plastic vials with protein extracts were warmed up for few mins in the water bath at 28 ^{o}C. Then 200 µl of Z buffer containing 4 mg/ml ONPG substrate (o-Nitrophenyl-ß-D-galactopyranosidase, *Sigma Aldrich*) were added to each glass tube every 10 seconds. Time in and time out was written down for each tube. The ONPG substrate yields a yellow product that was easily detectable in the visual range after stopping the reaction with 500 µl of 1M Sodium Carbonate. The time of reaction were stopped and was written down. Acryl Cuvettes 10×4×45 mm (*Sarstedt*) were used for reaction measurement at OD_{420}.

2.2.8 PROTEIN CONCENTRATION DETERMINATION

40 µl of yeast protein extract were added to the Acryl Cuvettes 10×4×45 mm (*Sarstedt*) and 960 µl of BioRad solution diluted 1:5 in distilled water were added. 5 mins later protein concentration was measured at OD_{595}. 1 ml of BioRad solution diluted 1:5 in water was used as a blank for measurement.

β galactosidase reaction was determined by an equation: **OD_{420}×0.04×10000/t×OD_{595}×0.2**

Where: **OD_{420}** is a β gal acitivity, **0.04** is a volume of protein extracts used for protein concentration measurement in mlillilitres, **t** is a time of β gal reaction in mins, **OD_{595}** is a concentration of proteins and **0.2** is a volume of proteins used for β gal reaction in millilitres.

2.3 CELL CULTURE

2.3.1 S2 CELL CULTURE TRANSFECTION AND RECOMBINANT PROTEIN EXPRESSION

S2 cells were grown in the S2 Schneider´s Drosophila Media (*Gibco*) containing Streptomycin and Penicilin antibiotics and Fetal Bovine Albumin (FBS) at 25 ^{o}C. 3x10^{6} of the cells were seeded per one well of the six 6-well plates and transfected with 300 ng of the corresponding vector (3xFLAG-*baz*, 3xFLAG-*cno* or *numb*-3xFLAG). Effectine transfection reagent protocol (*Qiagen*) was used for the transfection of the S2 cells. Cells were growing at 25 ^{o}C for 48 hours.

2.3.2 CELL LYSIS AND IMMUNOPRECIPITATION

After that cells were collected and washed once with cold PBS. Later the lysis procedure was performed. Cells were lysed in the 2 mL of RIPA buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1% (w/v) Sodium Deoxycholate) with protease inhibitors (0.5 mM NaF, Roche Complete protease inhibitor cocktail). Cell lysates were clarified by the centrifugation at maximum speed for 30 mins at 4 ^{o}C. 90 µl of the slurry FLAG-tagged beads (50/50 beads in the RIPA buffer) were used for the immunoprecipitation of the recombinant FLAG-tagged protein substrates. The lysates were incubated with the beads for 2 hours at 4 ^{o}C on the wheel. Beads then were washed 4 times with cold RIPA buffer.

2.3.3 RECOMBINANT PROTEIN ELUTION

FLAG-tagged substrates were eluted from the beads by the incubation with FLAG peptide incubation. 100 µl of the elution buffer (20mM HEPES, 5mM MgCl, 1mM DTT and protease and phosphatase inhibitors) containing FLAG peptide (150 ng/µl) were added to the beads and incubated for 30 mins at 4 ^{o}C on the wheel. Supernatants

were frozen at -80 °C after short speed and the concentration was measured comparing to the BSA (0.5mg/µl, 1 mg/µl, 2 mg/µl) by Western blot and BlueStain gel staining.

2.4 *IN VITRO* KINASE ASSAY

The kinase reaction was performed in the kinase buffer containing protease inhibitor cocktail (*Roche*) and phosphatase inhibitors 1 and 3 (1:100, *Roche*). Approximately 0.3 – 0.5 mg of the FLAG-substrate (*cno*, *baz,* and *numb*), 200 ng of the human LATS1/2 active kinase (*BioChem*), and 3µCi of the ^{32}P γ-ATP were mixed in the total volume of 25 µl. The kinase reaction was performed at 30 °C by shaking, after short centirufagation SDS loading dye was added. Samples were boiled for 5 mins prior to the gel loading and running. Gel was left to dry for 2 hours approximately. The gel was exposed against phosphoimager plate for an hour; the results were read with phosphoimager.

2.5 CO-IMMUNOPRECIPITATIONS IN VIVO

2.5.1 EMBRYO COLLECTION

Nearly 100 µl of 0-7 hour *Drosophila* embryos were homogenized in the same volume of the Lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 1 MM EDTA, 1% Triton X-100, protease inhibitors 1 mM NaF, 100 mM Na_3VO_4, 2 mM PMSF and Complete Protease Inhibitors cocktail (*Roche*) were freshly added prior to use) on the ice in glass homogenizer. Protein extracts were centrifuged at 18.700×g at 4 °C for 15 min then for 5 min in the same conditions to remove completely cell debris. 1/10 of protein extract was heated in the same volume of 2×laemmli buffer (*Fluka*) for 5 min at 95 °C and then was used in Western blot as an input.

2.5.2 IMMUNOPRECIPITATION

Protein extracts were incubated with 1:500 mouse anti-myc antibody (*BD lifescience*) to immunoprecipitate Myc-tagged forms of the proteins (*wts*::MYC) at 4 °C on the wheel overnight. Then 40 µl of G Sepharose beads (*Sigma*) were added for 2 hours at 4 °C on the wheel. Or GFP-tagged beads (*Abcam*) were incubated with the embryo extracts to immunoprecipitatie GFP-tagged forms of the proteins (*insc*-GFP, *baz*-GFP etc) at 4 °C on the wheel overnight. The beads then were washed three times with Lysis buffer without protease inhibitors, resuspended in the 2×Laemmli buffer (*Fluka*) and heated

for 5 min at 95 ^{o}C. All precipitates and the input were centrifuged at maximum speed for 2 mins prior to be resolved by SDS-PAGE. Spectra^{TM} Multicolor High Range Protein ladder (*Fermentas*) was used for protein molecular mass detection. Western blotting was performed as described previously. ECL Nitrocellulose filters were immunoblotted with rabbit anti-myc (*Abcam*) antibody in the concentration 1:5000 and mouse anti-GFP in the concentration 1:2000 (*BD Lifesciences*).

2.6 DROSOPHILA EMBRYO FIXATION

2.6.1 EMBRYO COLLECTION AND DECHORIONATION

All embryos were collected from apple juice laying plates daubed with yeast paste at intervals of 0-16 h at 18 ^{o}C, 25 ^{o}C or 29 ^{o}C (in UAS-Gal4 experiments) depending on required embryo stage. Materials such a small Nitex basket and paintbrush were used for the embryo collection. Intense washing with water was necessary to remove the rest of yeast. Embryo dechorionation was performed in the small basket using 100% commercial bleach for 2 mins at room temperature. Embryos were washed with sufficient amount of water to completely remove the rest of the bleach.

2.6.2 FORMALDEHYDE FIXATION

After embryo collection and dechorionation embryos were passed to glass vials and then fixed in 1:1 heptane and 4% of formaldehyde for 20 mins in the shaker. Then formaldehyde was removed by Pasteur pipette from inferior part of the glass vial. The same volume of 100% methanol was added to the vial with embryos that were found in the upper heptane phase. Embryo devitellination was performed by hard shaking of the tube for 30 seconds. Embryos without vitelline membrane were felt down to the bottom of the vial. Embryos then were transferred to 1.5 ml eppendorf tube and washed with 100% methanol for three times and then were stored at –20^{o}C or directly used for immunostaining experiments.

2.6.3 HEAT FIXATION

After collection and dechorionation embryos were transferred to a nickel mesh and fixed for 16 seconds in E-wash (70 mM NaCl, 0.1% Triton X-100) at 80°C. Embryos were chilled with an excess volume of ice-cold E-wash and incubated on ice for 2 min (Tepass 1996). Fixed embryos were then gently transferred using paintbrush into the

glass vial with heptane and the same aliquot of methanol was added and the process of devitillination was done the same way as after formaldehyde fixation.

2.7 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

2.7.1 ANTIBODY STAINING

Fixated embryos were washed three times with PBS containing 0.3% Triton X-100 (PBT, pH 7.4). Then embryos were washed with 400 µl of PBT pH 7.4 for 1 hour in the rotator. PBT was replaced by 400 µl of PBT containing 0.1% BSA (Bovine Serum Albumine, *Sigma*) and embryos were incubated for one h in the rotator. From 100 to 400 µl of primary antibody diluted in PBT 0.1% BSA were added to the embryos and were incubated for 2 hs at room temperature (RT) or overnight at 4 ^{o}C. Diluted primary antibody after incubation containing thimerosal was stored at 4 ^{o}C. Embryos were washed with PBT pH7.4 for 1 h in the rotator. 1:200 of secondary antibody diluted in PBT 0.1% BSA in total volume of 400 µl was added to the embryos and was incubated for 1 h at RT in the rotator. Then embryos were washed with of PBT for 1 h. In the case of direct secondary antibody conjugated with fluorescent dyes such as Alexa 488 (green), Alexa 546 (red) and Alexa 633 (blue) (*Molecular Probes*) 30 µl of Vectashield mounting media for fluorescence (*Vector labs*) were directly added to the embryos. Embryos then were placed to the microscope slide and then 5 embryos were mounted in 3 to 5 rows in a lateral or a ventral position.

Biotinylated secondary antobodies (*Vector labs*) also were used in the same concentration as described previously. After 1 hour-washing with PBT embryos were incubated in Avidin and Biotin mixture (*Vectastain ABC kit, Vector laboratories*) for 1 h. Then embryos were washed with PBT for one h. Then embryos were incubated for 40 mins in 1:200 Streptavidine-488, 546 or 633 (*Molecular Probes*) diluted in 400 µl of PBT 0.1% BSA. Embryos then were washed in PBT for one h. 30 µl of Vestashield mounting media for fluorescence (*Vector laboratories*) was added to the embryos before placing them to the slide. Protein pattern was detected by fluorescence. Fluorescent images were recorded by using Leica upright DM_SL microscope (Leica Spectral Confocal acquisition software). All images were taken with an HCX PL APO 63×/1.32-0.6 oil CS objective. Images were assembled using Adobe Photoshop CS3 programme.

2.7.2 DAB HISTOCHEMISTRY

Fixed embryos were incubated with primary and biotin-conjugated secondary antibody as described previously. Then embryos were incubated in Avidin Biotin solution for 1 h. After 1 hour-washing with PBT embryos were passed to small collection basket. Almost all PBT was removed and replaced by 200 µl of 0.5 mg/ml of diaminobenzidene solution (DAB) in PBS pH 7.4 for 3 min. Protein expression was detected through oxidation of diaminobenzidine using 0.01% of peroxidase. This gave an orange-brown color. Adding ions of nickel Ni^{2+} to the DAB prior to peroxidase reaction gave dark-blue color. That helped to distinguish between two patterns of distinct proteins. Embryos were then dehydrated through a graded series of ethanol, 1 time for 10 min in 70 % ethanol, 2 times for 10 min in 100% ethanol, washed 2 times for 10 min in acetone and finally mounted in Aceton/Epon. HRP immunochemistry was visualised using Nomarski Optics on a Zeiss Axio Imager Microscope.

Images of ventral view embryos were taken with 63×/1.25 oil objective and images of lateral view embryos were taken with 40×/1.3 oil objective. Images were assembled using Adobe Photoshop CS3 programme.

2.7.3 IMMUNOFLUORSCENCE

The following primary antibodies were used: rabbit anti-β-glactosidase 1:1000 to 1:10000 (*Cappel*), mouse anti- β-glactosidase 1:1000 to 1:8000 (*Promega*), rabbit anti-Baz (Wodarz et al. 1999) 1:1000, guinea-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-Cno 1:400 (Speicher et al. 2008), rabbit anti-PKCζ 1:100 (C-20, *Santa Cruz*), rabbit anti-Scrib 1:4000 (a gift from Chris Doe), rabbit anti-Eve 1:3000 (Frasch et al. 1987), guinea-pig anti-Eve 1:200 (Kosman et al. 1998), rabbit anti-LATS1/2 (*Assay Biotech*) 1:20 to 1:100, rat anti-L´sc 1:800 to 1:2000 (Martin-Bermudo et al. 1991), Mouse anti-Pros (*Hybridoma bank*) 1:50, mouse anti-Wg 1:50 (Hybridoma bank), rabbit anti-Twist 1:1000 (a gift from Maria Leptin), rabbit anti-Zfh1 1:2500 (Lai et al. 1991), mouse anti-Eve 1:25 (2B8, (Patel et al. 1994), guinea-pig anti-Runt 1:400 (Kosman et al. 1998), rabbit anti-Insc (1:250) (a gift from (Kraut and Campos-Ortega 1996), rabbit anti-Mira 1:2000, rat anti-Yki69 1:100 (N. Tapon), rat anti-Hpo66 1:100 (N. Tapon), rabbit anti-Pins 1:200 (Parmentier et al. 2000), mouse anti-Nrt 1:100 (Speicher et al. 1998)

Fluorescent images were recorded by using an upright microscope (DM-SL with Spectral Confocal acquisition software; Leica). All images were taken with an HCX Plan Apochromat 63°—/1.32-0.6 NA oil confocal scanning objective. Images were assembled using Photoshop CS3.

2.8 DROSOPHILA STRAINS AND GENETICS

2.8.1 STOCKS

The following mutant stocks and fly lines were used for current work: *wts*^{x1} (Xu et al. 1995), *yki*^{B5} (a gift from María Dominguez), *mats*^{e03077}(BSC), *ft*$^{G\text{-}rv}$ (BSC), *ex*^{e1} (BSC), *cno*^{2} (BSC), *hpo*^{KS240} (BSC), *hpo*^{KC202} (BSC), *sav*^{3} (a gift from Y. N. Jan), *Mer*^{4} (BSC), *R*^{PS709} (a gift from Nick Brown), *hsFlp* (a gift from María Dominguez), *ΔGαi*^{KG01907} (BSC), *aPKC*^{K06403} (BSC), *insc*^{P49} (BSC), *pins*^{Δ50} (BSC), *scrib*^{1} (BSC), *ex*^{1} (BSC), *baz*^{4} (Schaefer et.al 2000), *l(2)gl4* (BSC), *pros*^{17} (BSC), *aur*$^{87Ac\text{-}3}$ (BSC), *Df(3R)*^{Exel8194} (BSC), mud^{4} (BSC)

2.8.2 GAL4-UAS SYSTEM

Ectopic expression was achieved with the GAL4-UAS system (Brand and Perrimon 1993) and the following fly lines were used: *maternal*-GAL4 V32 (a gift from Juergen Knoblich) and *twist-GAL4, dmef2-GAL4* (mesodermal expression), UAS-yki::GFP (BSC), UAS-yki-S168A::GFP (BSC), UAS-myc::wts (a gift from K. Irvine), UAS-cno::GFP (Slováková and Carmena, 2011), UAS-Insc::GFP (a gift from Y. Akiyama-Oda), UAS-Baz::GFP (a gift from A. Wodarz). All the crosses GAL4-UAS were carried out at 29°C. *yellow white* (*yw*) strain was used as the reference control wild-type strain. Balancer chromosomes containing different lacZ or GFP transgenes were used for identification of homozygous mutant embryos (absence of β-gal or GFP expression).

2.8.3 GENERATION OF THE GERMLINE CLONES

yw, hsFLP; *P{FRT (w hs)}82B, wts*^{x1}*/TM6B Tb* females were crossed with *w; P{ovo D1-18}3L P{FRT(w hs)}82B/TM3, Sb* males. Mitotic recombination was induced in 24–48-h larvae for 2h at 37°C. Virgins from this cross were mated with *yw; FRT82B wts*^{x1}*/TM6B, Tb* males, and the embryos (without maternal and zygotic Wts products) were used for the phenotypic analysis.

CHAPTER 3

OBJECTIVES

1. To analyze functional interactions between Cno, Wts and other components of the Hippo signalling pathway during the process of ACD
2. To analyze in detail a potential function of the Hippo/Wts pathway in ACD using as a model system *Drosophila* asymmetric NB division
3. To investigate the mechanism underlying Wts function in asymmetric NB division
4. To analyze whether the Hippo/Wts pathway function in ACD is conserved in other tissues

UNIVERSITAS
Miguel
Hernández

CHAPTER 4

RESULTS

4.1 BACKGROUND

4.1.1 WTS IS A POTENTIAL PARTNER OF CNO

Canoe was identified in our lab as a key regulator of asymmetric NB division (Speicher et al. 2008). Given that Cno is a PDZ domain-containing protein that has other protein-protein interaction domains (see Chapter 1, Introduction, part 1.2, Figure 11) it was interesting to identify other Cno-interacting partners during the process of asymmetric cell division (ACD). Recently our group demonstrated that the small Rap1 GTPase, an evolutionarily conserved Cno partner that bind the Cno RA domain (see introduction), contributes to regulate the ACD of the embryonic NBs (Carmena et al. 2011). Performing a yeast-two hybrid screening (see Chapter 2 Materials and Methods part 2.2), we also identified the kinase called Warts (Wts) as a possible Cno-interacting partner, with a binding consensus site for the PDZ domain of Cno. Given that Wts plays a role in organ growth and proliferation processes in a context of the Hippo tumor suppressor signalling pathway (See Chapter 1, part 1.3) and that its human counterpart LATS 1/2 has been linked to the cell cycle regulation (See Chapter 1, part 1.3.4) we were very interested in analyzing whether Wts had a functional interaction with Cno in ACD.

Our initial approach to analyze the functional interactions between Cno and Wts was to analyze the asymmetric division of the neural precursor GMC-1, whose lineage, the "RP2 lineage", is very well studied (Figure 15). The RP2 lineage is characterized by the expression of the specific neuronal marker Even-skipped (Eve) (Isshiki et al. 2001). At stage 11 NB divides asymmetrically giving rise to an identical NB and the GMC-1, in which Eve starts to be expressed. By stage 12, GMC-1 divides to give rise to two neurons, called RP2 and RP2 sibling (RP2sib), and Eve is still expressed in both of them. By late stage 16, only the RP2 neuron maintains this Eve expression, whereas in the RP2sib neuron the expression of Eve disappears. So by late stage 16 only one RP2 neuron appears per hemisegment expressing Eve in the embryonic CNS (Figure 15). Defects observed in the number of RP2 neurons at this stage indicate that the ACD of the NB and/or GMC failed at earlier stages.

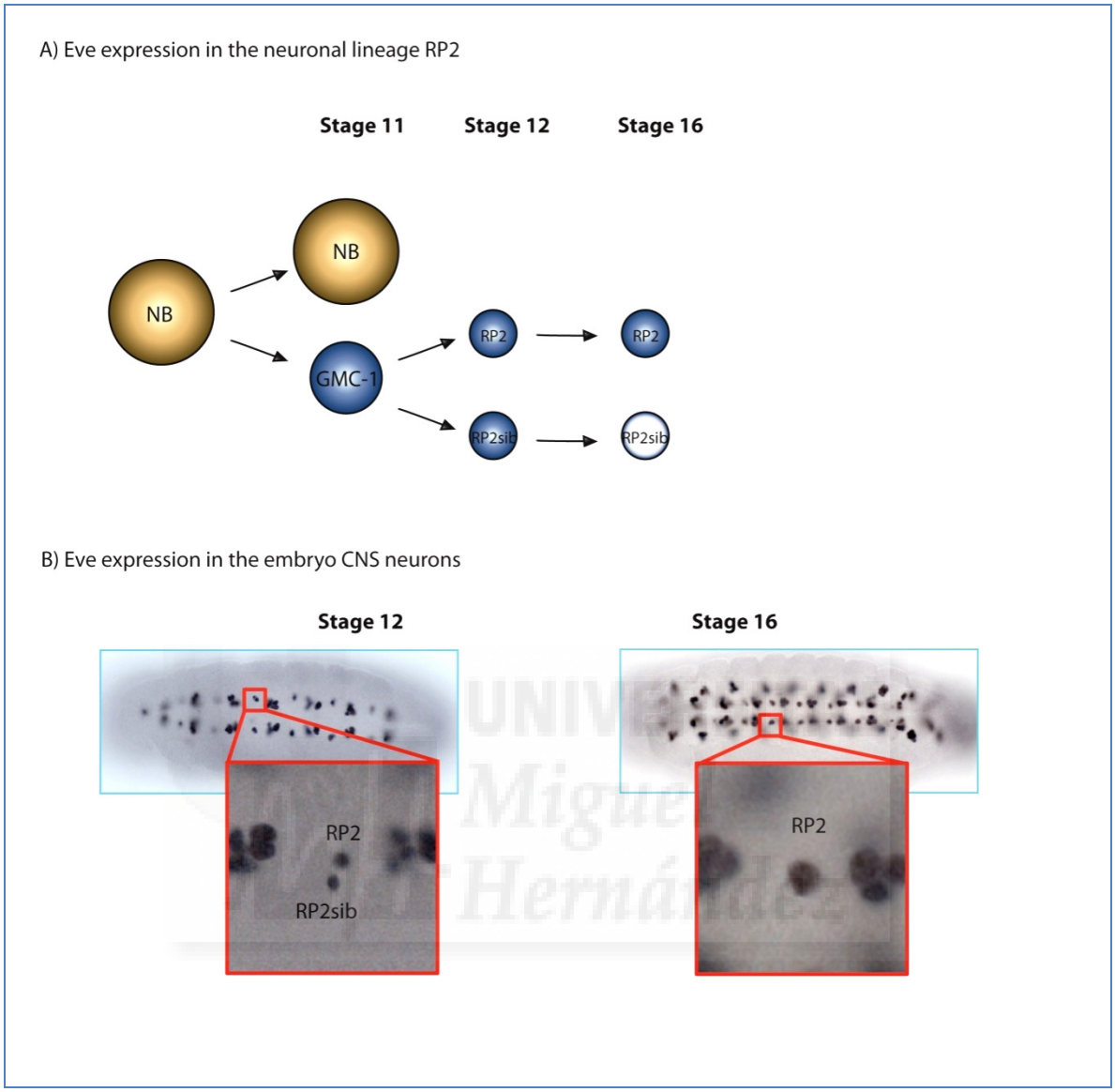


Figure 15. RP2 neuronal lineage A) Eve appears in the ganglion mother cell type 1 (GMC-1) and maintains its expression after GMC-1 division in neuron RP2 and RP2 sibling (RP2sib). By later stage 16 only RP2 neuron keeps the Eve expression**. B)** Ventral view of *Drosophila* embryo CNS is represented. In higher magnification of one hemisegment stage 12 (where both RP2 and RP2sib neurons express Eve), and stage 16 (here only the RP2 neuron maintains Eve expression) are illustrated. NB (neuroblast).

4.2 RESULTS

4.2.1 WTS FUNCTIONALLY INTERACTS WITH CNO DURING ACD

To analyze functional interactions between Cno and Wts and other components of the Hippo signalling pathway during the process of ACD, we analyzed the RP2 neuronal lineage in *cno*, *wts* double heterozygotes, a sensitized genetic background that

indicates whether two genes participate in the same biological process. We observed clear defects in the number of RP2/RP2sib neurons generated (Figure 16 B).

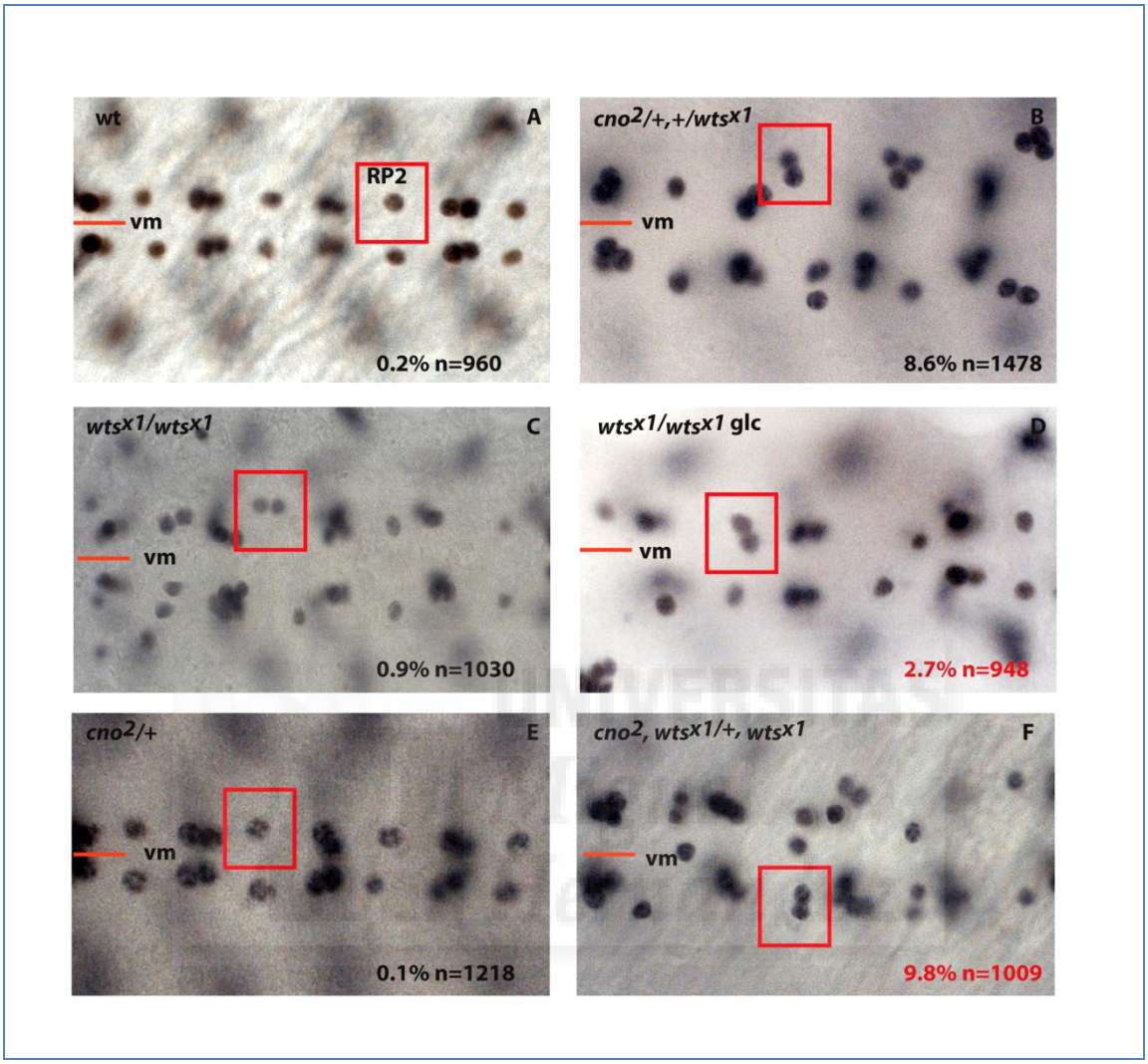
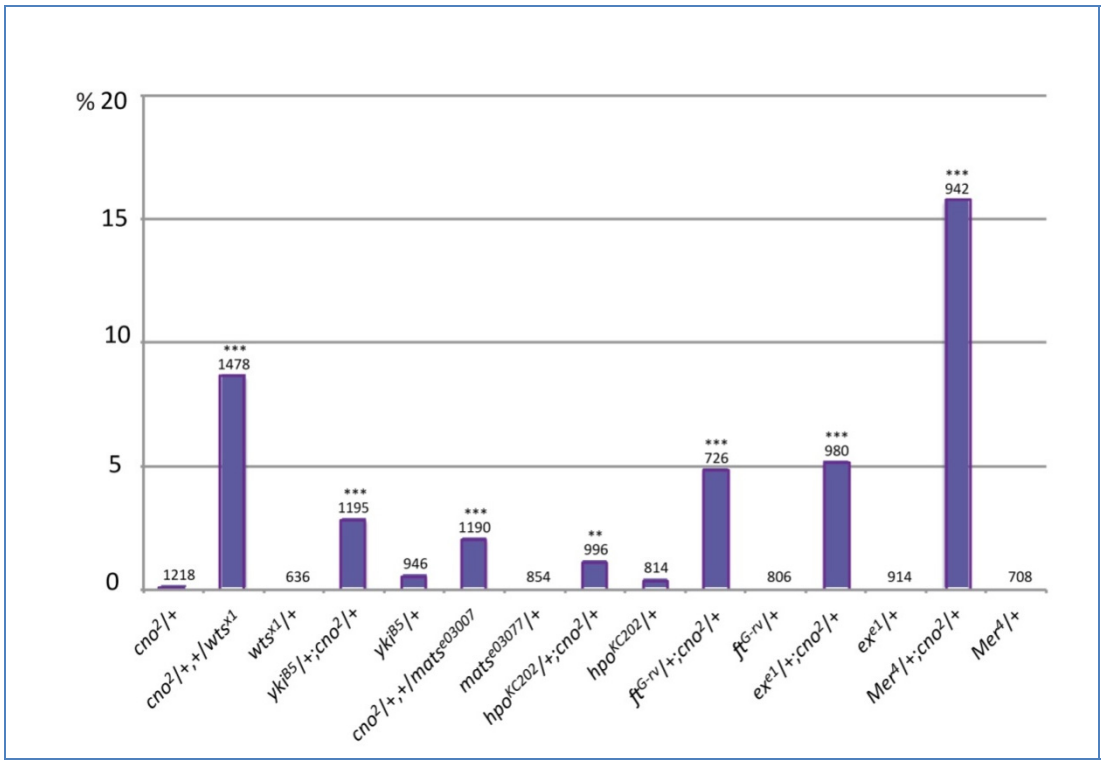


Figure 16. RP2 analysis A) Ventral view of the wildtype (wt) CNS stained with anti-Eve antibody, where one RP2 neuron is present per one hemisegment (red square). The percentage of defects in numbers of RP2 is shown in the left corner of the image, where **n** is total number of the hemisegments analyzed **B)** cno^{2},+/+, wts^{x1} double heterozygotes show the defect RP2 phenotype in 8.6% of the hemisegments analyzed. **C)** $wts^{x1/}$ wts^{x1} zygotic loss-of-function mutant shows 0.9% of the RP2 phenotype. **D)** The RP2 phenotype is increased in wts^{x1}/ wts^{x1} germline clone (glc) mutants three-fold. **E)** cno^{2}/+ heterozygote shows wildtype RP2 phenotype, when combined with wts^{x1x}/wts^{x1} zygotic mutant **C)** the phenotype is increased 10 times **F)**. vm= ventral midline

Hence, this result strongly suggested a functional interaction between Cno and Wts during the process of ACD. Cno showed functional interaction with all components of the Hippo pathway (Graph 1). Hence, we wanted to analyze next whether the Hippo pathway itself had a function in ACD.

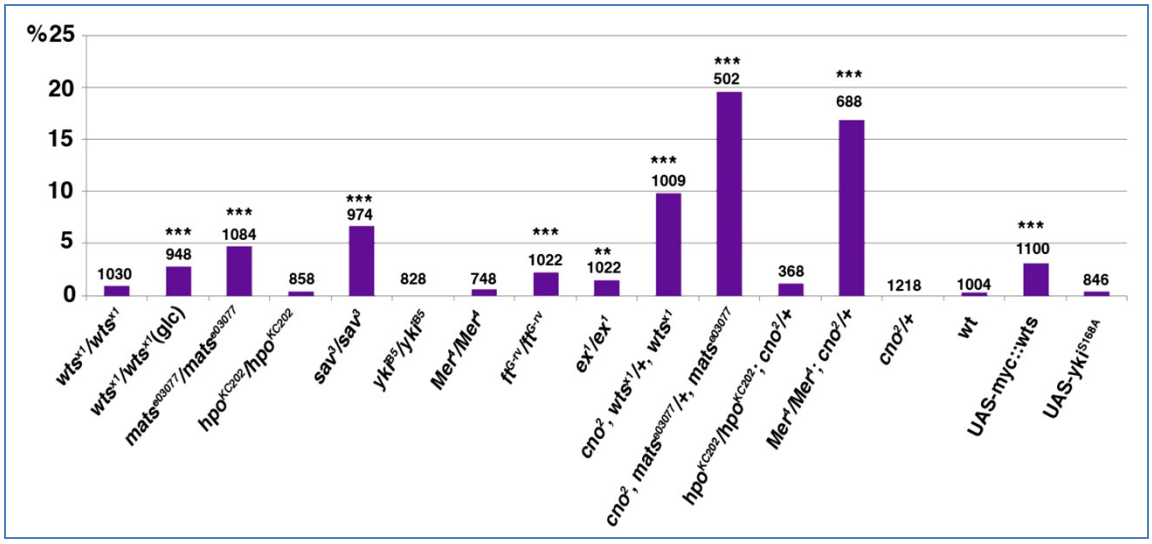


Graph 1 RP2 analysis of the double heterozygotes of Cno and different Hippo pathway components. *cno*^{2}/+ and *wts*^{x1}/+ heterozygotes separately don't show any phenotype in numbers of RP2 neurons, but when combined to each other the phenotype is 8.6%, which indicates that two genes are involved in the regulation of asymmetric cell division. Similar results were obtained with double heterozygotes of *cno* and other components of the Hippo signalling, such as *yki, mats*, *hpo*, *ft*, *ex*, and *mer*. Y scale represents the percentage of the hemisegments with defect RP2 phenotype of all hemisegments analyzed (numbers above the graphic). X scale represents the genotype of the analyzed embryos. Stars indicate that the phenotype is significant comparing to the sum of two heterozygote phenotypes (according to the Fisher´s test *** P<0.001, **P<0.01)

4.2.2 THE HIPPO SIGNALLING PATHWAY FUNCTIONS IN ACD

To analyze whether Wts and other Hippo pathway components have a function in ACD, we used the same RP2 neuronal lineage. Loss-of- function of all components of the Hippo pathway showed specific phenotypes (i.e loss or gain of RP2/RP2sib neurons) (Graph 2). However, even though the phenotype was very specific, the percentage of defects was relatively low (Figure 16 C). One of the reasons to explain this low percentage of the phenotype is the presence in the mutant embryos of what is called maternal contribution: very early in the oogenesis the mother provides some mRNAs of the gene in the oocyte, which can rescue the zygotic mutant phenotypes in the embryo. Thus, to remove the maternal contribution, we generated germ line clones (glc) for the *wts*^{x1} gene (See Chapter 2, part 2.8.3), thus removing both the zygotic and

the maternal product.



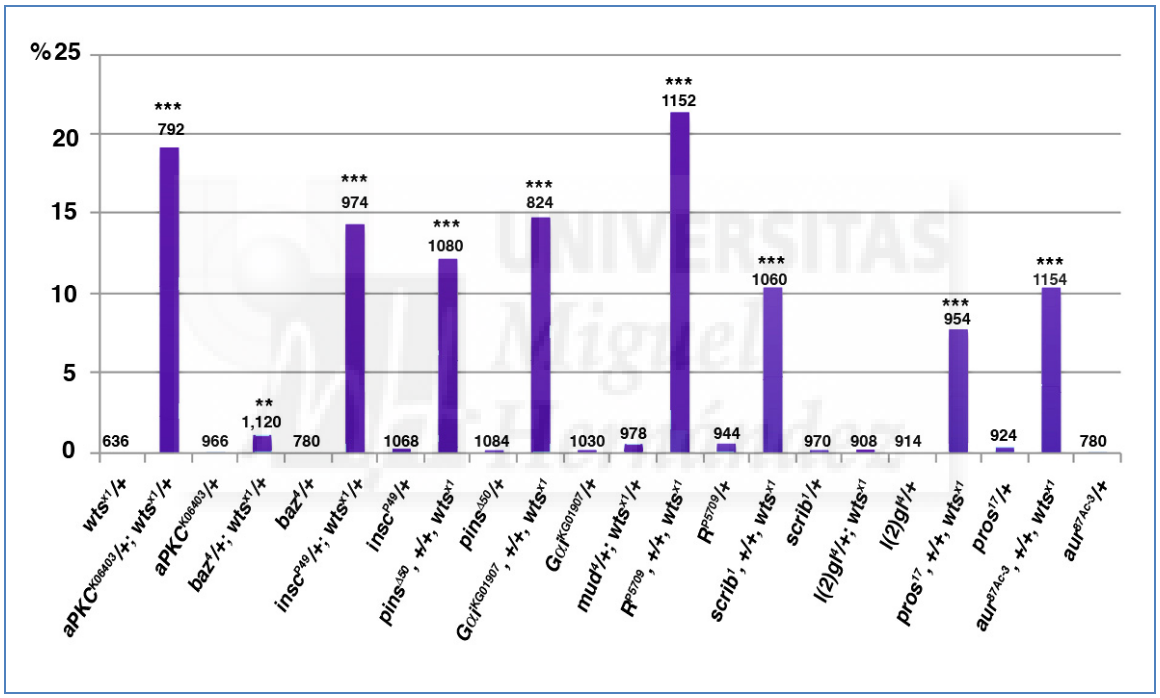
Graph 2 RP2 analysis of the Hippo pathway loss-of-function mutants. Loss-of-function mutants for *wts, mats, hpo, sav, ft* and ex show significant (***) phenotype of RP2 neurons. The phenotype of *mats*, *mer* and *wts* homozygote mutants is significantly increased in cno^2/+ heterozygous background. Gain-of-function mutant for *wts* (UAS-myc::wts) also show phenotypic defects in number of RP2 neurons. Y scale represents the percentage of the hemisegments with defect RP2 phenotype of all hemisegments analyzed (numbers above the graphic). X scale represents the genotype of the analyzed embryos. Stars indicate that the phenotype is significant comparing to the wildtype (wt) phenotype (according to the Fisher´s test *** P<0.001, **P<0.01)

In wts^{x1}/wts^{x1} glc mutants the phenotype increased three times (Figure 16 D, Graph 2).

The second reason for the low percentage of the phenotype is the high redundancy: multiple proteins contribute to the regulation of ACD and if only one component is removed, others can still compensate partially the loss of that regulator. Indeed, when we analyzed *wts, mats,* and *Mer* loss-of-function homozygous mutants in a sensitized genetic background, i.e. removing half of the dosage of *cno*, the phenotype was significantly increased (Figure 16 F, Graph 2). These results strongly suggested that the Hippo signalling pathway is involved in regulating the process of asymmetric NB division. Moreover, using the same RP2 analysis of double heterozygotes, we found that Wts is functionally interacting with multiple specific regulators of ACD (Graph 3).

To further support this observation, we decided to analyze in much more detail the role of the Wts kinase in asymmetric NB division, at earlier embryonic stages, when NBs start dividing asymmetrically.

To analyze in detail the function of Wts in the asymmetric division of embryonic NBs, we studied the orientation of the mitotic spindle along the apico-basal axis of cell polarity and the asymmetric localization of cell-fate determinants, two important features for the correct asymmetric NB division. Both processes are tightly regulated by a protein complex located at the apical pole of the NB. The apical complex consists of many proteins including the evolutionarily conserved Par polarity complex: Par6, Par3/Bazooka and the atypical protein kinase C (aPKC). The Par complex is linked by an adaptor-protein called Inscuteable (Insc) to the Partner of Insc (Pins) complex that regulates the mitotic spindle orientation (see Chapter 1, part 1.1.4.3.1).



Graph 3 RP2 analysis of the double heterozygotes for Wts and the asymmetric cell division regulating genes

aPKC^{K06403}/+ and *wts*^{x1}/+ heterozygotes separately don't show any phenotype in numbers of RP2 neurons, but when combined to each other, the RP2 phenotype is 19% which indicates that two genes are functionally interacting to each other during the process of asymmetric cell division. Double heterozygotes for *wts* and *baz, insc, pins, Gαi, rap1 (R), scrib, pros* and, *aur* showed RP2 mutant phenotypes. Y scale represents the percentage of the hemisegments with defect RP2 phenotype of all hemisegments analyzed (numbers above the graphic). X scale represents the genotype of the analyzed embryos. Stars indicate that the phenotype is significant comparing to the sum of two heterozygote phenotypes (according to the Fisher´s test *** P<0.001, **P<0.01)

Cell-fate dereminants that are regulated by the apical protein complex are Numb with its effector Pon, Prospero with its cargo Miranda and the translation regulator Brat at the basal membrane of the NB (see Chapter 1, part 1.1.4.3.2).

Thus, we analyzed in detail the localization of different apical proteins and basal cell-fate determinants in wts^{x1}/wts^{x1} glc null-mutants.

4.2.3.1 APICAL AND BASAL DETERMINANT LOCALIZATION IS IMAPIRED IN *WTS*^{x1} MUTANT NBS

The correct localization of the aPKC, Baz, Cno, Pros and Numb was very altered in wts^{x1}/wts^{x1} glc mutant NBs comparing to the wildtype (WT) NBs. The localization of Insc, Pins and Mira was less altered, and the Gαi apical localization was not affected (Table 6).

In dividing wt NBs, aPKC forms an apical crescent and Numb localizes at the basal membrane at metaphase (Figure 17 A, A′, A′′). Numb shows failures in its localization in 27.6% of the NB analyzed (n=76) and aPKC shows defects in 26.3% of the cases (n=76). Failures in the correct determinant localization or complete absence of the determinant are observed. Numb localization is failing and the aPKC localization is normal in 10.5% of the NBs analyzed (n=76) (Figure 17 B). Numb is normally distributed and aPKC fails to localize apically in 7.9% of the NBs analyzed (n=76) (Figure 17 D). Both Numb and aPKC show defects in their localization in 18.4% of the cases (n=76) (Figure 17 C).

Next pair of ACD regulating proteins that we analyzed was the apical polarity protein Bazooka (Baz) and the cell-fate determinant Prospero (Pros). In the wt dividing NB Baz forms the apical crescent, while Pros forms the cortical basal crescent (Figure 18 A, A′, A′′). In wts^{x1} glc null-mutant NBs, Pros fails to localize basally in 58% of the NBs analyzed (n=91): 43% of the NBs show cytoplasmic localization of Pros (Figure 18 B, B) and in 15% Pros is absent (Figure 18 C, C′). In the case of Baz localization, 37% of NBs show failures: Baz is absent in 15.5% and cortically mislocalized in 21.5% of all NBs analyzed (n=91) (Figure 18C′′). The localization of Baz and Pros failed simultaneously in 36.3% of the mutant NBs (n=91) (Figure 18 C).

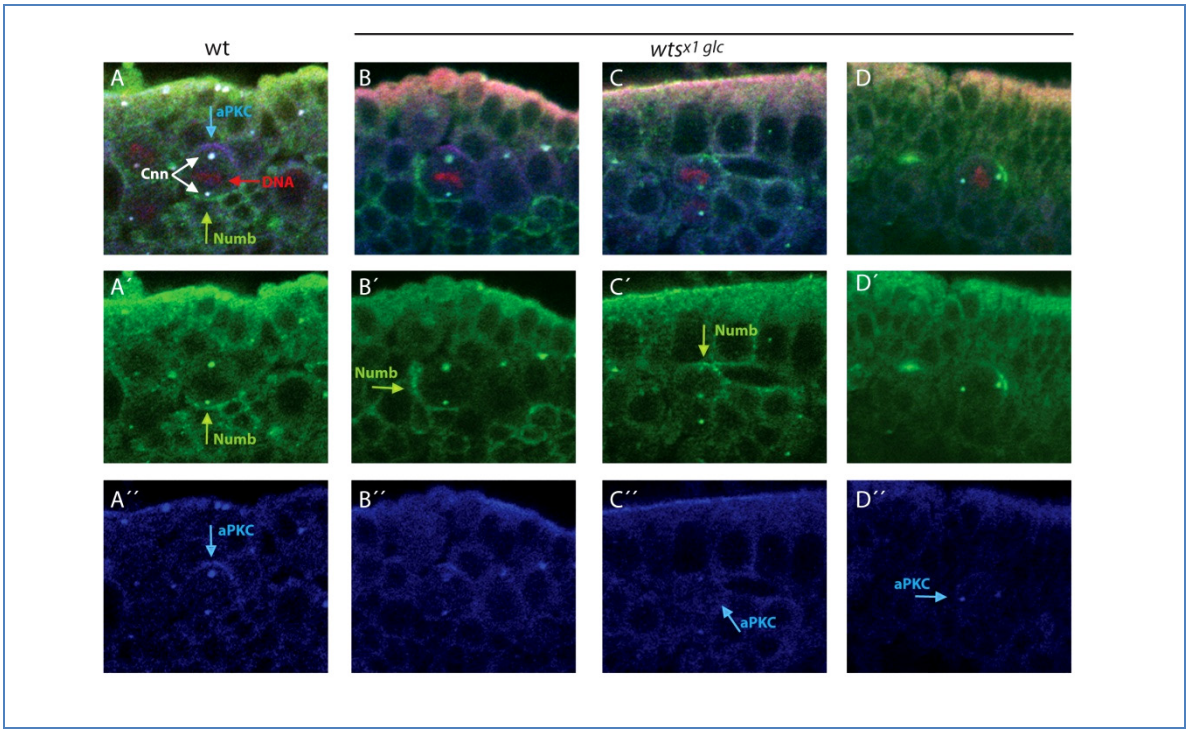


Figure 17. aPKC and Numb localization analysis in *wts*^{x1} glc mutant NBs at the metaphase.

A) aPKC is forming the apical crescent and Numb is forming the basal cresent in the wildtype (wt) NBs at the metaphase stage. Centrosomine (Cnn) labels centrosomes in white, DNA is shown in red. Numb is shown in green **(A´)** and aPKC is shown in blue **(A´´). B)** In *wts*^{x1} glc mutant NBs Numb is misocalized and aPKC is still forming the apical crescent. **B´)** Numb is laterally localized, where aPKC is found in apical crescent **(B´´). C)** Both aPKC and Numb show defect localization. **C´´)** Numb is found apically and aPKC is cytoplasmic **(C'')**. **D)** Numb is forming a crescent and aPKC is absent from the crescent. **D´´)** aPKC is abscent from the crescent, whereas Numb is forming the crescent **(D')**

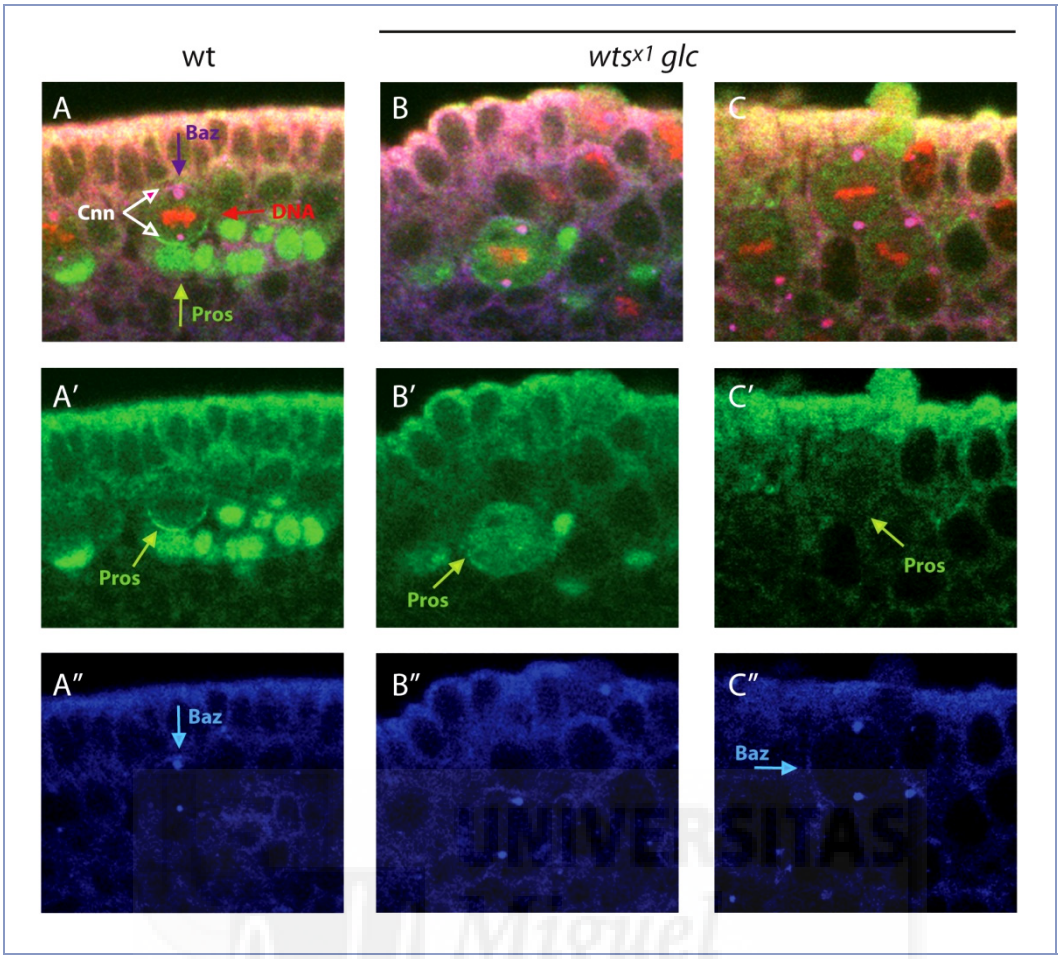


Figure 18. Baz and Pros localization analysis in *wts*^{x1} glc mutant NBs at the metaphase.

A) Baz is forming the apical crescent and Pros is forming the basal cresent in the wildtype (wt) NBs at the metaphase stage. Centrosomine (Cnn) labels centrosomes (magenta), PH3 labels the DNA (red) during division. Pros is shown in green **(A´)** and Baz is shown in blue **(A´´). B)** Pros shows the cytoplacmic localization and Baz is normally disrtributed in *wts*^{x1} glc mutant NBs. **B´)** Pros is cytoplasmically localized and Baz is still forming the apical crescent **(B´´). C)** Both Pros and Baz show defects in the localization in *wts*^{x1} glc mutant dividing NB. **C´)** Pros is absent from the crescent, and Baz is mislocalized laterally **(C'')**.

Given that Cno functionally interacted with Wts and came out from the yeast-two hybrid screening as a potential partner of Wts, we wondered whether the localization of Cno was also altered in *wts*^{x1} glc null-mutant NBs. Normally, Cno is forming the apical rescent in wt NBs (A, A´). In fact, in 24% of all mutant NB analyzed (n=108) Cno was cytoplasmic or absent (Figure 19 E, E). Other apical proteins, such as Insc and Pins as well as the cell-fate determinant Miranda (Mira) were also analyzed in *wts*^{x1} glc null-mutant NBs (Figure 19 B, C, D). Whereas Insc is forming an apical crescent in wt NBs, some cases of basal localization of Insc are detected in the mutants in 6.5% of all NB

analyzed (n=77) (Figure 19 F, F´.

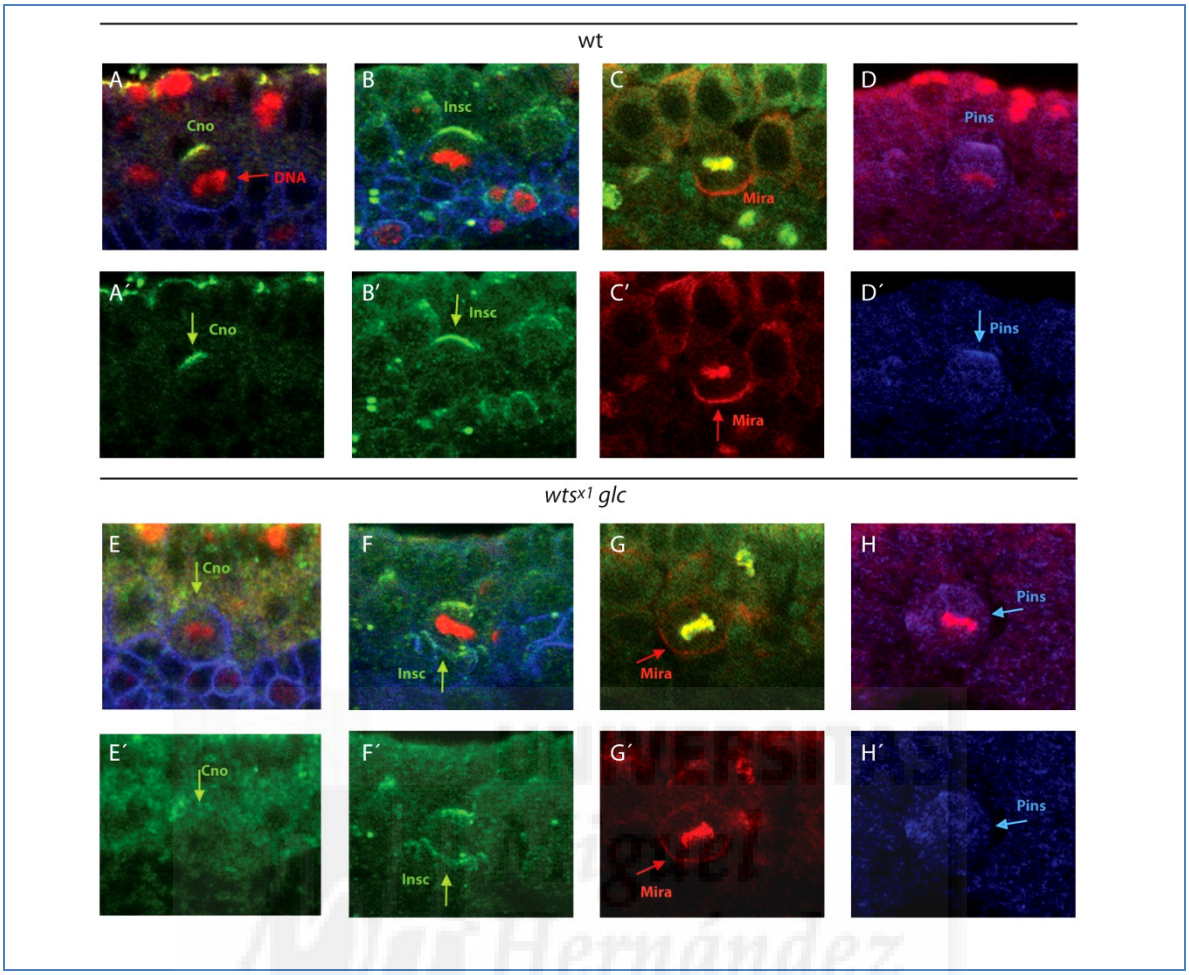


Figure 19. Cno, Insc, Mira, and Pins analysis in *wts*^{x1} glc mutant NBs at the metaphase.

A) Cno is forming an apical crescent in the wildtype (wt) NBs at the metaphase stage. DNA (red) is labeled by PH3 and the membrane is labeled by Nrt. Cno is showed in green **(A')**. **E)** Cno is absent from the apical crescent in *wts*^{x1} glc mutant NBs. **B)** Insc (green, **B´**) is always found in the apical crescent at the mitotic stage of the wt NBs. **F)** Basal localization of the Insc is found in *wts*^{x1} glc mutant NBs. **C)** Mira (red, **C´**) is basally localized in wt NBs, whereas in *wts*^{x1} glc mutant NBs **(G)** Mira is uniformly distributed along the cortex. **D)** In wt NBs Pins (blue, **D´´**) localizes apically, and in *wts*^{x1} glc mutant NBs **(H)** Pins shows the cytoplasmic localization.

Mira, which is forming a basal crescent in mitotic wt NBs, in *wts*^{x1} glc null-mutant NBs the fails to localize basally in 6.5% of NBs analyzed (n= 46) (Figure 19 G, G´). In 5.12 % of mutant NBs Pins was cytoplasmically distirbuted, comparing to its normal apical localization in the wt NBs (n=78) (Figure 19 H). We classified the phenotypes that we observed in to the three major groups: when the protein is abscent or less expressed, when the protein is mislocalized, and when it is cytoplasmic. See also Table 6 for a

summary of the observed phenotypes, and Table 7 for the summary of the detailed phenotypes.

Table 6. Summary of ACD proteins distribution in *wts*^{x1} glc null-mutants.

ACD proteins	n=NBs in WT	WT %	n=NBs in *wts*^{x1}/*wts*^{x1} *glc*	*wts*^{x1}/*wts*^{x1} *glc* phenotype %
aPKC	66	3.85	76	**26.3**
Baz	67	6	91	**37**
G α i	49	0	66	0
Cno	57	1.75	108	**24**
Insc	74	0	77	6.5
Pins	60	0	78	5.1
Mira	42	0	46	6.5
Pros	67	10	91	**58**
Numb	66	0	76	**27.6**

Table 7. Characteristics of the phenotype of the ACD proteins in the *wts*^{x1} glc null-mutants.

Phenotype	aPKC	Baz	Cno	Insc	Pins	Mira	Numb	Pros
absent or less	55%	44%	88%	0%	0	33.3%	47.5%	19%
mislocalized	45%	56%	0	100%	0	33.3%	52.5%	7.5%
cytoplasmic	0	0	12%	0	100%	33.3%	0	73.5%

4.2.3.2 CELL FATE DETERMINANTS FAIL TO SEGREGATE CORRECTLY IN *WTS*^{X1} MUTANT NBS DURING THE TELOPHASE

There is a further step of regulation of ACD, a process called "telophase rescue", which allows to repair defects occurred during the metaphase in order to ensure a normal asymmetric division. Hence, we wondered whether the phenotypes observed in *wts*^{x1} mutant NBs at metaphase were rescued at telophase.

In wt embryos, Pros is forming a basal crescent in telophase NBs (Figure 20 A, A´). Thus, it only segregates into the smaller ganglion mother cell where Pros will enter the nucleus and will promote the transcription of differentiation genes. In *wts*^{x1} mutant NBs a partial telophase rescue was observed. Whereas in metaphase 58% of Pros defects were detected in mutant NBs (see above), now at telophase Pros failed in 20.6% of all NB analyzed (n=36) (Figure 20 B, B´). Numb forms a basal crescent in wt NB at telophase (Figure 20 C, C´). A similar partial telophase rescue was observed for Numb localization in *wts*^{x1} mutants: in metaphase 27.6% of defects were found in wts mutants (see above) and only 7.4% of mutant NBs analyzed at telophase showed failures of Numb localization (n=54) (Figure 20 D, D´).

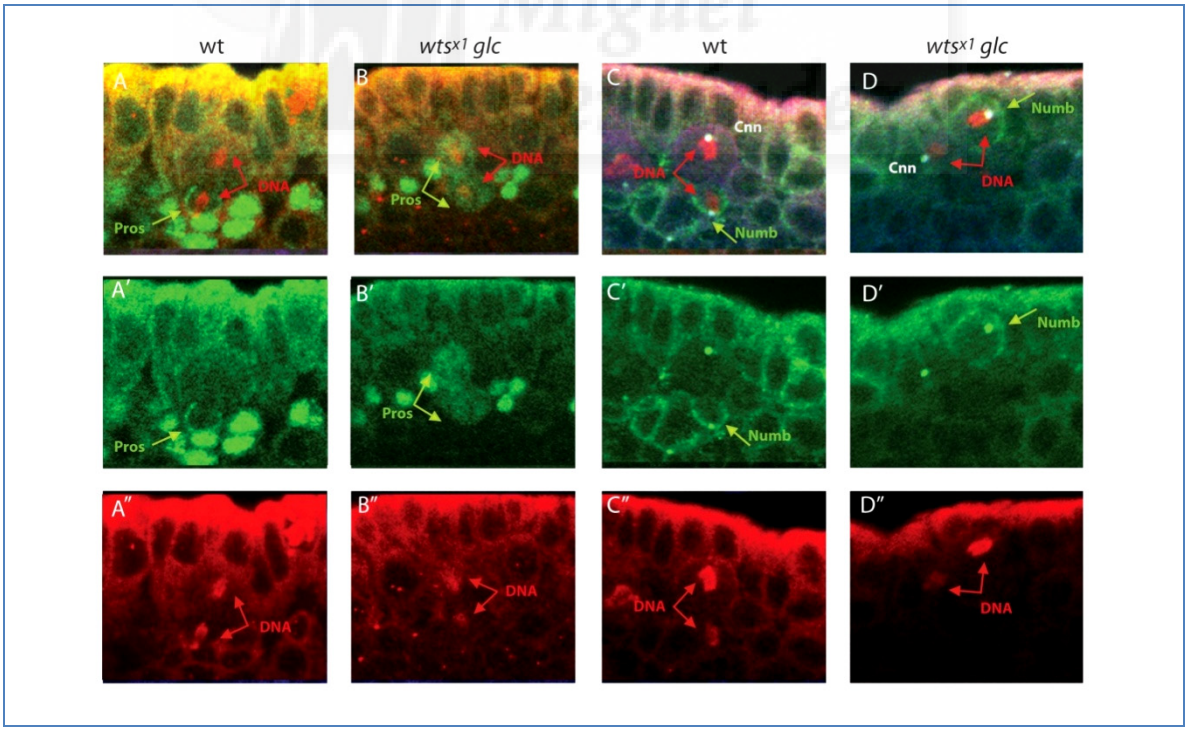


Figure 20. Pros and Numb analysis in *wts*^{x1} glc mutant NBs at the telophase. A) Pros is forming the basal crescent of the budding gmc. **B)** Pros is cytoplasmically localized in both NB and gmc Pros is shown in green (**A´, B´**) and DNA in red (**A´´, B´**). **C)** Numb is segregating into the budding gmc during the telophase. **D)** Numb is apically localized in the dividing NB at the telophase. Numb is shown in green

(**C´, D´**), DNA in red (**C´´, D´´**), Cnn-centrosomine.

Another characteristic feature of asymmetric NB division is the generation of unequal-sized daughter cells: a larger NB and a smaller GMC (Figure 20 A). In *wts*^{x1} loss-of-function mutants equal-sized daughter cells appear in 18.2% of the telophase NBs analyzed (n=88) (Figure 20 B).

4.2.3.3 MITOTIC SPINDLE ORIENTATION FAILS IN *WTS*^{X1} MUTANT NBS

As mentioned before, the localization of the mitotic spindle along the apico-basal cell polarity axis is a fundamental requirement for an ACD. We analyzed the spindle position in the *wts*^{x1} glc null-mutant NBs at metaphase, using Cnn as a centrosome marker that allows determining the spindle position and PH3 to determine the metaphase stage. The spindle is located perpendicularly to the neuroectoderm (NE) and any fluctuations in the mitotic spindle orientation can lead to defects in the ACD (Figure 21 A, B). We measured the angle between the mutant spindle position and the normal spindle position (Figure 21 A, B). In *wts*^{x1} glc mutants clear defects in spindle position are observed in 40% of metaphase NBs analyzed (n=76) (see the diagram on the figure 21 A, C for the details).

Altogether this data indicate that the Ser/Thr kinase Wts is crucial to regulate the most characteristic features of an asymmetric NB division: the localization of apical proteins and cell-fate determinants, the correct orientation of the mitotic spindle orientation as well as the generation of unequal-sized daughter cells.

4.2.3.4 WTS IS EXPRESSED IN EMBRYONIC NBS

To understand the mechanism of action of the Wts kinase during the process of ACD we analyzed the expression of Wts and other Hippo pathway components in the embryonic NBs.

We started checking whether a commercial human anti-LATS1/2 antibody was able to recognize Drosophila Wts. For that, we stained *wt* embryos, *Df (3R)* ^{Excel8194} embryos (a deficiency that eliminates *wts*), and embryos overexpressing Wts using UAS-*wts* under the *wingless (wg)*-Gal4 driver, which is expressed in a very characteristic stripe pattern (Figure 22 A, B, C). These experiments showed the specificity of the LATS1/2 antibody. Then, we labeled early stage 9 wt embryos with Wts and specific markers of

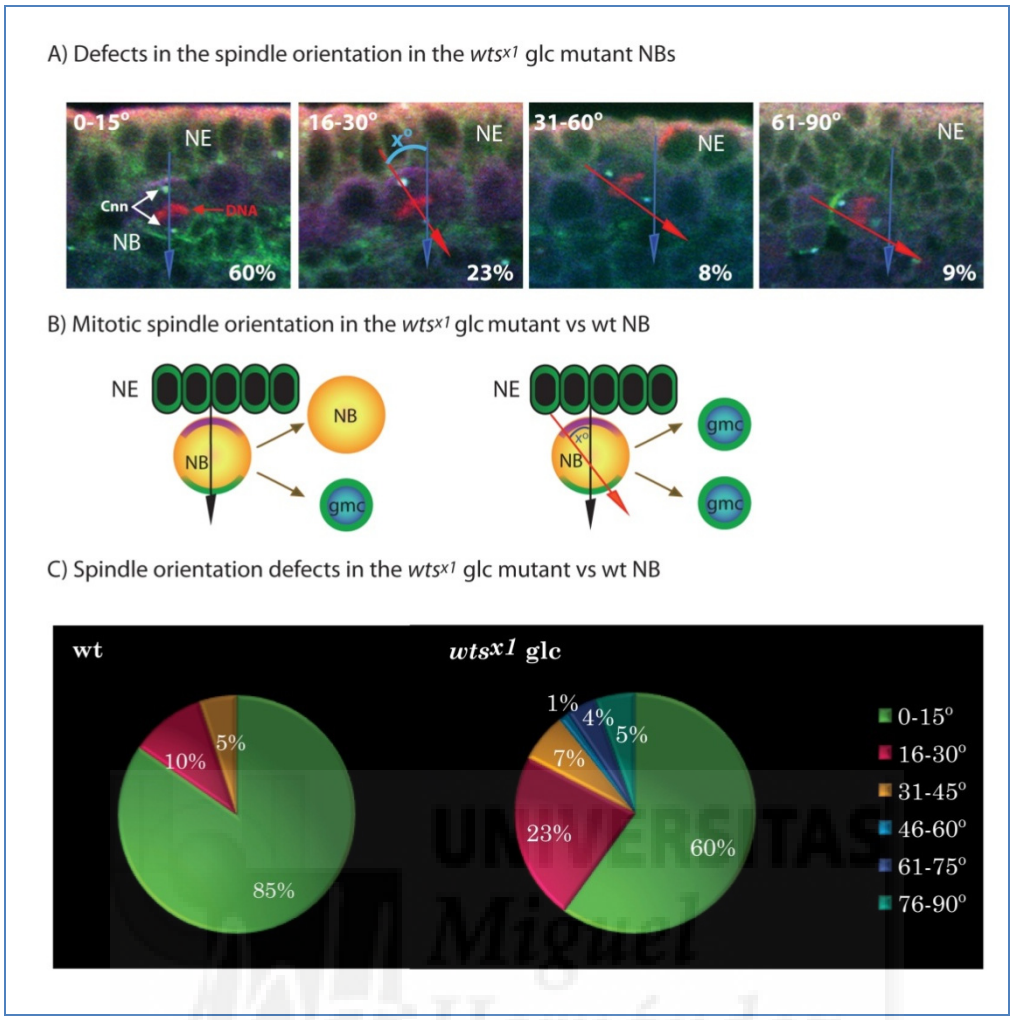


Figure 21. The mitotic spindle position analysis in the *wts*^{x1} glc mutant NBs

A) Defects in the spindle orientation in wts glc null-mutant NBs. In 60% of the cases the localization of the mitotic spindle is perpendicular to the NE. The angle between the normal spindle and abnormally localized spindle is between 16-30^{o} in 23% of the NBS analyzed. The angle between 31-60^{o} and 61-90^{o} was observed in 8% and in 9% of the NBS analyzed, respectively. **B) Mitotic spindle orientation in the mutant vs wt NB.** Correct spindle orientation results in the correct determinants segregation and normal ACD. The defects in the mitotic spindle orientation lead to the defects in the cell-fate determinants segregation and results in the incorrect ACD. **C) The spindle orientation analysis of the wt NBs and *wts*^{x1} glc mutant NBs.** The defect spindle orientation angle between 16-30^{0} and between 31-45^{o} is found in 10% and 5% of the all wt NBs analyzed, respectively. Totally 40% of the mutant NBs show the defects in the mitotic spindle orientation represented on the diagram. NE=neuroepithelium, NB=neuroblast, gmc=ganglion mother cell

NBs, including the proneural gene Lethal of Scute (L'sc), expressed in all NBs (Martin-Bermudo M, 1991 Dev), and the secretable protein Wingless/Wnt (Wg) expressed in a subset of NBs (Chu-LaGraff and Doe 1993). We observed the expression of Wts in most of the NBs of early stage 9 embryos (Figure 22 D, D´).

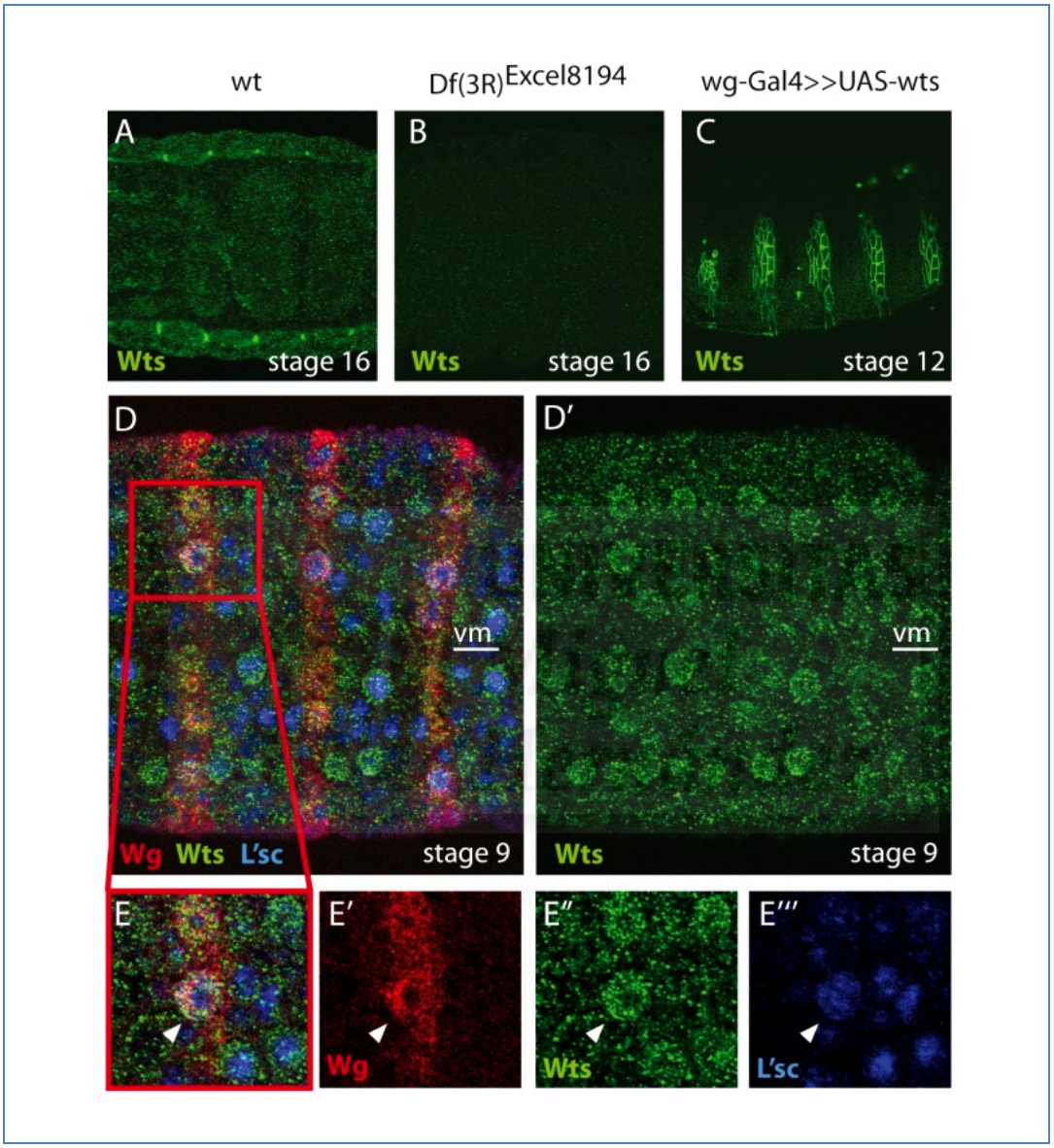


Figure 22. Wts expression in the NBs A) Late stage 16 embryo ventral view with the human anti-LATS1/2 antibody staining **B)** Loss-of-function of *wts* using the deficient allele *Df(3R)*^{Excel8194} at stage 16 labeled with human anti- LATS1/2 antibody **C)** Wts overexpression using *UAS-wts* and *wg-Gal4*, labeled with human anti-LATS1/2 antibody that shows its expression in wg-specific stripes at stage 12. **D)** Embryo ventral view at stage 9 stained with Wts (green), L´sc (blue), and Wg (red). **D´)** Wts expression in green single-channel. **E)** Magnification of one hemisegment stained with all three markers. **E´)** Cytoplasmic expression of Wg in the NB (red single-channel). **E´´)** Cytoplasmic expression of Wts in the NB (green single-channel). **E´´´)** Nuclear expression of L´sc in the NB (blue single-channel). vm= ventral midline

Wts showed an homogenous cytoplasmic expresión along with Wg in L´sc expressing NBs (Figure 22 E, E´, E´´, E´´´). We wondered whether the localization of Wts changed in the dividing NBs at the different mitotic stages (Figure 23). We observed that the localization of Wts is mainly homogeneous at the cytoplasm in all mitotic stages (Figure 23).

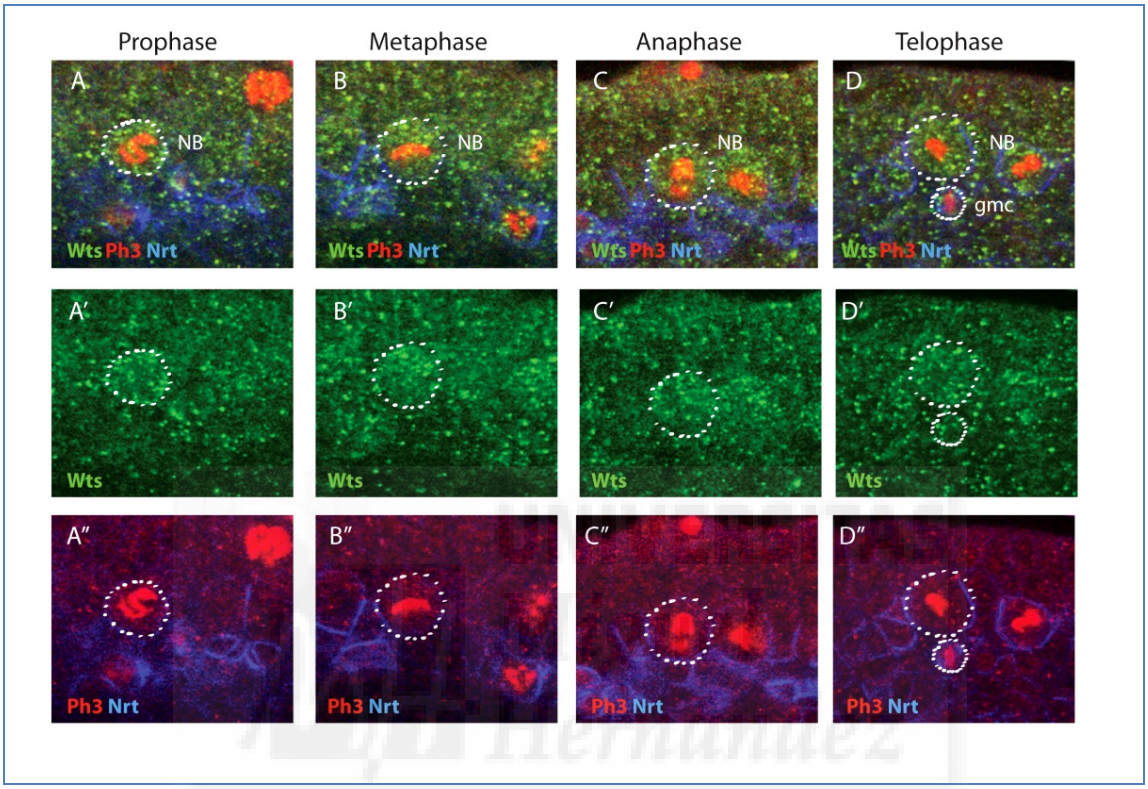


Figure 23. Wts expression in the NBs at the different mitotic stages A) Prophase stage of the NB Wts is homogeneously distributed in the cytoplasm. Wts (green, **A´**), Ph3 (red, **A´´**) and Nrt (blue, **A´´**). **B)** Wts is homogeneously expressed in the cytoplasm of the dividing NB at the metaphase. Wts **(B´),** Ph3 and Nrt **(B´´)**. **C)** Cytoplasmic localization of Wts during anaphase, Wts **(C´)** Nrt and Ph3 **(C´´)**. **D)** Cytoplasmic localization of Wts during the telophase. Wts **(D´)**, Ph3 and Nrt **(D´´)** NB=neuroblast, gmc=ganglion mother cell

Then, we checked whether other components of the Hippo pathway, such as co-transcriptional activator Yki and the Hpo kinase were also expressed in the dividing NBs (Figure 24).

The expression of Hpo and Yki, similarly to the Wts expression, was homogeneous in the cytoplasm of NBs at all mitotic stages (Figure 24).

We next wondered whether Wts was forming a complex with some apical or basal determinants required for ACD. To answer this question we performed co-

immunoprecipitation assays *in vivo* (see Chapter 2, part 2.5) from *Drosophila* embryo protein extracts.

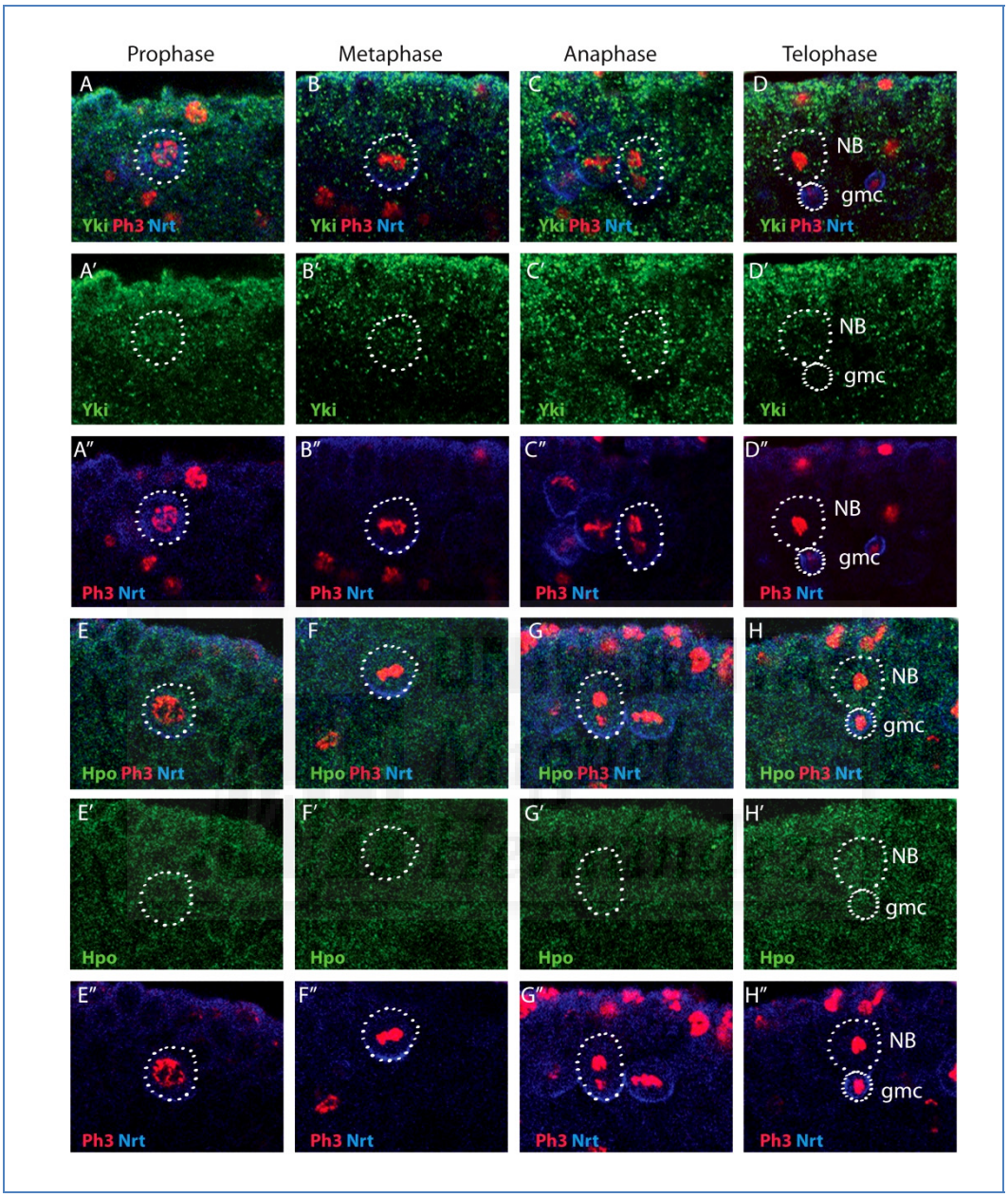


Figure 24. Hpo and Yki expression in the NBs at the different mitotic stages A, E) Prophase stage. **B, F)** Metaphase stage. **C, G)** Anaphase stage. **D, H)** Telophase stage. Yki is shown in green **(A′, B′, C′, D′)**, Ph3 and Nrt are shown in red and blue, respectively **(A′′, B′′, C′′, D′′, E′′, F′′, G′′, H′′)**. Hpo is shown in green **(E′, F′, G′, H′)**. NB=neuroblast, gmc=ganglion mother cell

4.2.3.5 WTS FORMS A COMPLEX IN VIVO WITH BAZ AND INSC

We overexpressed UAS-Insc::GFP and UAS-Wts::Myc in the embryos using the maternally expressing general driver V32-GAL4. The same experiment we performed with embryos overexpressing UAS-Baz::GFP and UAS-Wts::Myc. We immunoprecipitated Insc and Baz using an α-GFP antibody (Figure 25 C and D) and detected Wts::Myc from the immpunoprecipitation lysate using anti-α-Myc antibody (Figure 25 A, B). aPKC, Cno, Pins, Gαi, Rap1 and Pros did not co-immunoprecipitate with Wts.

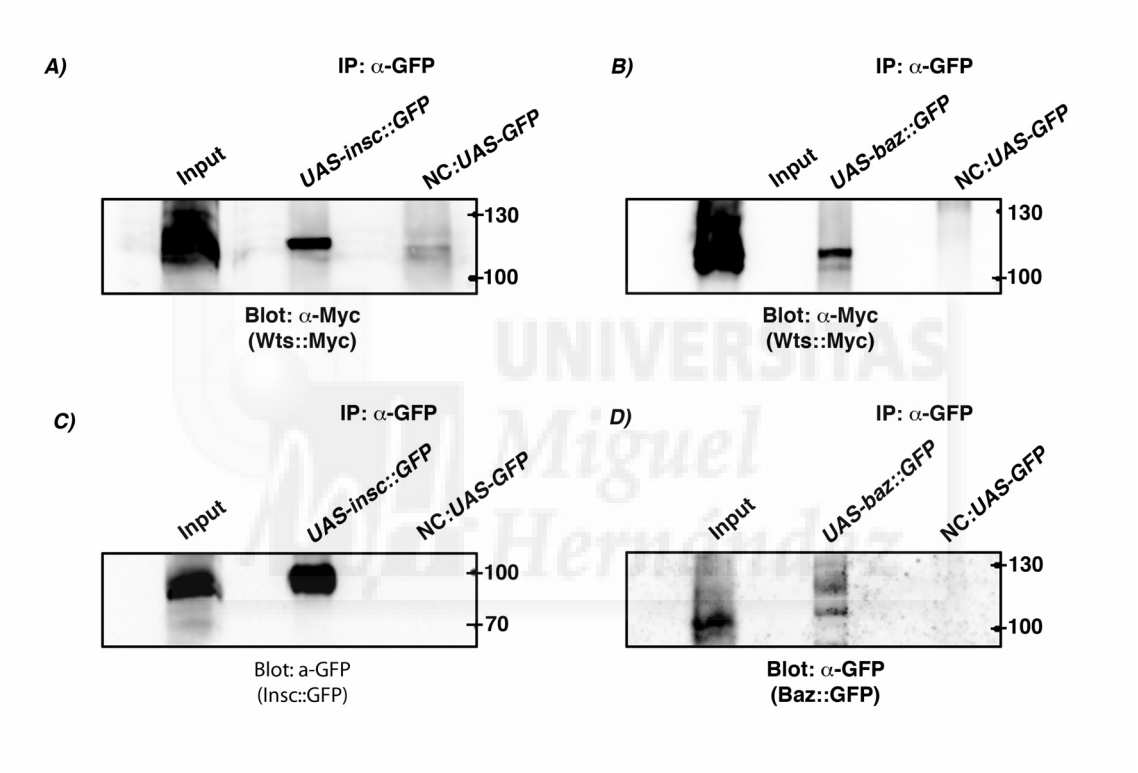


Figure 25. Baz and Insc are forming a compex with Wts *in vivo.* A) Co-immunoprecipitation (co-IP) experiment from the embryo lysates expressing both UAS-Insc::GFP and UAS-Wts:myc. Western blot (WB) with anti-Myc antibody. Wts:myc was detected from the immunoprecipitated (IP) protein complex of Insc:GFP. **B)** co-IP experiment with anti-GFP beads from the embryo lysates expressing both UAS-Baz:GFP and Wts::myc. WB with anti-Myc antibody revealing the co-IP between Wts::myc and Baz::GFP. **C)** IP control with anti-GFP beads from the embryo extracts expressing both UAS-Insc::GFP and UAS-Wts::myc. WB with anti-GFP antibody. **D)** IP control with anti-GFP beads from embryo extracts expressing both Baz::GFP and Wts::myc. WB with anti-GFP antibody. Negative control (Neg) is co-expression of UAS-GFP with UAS-Wts::Myc

Thus, co-immunoprecipitation experiments indicated that Wts is forming a complex

with Insc and Baz *in vivo*. However the mechanism of the action of Wts was still unclear. Knowing that Wts is a Ser/Thr kinase we wondered whether some ACD proteins could be direct phosphorylation substrates of Wts.

4.2.3.6 WTS DIRECTLY PHOSPHORYLATES CNO AND BAZ

As phosphorylation/dephosphorylation is an important mechanism for the correct localization of apical proteins and cell-fate determinants, we wondered whether Wts was phosphorylating any of these proteins, whose localization is affected in wts^{x1} glc mutants. Intriguingly, when analyzing Wts kinase phosphorylation consensus sequence Hx(R/H/K)xx(S/T) (Hao et al. 2008) in different apical proteins and cell-fate determinants, we identified possible Wts phosphorylation sites in the C-terminus of Baz/Par3 (7), in Cno (1), Pins (1), Mud (1), Polo (1), Brat (2), Pros (2) and Numb (1). Interestingly the phosphorylation consensus in Cno is conserved between species maintaining its position, whereas the phosphorylation sites in Baz did not show any conservation between species. Performing phosphorylation assays using standard band-shift assays Wts seemed to phosphorylate Cno. Phosphorylated Cno was shifted in the presence of Hpo and in the presence of Wts and its effector Mats, while in the presence of GFP Cno was not shifting (Figure 26).

S2 cells were transfected with plasmid expressing the FLAG-tagged truncated form of Cno at the aminoacid (aa) position 1100 together with plasmids expressing Hpo, Wts and Mats, and GFP. In a presense of HA-tagged Hpo, Wts together with Mats, Cno was shifting on the SDS-PAGE gel, whereas in the presence of the GFP, Cno was not shifting (red arrowheads in the Figure 26). HA-tagged Yki has been chosen as a positive phosphorylation control. In a presence of Wts and Mats compairing to GFP Yki is shifting. Baz and Numb didn´t show any shift on the SDS-PAGE gel.

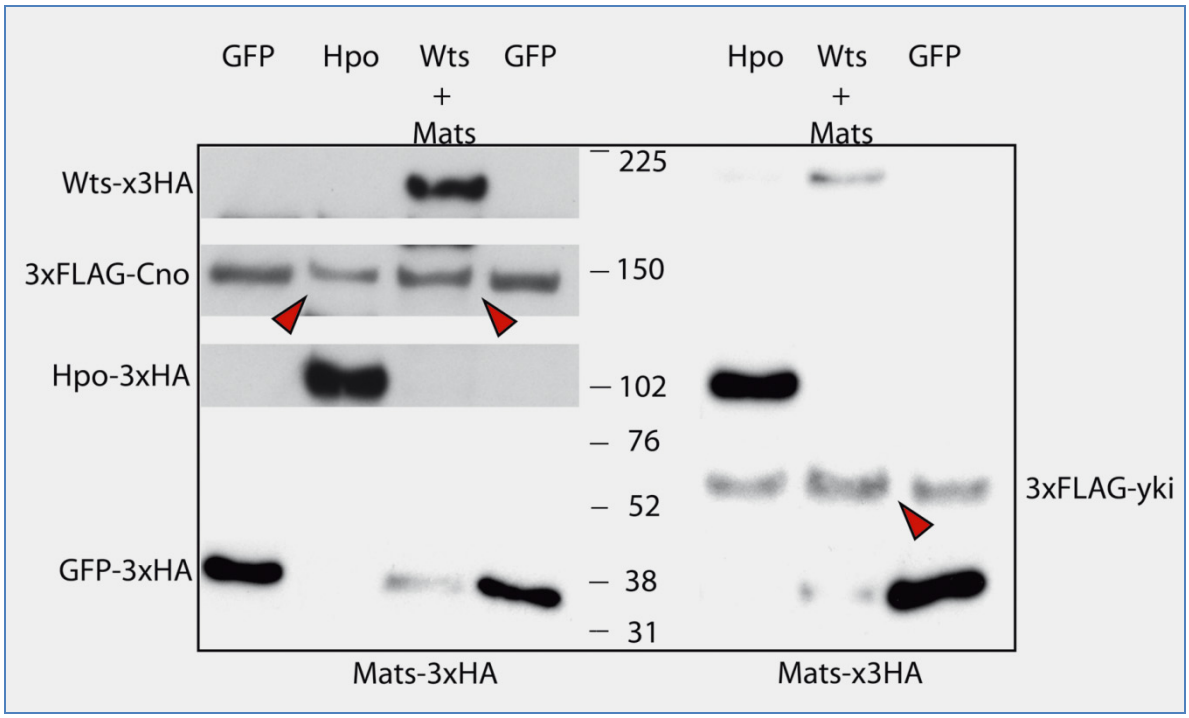


Figure 26. C-terminal part of Cno shifts in a presence of the Hpo and Wts. In a presence of HA-tagged Hpo C-terminal FLAG-tagged part of Cno is shifting (red arrowhead) comparing to the control in a presence of HA-tagged GFP. C-terminal Cno is shifting in a presence of Wts-3xHA cotransfected with Mats-3xHA (red arrowhead) comparing to the GFP-3xHA control. 3xFALG-yki is shifting in a presence of Hpo and Wts together with Mats comparing to the GFP control (red arrowhead).

To further confirm this result, we performed *in vitro* kinase assays using an active form of the human LATS1 kinase. In the presence of LATS1 Cno and Baz were both phosphorylated, whereas in the absence of the kinase no phosphorylation of C-terminal part of Cno and lower phosphorylation of Baz was detected (Figure 27). Numb showed LATS1-independent phosphorylation in the presence and the absence of LATS1 kinase.

The mutant form of C-terminal Cno with the mutation in the aa position 1196 from Ser to Ala (Cno-mut) was still phosphorylated by LATS1 kinase. The mutant form of Baz with mutations in the aa positions 757, 759, 1049, 1308, 1366, 1367, 1368 from Ser to Ala (Baz-mut) was still slightly phosphorylated comparing to the Baz wt. In the absence of LATS1 in Baz-mut no phosphorylation was detected (see figure 27).

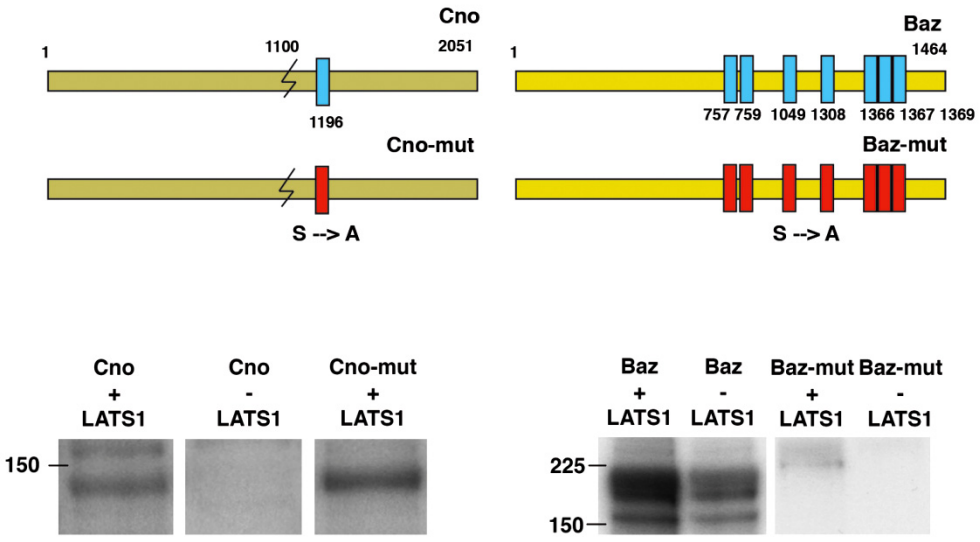


Figure 27. LATS1 phosphorylates *in vitro* Cno and Baz. Schematic representation of the Cno C-terminal part (Cno and Cno-mut), truncatied at the 1100 aa position with the mutation to alanine (A) at the serine (S) position 1196 are shown. Baz full-length shematic representation with 7 phosphorylation sites for Wts at the S positions 757, 759, 1049, 1308, 1366, 1367, 1368, all mutated to the A in the Baz-mut are shown. Truncated form of Cno and Cno-mut are phosphorylated by LATS1 kinase *in vitro*. LATS1 phosphorylates Baz in all predicted 7 sites of phosphorylation. In the absence of LATS Baz is phosphorylated by other kinases. In the absence of LATS1 the Baz-mut is not phosphorylated.

All these data unveil a novel role for the Wts kinase in a process of ACD.

Finally, we wondered whether the function of Wts and Hippo signalling regulating ACD was conserved in other tissues.

4.2.4 THE FUNCTION OF WTS AND HIPPO SIGNALLING IN ACD IS CONSERVED IN DIFFERENT TISSUES

4.2.4.1 MUSCLE/HEART PROGENITOR ASYMMETRIC DIVISION IS IMPAIRED IN HIPPO PATHWAY MUTANTS

Muscle and heart progenitors divide asymmetrically and Cno is also involved in regulating this division (Carmena et al. 1998a, Speicher et al. 2008). Hence, we wondered whether the Hippo pathway had a conserved function in this system as well. To analyze it we focused on two particular muscle/heart lineages: those from progenitors 2 and 15 that express the previously described marker Eve (Figure 28 A). Normally, by stage 12 three or four Eve-positive cells appear per hemisegment as a

result of the correct asymmetric division of progenitors 2 and 15 (Figure 28 A and C). *wts*^{x1} and *mats*^{e03077} loss-of-function mutant embryos showed a strong phenotype at this stage in the mesoderm (Figure 28 F, I) comparing with wt embryos (Figure 28 C). To analyze this division in more detail, we used specific markers to distinguish between the two daughter cells of these muscle/heart progenitors. Specifically, in the progeny of progenitor 2, the transcription factor Runt is expressed in only one daughter cell, the muscle founder cell of the dorsal obligue 2 (DO2) muscle (F_{DO2}), whereas Zfh1 is exclusively expressed in the other daughter cell, the founder of the Eve Pericardial Cells (E_{EPCs}) that will divide giving rise to two Eve-positive pericardial cells. (Figure 28 A, E). In *wts*^{x1} and *mats*^{e03077} loss-of-function mutant embryos were detected failures in the asymmetric division of this progenitor, where two Runt-positive daughter cells F_{DO2} appear in 8,3% (n =306, in the case of *wts*^{x1} mutant) and 11,3% (n=479, in the case of *mats*^{e03077}) of all hemisegments analyzed (Figure 27 E, H). We predicted that failures in the asymmetric muscle/heart progenitor division would lead to defects in muscle/heart formation at later stages. Indeed, In *wts*^{x1} and *mats*^{e03077} loss-of-function mutant embryos we observed 20,8% (n=318) and 17,55% (n=262), respectively, of defects in muscle formation (absence or duplications) (Figure 28 G, J). We also detected failures in the number of muscle/heart daughter cells in multiple loss-of-function mutants of genes belonging to the Hippo pathway, as well as in *wts* and *yki* gain-of-function mutants (Graph 4).

When analyzing the Eve, Zfh1 and Runt expression in muscle- and heart founder cells in Hippo pathway mutants (Figure 28 F, I) we sometimes detected a dramatic increase in the number of Eve- positive and Runt-positive founder cells, which suggested that not only the process of ACD is failing in those mutants (Figure 29 B).

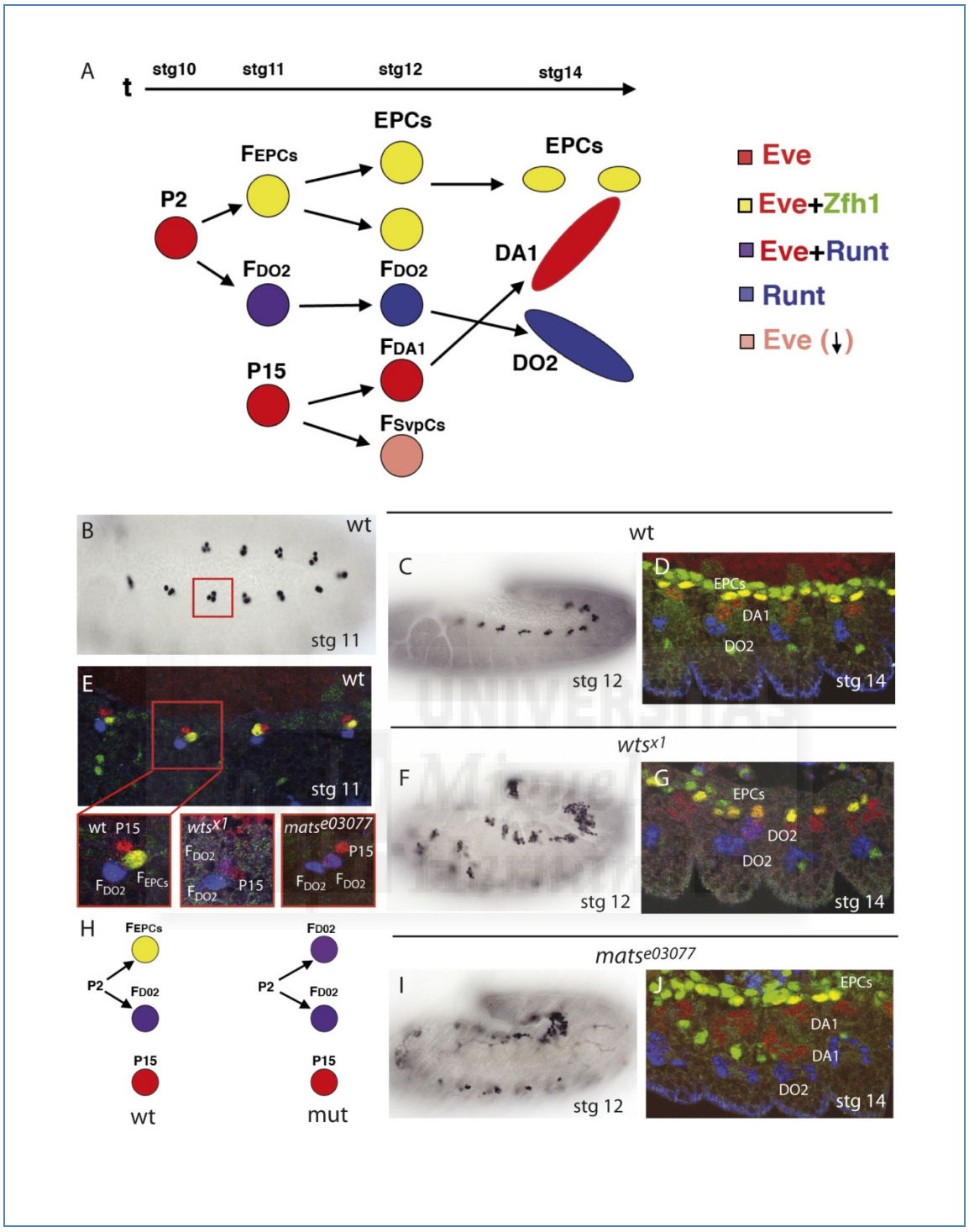
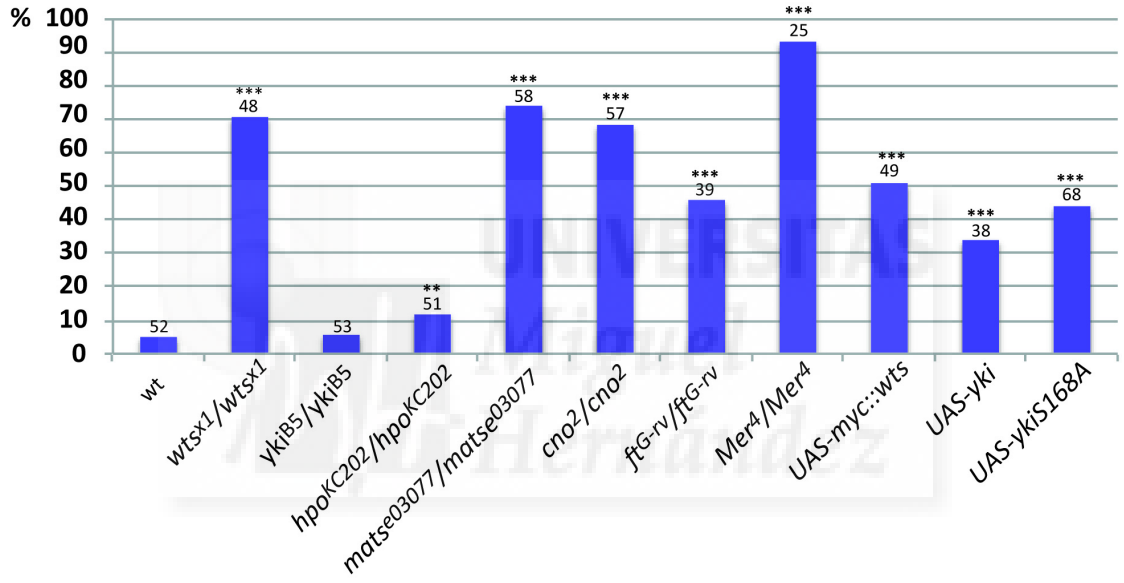


Figure 28. Muscle and heart formation from the lineages 2 and 15 A) At the stage 11 Progenitor 2 (P2) is dividing asymmetrically to two muscle and heart founder cells F_{DO2}, that express markers Eve and Zfh1 and F_{EPCs}, that express markers Eve and Runt. At the same time the progenitor 15 (p15) is specified and expresses Eve. By the stage 12 F_{EPCs} divides symmetrically to two pericardial cells EPCs and the Eve expression disappears from the F_{DO2} cell. P15 divides giving rise to the muscle founder cell F_{DA1} and F_{SvpCs} By the stage 14 two pericardial cells are formed EPCs and express Eve and Zfh1, muscle dorsal obligue 2 (DO2) and dorsal acute 1 (DA1) express Runt and Eve, respectively. **B)** Lateral view of the embryo mesoderm at stage 11 is represented with the anti-Eve staining, where three Eve-positive cells

should appear. **C)** At the stage 12 maximum 4 or 5 cells express Eve in the wt. **D)** By the stage 14 two EPCs (yellow), muscle DA1 (red) and muscle DO2 (blue) appear per hemisegment of wt. **E)** Stage 11 embryo stained with three markers Eve (red) Zfh1 (green) and Runt (blue). The co-expression of Zfh1 and Eve in the one daughter cell gives the yellow color. In the red square in a higher magnification one hemisegment is represented with Eve-expressing P15 (red), Zfh1- and Eve expressing F_{EPCs} (yellow), Runt-expressing F_{DO2} (blue). In *wts*^{x1} and *mats*^{e03077} mutants two Runt-expressing F_{DO2} cells appear and one Eve-expressing P15. **F)** Lateral view of the *wts*^{x1} mutant embryo mesoderm at stage 12 is represented with the anti-Eve staining **G)** At the stage 14 of the *wts*^{x1} mutants two DO2 muscles are formed and DA1 muscle is absent. **H)** Schematic representation of the P2 asymmetric division in wt and in the mutant (mut): instead of dividing asymmetrically theP2 divides symmetrically to two F_{DO2} cells. **I)** Lateral view of the *mats*^{e03077} mutant embryo mesoderm at stage 12 is represented with the anti-Eve staining. **J)** At the stage 14 of the *mats*^{e03077}mutants two DA1 muscles are formed.



Graph 4. Muscle and heart lineage analysis with anti-Eve antibody staining. Loss-of-function mutants for *wts, yki, mats, cno, ft,* and *Mer* show significant (***) phenotype, which consists in the defects in the number of muscle progenitor daughter cells. Gain-of-function mutants for *wts* (UAS-myc::wts) and for *yki* (UAS-yki) and *yki* active form (UAS-ykiS168A) using *twi*-Gal4, *dmef2*-Gal4 mesodermal Gal4 drivers also show phenotypic defects in mesoderm. Y scale represents the percentage of the embryos with defect phenotype of all embryos analyzed (numbers above the graphics). X scale represents the genotype of the analyzed embryos. Stars indicate that the phenotype is significant comparing to the wildtype (wt) phenotype (according to the Fisher´s test *** P<0.001, **P<0.01).

4.2.5 MUSCLE AND HEART PROGENITOR SPECIFICATION IS ALTERED IN HIPPO PATHWAY MUTANT COMPONENTS

Muscle and heart progenitor 2 (P2) is specified in the dorsal part of the mesoderm from equivalence cluster of cells, the “promuscle clusters”. All cells in the cluster initially

express the transcriptional factor L'sc; later, the expression of L'sc is inhibited in the whole cluster but in one cell, which is singled out from the cluster as the progenitor (Carmena et al. 1995) (Figure 29 A, C).

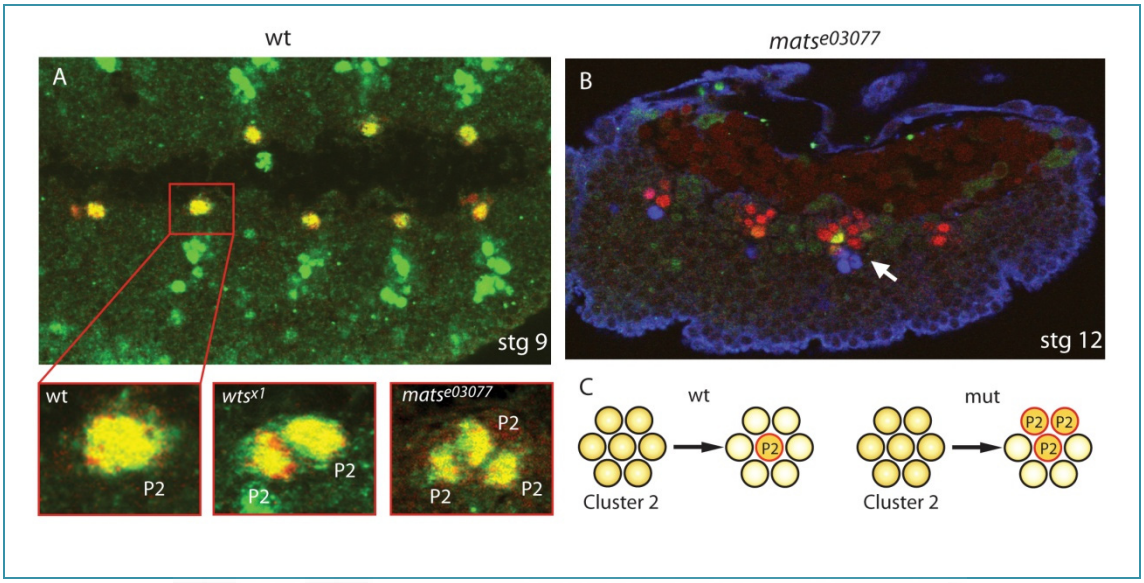


Figure 29. P2 specification analysis in *wts*^{x1} and *mats*^{03077} mutants A) In wt embryos one progenitor 2 (yellow, P2) is specified from the cluster per one hemisegment at the stage 9. L´sc is shown in green and Eve in red. On the magnification of one hemisegment in the wildtype (wt) only one P2 is specified, whereas in the *wts*^{x1} and *mats*^{03077} mutants there are two or even three P2 appearing. **B)** The number of the muscle and heart founder cells is affected (arrow) in the mutants **C)** Schematic representation of the P2 specification from the cell cluster 2. In the wt situation only one P2 is singled-out from the cluster. In the mutant more than one P2 are specified from the cell cluster and express L´sc and Eve markers.

The process of the progenitor specification is precisely controlled by the Notch pathway, and the process is known as "lateral inhibition" (Rusconi and Corbin 1998, Frasch 1999a, b). In the figure 29 B P2 specification is represented, where only one P2 is specified per hemisegment. In *wts*^{x1} and *mats*^{e03077} loss-of-function mutants the number of P2 was higher than one, in 14% (n=302) and in 25,4% (n=315) of all hemisegments analyzed, respectively (Figure 28 A). Phenotype consists of the double, triple or even quadruple P2, indicating that the specification process is failing in *wts*^{x1} and *mats*^{e03077} zygotic loss-of-function mutants.

UNIVERSITAS
Miguel
Hernández

CHAPTER 5

DISCUSSION

During this thesis research project, it has been discovered a novel function of the Hippo/Wts signalling pathway in the process of ACD (Figure 30). Moreover, we have shown that the function of the Hippo pathway during ACD is conserved in different tissues.

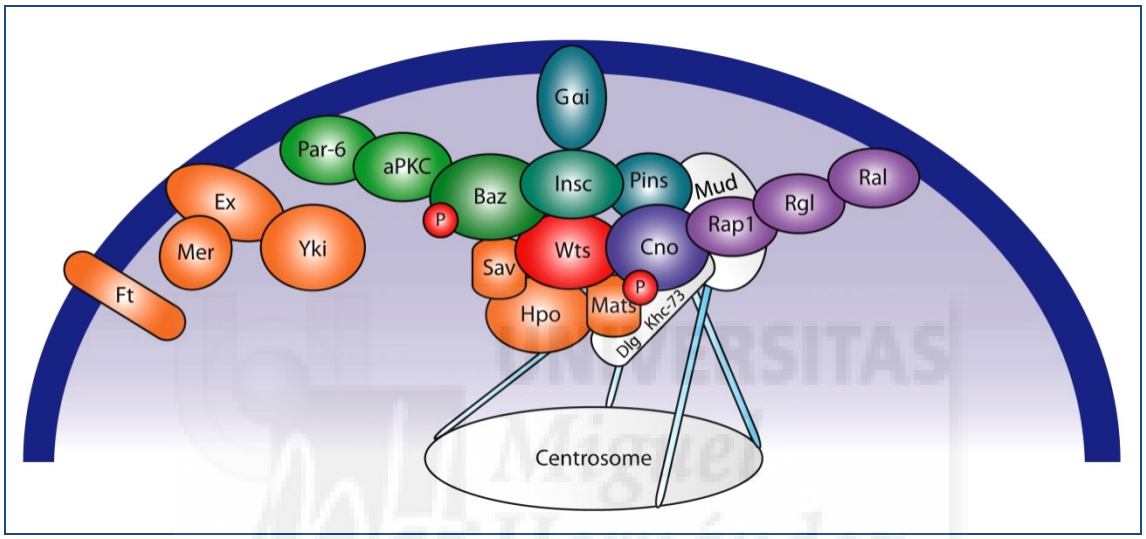


Figure 30. The Hippo pathway is a novel regulator of ACD. The Par complex (shown in green), which consists of Par-6, aPKC and Baz, is linked through the adaptor protein Insc (greenish blue) to the spindle position regulator-complex (shown in grey) via the interaction with Gαi at the membrane and with Pins. Spindle regulator-complex contains Mud that is linked to the Pins and to the spindle microtubules through Lyssein-dynein-dynactin complex (not shown in the diagram). Dlg together with Khc-73 also links the Pins complex to the microtubules. Cno and Rap1-Rgl-Ral network contributes to the mitotic spindle orientation and are linked through the interaction with Pins protein. Hippo pathway components (shown in orange), and Wts kinase (in red) are represented in the scheme according to the genetic and/or to the physical interactions between the proteins. Wts is forming a complex with Baz and Insc, and then Wts is able to phosphorylate Baz and Cno (phosphate is shown in red circle).

ACD is a crucial process for generating cell diversity during development and, in addition, an intriguing connection between tumorigenesis and ACD has emerged over the past years. Most genes involved in regulating ACD, such as cell polarity proteins

also behave as tumor suppressor genes and vice versa. Indeed, the Hippo pathway is a tumor suppressor pathway whose deregulation leads to organ overgrowth, due to an increase in cell proliferation, and to a reduced cell death. It has been shown that mutations in different components of the Hippo pathway lead to the formation of different human cancers (Harvey et al. 2013). The new function of the Hippo pathway in ACD observed in this thesis research opens a new field for the further investigation of the role of Hippo pathway in human cancers. It has been previously shown that stem cells in different organs divide asymmetrically producing different cell types within the tissue. And the defects in the process of asymmetric stem cell division may lead to the tumour formations (Knoblich 2010). Given that the Hippo pathway function in ACD is conserved in different *Drosophila* tissues (the CNS and the mesoderm), the function of the Hippo pathway in ACD in different human tissues in may be also conserved.

Interestingly, the regulator core of the Hippo pathway is a "kinase cassette" and phosphorylation is one of the main mechanisms to modulate the asymmetric localization of determinants during ACD (Sousa-Nunes and Somers 2010, Keder and Carmena 2013). For example, the phosphorylation of Numb by aPKC promotes its exclusion of the membrane; and the activation of aPKC depends in turn on the phosphorylation of Par6 by the Aurora-A kinase (Lee et al. 2006b, Wang et al. 2006, Smith et al. 2007, Wirtz-Peitz et al. 2008). aPCK, once activated, also phosphorylates Lgl and this event leads to the replacement of Lgl by Baz, in a complex with aPKC and Par6 (Betschinger et al. 2003). Likewise, the Polo kinase phosphorylates Partner of Numb (Pon) and this is key for both Pon and Numb asymmetric localization (Wang et al. 2007). Here, we observed that the kinase Wts phosphorylates Baz and Cno, two key regulators of ACD. Numb, like Baz and Cno showed a presence of the Wts consensus phosphorylation site. Surprisingly, the Wts phosphorylation consensus in the Numb containted the same serine at the position 161 that is phosphorylated by the aPKC kinase (Knoblich et al. 1997). Then, we tried to test whether Numb was another Wts-phosphorylation substrate during asymmetric NB division. However, we did not detect any change in Numb phosphorylation with and without adding human Wts homolog LATS1. There are two possible interpretations of this result (1) Numb is only phosphorylated by aPKC or (2) Wts kinase also can phosphorylate Numb and it competes for the accessibility to the serine 161, thus, in the absence of the Wts kinase, Numb is phosphorylated by aPKC. To answer the question whether Numb is Wts-

phosphorylation substrate more investigation work has to be done. While analyzing different ACD components for the presence of Wts-phosphorylation consensus, many proteins came out in this analysis, such as Pins, Mud, Lgl, Polo, Brat and Pros. To better understand the role of Wts kinase in the process of ACD it would be necessary to test all of the potential Wts-phosphorylation substrates. It would be interesting to analyze also if the Wts activation as a kinase requires the adaptor protein Mats (that has been shown to act in the asymmetric muscle progenitor division), or previous Wts activation by the upstream kinase Hippo.

wts loss of function mutant embryos showed very specific defects in fundamental aspects of ACD, such as the localization of apical proteins, including aPKC, Baz and Cno. Hence, phosphorylation of key regulators of this process is a potential mechanism by which Wts is regulating ACD. In the case of Cno, even though the kinase assays strikingly showed that Wts phosphorylates Cno, the mutation in its most conserved phosphorylation consensus site by Wts (HxHxxS) did not have any apparent effect. However, we have observed that there is another much less frequent consensus sequence (HxHxxT) in Cno. Intriguingly, this site is very close to the most conserved one and might have been evolved to be phosphorylated by Wts because, for example, structural constrains and inaccessibility of Wts to the other site. Additional research has to be done in order to describe the Wts-phosphorylation sites in Cno.

In vivo coimmunoprecipitation experiments clearly demostrated that Wts forms a complex with Baz and Insc, but not with Cno. How the phosphorylation of those substrates by the Wts kinase is possible? Is there any physical interaction between Wts and Baz and Insc, which are in a complex with Pins and thus can bring Cno closer to Wts to enable the phosphorylation of Cno by this kinase? To answer this question we analyzed in more detail physical interactions between the Hippo pathway components. In this context, it is interesting to mention the high prevalence of the interactions between WW domains (20-23 amino acids flanked by two conserved trytophan [W] residues) and proline rich motifs PPxY within the Hippo pathway. Wts contains 5 PPxY motifs to physically interact with Sav and Yki and both of them contain WW domains. Hpo, the cadherin Fat contain one PPxY motif, whereas Ds and Ex contain 3 PPxY motifs, and Kibra contains both PPxY and WW domains. This has led to propose that other proteins containing these domains will be probably involved in the Hippo pathway regulation (Sudol and Hunter 2000, Salah and Aqeilan 2011). Moreover, because the

last amino acid of the PPxY domain is a tyrosine, it has been suggested that WW-PPxY interactions might be regulated by phosphorylation. Given the relationships found in this work between proteins involved in the Hippo pathway and proteins required for ACD, we analyzed in these last proteins the presence of WW and PPxY domains. No WW domains were found in any of them, but intriguingly both Baz and Insc, which were found in a complex *in vivo* with Wts, contain the PPxY motif. Within the Hippo pathway, Sav, Kibra and Yki are the only ones that have WW domains, and are in a complex with Wts kinase. Hence, a possible scenario would be that the scaffolding protein Sav binds Baz and/or Insc, linking them to Wts, and facilitates in this way the phosphorylation of Baz and Cno by Wts (Figure 30). However more data need to be shown to explain that possibility.

In addition, we have shown that Wts along with its effector Mats function not only in ACD but also in the specification of the muscle/heart progenitors within the mesoderm. This finding opens a new field for further investigating the function of the Hippo pathway in this process and its relationships and cross-communication with the different signalling pathways that are involved in this process, such as Notch, Ras/MAPK and Wg/Wnt (Carmena et al. 1995, Carmena et al. 1998b, Frasch 1999a, b, Carmena et al. 2006).

Altogether, this work has revealed a new functional connection between the Hippo pathway, an organ growth regulatory pathway highly conserved form flies to humans, and the process of ACD, a universal process in developmental, stem cell and cancer biology. Future perspectives of the research would be to discover more crosslinks between fundamental signalling pathways and ACD.

CHAPTER 6

CONCLUSIONS

1. The Hippo/Wts signaling pathway functionally interacts with Cno during the process of ACD.

2. The Hippo/Wts pathway is a novel regulator of asymmetric NB division.

3. The kinase Wts forms a complex *in vivo* with the apical proteins Baz and Insc, both key regulators of ACD.

4. Human LATS1 kinase, an ortholog of the Wts kinase, phosphorylates Cno and Baz *in vitro.*

5. The novel function of the Hippo pathway in ACD is conserved in different tissues.

6. The Hippo/Wts pathway is additionally required for the muscle/heart progenitor specification.

CONCLUCIONES

1. La vía de señalización Warts/Hippo interacciona genéticamente con la proteína Canoe y con otros múltiples moduladores del proceso de división asimétrica.
2. La vía de Hippo desempeña una función reguladora en el proceso de división asimétrica de neuroblastos
3. La proteín kinasa Warts forma un complejo *in vivo* con las proteínas Bazooka e Inscutable, ambas reguladoras clave del proceso de división asimétrica.
4. La kinasa LATS1 (ortólogo humano del Warts) fosforila a Canoe y a Bazooka *in vitro*.
5. La nueva función de la vía de Hippo en división asimétrica está conservada en distintos tejidos.
6. La vía de Warts/Hippo está adicionalmente involucrada en la especificación de los progenitores musculares y cardiales.

BIBLIOGRAPHY

Abe, Y., M. Ohsugi, K. Haraguchi, J. Fujimoto, and T. Yamamoto. 2006. LATS2-Ajuba complex regulates gamma-tubulin recruitment to centrosomes and spindle organization during mitosis. FEBS letters 580: 782-788.

Alarcon, C., A. I. Zaromytidou, Q. Xi, S. Gao, J. Yu, S. Fujisawa, A. Barlas, A. N. Miller, K. Manova-Todorova, M. J. Macias, G. Sapkota, D. Pan, and J. Massague. 2009. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. Cell 139: 757-769.

Albertson, R., C. Chabu, A. Sheehan, and C. Q. Doe. 2004. Scribble protein domain mapping reveals a multistep localization mechanism and domains necessary for establishing cortical polarity. Journal of cell science 117: 6061-6070.

Atwood, S. X., and K. E. Prehoda. 2009. aPKC phosphorylates Miranda to polarize fate determinants during neuroblast asymmetric cell division. Current biology : CB 19: 723-729.

Atwood, S. X., C. Chabu, R. R. Penkert, C. Q. Doe, and K. E. Prehoda. 2007. Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6 aPKC. Journal of cell science 120: 3200-3206.

Avruch, J., D. Zhou, J. Fitamant, N. Bardeesy, F. Mou, and L. R. Barrufet. 2012. Protein kinases of the Hippo pathway: regulation and substrates. Seminars in cell & developmental biology 23: 770-784.

Azoury, J., K. W. Lee, V. Georget, P. Hikal, and M. H. Verlhac. 2011. Symmetry breaking in mouse oocytes requires transient F-actin meshwork destabilization. Development 138: 2903-2908.

Balasubramanian, M. K., D. McCollum, L. Chang, K. C. Wong, N. I. Naqvi, X. He, S. Sazer, and K. L. Gould. 1998. Isolation and characterization of new fission yeast cytokinesis mutants. Genetics 149: 1265-1275.

Barros, C. S., C. B. Phelps, and A. H. Brand. 2003. Drosophila nonmuscle myosin II promotes the asymmetric segregation of cell fate determinants by cortical exclusion rather than active transport. Developmental cell 5: 829-840.

Baumgartner, R., I. Poernbacher, N. Buser, E. Hafen, and H. Stocker. 2010. The WW domain protein Kibra acts upstream of Hippo in Drosophila. Developmental cell 18: 309-316.

Bellaiche, Y., M. Gho, J. A. Kaltschmidt, A. H. Brand, and F. Schweisguth. 2001a. Frizzled regulates localization of cell-fate determinants and mitotic spindle rotation during asymmetric cell division. Nature cell biology 3: 50-57.

Bellaiche, Y., A. Radovic, D. F. Woods, C. D. Hough, M. L. Parmentier, C. J. O'Kane, P. J. Bryant, and F. Schweisguth. 2001b. The Partner of Inscuteable/Discs-large complex is required to establish planar polarity during asymmetric cell division in Drosophila. Cell 106: 355-366.

Bennett, F. C., and K. F. Harvey. 2006. Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. Current biology : CB 16: 2101-2110.

Berdnik, D., and J. A. Knoblich. 2002. Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Current biology : CB 12: 640-647.

Berdnik, D., T. Torok, M. Gonzalez-Gaitan, and J. A. Knoblich. 2002. The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. Developmental cell 3: 221-231.

Betschinger, J., and J. A. Knoblich. 2004. Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Current biology : CB 14: R674-685.

Betschinger, J., K. Mechtler, and J. A. Knoblich. 2003. The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature 422: 326-330.

Betschinger, J., K. Mechtler, and J. A. Knoblich. 2006. Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell 124: 1241-1253.

Bidlingmaier, S., E. L. Weiss, C. Seidel, D. G. Drubin, and M. Snyder. 2001. The Cbk1p pathway is important for polarized cell growth and cell separation in Saccharomyces cerevisiae. Molecular and cellular biology 21: 2449-2462.

Boedigheimer, M. J., K. P. Nguyen, and P. J. Bryant. 1997. Expanded functions in the apical cell domain to regulate the growth rate of imaginal discs. Developmental genetics 20: 103-110.

Boettner, B., P. Harjes, S. Ishimaru, M. Heke, H. Q. Fan, Y. Qin, L. Van Aelst, and U. Gaul. 2003. The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the Drosophila embryo. Genetics 165: 159-169.

Bothos, J., R. L. Tuttle, M. Ottey, F. C. Luca, and T. D. Halazonetis. 2005. Human LATS1 is a mitotic exit network kinase. Cancer research 65: 6568-6575.

Bowman, S. K., R. A. Neumuller, M. Novatchkova, Q. Du, and J. A. Knoblich. 2006. The Drosophila NuMA Homolog Mud regulates spindle orientation in asymmetric cell division. Developmental cell 10: 731-742.

Boyd, L., S. Guo, D. Levitan, D. T. Stinchcomb, and K. J. Kemphues. 1996. PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in C. elegans embryos. Development 122: 3075-3084.

Brand, A. H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401-415.

Bultje, R. S., D. R. Castaneda-Castellanos, L. Y. Jan, Y. N. Jan, A. R. Kriegstein, and S. H. Shi. 2009. Mammalian Par3 regulates progenitor cell asymmetric

division via notch signaling in the developing neocortex. Neuron 63: 189-202.

Cai, Y., F. Yu, S. Lin, W. Chia, and X. Yang. 2003. Apical complex genes control mitotic spindle geometry and relative size of daughter cells in Drosophila neuroblast and pI asymmetric divisions. Cell 112: 51-62.

Callus, B. A., A. M. Verhagen, and D. L. Vaux. 2006. Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. The FEBS journal 273: 4264-4276.

Carmena, A., M. Bate, and F. Jimenez. 1995. Lethal of scute, a proneural gene, participates in the specification of muscle progenitors during Drosophila embryogenesis. Genes & development 9: 2373-2383.

Carmena, A., S. Speicher, and M. Baylies. 2006. The PDZ protein Canoe/AF-6 links Ras-MAPK, Notch and Wingless/Wnt signaling pathways by directly interacting with Ras, Notch and Dishevelled. PloS one 1: e66.

Carmena, A., A. Makarova, and S. Speicher. 2011. The Rap1-Rgl-Ral signaling network regulates neuroblast cortical polarity and spindle orientation. The Journal of cell biology 195: 553-562.

Carmena, A., B. Murugasu-Oei, D. Menon, F. Jimenez, and W. Chia. 1998a. Inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. Genes & development 12: 304-315.

Carmena, A., S. Gisselbrecht, J. Harrison, F. Jimenez, and A. M. Michelson. 1998b. Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. Genes & development 12: 3910-3922.

Chan, E. H., M. Nousiainen, R. B. Chalamalasetty, A. Schafer, E. A. Nigg, and H. H. Sillje. 2005. The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. Oncogene 24: 2076-2086.

Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger, and S. L. Reiner. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science 315: 1687-1691.

Chartrand, P., R. H. Singer, and R. M. Long. 2001. RNP localization and transport in yeast. Annual review of cell and developmental biology 17: 297-310.

Chen, C. L., K. M. Gajewski, F. Hamaratoglu, W. Bossuyt, L. Sansores-Garcia, C. Tao, and G. Halder. 2010. The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 107: 15810-15815.

Chen, D., and D. M. McKearin. 2003. A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. Development 130: 1159-1170.

Cho, E., Y. Feng, C. Rauskolb, S. Maitra, R. Fehon, and K. D. Irvine. 2006. Delineation of a Fat tumor suppressor pathway. Nature genetics 38: 1142-1150.

Chu-LaGraff, Q., and C. Q. Doe. 1993. Neuroblast specification and formation regulated by wingless in the Drosophila CNS. Science 261: 1594-1597.

Codelia, V. A., and K. D. Irvine. 2012. Hippo signaling goes long range. Cell 150: 669-670.

Colombo, K., S. W. Grill, R. J. Kimple, F. S. Willard, D. P. Siderovski, and P. Gonczy. 2003. Translation of polarity cues into asymmetric spindle positioning in Caenorhabditis elegans embryos. Science 300: 1957-1961.

Cordenonsi, M., F. Zanconato, L. Azzolin, M. Forcato, A. Rosato, C. Frasson, M. Inui, M. Montagner, A. R. Parenti, A. Poletti, M. G. Daidone, S. Dupont, G. Basso, S. Bicciato, and S. Piccolo. 2011. The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. Cell 147: 759-772.

Coumailleau, F., M. Furthauer, J. A. Knoblich, and M. Gonzalez-Gaitan. 2009. Directional Delta and Notch trafficking in Sara endosomes during asymmetric cell division. Nature 458: 1051-1055.

Das Thakur, M., Y. Feng, R. Jagannathan, M. J. Seppa, J. B. Skeath, and G. D. Longmore. 2010. Ajuba LIM proteins are negative regulators of the Hippo signaling pathway. Current biology : CB 20: 657-662.

Degoutin, J. L., C. C. Milton, E. Yu, M. Tipping, F. Bosveld, L. Yang, Y. Bellaiche, A. Veraksa, and K. F. Harvey. 2013. Riquiqui and minibrain are regulators of the hippo pathway downstream of Dachsous. Nature cell biology 15: 1176-1185.

do Carmo Avides, M., and D. M. Glover. 1999. Abnormal spindle protein, Asp, and the integrity of mitotic centrosomal microtubule organizing centers. Science 283: 1733-1735.

do Carmo Avides, M., A. Tavares, and D. M. Glover. 2001. Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. Nature cell biology 3: 421-424.

Dong, J., G. Feldmann, J. Huang, S. Wu, N. Zhang, S. A. Comerford, M. F. Gayyed, R. A. Anders, A. Maitra, and D. Pan. 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell 130: 1120-1133.

Du, Q., P. T. Stukenberg, and I. G. Macara. 2001. A mammalian Partner of inscuteable binds NuMA and regulates mitotic spindle organization. Nature cell biology 3: 1069-1075.

Duncan, F. E., S. B. Moss, R. M. Schultz, and C. J. Williams. 2005. PAR-3 defines a central subdomain of the cortical actin cap in mouse eggs. Developmental biology 280: 38-47.

Emoto, K., J. Z. Parrish, L. Y. Jan, and Y. N. Jan. 2006. The tumour suppressor Hippo acts with the NDR kinases in dendritic tiling and maintenance. Nature 443: 210-213.

Erben, V., M. Waldhuber, D. Langer, I. Fetka, R. P. Jansen, and C. Petritsch. 2008. Asymmetric localization of the adaptor protein Miranda in neuroblasts is achieved by diffusion and sequential interaction of Myosin II and VI. Journal of cell science 121: 1403-1414.

Etemad-Moghadam, B., S. Guo, and K. J. Kemphues. 1995. Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early C. elegans embryos. Cell 83: 743-752.

Feng, Y., and K. D. Irvine. 2007. Fat and expanded act in parallel to regulate growth through warts. Proceedings of the National Academy of Sciences of the United States of America 104: 20362-20367.

Fernandez, B. G., P. Gaspar, C. Bras-Pereira, B. Jezowska, S. R. Rebelo, and F. Janody. 2011. Actin-Capping Protein and the Hippo pathway regulate F-actin and tissue growth in Drosophila. Development 138: 2337-2346.

Fietz, S. A., I. Kelava, J. Vogt, M. Wilsch-Brauninger, D. Stenzel, J. L. Fish, D. Corbeil, A. Riehn, W. Distler, R. Nitsch, and W. B. Huttner. 2010. OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nature neuroscience 13: 690-699.

Frasch, M. 1999a. Controls in patterning and diversification of somatic muscles during Drosophila embryogenesis. Current opinion in genetics & development 9: 522-529.

Frasch, M. 1999b. Intersecting signalling and transcriptional pathways in Drosophila heart specification. Seminars in cell & developmental biology 10: 61-71.

Frasch, M., T. Hoey, C. Rushlow, H. Doyle, and M. Levine. 1987. Characterization and localization of the even-skipped protein of Drosophila. The EMBO journal 6: 749-759.

Fuentealba, L. C., E. Eivers, D. Geissert, V. Taelman, and E. M. De Robertis. 2008. Asymmetric mitosis: Unequal segregation of proteins destined for degradation. Proceedings of the National Academy of Sciences of the United States of America 105: 7732-7737.

Fuller, M. T., and A. C. Spradling. 2007. Male and female Drosophila germline stem cells: two versions of immortality. Science 316: 402-404.

Fuse, N., K. Hisata, A. L. Katzen, and F. Matsuzaki. 2003. Heterotrimeric G proteins regulate daughter cell size asymmetry in Drosophila neuroblast divisions. Current biology : CB 13: 947-954.

Gaengel, K., and M. Mlodzik. 2003. Egfr signaling regulates ommatidial rotation and cell motility in the Drosophila eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6. Development 130: 5413-5423.

Garner, C. C., J. Nash, and R. L. Huganir. 2000. PDZ domains in synapse assembly and signalling. Trends in cell biology 10: 274-280.

Genevet, A., M. C. Wehr, R. Brain, B. J. Thompson, and N. Tapon. 2010. Kibra is a regulator of the Salvador/Warts/Hippo signaling network. Developmental cell 18: 300-308.

Gerard, A., A. E. Mertens, R. A. van der Kammen, and J. G. Collard. 2007. The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. The Journal of cell biology 176: 863-875.

Gho, M., and F. Schweisguth. 1998. Frizzled signalling controls orientation of asymmetric sense organ precursor cell divisions in Drosophila. Nature 393: 178-181.

Gomez-Lopez, S., R. G. Lerner, and C. Petritsch. 2013. Asymmetric cell division of stem and progenitor cells during homeostasis and cancer. Cellular and molecular life sciences : CMLS.

Gotta, M., Y. Dong, Y. K. Peterson, S. M. Lanier, and J. Ahringer. 2003. Asymmetrically distributed C. elegans homologs of AGS3/PINS control spindle position in the early embryo. Current biology : CB 13: 1029-1037.

Goulev, Y., J. D. Fauny, B. Gonzalez-Marti, D. Flagiello, J. Silber, and A. Zider. 2008. SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. Current biology : CB 18: 435-441.

Grzeschik, N. A., L. M. Parsons, M. L. Allott, K. F. Harvey, and H. E. Richardson. 2010. Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. Current biology : CB 20: 573-581.

Guedes, S., and J. R. Priess. 1997. The C. elegans MEX-1 protein is present in germline blastomeres and is a P granule component. Development 124: 731-739.

Guo, M., L. Y. Jan, and Y. N. Jan. 1996. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17: 27-41.

Guo, S., and K. J. Kemphues. 1995. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 81: 611-620.

Guo, X., and S. Gao. 2009. Pins homolog LGN regulates meiotic spindle organization in mouse oocytes. Cell research 19: 838-848.

Halet, G., and J. Carroll. 2007. Rac activity is polarized and regulates meiotic spindle stability and anchoring in mammalian oocytes. Developmental cell 12: 309-317.

Hamaratoglu, F., M. Willecke, M. Kango-Singh, R. Nolo, E. Hyun, C. Tao, H. Jafar-Nejad, and G. Halder. 2006. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nature cell biology 8: 27-36.

Hansen, D. V., J. H. Lui, P. R. Parker, and A. R. Kriegstein. 2010. Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature 464: 554-561.

Hao, Y., A. Chun, K. Cheung, B. Rashidi, and X. Yang. 2008. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. The Journal of biological chemistry 283: 5496-5509.

Hartenstein, V. 1993. Early pattern of neuronal differentiation in the Xenopus embryonic brainstem and spinal cord. The Journal of comparative neurology 328: 213-231.

Harvey, K. F., X. Zhang, and D. M. Thomas. 2013. The Hippo pathway and human cancer. Nature reviews. Cancer 13: 246-257.

Henderson, K. A., and D. E. Gottschling. 2008. A mother's sacrifice: what is she keeping for herself? Current opinion in cell biology 20: 723-728.

Hergovich, A. 2013. Regulation and functions of mammalian LATS/NDR kinases: looking beyond canonical Hippo signalling. Cell & bioscience 3: 32.

Homem, C. C., and J. A. Knoblich. 2012. Drosophila neuroblasts: a model for stem cell biology. Development 139: 4297-4310.

Huang, J., S. Wu, J. Barrera, K. Matthews, and D. Pan. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell 122: 421-434.

Hubbard, E. J., and D. Greenstein. 2005. Introduction to the germ line. WormBook : the online review of C. elegans biology: 1-4.

Hung, T. J., and K. J. Kemphues. 1999. PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in Caenorhabditis elegans embryos. Development 126: 127-135.

Hutterer, A., and J. A. Knoblich. 2005. Numb and alpha-Adaptin regulate Sanpodo endocytosis to specify cell fate in Drosophila external sensory organs. EMBO reports 6: 836-842.

Hutterer, A., D. Berdnik, F. Wirtz-Peitz, M. Zigman, A. Schleiffer, and J. A. Knoblich. 2006. Mitotic activation of the kinase Aurora-A requires its binding partner Bora. Developmental cell 11: 147-157.

Hyenne, V., N. T. Chartier, and J. C. Labbe. 2010. Understanding the role of asymmetric cell division in cancer using C. elegans. Developmental dynamics : an official publication of the American Association of Anatomists 239: 1378-1387.

Inaba, M., and Y. M. Yamashita. 2012. Asymmetric stem cell division: precision for robustness. Cell stem cell 11: 461-469.

Isshiki, T., B. Pearson, S. Holbrook, and C. Q. Doe. 2001. Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106: 511-521.

Izumi, Y., N. Ohta, A. Itoh-Furuya, N. Fuse, and F. Matsuzaki. 2004. Differential functions of G protein and Baz-aPKC signaling pathways in Drosophila neuroblast asymmetric division. The Journal of cell biology 164: 729-738.

Jordan, J. D., E. M. Landau, and R. Iyengar. 2000. Signaling networks: the origins of cellular multitasking. Cell 103: 193-200.

Justice, R. W., O. Zilian, D. F. Woods, M. Noll, and P. J. Bryant. 1995. The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes & development 9: 534-546.

Kaltschmidt, J. A., C. M. Davidson, N. H. Brown, and A. H. Brand. 2000. Rotation and asymmetry of the mitotic spindle direct asymmetric cell division in the developing central nervous system. Nature cell biology 2: 7-12.

Kanai-Azuma, M., J. S. Mattick, K. Kaibuchi, and S. A. Wood. 2000. Co-localization of FAM and AF-6, the mammalian homologues of Drosophila faf and canoe, in mouse eye development. Mechanisms of development 91: 383-386.

Keder, A., and A. Carmena. 2013. Cytoplasmic protein motility and polarized sorting during asymmetric cell division. Wiley interdisciplinary reviews. Developmental biology 2: 797-808.

Kelsom, C., and W. Lu. 2012. Uncovering the link between malfunctions in Drosophila neuroblast asymmetric cell division and tumorigenesis. Cell & bioscience 2: 38.

Kiger, A. A., D. L. Jones, C. Schulz, M. B. Rogers, and M. T. Fuller. 2001. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science 294: 2542-2545.

Knoblich, J. A. 2008. Mechanisms of asymmetric stem cell division. Cell 132: 583-597.

Knoblich, J. A. 2010. Asymmetric cell division: recent developments and their implications for tumour biology. Nature reviews. Molecular cell biology 11: 849-860.

Knoblich, J. A., L. Y. Jan, and Y. N. Jan. 1995. Asymmetric segregation of Numb and Prospero during cell division. Nature 377: 624-627.

Knoblich, J. A., L. Y. Jan, and Y. N. Jan. 1997. The N terminus of the Drosophila Numb protein directs membrane association and actin-dependent

asymmetric localization. Proceedings of the National Academy of Sciences of the United States of America 94: 13005-13010.

Kosman, D., S. Small, and J. Reinitz. 1998. Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. Development genes and evolution 208: 290-294.

Krahn, M. P., D. Egger-Adam, and A. Wodarz. 2009. PP2A antagonizes phosphorylation of Bazooka by PAR-1 to control apical-basal polarity in dividing embryonic neuroblasts. Developmental cell 16: 901-908.

Kraut, R., and J. A. Campos-Ortega. 1996. inscuteable, a neural precursor gene of Drosophila, encodes a candidate for a cytoskeleton adaptor protein. Developmental biology 174: 65-81.

Kraut, R., W. Chia, L. Y. Jan, Y. N. Jan, and J. A. Knoblich. 1996. Role of inscuteable in orienting asymmetric cell divisions in Drosophila. Nature 383: 50-55.

Kremerskothen, J., C. Plaas, K. Buther, I. Finger, S. Veltel, T. Matanis, T. Liedtke, and A. Barnekow. 2003. Characterization of KIBRA, a novel WW domain-containing protein. Biochemical and biophysical research communications 300: 862-867.

Kuchinke, U., F. Grawe, and E. Knust. 1998. Control of spindle orientation in Drosophila by the Par-3-related PDZ-domain protein Bazooka. Current biology : CB 8: 1357-1365.

Kumfer, K. T., S. J. Cook, J. M. Squirrell, K. W. Eliceiri, N. Peel, K. F. O'Connell, and J. G. White. 2010. CGEF-1 and CHIN-1 regulate CDC-42 activity during asymmetric division in the Caenorhabditis elegans embryo. Molecular biology of the cell 21: 266-277.

Kuriyama, M., N. Harada, S. Kuroda, T. Yamamoto, M. Nakafuku, A. Iwamatsu, D. Yamamoto, R. Prasad, C. Croce, E. Canaani, and K. Kaibuchi. 1996. Identification of AF-6 and canoe as putative targets for Ras. The Journal of biological chemistry 271: 607-610.

Kusek, G., M. Campbell, F. Doyle, S. A. Tenenbaum, M. Kiebler, and S. Temple. 2012. Asymmetric segregation of the double-stranded RNA binding protein Staufen2 during mammalian neural stem cell divisions promotes lineage progression. Cell stem cell 11: 505-516.

Lai, Z. C., M. E. Fortini, and G. M. Rubin. 1991. The embryonic expression patterns of zfh-1 and zfh-2, two Drosophila genes encoding novel zinc-finger homeodomain proteins. Mechanisms of development 34: 123-134.

Lai, Z. C., X. Wei, T. Shimizu, E. Ramos, M. Rohrbaugh, N. Nikolaidis, L. L. Ho, and Y. Li. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell 120: 675-685.

Le Borgne, R., and F. Schweisguth. 2003. Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. Developmental cell 5: 139-148.

Lechler, T., and E. Fuchs. 2005. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 437: 275-280.

Lee, C. Y., B. D. Wilkinson, S. E. Siegrist, R. P. Wharton, and C. Q. Doe. 2006a. Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Developmental cell 10: 441-449.

Lee, C. Y., R. O. Andersen, C. Cabernard, L. Manning, K. D. Tran, M. J. Lanskey, A. Bashirullah, and C. Q. Doe. 2006b. Drosophila Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. Genes & development 20: 3464-3474.

Lee, J., T. Miyano, and R. M. Moor. 2000. Spindle formation and dynamics of gamma-tubulin and nuclear mitotic apparatus protein distribution during meiosis in pig and mouse oocytes. Biology of reproduction 62: 1184-1192.

Lei, Q. Y., H. Zhang, B. Zhao, Z. Y. Zha, F. Bai, X. H. Pei, S. Zhao, Y. Xiong, and K. L. Guan. 2008. TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. Molecular and cellular biology 28: 2426-2436.

Li, P., X. Yang, M. Wasser, Y. Cai, and W. Chia. 1997. Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during Drosophila neuroblast cell divisions. Cell 90: 437-447.

Liu, C. Y., Z. Y. Zha, X. Zhou, H. Zhang, W. Huang, D. Zhao, T. Li, S. W. Chan, C. J. Lim, W. Hong, S. Zhao, Y. Xiong, Q. Y. Lei, and K. L. Guan. 2010. The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. The Journal of biological chemistry 285: 37159-37169.

Lu, B., M. Rothenberg, L. Y. Jan, and Y. N. Jan. 1998. Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in Drosophila neural and muscle progenitors. Cell 95: 225-235.

Lu, B., L. Ackerman, L. Y. Jan, and Y. N. Jan. 1999. Modes of protein movement that lead to the asymmetric localization of partner of Numb during Drosophila neuroblast division. Molecular cell 4: 883-891.

Lucas, E. P., I. Khanal, P. Gaspar, G. C. Fletcher, C. Polesello, N. Tapon, and B. J. Thompson. 2013. The Hippo pathway polarizes the actin cytoskeleton during collective migration of Drosophila border cells. The Journal of cell biology 201: 875-885.

Mandai, K., Y. Rikitake, Y. Shimono, and Y. Takai. 2013. Afadin/AF-6 and canoe: roles in cell adhesion and beyond. Progress in molecular biology and translational science 116: 433-454.

Mao, Y., B. Kucuk, and K. D. Irvine. 2009. Drosophila lowfat, a novel modulator of Fat signaling. Development 136: 3223-3233.

Martin-Bermudo, M. D., C. Martinez, A. Rodriguez, and F. Jimenez. 1991. Distribution and function of the lethal of scute gene product during early neurogenesis in Drosophila. Development 113: 445-454.

Matakatsu, H., and S. S. Blair. 2008. The DHHC palmitoyltransferase approximated regulates Fat signaling and Dachs localization and activity. Current biology : CB 18: 1390-1395.

Matsuo, T., K. Takahashi, S. Kondo, K. Kaibuchi, and D. Yamamoto. 1997. Regulation of cone cell formation by Canoe and Ras in the developing Drosophila eye. Development 124: 2671-2680.

Maurange, C., and A. P. Gould. 2005. Brainy but not too brainy: starting and stopping neuroblast divisions in Drosophila. Trends in neurosciences 28: 30-36.

McCartney, B. M., R. M. Kulikauskas, D. R. LaJeunesse, and R. G. Fehon. 2000. The neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation. Development 127: 1315-1324.

Mello, C. C., C. Schubert, B. Draper, W. Zhang, R. Lobel, and J. R. Priess. 1996. The PIE-1 protein and germline specification in C. elegans embryos. Nature 382: 710-712.

Miller, E., J. Yang, M. DeRan, C. Wu, A. I. Su, G. M. Bonamy, J. Liu, E. C. Peters, and X. Wu. 2012. Identification of serum-derived sphingosine-1-phosphate as a small molecule regulator of YAP. Chemistry & biology 19: 955-962.

Mo, J. S., F. X. Yu, R. Gong, J. H. Brown, and K. L. Guan. 2012. Regulation of the Hippo-YAP pathway by protease-activated receptors (PARs). Genes & development 26: 2138-2143.

Motegi, F., and A. Sugimoto. 2006. Sequential functioning of the ECT-2 RhoGEF, RHO-1 and CDC-42 establishes cell polarity in Caenorhabditis elegans embryos. Nature cell biology 8: 978-985.

Munro, E., and B. Bowerman. 2009. Cellular symmetry breaking during Caenorhabditis elegans development. Cold Spring Harbor perspectives in biology 1: a003400.

Na, J., and M. Zernicka-Goetz. 2006. Asymmetric positioning and organization of the meiotic spindle of mouse oocytes requires CDC42 function. Current biology : CB 16: 1249-1254.

Neumuller, R. A., and J. A. Knoblich. 2009. Dividing cellular asymmetry: asymmetric cell division and its implications for stem cells and cancer. Genes & development 23: 2675-2699.

Nishimura, T., and K. Kaibuchi. 2007. Numb controls integrin endocytosis for directional cell migration with aPKC and PAR-3. Developmental cell 13: 15-28.

Nishiyama, Y., T. Hirota, T. Morisaki, T. Hara, T. Marumoto, S. Iida, K. Makino, H. Yamamoto, T. Hiraoka, N. Kitamura, and H. Saya. 1999. A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. FEBS letters 459: 159-165.

Ogawa, H., N. Ohta, W. Moon, and F. Matsuzaki. 2009. Protein phosphatase 2A negatively regulates aPKC signaling by modulating phosphorylation of Par-6 in Drosophila neuroblast asymmetric divisions. Journal of cell science 122: 3242-3249.

Oh, H., and K. D. Irvine. 2008. In vivo regulation of Yorkie phosphorylation and localization. Development 135: 1081-1088.

Oh, H., and K. D. Irvine. 2009. In vivo analysis of Yorkie phosphorylation sites. Oncogene 28: 1916-1927.

Oh, H., and K. D. Irvine. 2010. Yorkie: the final destination of Hippo signaling. Trends in cell biology 20: 410-417.

Oh, H., and K. D. Irvine. 2011. Cooperative regulation of growth by Yorkie and Mad through bantam. Developmental cell 20: 109-122.

Pantalacci, S., N. Tapon, and P. Leopold. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nature cell biology 5: 921-927.

Parmentier, M. L., D. Woods, S. Greig, P. G. Phan, A. Radovic, P. Bryant, and C. J. O'Kane. 2000. Rapsynoid/partner of inscuteable controls asymmetric division of larval neuroblasts in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience 20: RC84.

Parsons, L. M., N. A. Grzeschik, M. L. Allott, and H. E. Richardson. 2010. Lgl/aPKC and Crb regulate the Salvador/Warts/Hippo pathway. Fly 4: 288-293.

Patel, N. H., B. G. Condron, and K. Zinn. 1994. Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. Nature 367: 429-434.

Peng, C. Y., L. Manning, R. Albertson, and C. Q. Doe. 2000. The tumour-suppressor genes lgl and dlg regulate basal protein targeting in Drosophila neuroblasts. Nature 408: 596-600.

Peng, H. W., M. Slattery, and R. S. Mann. 2009. Transcription factor choice in the Hippo signaling pathway: homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the Drosophila eye imaginal disc. Genes & development 23: 2307-2319.

Pereira, G., and Y. M. Yamashita. 2011. Fly meets yeast: checking the correct orientation of cell division. Trends in cell biology 21: 526-533.

Peterson, F. C., R. R. Penkert, B. F. Volkman, and K. E. Prehoda. 2004. Cdc42 regulates the Par-6 PDZ domain through an allosteric CRIB-PDZ transition. Molecular cell 13: 665-676.

Petritsch, C., G. Tavosanis, C. W. Turck, L. Y. Jan, and Y. N. Jan. 2003. The Drosophila myosin VI Jaguar is required for basal protein targeting and correct spindle orientation in mitotic neuroblasts. Developmental cell 4: 273-281.

Petronczki, M., and J. A. Knoblich. 2001. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. Nature cell biology 3: 43-49.

Postiglione, M. P., C. Juschke, Y. Xie, G. A. Haas, C. Charalambous, and J. A. Knoblich. 2011. Mouse inscuteable induces apical-basal spindle orientation to facilitate intermediate progenitor generation in the developing neocortex. Neuron 72: 269-284.

Poulson, N. D., and T. Lechler. 2010. Robust control of mitotic spindle orientation in the developing epidermis. The Journal of cell biology 191: 915-922.

Praskova, M., F. Xia, and J. Avruch. 2008. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Current biology : CB 18: 311-321.

Racki, W. J., A. M. Becam, F. Nasr, and C. J. Herbert. 2000. Cbk1p, a protein similar to the human myotonic dystrophy kinase, is essential for normal morphogenesis in Saccharomyces cerevisiae. The EMBO journal 19: 4524-4532.

Rauskolb, C., G. Pan, B. V. Reddy, H. Oh, and K. D. Irvine. 2011. Zyxin links fat signaling to the hippo pathway. PLoS biology 9: e1000624.

Ray, S., and T. Lechler. 2011. Regulation of asymmetric cell division in the epidermis. Cell division 6: 12.

Reichert, H. 2011. Drosophila neural stem cells: cell cycle control of self-renewal, differentiation, and termination in brain development. Results and problems in cell differentiation 53: 529-546.

Rhyu, M. S., L. Y. Jan, and Y. N. Jan. 1994. Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76: 477-491.

Ribeiro, P. S., F. Josue, A. Wepf, M. C. Wehr, O. Rinner, G. Kelly, N. Tapon, and M. Gstaiger. 2010. Combined functional genomic and proteomic approaches identify a PP2A complex as a negative regulator of Hippo signaling. Molecular cell 39: 521-534.

Robinson, B. S., J. Huang, Y. Hong, and K. H. Moberg. 2010. Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein Expanded. Current biology : CB 20: 582-590.

Rogulja, D., C. Rauskolb, and K. D. Irvine. 2008. Morphogen control of wing growth through the Fat signaling pathway. Developmental cell 15: 309-321.

Royer, C., and X. Lu. 2011. Epithelial cell polarity: a major gatekeeper against cancer? Cell death and differentiation 18: 1470-1477.

Rusconi, J. C., and V. Corbin. 1998. Evidence for a novel Notch pathway required for muscle precursor selection in Drosophila. Mechanisms of development 79: 39-50.

Salah, Z., and R. I. Aqeilan. 2011. WW domain interactions regulate the Hippo tumor suppressor pathway. Cell Death Dis 2: e172.

Sansores-Garcia, L., W. Bossuyt, K. Wada, S. Yonemura, C. Tao, H. Sasaki, and G. Halder. 2011. Modulating F-actin organization induces organ growth by affecting the Hippo pathway. The EMBO journal 30: 2325-2335.

Sawyer, J. K., N. J. Harris, K. C. Slep, U. Gaul, and M. Peifer. 2009. The Drosophila afadin homologue Canoe regulates linkage of the actin cytoskeleton to adherens junctions during apical constriction. The Journal of cell biology 186: 57-73.

Schaefer, M., A. Shevchenko, A. Shevchenko, and J. A. Knoblich. 2000. A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in Drosophila. Current biology : CB 10: 353-362.

Schaefer, M., M. Petronczki, D. Dorner, M. Forte, and J. A. Knoblich. 2001. Heterotrimeric G proteins direct two modes of asymmetric cell division in the Drosophila nervous system. Cell 107: 183-194.

Schatten, H., and Q. Y. Sun. 2011. Centrosome dynamics during mammalian oocyte maturation with a focus on meiotic spindle formation. Molecular reproduction and development 78: 757-768.

Schober, M., M. Schaefer, and J. A. Knoblich. 1999. Bazooka recruits Inscuteable to orient asymmetric cell divisions in Drosophila neuroblasts. Nature 402: 548-551.

Schonegg, S., and A. A. Hyman. 2006. CDC-42 and RHO-1 coordinate acto-myosin contractility and PAR protein localization during polarity establishment in C. elegans embryos. Development 133: 3507-3516.

Schubert, C. M., R. Lin, C. J. de Vries, R. H. Plasterk, and J. R. Priess. 2000. MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos. Molecular cell 5: 671-682.

Seydoux, G., C. C. Mello, J. Pettitt, W. B. Wood, J. R. Priess, and A. Fire. 1996. Repression of gene expression in the embryonic germ lineage of C. elegans. Nature 382: 713-716.

Shen, C. P., L. Y. Jan, and Y. N. Jan. 1997. Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. Cell 90: 449-458.

Sheng, M., and C. Sala. 2001. PDZ domains and the organization of supramolecular complexes. Annual review of neuroscience 24: 1-29.

Shitamukai, A., and F. Matsuzaki. 2012. Control of asymmetric cell division of mammalian neural progenitors. Development, growth & differentiation 54: 277-286.

Siegrist, S. E., and C. Q. Doe. 2005. Microtubule-induced Pins/Galphai cortical polarity in Drosophila neuroblasts. Cell 123: 1323-1335.

Siller, K. H., and C. Q. Doe. 2009. Spindle orientation during asymmetric cell division. Nature cell biology 11: 365-374.

Silva, E., Y. Tsatskis, L. Gardano, N. Tapon, and H. McNeill. 2006. The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. Current biology : CB 16: 2081-2089.

Simon, M. A., A. Xu, H. O. Ishikawa, and K. D. Irvine. 2010. Modulation of fat:dachsous binding by the cadherin domain kinase four-jointed. Current biology : CB 20: 811-817.

Simons, M., and M. Mlodzik. 2008. Planar cell polarity signaling: from fly development to human disease. Annual review of genetics 42: 517-540.

Skerker, J. M., and M. T. Laub. 2004. Cell-cycle progression and the generation of asymmetry in Caulobacter crescentus. Nature reviews. Microbiology 2: 325-337.

Slack, C., P. M. Overton, R. I. Tuxworth, and W. Chia. 2007. Asymmetric localisation of Miranda and its cargo proteins during neuroblast division requires the anaphase-promoting complex/cyclosome. Development 134: 3781-3787.

Slaughter, B. D., S. E. Smith, and R. Li. 2009. Symmetry breaking in the life cycle of the budding yeast. Cold Spring Harbor perspectives in biology 1: a003384.

Slovakova, J., and A. Carmena. 2011. Canoe functions at the CNS midline glia in a complex with Shotgun and Wrapper-Nrx-IV during neuron-glia interactions. Development 138: 1563-1571.

Slovakova, J., S. Speicher, N. Sanchez-Soriano, A. Prokop, and A. Carmena. 2012. The actin-binding protein Canoe/AF-6 forms a complex with Robo and is required for Slit-Robo signaling during axon pathfinding at the CNS midline. The Journal of neuroscience : the official journal of the Society for Neuroscience 32: 10035-10044.

Smith, C. A., K. M. Lau, Z. Rahmani, S. E. Dho, G. Brothers, Y. M. She, D. M. Berry, E. Bonneil, P. Thibault, F. Schweisguth, R. Le Borgne, and C. J. McGlade. 2007. aPKC-mediated phosphorylation regulates asymmetric membrane

localization of the cell fate determinant Numb. The EMBO journal 26: 468-480.

Song, X., M. D. Wong, E. Kawase, R. Xi, B. C. Ding, J. J. McCarthy, and T. Xie. 2004. Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development 131: 1353-1364.

Sonoda, J., and R. P. Wharton. 2001. Drosophila Brain Tumor is a translational repressor. Genes & development 15: 762-773.

Sopko, R., E. Silva, L. Clayton, L. Gardano, M. Barrios-Rodiles, J. Wrana, X. Varelas, N. I. Arbouzova, S. Shaw, S. Saburi, H. Matakatsu, S. Blair, and H. McNeill. 2009. Phosphorylation of the tumor suppressor fat is regulated by its ligand Dachsous and the kinase discs overgrown. Current biology : CB 19: 1112-1117.

Sousa-Nunes, R., and W. G. Somers. 2010. Phosphorylation and dephosphorylation events allow for rapid segregation of fate determinants during Drosophila neuroblast asymmetric divisions. Commun Integr Biol 3: 46-49.

Spana, E. P., and C. Q. Doe. 1995. The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. Development 121: 3187-3195.

Sparks, C. A., M. Morphew, and D. McCollum. 1999. Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. The Journal of cell biology 146: 777-790.

Speicher, S., A. Fischer, J. Knoblich, and A. Carmena. 2008. The PDZ protein Canoe regulates the asymmetric division of Drosophila neuroblasts and muscle progenitors. Current biology : CB 18: 831-837.

Speicher, S., L. Garcia-Alonso, A. Carmena, M. D. Martin-Bermudo, S. de la Escalera, and F. Jimenez. 1998. Neurotactin functions in concert with other identified CAMs in growth cone guidance in Drosophila. Neuron 20: 221-233.

Srinivasan, D. G., R. M. Fisk, H. Xu, and S. van den Heuvel. 2003. A complex of LIN-5 and GPR proteins regulates G protein signaling and spindle function in C elegans. Genes & development 17: 1225-1239.

Staley, B. K., and K. D. Irvine. 2012. Hippo signaling in Drosophila: recent advances and insights. Developmental dynamics : an official publication of the American Association of Anatomists 241: 3-15.

Strome, S., and W. B. Wood. 1983. Generation of asymmetry and segregation of germ-line granules in early C. elegans embryos. Cell 35: 15-25.

Subramanian, K., M. R. Paul, and J. J. Tyson. 2013. Potential role of a bistable histidine kinase switch in the asymmetric division cycle of Caulobacter crescentus. PLoS computational biology 9: e1003221.

Sudol, M., and T. Hunter. 2000. NeW wrinkles for an old domain. Cell 103: 1001-1004.

Tabara, H., R. J. Hill, C. C. Mello, J. R. Priess, and Y. Kohara. 1999. pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans. Development 126: 1-11.

Tabuse, Y., Y. Izumi, F. Piano, K. J. Kemphues, J. Miwa, and S. Ohno. 1998. Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. Development 125: 3607-3614.

Takahashi, K., T. Matsuo, T. Katsube, R. Ueda, and D. Yamamoto. 1998. Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in Drosophila morphogenesis. Mechanisms of development 78: 97-111.

Takai, Y., J. Miyoshi, W. Ikeda, and H. Ogita. 2008. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. Nature reviews. Molecular cell biology 9: 603-615.

Tapon, N., K. F. Harvey, D. W. Bell, D. C. Wahrer, T. A. Schiripo, D. Haber, and I. K. Hariharan. 2002. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell 110: 467-478.

Tepass, U. 1996. Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of Drosophila. Developmental biology 177: 217-225.

Toyn, J. H., and L. H. Johnston. 1994. The Dbf2 and Dbf20 protein kinases of budding yeast are activated after the metaphase to anaphase cell cycle transition. The EMBO journal 13: 1103-1113.

Truman, J. W., and M. Bate. 1988. Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. Developmental biology 125: 145-157.

Tsokos, C. G., and M. T. Laub. 2012. Polarity and cell fate asymmetry in Caulobacter crescentus. Current opinion in microbiology 15: 744-750.

Tulina, N., and E. Matunis. 2001. Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science 294: 2546-2549.

Udan, R. S., M. Kango-Singh, R. Nolo, C. Tao, and G. Halder. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nature cell biology 5: 914-920.

Uemura, T., S. Shepherd, L. Ackerman, L. Y. Jan, and Y. N. Jan. 1989. numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell 58: 349-360.

Varelas, X., P. Samavarchi-Tehrani, M. Narimatsu, A. Weiss, K. Cockburn, B. G. Larsen, J. Rossant, and J. L. Wrana. 2010. The Crumbs complex couples

cell density sensing to Hippo-dependent control of the TGF-beta-SMAD pathway. Developmental cell 19: 831-844.

Verde, F., D. J. Wiley, and P. Nurse. 1998. Fission yeast orb6, a ser/thr protein kinase related to mammalian rho kinase and myotonic dystrophy kinase, is required for maintenance of cell polarity and coordinates cell morphogenesis with the cell cycle. Proceedings of the National Academy of Sciences of the United States of America 95: 7526-7531.

Verghese, S., I. Waghmare, H. Kwon, K. Hanes, and M. Kango-Singh. 2012. Scribble acts in the Drosophila fat-hippo pathway to regulate warts activity. PloS one 7: e47173.

Vessey, J. P., G. Amadei, S. E. Burns, M. A. Kiebler, D. R. Kaplan, and F. D. Miller. 2012. An asymmetrically localized Staufen2-dependent RNA complex regulates maintenance of mammalian neural stem cells. Cell stem cell 11: 517-528.

Vinot, S., T. Le, B. Maro, and S. Louvet-Vallee. 2004. Two PAR6 proteins become asymmetrically localized during establishment of polarity in mouse oocytes. Current biology : CB 14: 520-525.

Visintin, R., and A. Amon. 2001. Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. Molecular biology of the cell 12: 2961-2974.

Wai, P., B. Truong, and K. M. Bhat. 1999. Cell division genes promote asymmetric interaction between Numb and Notch in the Drosophila CNS. Development 126: 2759-2770.

Wang, C., K. C. Chang, G. Somers, D. Virshup, B. T. Ang, C. Tang, F. Yu, and H. Wang. 2009. Protein phosphatase 2A regulates self-renewal of Drosophila neural stem cells. Development 136: 2287-2296.

Wang, H., Y. Ouyang, W. G. Somers, W. Chia, and B. Lu. 2007. Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. Nature 449: 96-100.

Wang, H., G. W. Somers, A. Bashirullah, U. Heberlein, F. Yu, and W. Chia. 2006. Aurora-A acts as a tumor suppressor and regulates self-renewal of Drosophila neuroblasts. Genes & development 20: 3453-3463.

Wang, X., J. W. Tsai, B. LaMonica, and A. R. Kriegstein. 2011. A new subtype of progenitor cell in the mouse embryonic neocortex. Nature neuroscience 14: 555-561.

Watt, F. M. 2002. Role of integrins in regulating epidermal adhesion, growth and differentiation. The EMBO journal 21: 3919-3926.

Watts, J. L., B. Etemad-Moghadam, S. Guo, L. Boyd, B. W. Draper, C. C. Mello, J. R. Priess, and K. J. Kemphues. 1996. par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3. Development 122: 3133-3140.

Wee, B., C. A. Johnston, K. E. Prehoda, and C. Q. Doe. 2011. Canoe binds RanGTP to promote Pins(TPR)/Mud-mediated spindle orientation. The Journal of cell biology 195: 369-376.

Wei, S. Y., L. M. Escudero, F. Yu, L. H. Chang, L. Y. Chen, Y. H. Ho, C. M. Lin, C. S. Chou, W. Chia, J. Modolell, and J. C. Hsu. 2005. Echinoid is a component of adherens junctions that cooperates with DE-Cadherin to mediate cell adhesion. Developmental cell 8: 493-504.

Willecke, M., F. Hamaratoglu, M. Kango-Singh, R. Udan, C. L. Chen, C. Tao, X. Zhang, and G. Halder. 2006. The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. Current biology : CB 16: 2090-2100.

Wirtz-Peitz, F., T. Nishimura, and J. A. Knoblich. 2008. Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. Cell 135: 161-173.

Wodarz, A., and W. B. Huttner. 2003. Asymmetric cell division during neurogenesis in Drosophila and vertebrates. Mechanisms of development 120: 1297-1309.

Wodarz, A., A. Ramrath, U. Kuchinke, and E. Knust. 1999. Bazooka provides an apical cue for Inscuteable localization in Drosophila neuroblasts. Nature 402: 544-547.

Wodarz, A., A. Ramrath, A. Grimm, and E. Knust. 2000. Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. The Journal of cell biology 150: 1361-1374.

Wu, S., J. Huang, J. Dong, and D. Pan. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell 114: 445-456.

Wu, S., Y. Liu, Y. Zheng, J. Dong, and D. Pan. 2008. The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Developmental cell 14: 388-398.

Xu, T., W. Wang, S. Zhang, R. A. Stewart, and W. Yu. 1995. Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development 121: 1053-1063.

Yu, F., X. Morin, Y. Cai, X. Yang, and W. Chia. 2000. Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization. Cell 100: 399-409.

Yu, F., Y. Cai, R. Kaushik, X. Yang, and W. Chia. 2003. Distinct roles of Galphai and Gbeta13F subunits of the heterotrimeric G protein complex in the mediation of Drosophila neuroblast asymmetric divisions. The Journal of cell biology 162: 623-633.

Yu, F. X., and K. L. Guan. 2013. The Hippo pathway: regulators and regulations. Genes & development 27: 355-371.

Yu, F. X., B. Zhao, N. Panupinthu, J. L. Jewell, I. Lian, L. H. Wang, J. Zhao, H. Yuan, K. Tumaneng, H. Li, X. D. Fu, G. B. Mills, and K. L. Guan. 2012. Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. Cell 150: 780-791.

Yu, J., Y. Zheng, J. Dong, S. Klusza, W. M. Deng, and D. Pan. 2010. Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. Developmental cell 18: 288-299.

Zhao, B., L. Li, K. Tumaneng, C. Y. Wang, and K. L. Guan. 2010. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). Genes & development 24: 72-85.

Zhao, B., L. Li, L. Wang, C. Y. Wang, J. Yu, and K. L. Guan. 2012. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes & development 26: 54-68.

Zhao, B., X. Wei, W. Li, R. S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z. C. Lai, and K. L. Guan. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes & development 21: 2747-2761.

Ziff, E. B. 1997. Enlightening the postsynaptic density. Neuron 19: 1163-1174.

The Rap1–Rgl–Ral signaling network regulates neuroblast cortical polarity and spindle orientation

Ana Carmena, Aljona Makarova, and Stephan Speicher

Instituto de Neurociencias de Alicante, Centro Superior de Investigaciones Científicas/Universidad Miguel Hernández, 03550 Alicante, Spain

A crucial first step in asymmetric cell division is to establish an axis of cell polarity along which the mitotic spindle aligns. *Drosophila melanogaster* neural stem cells, called neuroblasts (NBs), divide asymmetrically through intrinsic polarity cues, which regulate spindle orientation and cortical polarity. In this paper, we show that the Ras-like small guanosine triphosphatase Rap1 signals through the Ral guanine nucleotide exchange factor Rgl and the PDZ protein Canoe (Cno; AF-6/Afadin in vertebrates) to modulate the NB division axis and its apicobasal cortical polarity. Rap1 is slightly enriched at the apical pole of metaphase/anaphase NBs and was found in a complex with atypical protein kinase C and Par6 in vivo. Loss of function and gain of function of Rap1, Rgl, and Ral proteins disrupt the mitotic axis orientation, the localization of Cno and Mushroom body defect, and the localization of cell fate determinants. We propose that the Rap1–Rgl–Ral signaling network is a novel mechanism that cooperates with other intrinsic polarity cues to modulate asymmetric NB division.

Introduction

Asymmetric cell division is a key process in development and stem cell biology. In an asymmetric cell division, one daughter cell retains the self-renewal capacity of the mother stem cell and keeps on dividing, whereas the other daughter cell is committed to initiating a differentiation program. A crucial first step in an asymmetric cell division is to establish an axis of cell polarity along which the mitotic spindle aligns. Extrinsic and intrinsic mechanisms regulate the spindle orientation and the final asymmetry of the division. *Drosophila melanogaster* stem cells have been extensively studied during the last few decades, providing a deep insight into both types of mechanisms (Doe, 2008; Knoblich, 2008; Morrison and Spradling, 2008). *Drosophila* neural stem cells, called neuroblasts (NBs), divide asymmetrically, mainly through intrinsic polarity cues. In the embryonic central nervous system (CNS), NBs delaminate from the neuroectoderm (NE) inheriting the apicobasal polarity of the neuroepithelial cells. Intrinsic signals, mostly polarized at the apical NB cortex, tightly couple the spindle orientation along the apicobasal axis with the asymmetric location of cell fate determinants at the basal pole of the NB. In this way, these determinants are secreted to the basal and smaller daughter cell, called the ganglion mother cell (GMC). The apical and bigger daughter cell continues dividing as an NB, always budding off smaller GMCs into the embryo in the same, highly stereotyped, basal orientation (Wodarz and Huttner, 2003; Chia et al., 2008; Knoblich, 2008; Siller and Doe, 2009). Extrinsic signals emanating from the NE also participate in regulating spindle orientation and cortical polarity in the NB, though the nature of these signals remains elusive (Siegrist and Doe, 2006).

Here, we show that the Ras-like small GTPase Rap1 contributes to regulate asymmetric NB division through the Ral guanine nucleotide exchange factor Rgl, Ral, and the PDZ domain–containing protein Canoe (Cno; AF-6/Afadin in vertebrates; Miyamoto et al., 1995; Asha et al., 1999; Mirey et al., 2003). Rap1 has a key and evolutionary conserved role in regulating morphogenesis, integrin- as well as cadherin-mediated cell–cell adhesion, and junction formation. In addition, Rap1 has adhesion-independent functions that suggest a central function of Rap1 in signal transduction (Asha et al., 1999; Knox and Brown, 2002; Caron, 2003; Mirey et al., 2003; Price et al., 2004; Wang et al., 2006; Kooistra et al., 2007; O'Keefe et al., 2009). Ral proteins are Ras-like GTPases that can be activated through a Ras-dependent mechanism in mammalian cell lines (Yaffe et al., 2001) and downstream of Rap1–Rgl in *Drosophila*

Correspondence to Ana Carmena: acarmena@umh.es

Abbreviations used in this paper: aPKC, atypical PKC; Baz, Bazooka; Cnn, centrosomine; Cno, Canoe; CNS, central nervous system; DE-cad, *Drosophila* E-cadherin; GMC, ganglion mother cell; Insc, Inscuteable; L'sc, Lethal of Scute; Mira, Miranda; mNB, metaphase NB; Mud, Mushroom body defect; NB, neuroblast; NE, neuroectoderm; Pins, Partner of Insc; Pros, Prospero; Scrib, Scribbled; WT, wild type.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 195 No. 4 553–562
www.jcb.org/cgi/doi/10.1083/jcb.201108112

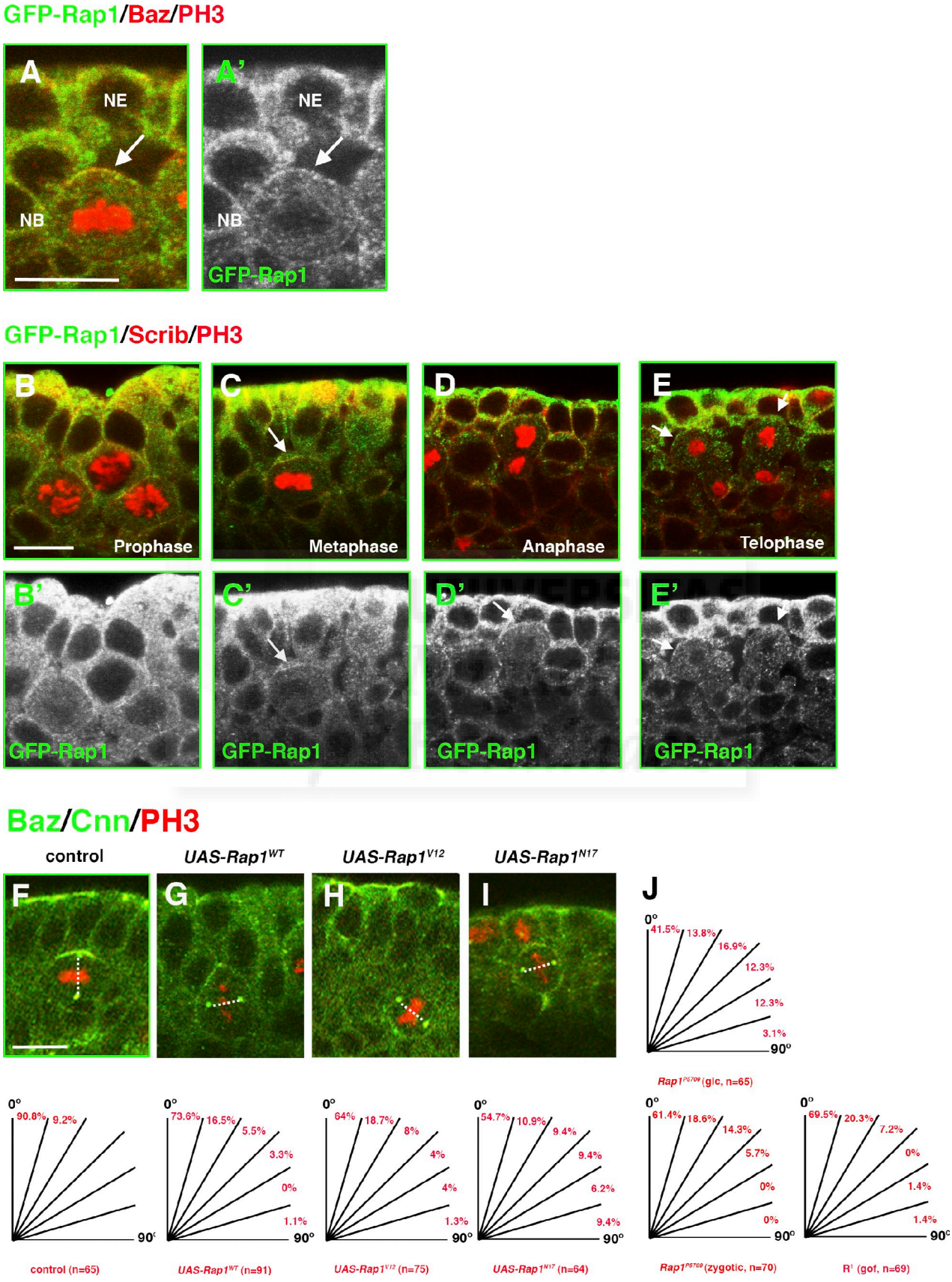


Figure 1. **Rap1 is present in mNBs, and it is required for spindle orientation.** (A and A′) GFP-Rap1 appears in a punctate pattern, which is uniformly distributed in the NE and enriched at the apical pole of NBs (arrows), where Rap1 colocalizes with Baz. The DNA (PH3) is in red. (B–E′) GFP-Rap1 location throughout mitosis. (B and B′) At prophase, GFP-Rap1 is mainly cytoplasmic. (C–D′) At metaphase, it accumulates at the NB apical pole (C and C′),

(Mirey et al., 2003). The Rap/Ras–Rgl–Ral GTPase signaling network is highly conserved between *Drosophila* and mammals (Moskalenko et al., 2001; Mirey et al., 2003). Intriguingly, Rap1 interacts physically with Cno/AF-6, and the Ral guanine nucleotide exchange factor Rgl has been predicted as a potential partner of Cno (Drosophila Interactions Database; Boettner et al., 2000, 2003), a novel regulator of asymmetric NB division (Speicher et al., 2008). Our results now show that loss and gain of function of Rap1, Rgl, and Ral proteins affect the NB spindle orientation, the generation of unequal-sized progeny, and the localization of apical proteins, such as Cno and the microtubule-associated protein Mushroom body defect (Mud; Numa in vertebrates; Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Bazooka (Baz; Par3 in vertebrates) and the atypical PKC (aPKC; Schober et al., 1999; Wodarz et al., 1999, 2000) were affected to a lesser degree. Failures in the basal localization of the cell fate determinants Numb, Prospero (Pros), and its adaptor protein Miranda (Mira; Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Schuldt et al., 1998) were also detected in *Rap1* and *Ral* mutants. Moreover, co-immunoprecipitation experiments from embryo extracts showed that Rap1 is in a complex with aPKC and Par6. Additionally, Rgl synergistically cooperated with other apical proteins, such as Partner of Inscuteable (Insc; Pins), Insc, and Mud to regulate spindle orientation. Taking all data into account, we propose that the Rap1–Rgl–Ral signaling network is a novel intrinsic mechanism that cooperates with other apical proteins to regulate cortical polarity and spindle orientation in NBs.

Results and discussion

Rap1 is present in the embryonic neuroepithelium and in NBs

In an attempt to further characterize the protein network that along with Cno modulates asymmetric NB division (Speicher et al., 2008), we wanted to analyze in detail the expression and function of the Cno-interacting partner Rap1 (Boettner et al., 2000). GFP-Rap1, a fusion protein that is expressed under the control of the endogenous *Rap1* promoter (Knox and Brown, 2002), was detected throughout the NE, evenly distributed in the cytoplasm, and in the delaminated metaphase NBs (mNBs), with a slight enrichment at the apical pole along with the Baz apical crescent (Fig. 1, A and A′). Looking in more detail throughout the NB mitotic cycle, GFP-Rap1 was found uniformly distributed at prophase and started to accumulate apically at metaphase (Fig. 1, B–C′). During anaphase, this apical enrichment was still detected, and by telophase, GFP-Rap1 began to delocalize (Fig. 1, D–E′). Hence, Rap1 was a potential candidate for regulating the process of asymmetric NB division.

Rap1 is required for the mitotic axis orientation and apical proteins localization in mNBs

To determine a possible function of Rap1 in the cell division axis orientation, we analyzed the effect of expressing wild-type (WT), constitutively active (V12), and dominant-negative (N17) forms of Rap1 (hereafter referred to as *Rap1* mutants). A maternal Gal-4 line (V32) was used to drive expression of these transgenes. The spindle orientation was altered when the Rap1 signal was impaired, in clear contrast with control embryos, in which most NBs showed a normal spindle orientation along the apicobasal axis of cell polarity (Fig. 1, F–I). To further support these results, we decided to look at additional *Rap1* mutant conditions: $Rap1^{P5709}$ germline clones, a complete loss of maternal and zygotic product, $Rap1^{P5709}$-only zygotic loss, and R^{1} (*Roughened*^{1}), a gain-of-function mutation in the *Rap1* locus (Hariharan et al., 1991). Clear defects in the spindle orientation were detected in all these mutants (Fig. 1 J). Apical cortical polarity was also affected in *Rap1* mutants. The Par complex proteins Baz and aPKC were mislocalized in 10.1% of mNBs (n = 178) and 1.4% (n = 143) in *UAS-*$Rap1^{WT}$ embryos, respectively, in 11.2% (n = 161) and 11.3% (n = 115) in *UAS-*$Rap1^{V12}$ embryos, and in 28.8% (n = 145) and 19.8% (n = 101) in *UAS-*$Rap1^{N17}$ embryos (Fig. 1, G–I). No defects in Baz (n = 65 mNBs) or aPKC (n = 66) were observed in control embryos. Much more penetrant phenotypes were observed for the apical proteins Cno and Mud in those mutant backgrounds (Fig. 2, A–I). The localization of the Gαi subunit and Pins, other apical proteins key for regulating spindle alignment (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000), were, however, not affected (Fig. 2, J–M; and not depicted). Hence, Rap1 is required for the correct establishment of cortical polarity and spindle orientation through a pathway that includes Cno and Mud but is Gαi and Pins independent.

Rap1 mutants show cell fate determinant mislocalization and equal-sized daughter cells

Given the defective localization of apical proteins we observed in *Rap1* mutants, we predicted that cell fate determinants would be misplaced in mNBs. In fact, whereas in control embryos Numb was found in basal crescents in most mNBs, clear defects in Numb localization were detected in *Rap1* mutants, including $Rap1^{P5709}$ germline clones and R^{1} mutant conditions (Fig. 3, A–Q). Another cell fate determinant, the transcription factor Pros and its adaptor protein Mira also showed altered location in a significant number of the mNBs analyzed compared with control embryos (Fig. 3, R–Y). These defects were partially or completely rescued at telophase (compensatory mechanism known as telophase rescue; Fig. 3 Y). Therefore, Rap1 is required for the correct establishment of apical polarity in the NB and for the proper location

where it still can be detected at anaphase (D and D′). (E and E′) At telophase, GFP-Rap1 starts to delocalize. (F–I, top) Cnn at the centrosomes reveals spindle orientation. Arrows point to GFP-Rap1 at the apical pole of the dividing NB. (F) In control embryos, the mitotic spindle (depicted by dotted lines) orientates along the apicobasal axis. (G–I) In *Rap1* mutants, the spindle was misorientated. (F–I, bottom) Percentages of spindles aligned in each 15° bin for the genotypes indicated. (J) The same analysis is shown in additional *Rap1* mutant conditions (see also Rap1 is required for the mitotic axis orientation and apical protein localization in mNBs in the Results). glc, germline clone; gof, gain of function. Bars, 10 µm.

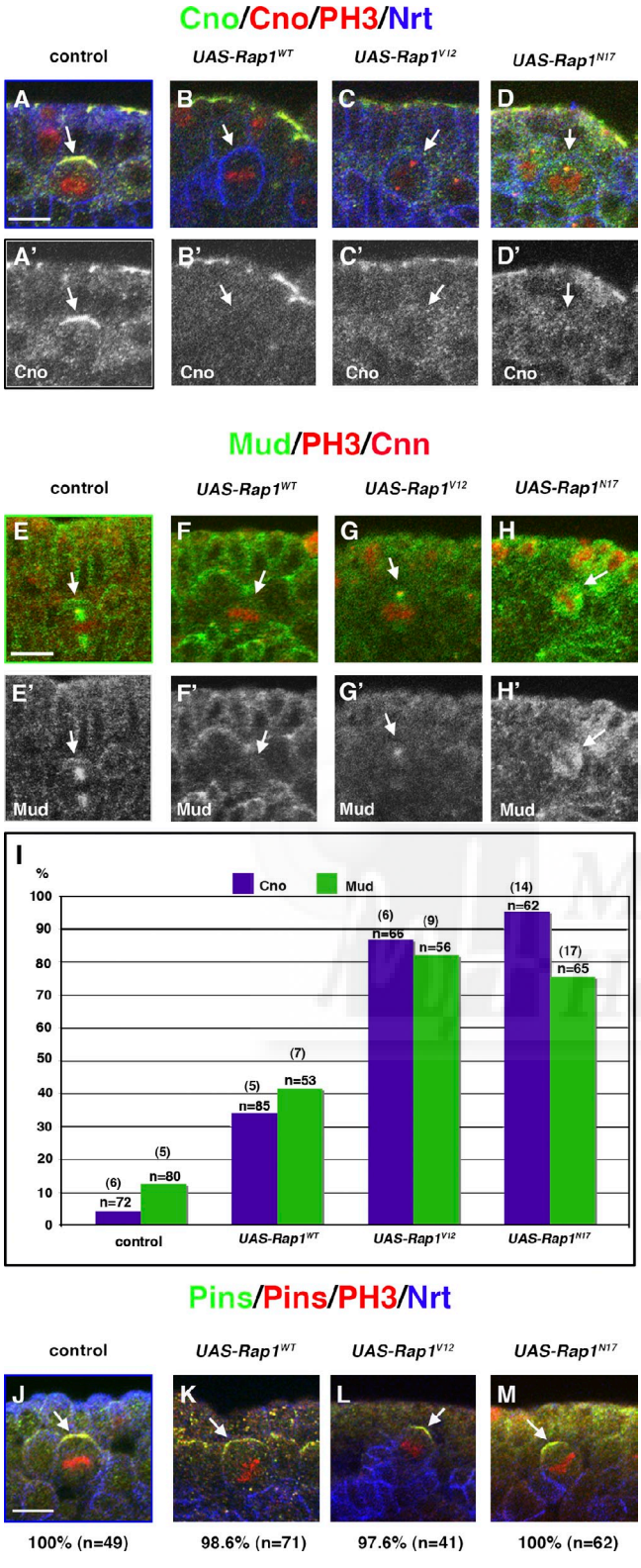


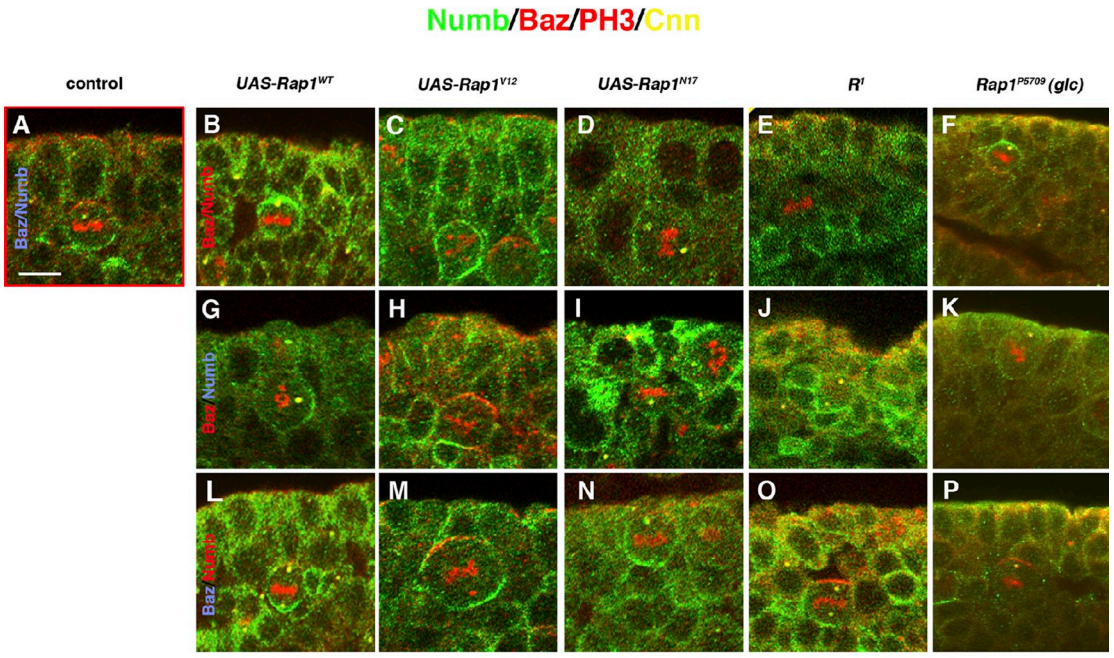
Figure 2. **Rap1 is required for the proper localization of apical proteins in mNBs.** (A and A′) In control embryos, Cno (in yellow: green plus red channels) appears in an apical crescent in mNBs (arrows). (B–D′) In *Rap1* mutants, Cno was absent (B–C′) or mislocalized (D and D′). Note that Cno location is not affected in the NE. Cnn is only shown in C and D. Neurotactin (Nrt) labels membranes. (E and E′) In control embryos, Mud appears apically in mNBs (arrows) as well as associated with centrosomes and microtubules. (F–H′) In *Rap1* mutants, the apical location of Mud frequently fails (arrows). (I) Quantitations of Cno- and Mud-defective localization in *Rap1* mutants. The experiment was completed once (n = total number of mNBs analyzed; number of embryos analyzed in each case is shown between brackets). (J–M) Pins location is not altered in *Rap1* mutants. Arrows point to Pins at the apical pole of the mNB. Percentages indicate the frequency of Pins correct localization. Bars, 10 µm.

of asymmetrically segregating factors. Mud, whose distribution was altered in *Rap1* mutants, is not required for apicobasal cortical polarity (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Cabernard and Doe, 2009). Hence, the failure in cell fate determinant location in *Rap1* mutants was caused, at least in part, by the mislocalization in these mutants of Cno and aPKC/Baz, all key factors for the proper formation of determinant basal crescents in mNBs (Speicher et al., 2008; Wirtz-Peitz et al., 2008). Intriguingly, another distinctive feature of asymmetric NB division, the generation of unequal-sized daughter cells, was also altered in *Rap1* mutants (Fig. S1, A–D). In addition, the asymmetric division within the well-characterized RP2 neuron lineage (Broadus et al., 1995) was affected in these mutants (Fig. S1, E–H). Hence, Rap1 regulates multiple aspects of the asymmetric NB division.

Given that Rap1 is required for a proper cell–cell adhesion in epithelial tissues and that this might influence the analysis of the underlying NBs in *Rap1* mutants, we looked in detail to epithelial cell polarity in $Rap1^{V12}$ and $Rap1^{N17}$ mutant embryos (Fig. S2). Different polarity proteins, such as *Drosophila* E-cadherin (DE-cad), aPKC, and Scribble (Scrib), were analyzed. In $Rap1^{V12}$ mutants, aPKC was reduced, but DE-cad and Scrib were not affected. Similar results were found in $Rap1^{N17}$, though in this case, clear defects in the morphology/integrity of the epithelia were observed. Remarkably, however, no correlation was found between epithelial morphology and NB polarity/orientation defects in these mutants (for an example see Fig. S2, C, C′, F, and F′). From this analysis, we conclude that Rap1 is required for both processes, epithelial integrity and NB polarity, and that these functions are independent. Additionally, to discard that the defects observed in *Rap1* mutant NBs were caused by earlier defects in NB specification, we analyzed in detail the process of NB delamination at stage 9 in these mutants. In $Rap1^{V12}$, NBs were organized in the windowlike arrangement typical of this stage in WT embryos (Fig. S3, A and D). About 31% of $Rap1^{N17}$ mutant embryos showed NB disorganization, probably caused by the early role of Rap1 in morphogenetic events, such as gastrulation (Fig. S3, G and H; see also Fig. 5 in Asha et al. [1999]). In a percentage of $Rap1^{V12}$ and $Rap1^{N17}$ mutant embryos, the number of NBs seemed also to be affected, but importantly, NB delamination was properly achieved in both cases (Fig. S3, compare E, F, and I–K with B and C). Thus, NB alterations in polarity and spindle orientation are not merely a consequence of an impaired NB specification process.

Rgl and *Ral* mutants display abnormal NB spindle orientation and apicobasal cortical polarity

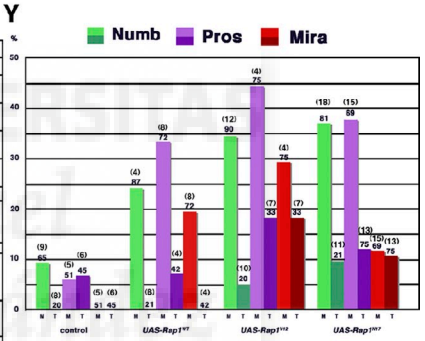
Given the phenotypes we found in *Rap1* mutants in the CNS NBs, we wondered whether the Rap1 effectors Rgl and Ral also had a function in this system. We found that *ΔRgl* mutant embryos



Q

	control	UAS-Rap1WT	UAS-Rap1V12	UAS-Rap1N17	R1	Rap1P5709 (glc)
n (#NBs)	65	87	90	81	69	65
Baz/Numb	90.8% (59/65)	73.6% (64/87)	64.4% (58/90)	61.7% (50/81)	53.6% (37/69)	41.5% (27/65)
Baz/Numb	9.2% (6/65)	0% (0/6)	26.4% (23/87)	47.8% (11/23)	35.5% (32/90)	31.2% (10/32)
Baz/Numb		8.7% (2/23)		3.1% (1/32)		3.2% (1/31)
Baz/Numb		43.5% (10/23)		68.8% (21/32)		22.6% (7/31)
Baz/Numb		0% (0/6)		74.2% (23/31)	38.3% (31/90)	3.2% (1/31)
Baz/Numb		100% (6/6)		25.8% (8/31)	46.4% (32/69)	3.1% (1/32)
Baz/Numb				18.7% (6/32)		78.1 (25/32)
Baz/Numb				68.4% (26/38)	58.5% (38/65)	5.3% (2/38)
Baz/Numb				31.6% (12/38)		26.3% (10/38)
Baz Failures	0% (0/65)	14.9% (13/87)	12.2% (11/90)	29.6% (24/81)	10.1% (7/69)	43.1% (28/65)
	NP CP CM	NP CP CM	NP CP CM	NP CP CM	NP CP CM	NP CP CM
	0 0 0	13 0 0	6 0 5	22 0 2	6 0 1	21 0 7
Numb Failures	9.2% (6/65)	24.1% (21/87)	34.4% (31/90)	37% (30/81)	44.9% (31/69)	55.4% (36/65)
	NP CP CM	NP CP CM	NP CP CM	NP CP CM	NP CP CM	NP CP CM
	1 0 5	5 0 16	1 0 30	6 3 21	10 0 21	22 1 13

Y



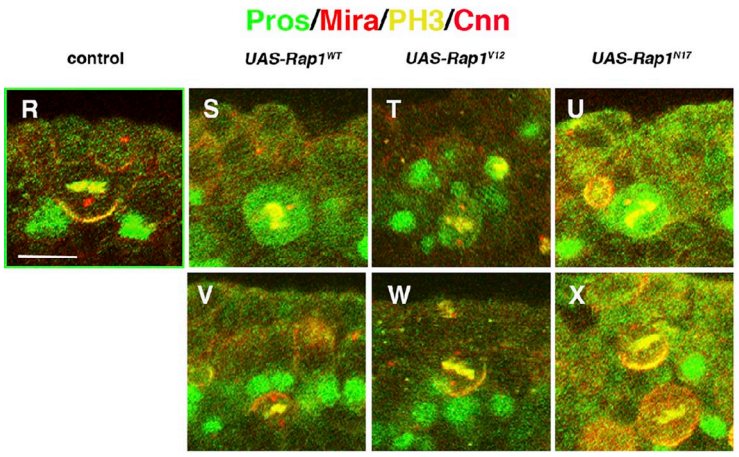


Figure 3. ***Rap1* mutants show cell fate determinant mislocalization.** (A) In control embryos, Numb appears at basal crescents in mNBs, just opposite to the Baz apical crescent. (B–P) In *Rap1* mutants, Numb location failed. Three examples of each mutant phenotype are shown: (1) Baz and Numb have wrong locations (Baz/Numb, first row); (2) only Baz is mislocalized (Baz/Numb, second row); and (3) only Numb is mislocalized (Baz/Numb, third row). (Q) Quantitations of these three phenotypes. The particular defects observed in the *Rap1* mutants specified are described: NP, not present; CP, cytoplasmic; CM, cortical mislocalization. (R) In control embryos, Pros and Mira colocalize in a basal crescent in mNBs. PH3 is in red/green channels. (S–X) In *Rap1* mutants (two examples are shown of each mutant genotype), Pros and Mira localization was altered. They were found at the cytoplasm (S–U and X), not present (S–V), or mislocalized (V and W). (Y) Quantitations of Pros, Mira, and Numb location failures in *Rap1* mutants at metaphase and at telophase (telophase rescue is shown by darker color bars). The experiment was completed once (n = total number of mNBs analyzed; number of embryos analyzed in each case is shown between brackets). M, metaphase; T, telophase; glc, germline clone. Bars, 10 μm.

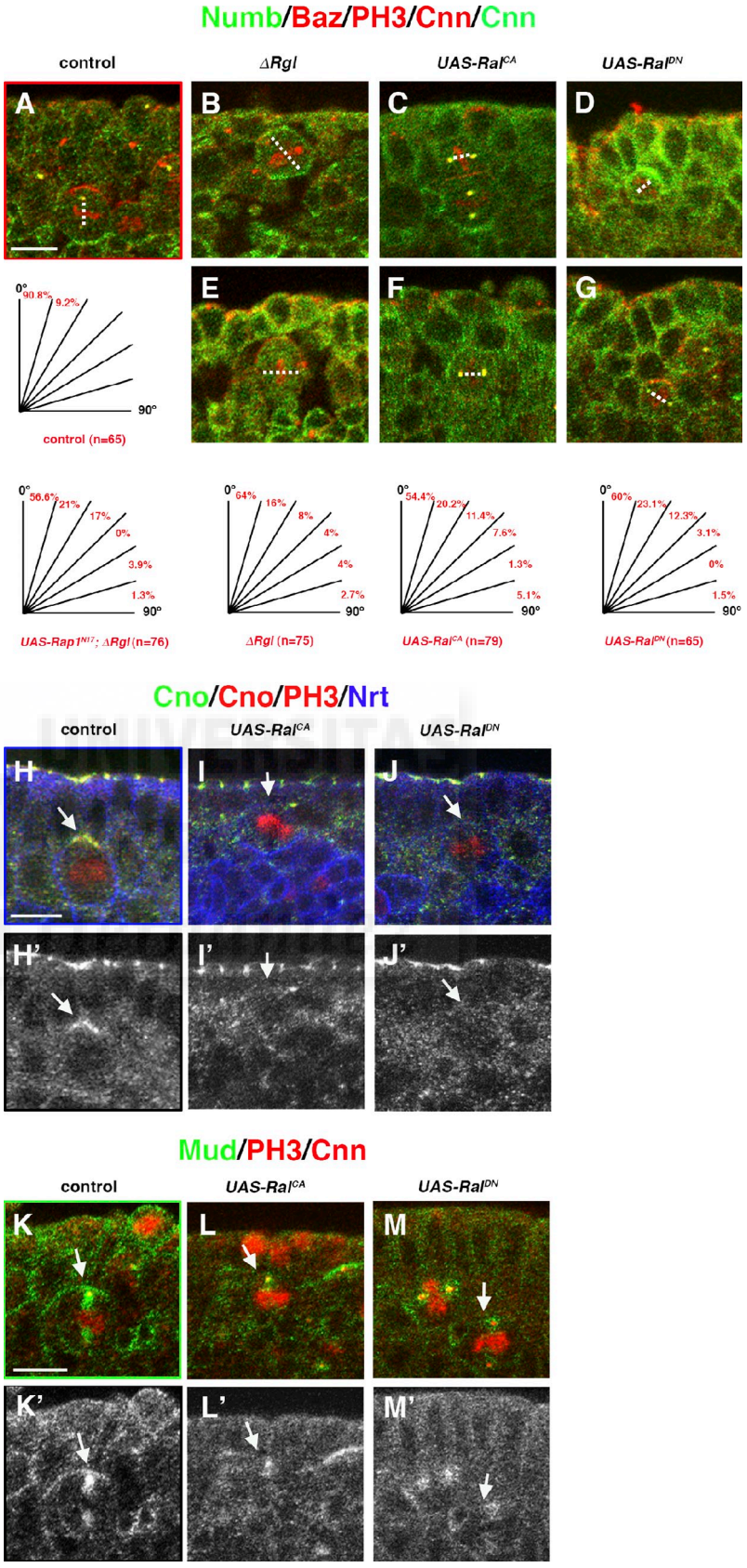


Figure 4. ***Rgl* and *Ral* mutants display abnormal NB spindle orientation and apicobasal cortical polarity.** (A) In control embryos, Baz and Numb form crescents at the apical and basal poles of mNBs, respectively, and the spindle aligns along the apicobasal axis (dotted lines). (B–G) In *Rgl* and *Ral* mutants, the localization of Baz and Numb was altered (two different examples of each genotype are shown). The spindle orientation also failed in *Rgl* and *Ral* mutants. Bottom diagrams show the percentages of spindles aligned in each 15° bin for each genotype analyzed. This analysis was also performed in *UAS-Rap1*^{N17}*; ΔRgl* double mutant embryos (see also *Rgl* and *Ral* mutants display abnormal NB spindle orientation and apicobasal cortical polarity in the Results). (H–M′) The location of Cno (H–J′) and Mud (K–M′) failed in *Ral* mutants. Arrows point to the apical pole of the mNB. Nrt, Neurotactin. Bars, 10 µm.

(Mirey et al., 2003) displayed mitotic spindle misorientation (Fig. 4, B and E), the same phenotype that showed embryos expressing activated (*Ral*^{CA}) and dominant-negative forms (*Ral*^{DN}) of Ral (Fig. 4, C, D, F, and G). Moreover, double mutants *Rap1*^{N17}; *ΔRgl* showed a spindle phenotype more similar to that shown by *ΔRgl* single mutants (Fig. 4), suggesting that Rgl is

acting in the same pathway of Rap1, downstream of it (Fig. 1 I). Additionally, in both *ΔRgl* and *Ral* mutants, the apicobasal cortical polarity was altered. Baz was misplaced in 9.3% of the *ΔRgl* NBs analyzed (n = 75; 0% in control embryos, n = 65) and in 16.4% (n = 79) and 15.4% (n = 65) of NBs in Ral^{CA} or Ral^{DN} embryos (Fig. 4, B–G). The localization of Cno and Mud also showed (similar to that observed in *Rap1* mutants) more penetrant phenotypes. Although in control embryos, 4.2 and 12.5% of mNBs displayed failures in Cno and Mud localization, respectively, in Ral^{CA} mutants, the percentages were 71% of NBs (n = 62) for Cno localization and 51.2% for Mud (n = 80; Fig. 4, H–I′ and K–L′). In Ral^{DN} mutant embryos, the failures observed corresponded to 76.8% (n = 56) for Cno and 64.3% (n = 56) for Mud (Fig. 4, J, J′, M, and M′). Finally, the basal localization of Numb was also affected in *ΔRgl*, in 48% of mNBs (n = 75; Fig. 4, B and E), and in Ral mutants. Specifically, in Ral^{CA} mutants, Numb failures were 27.8% (n = 79) and 29.2% (n = 65) in Ral^{DN} mutant embryos (Fig. 4, C, D, F, and G). Thus, Rgl–Ral functions in the embryonic NBs of the CNS to regulate cortical polarity and spindle orientation downstream of Rap1.

The Rap1–Rgl–Ral signaling network functions in a complex with aPKC and Par6 cooperating with other apical proteins to regulate asymmetric NB division

Here, we have shown that Rap1 functions in asymmetric NB division in the embryonic CNS to modulate NB apicobasal cortical polarity and mitotic axis orientation. Rap1 would act through the Rgl–Ral–Cno signaling network to regulate Mud localization and, hence, spindle alignment. The localization of the apical protein Pins, which is attached to the cortex through the heterotrimeric Gαi subunit (Fig. 5 F; Schaefer et al., 2000; Nipper et al., 2007), was not dependent on Rap1 signaling and neither was the location on the Gαi subunit. Pins, through Discs Large and Khc-73, is key for spindle location (Siegrist and Doe, 2005; Johnston et al., 2009). Hence, both pathways, Rap1–Rgl–Ral and Gαi–Pins could cooperate to properly orientate the mitotic axis. To test this possibility, we analyzed the spindle orientation in double mutants *ΔRgl*, $pins^{\Delta 50}$. In this genetic background, 46.4% of the mNBs (n = 69) showed a correct spindle alignment (0–15° window) compared with the 64.0% (n = 75; P = 0.0439) and the 66.1% (n = 56; P = 0.0314) of mNB defects found in *ΔRgl* or $pins^{\Delta 50}$ single mutants, respectively (Fig. 5 A). In addition, $insc^{P49}$; *ΔRgl* and mud^{4}; *ΔRgl* double mutants also showed a strong cooperation in regulating spindle orientation (Fig. 5, B and C). In $insc^{P49}$; *ΔRgl* double mutants, 36.1% of the mNBs (n = 72) displayed a WT spindle orientation (0–15° window) versus the 64.0% (n = 75; P = 0.0009) and the 57.9% (n = 57; P = 0.0204) of mNB failures observed in the single mutants *ΔRgl* and $insc^{P49}$, respectively. The interaction between *Rgl* and *mud* was statistically significant when comparing the expressivity, not the penetrance, of the phenotype. In other words, mud^{4}; *ΔRgl* double mutants showed many more cases of spindle alignment defects in the 75–90° window (11.8%, n = 59) compared with the single mutants *ΔRgl* (2.7%, n = 75; P = 0.0429) and mud^{4} (1.6%, n = 63; P = 0286). This last interaction between *mud* and *Rgl* suggests that the Rap1–Rgl–Ral pathway is modulating something else, independent of Mud, which is important for spindle alignment. It has previously been reported that there are two independent and redundant apical pathways in NBs. One of these pathways is formed by Baz, aPKC, Par6, and Insc, and the other pathway is formed by Gαi–Pins (Cai et al., 2003). Even though there are some interdependence between both pathways at prophase for their apical location (Yu et al., 2000), they become much more independent from metaphase onwards. For example, Pins localizes asymmetrically in 81% of the *insc* mutant mNBs analyzed (Cai et al., 2003). This would explain why the Baz and aPKC failures found in *Rap1* mutants, only at metaphase, are not affecting Pins location. Hence, the Rap1–Rgl–Ral pathway synergistically cooperates with other apical polarity cues to correctly position the mitotic spindle.

The Rap1 signaling network also contributes to the establishment of the cortical polarity and, consequently, to the proper segregation of determinants at the basal NB pole. This effect seems to be mainly mediated through its interacting partner Cno, which has been shown to be required for this process (Speicher et al., 2008). Trying to understand how Rap1 is initially polarized at the apical NB pole, we performed in vivo coimmunoprecipitation assays with GFP-Rap1 and different apical proteins. Although Pins, Gαi, or Mud did not show any positive result, we found that both aPKC and Par6 were able to coimmunoprecipitate with Rap1 (Fig. 5 D). Thus, these Par complex proteins can help to locate Rap1 at the apical NB pole. The localization of these proteins, Baz and aPKC, also key for cell fate determinant localization, were altered to a lesser degree in *Rap1* mutant mNBs. This effect of Rap1–Rgl–Ral on Baz and aPKC may respond to the establishment in normal conditions of a positive feedback loop of the Rap1 pathway on the Par protein complex (Par6-Baz/Par3–aPKC) to facilitate their stabilization at metaphase (Fig. 5 E). Intriguingly, Rap1B has been shown to act upstream of this complex and of Cdc42 to regulate neuronal polarity in mammalian cell cultures (Schwamborn and Püschel, 2004). The low penetrant phenotypes observed for Baz and aPKC location in *Rap1* mutants suggests though that Rap1 signal is not the major driving force initially positioning the Par proteins. This might be driven, at least in part, by extrinsic signals coming from the NE (Siegrist and Doe, 2006). The nature of those extrinsic cues remains, however, elusive.

Materials and methods

Drosophila strains and genetics

The following mutant stocks and fly lines were used: *GFP-Rap1* (Knox and Brown, 2002), *UAS-Rap1*^{WT}, *UAS-Rap1*^{V12}, and *UAS-Rap1*^{N17} (a gift from R. Reuter, Universität Tübingen, Tübingen, Germany), $Rap1^{P5709}$ (a gift from N. Brown, University of Cambridge, Cambridge, England, UK), R^{1} (Bloomington Stock Center), $pins^{\Delta 50}$ (Schaefer et al., 2000), $insc^{P49}$ (Bloomington Stock Center), mud^{4} (Bloomington Stock Center), *UAS-Ral*^{CA}, *UAS-Ral*^{DN}, *ΔRgl* (a gift from J. Camonis, Institut Curie, Institut National de la Santé et de la Recherche Médicale, Paris, France; Mirey et al., 2003), and *maternal-GAL4 V32* (a gift from J.A. Knoblich, Institute of Molecular Biotechnology, Vienna, Austria). All the crosses of *GAL4-UAS* were performed at 29°C. *yw* was used as the reference control WT strain. Balancer chromosomes containing different *lacZ* or *GFP* transgenes were used for identifying homozygous mutant embryos.

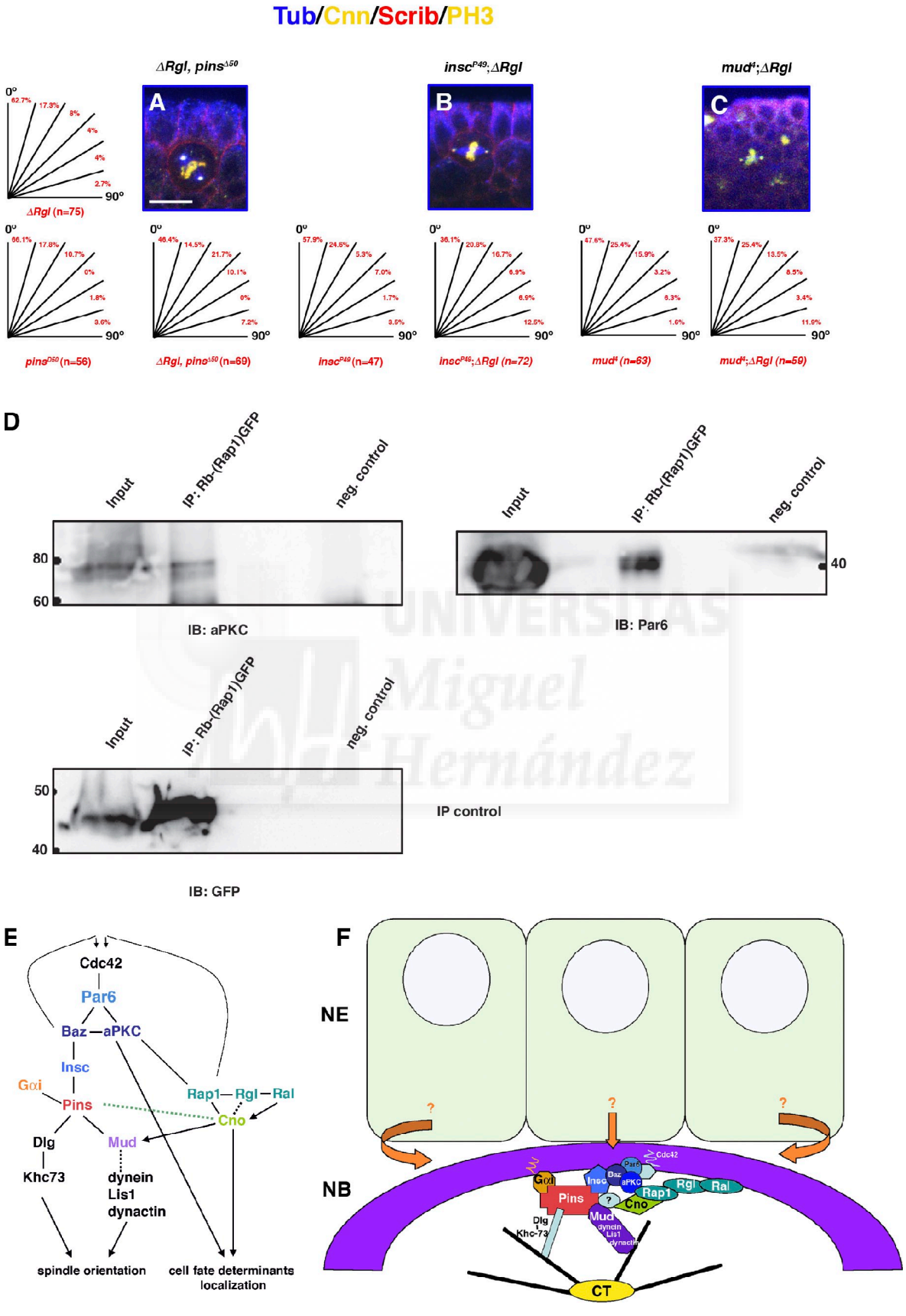


Figure 5. **Rap1–Rgl–Ral network functions in a complex with aPKC and Par6 cooperating with other apical proteins to regulate asymmetric NB division.** (A–C) Spindle orientation fails in a statistically significant higher degree in *ΔRgl, pins*$^{\Delta 50}$, *insc*^{P49}*; ΔRgl, and mud*^{4}*; ΔRgl* double mutant mNBs compared with *pins*$^{\Delta 50}$, *insc*^{P49}, *mud*^{4}, and *ΔRgl* single mutants, respectively. Percentages of spindles aligned in each 15° bin for the genotypes indicated are shown.

Generation of germline clones

w; pr pwn P {ry$^{+t7.2}$=hsFLP}38/Cyo; Rap1^{P5709} P{FRT (w^{hs})}2A/TM2 females were crossed with *w; P{ovo^{D1}-18}3L P{FRT(w^{hs})}2A/TM3, Sb* males. Mitotic recombination was induced in 24–48-h larvae for 2 h at 37°C. Virgins from this cross were mated with *Rap1^{P5709}/TM6lacZ* males, and the embryos (without maternal and zygotic Rap1 products) were used for the phenotypic analysis.

Immunofluorescence

Embryo fixation and antibody staining were performed by standard protocols (4% formaldehyde for 20 min) with the exceptions mentioned at the end of this paragraph. The following primary antibodies were used: sheep anti-GFP at 1:400 (Osenses); rabbit anti–β-galactosidase at 1:1,000–1:10,000 (Cappel); mouse anti–β-galactosidase at 1:8,000 (Promega); rabbit anti–PKC-ζ at 1:1,000 (C-20; Santa Cruz Biotechnology, Inc.); rabbit anti-Cno at 1:400 (Speicher et al., 2008); guinea pig anti-Numb at 1:250 (a gift from Y.-N. Jan, University of California, San Francisco, San Francisco, CA; Rhyu et al., 1994); rabbit anti-PH3 at 1:400 (Millipore); rabbit anticentrosomine (Cnn) at 1:400 (a gift from T.C. Kaufman, Indiana University, Bloomington, IN); mouse anti-Neurotactin at 1:200 (Speicher et al., 1998); rabbit anti-Baz at 1:200 (a gift from A. Wodarz, Georg-August-Universität Göttingen, Göttingen, Germany; Wodarz et al., 1999); rabbit anti-Mira at 1:2,000 (a gift from F. Matsuzaki, RIKEN Center for Developmental Biology, Kobe, Japan; Ikeshima-Kataoka et al., 1997); mouse anti-Pros at 1:100 (Developmental Studies Hybridoma Bank); mouse anti-Mud at 1:100 (a gift from F. Matsuzaki; Izumi et al., 2006); rabbit anti-Scrib at 1:4,000 (a gift from C. Doe, University of Oregon, Eugene, OR); rabbit anti–Even-skipped at 1:3,000 (Frasch et al., 1987); rat anti–DE-cad at 1:20 (Developmental Studies Hybridoma Bank); rabbit anti-Pins at 1:200 and rabbit anti-Gαi at 1:1,000 (both gifts from J.A. Knoblich); mouse anti–α-tubulin at 1:1,000 (Sigma-Aldrich); rat anti–Lethal of Scute (L'sc) at 1:2,000 (Martín-Bermudo et al., 1991); and mouse anti-Wingless at 1:50 (Developmental Studies Hybridoma Bank). Secondary antibodies coupled to biotin (Vector laboratories) plus streptavidin 488 (Invitrogen), Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 633 (Invitrogen) were used. For immunostaining with the anti-Cno antibody, embryos were fixed by using the heat–methanol method (Tepass, 1996). For α-tubulin staining, embryos were fixed with 37% formaldehyde for 1 min. L'sc signal was enhanced by use of reagents (Tyramide Signal Amplification; DuPont).

Spindle orientation analysis

Taking as a reference the overlying epithelia, angles formed between the axis delineated by the NB spindle and the apicobasal polarity axis of epithelial cells were measured using Photoshop (Adobe).

Microscope image acquisition

Fluorescent images were recorded by using an upright microscope (DM-SL with Spectral Confocal acquisition software; Leica). All images were taken with an HCX Plan Apochromat 63×/1.32-0.6 NA oil confocal scanning objective. Figs. 1 (A–E′) and 5 (A–C) were acquired with an additional electronic zoom (z = 4). Fig. S1 (E–H) was recorded by using a microscope (Axio Imager.A1; EC Plan Neofluar 63×/1.25 NA oil objective; Carl Zeiss) and a camera (AxioCam HRc; Carl Zeiss). Images were assembled by using Photoshop CS3.

Coimmunoprecipitations

For immunoprecipitations, 0–7-h embryos were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 100 µM Na_3VO_4, 2 mM PMSF, and protease inhibitors [Complete; Roche]). Embryo extracts were centrifuged at 4°C for 15 min at 14,000 rpm (18,700 *g*) and then for 5 min in the same conditions. Precleared extracts were incubated with rabbit polyclonal antibody to GFP Sepharose beads (Abcam) for 2 h at 4°C. The beads then were washed three times with lysis buffer without inhibitors, resuspended in protein set buffer (Fluka), and heated at 95°C for 5 min. Precipitates were resolved by SDS-PAGE and immunoblotted with mouse anti-GFP (Takara Bio, Inc.) at 1:2,000, rabbit anti–PKC-ζ at 1:500, or rabbit anti-Par6 (a gift from J.A. Knoblich) at 1:2,000. Each experiment was repeated at least three times.

Online supplemental material

Fig. S1 shows that *Rap1* mutants show equal-sized daughter cells and defects in the RP2 lineage. Fig. S2 shows that epithelial polarity defects in *Rap1* mutants are not correlated with NB polarity and spindle orientation failures. Fig. S3 shows that NB delamination is not affected in *Rap1* mutant embryos. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201108112/DC1.

We thank Rolf Reuter, Jacques Camonis, Nick Brown, Andreas Wodarz, Fumio Matsuzaki, Juergen Knoblich, Yuh-Nung Jan, Thomas Kaufman, Chris Doe, the Bloomington *Drosophila* Stock Center at the University of Indiana, and the Developmental Studies Hybridoma Bank at the University of Iowa for kindly providing fly strains and antibodies.

This work was supported by grants to our laboratory from the Spanish government (BFU2006-09130, BFU2009-08833, and CONSOLIDER-INGENIO 2010 CSD2007-00023).

Submitted: 18 August 2011
Accepted: 17 October 2011

References

Asha, H., N.D. de Ruiter, M.G. Wang, and I.K. Hariharan. 1999. The Rap1 GTPase functions as a regulator of morphogenesis in vivo. *EMBO J.* 18:605–615. http://dx.doi.org/10.1093/emboj/18.3.605

Boettner, B., E.E. Govek, J. Cross, and L. Van Aelst. 2000. The junctional multidomain protein AF-6 is a binding partner of the Rap1A GTPase and associates with the actin cytoskeletal regulator profilin. *Proc. Natl. Acad. Sci. USA.* 97:9064–9069. http://dx.doi.org/10.1073/pnas.97.16.9064

Boettner, B., P. Harjes, S. Ishimaru, M. Heke, H.Q. Fan, Y. Qin, L. Van Aelst, and U. Gaul. 2003. The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the *Drosophila* embryo. *Genetics.* 165:159–169.

Bowman, S.K., R.A. Neumüller, M. Novatchkova, Q. Du, and J.A. Knoblich. 2006. The *Drosophila* NuMA Homolog Mud regulates spindle orientation in asymmetric cell division. *Dev. Cell.* 10:731–742. http://dx.doi.org/10.1016/j.devcel.2006.05.005

Broadus, J., J.B. Skeath, E.P. Spana, T. Bossing, G. Technau, and C.Q. Doe. 1995. New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. *Mech. Dev.* 53:393–402. http://dx.doi.org/10.1016/0925-4773(95)00454-8

Cabernard, C., and C.Q. Doe. 2009. Apical/basal spindle orientation is required for neuroblast homeostasis and neuronal differentiation in *Drosophila*. *Dev. Cell.* 17:134–141. http://dx.doi.org/10.1016/j.devcel.2009.06.009

Cai, Y., F. Yu, S. Lin, W. Chia, and X. Yang. 2003. Apical complex genes control mitotic spindle geometry and relative size of daughter cells in *Drosophila* neuroblast and pI asymmetric divisions. *Cell.* 112:51–62. http://dx.doi.org/10.1016/S0092-8674(02)01170-4

Caron, E. 2003. Cellular functions of the Rap1 GTP-binding protein: a pattern emerges. *J. Cell Sci.* 116:435–440. http://dx.doi.org/10.1242/jcs.00238

Chia, W., W.G. Somers, and H. Wang. 2008. *Drosophila* neuroblast asymmetric divisions: cell cycle regulators, asymmetric protein localization, and tumorigenesis. *J. Cell Biol.* 180:267–272. http://dx.doi.org/10.1083/jcb.200708159

Doe, C.Q. 2008. Neural stem cells: balancing self-renewal with differentiation. *Development.* 135:1575–1587. http://dx.doi.org/10.1242/dev.014977

Frasch, M., T. Hoey, C. Rushlow, H. Doyle, and M. Levine. 1987. Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J.* 6:749–759.

Bar, 10 µm. (D) Rap1 forms a complex in vivo with aPKC and Par6. GFP-Rap1 embryo lysates were subject to immunoprecipitation (IP) with rabbit anti-GFP antibody bound to beads and probed on immunoblots (IB) with anti-aPKC, anti-Par6, and with anti-GFP (as an immunoprecipitation control). In negative (neg.) controls, WT embryo lysates were immunoprecipitated with the same rabbit anti-GFP antibody bound to beads. Each coimmunoprecipitation was repeated at least three times. Molecular masses are given in kilodaltons. (E and F) Diagram (E) shows the genetic and physical relationships among the apical proteins represented. Continuous line indicates a physical interaction. Dotted lines indicate potential physical interactions. Cno and Pins are in a complex in vivo, but they do not physically interact (green dotted line). Arrows indicate genetic relationships. (F) Location of proteins at the apical mNB cortex and their link with the mitotic spindle. Orange arrows represent unknown extrinsic signals coming from the NE. CT, centrosome; Dlg, Discs Large; Tub, tubulin.

Hariharan, I.K., R.W. Carthew, and G.M. Rubin. 1991. The *Drosophila* roughened mutation: activation of a rap homolog disrupts eye development and interferes with cell determination. *Cell*. 67:717–722. http://dx.doi.org/10.1016/0092-8674(91)90066-8

Hirata, J., H. Nakagoshi, Y. Nabeshima, and F. Matsuzaki. 1995. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature*. 377:627–630. http://dx.doi.org/10.1038/377627a0

Ikeshima-Kataoka, H., J.B. Skeath, Y. Nabeshima, C.Q. Doe, and F. Matsuzaki. 1997. Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions. *Nature*. 390:625–629. http://dx.doi.org/10.1038/37641

Izumi, Y., N. Ohta, K. Hisata, T. Raabe, and F. Matsuzaki. 2006. *Drosophila* Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization. *Nat. Cell Biol.* 8:586–593. http://dx.doi.org/10.1038/ncb1409

Johnston, C.A., K. Hirono, K.E. Prehoda, and C.Q. Doe. 2009. Identification of an Aurora-A/PinsLINKER/Dlg spindle orientation pathway using induced cell polarity in S2 cells. *Cell*. 138:1150–1163. http://dx.doi.org/10.1016/j.cell.2009.07.041

Knoblich, J.A. 2008. Mechanisms of asymmetric stem cell division. *Cell*. 132:583–597. http://dx.doi.org/10.1016/j.cell.2008.02.007

Knoblich, J.A., L.Y. Jan, and Y.N. Jan. 1995. Asymmetric segregation of Numb and Prospero during cell division. *Nature*. 377:624–627. http://dx.doi.org/10.1038/377624a0

Knox, A.L., and N.H. Brown. 2002. Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science*. 295:1285–1288. http://dx.doi.org/10.1126/science.1067549

Kooistra, M.R., N. Dubé, and J.L. Bos. 2007. Rap1: a key regulator in cell-cell junction formation. *J. Cell Sci.* 120:17–22. http://dx.doi.org/10.1242/jcs.03306

Martín-Bermudo, M.D., C. Martínez, A. Rodríguez, and F. Jiménez. 1991. Distribution and function of the lethal of scute gene product during early neurogenesis in *Drosophila*. *Development*. 113:445–454.

Mirey, G., M. Balakireva, S. L'Hoste, C. Rossé, S. Voegeling, and J. Camonis. 2003. A Ral guanine exchange factor-Ral pathway is conserved in *Drosophila melanogaster* and sheds new light on the connectivity of the Ral, Ras, and Rap pathways. *Mol. Cell. Biol.* 23:1112–1124. http://dx.doi.org/10.1128/MCB.23.3.1112-1124.2003

Miyamoto, H., I. Nihonmatsu, S. Kondo, R. Ueda, S. Togashi, K. Hirata, Y. Ikegami, and D. Yamamoto. 1995. canoe encodes a novel protein containing a GLGF/DHR motif and functions with Notch and scabrous in common developmental pathways in *Drosophila. Genes Dev*. 9:612–625. http://dx.doi.org/10.1101/gad.9.5.612

Morrison, S.J., and A.C. Spradling. 2008. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell*. 132:598–611. http://dx.doi.org/10.1016/j.cell.2008.01.038

Moskalenko, S., D.O. Henry, C. Rosse, G. Mirey, J.H. Camonis, and M.A. White. 2001. The exocyst is a Ral effector complex. *Nat. Cell Biol.* 4:66–72. http://dx.doi.org/10.1038/ncb728

Nipper, R.W., K.H. Siller, N.R. Smith, C.Q. Doe, and K.E. Prehoda. 2007. Galphai generates multiple Pins activation states to link cortical polarity and spindle orientation in *Drosophila* neuroblasts. *Proc. Natl. Acad. Sci. USA*. 104:14306–14311. http://dx.doi.org/10.1073/pnas.0701812104

O'Keefe, D.D., E. Gonzalez-Niño, M. Burnett, L. Dylla, S.M. Lambeth, E. Licon, C. Amesoli, B.A. Edgar, and J. Curtiss. 2009. Rap1 maintains adhesion between cells to affect Egfr signaling and planar cell polarity in *Drosophila*. *Dev. Biol.* 333:143–160. http://dx.doi.org/10.1016/j.ydbio.2009.06.032

Parmentier, M.L., D. Woods, S. Greig, P.G. Phan, A. Radovic, P. Bryant, and C.J. O'Kane. 2000. Rapsynoid/partner of inscuteable controls asymmetric division of larval neuroblasts in *Drosophila*. *J. Neurosci.* 20:RC84.

Price, L.S., A. Hajdo-Milasinovic, J. Zhao, F.J. Zwartkruis, J.G. Collard, and J.L. Bos. 2004. Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J. Biol. Chem.* 279:35127–35132. http://dx.doi.org/10.1074/jbc.M404917200

Rhyu, M.S., L.Y. Jan, and Y.N. Jan. 1994. Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell*. 76:477–491. http://dx.doi.org/10.1016/0092-8674(94)90112-0

Schaefer, M., A. Shevchenko, A. Shevchenko, and J.A. Knoblich. 2000. A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in *Drosophila. Curr. Biol.* 10:353–362. http://dx.doi.org/10.1016/S0960-9822(00)00401-2

Schober, M., M. Schaefer, and J.A. Knoblich. 1999. Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature*. 402:548–551. http://dx.doi.org/10.1038/990135

Schuldt, A.J., J.H. Adams, C.M. Davidson, D.R. Micklem, J. Haseloff, D. St Johnston, and A.H. Brand. 1998. Miranda mediates asymmetric protein and RNA localization in the developing nervous system. *Genes Dev.* 12:1847–1857. http://dx.doi.org/10.1101/gad.12.12.1847

Schwamborn, J.C., and A.W. Püschel. 2004. The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.* 7:923–929. http://dx.doi.org/10.1038/nn1295

Shen, C.P., L.Y. Jan, and Y.N. Jan. 1997. Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila. Cell*. 90:449–458. http://dx.doi.org/10.1016/S0092-8674(00)80505-X

Siegrist, S.E., and C.Q. Doe. 2005. Microtubule-induced Pins/Galphai cortical polarity in *Drosophila* neuroblasts. *Cell*. 123:1323–1335. http://dx.doi.org/10.1016/j.cell.2005.09.043

Siegrist, S.E., and C.Q. Doe. 2006. Extrinsic cues orient the cell division axis in *Drosophila* embryonic neuroblasts. *Development*. 133:529–536. http://dx.doi.org/10.1242/dev.02211

Siller, K.H., and C.Q. Doe. 2009. Spindle orientation during asymmetric cell division. *Nat. Cell Biol.* 11:365–374. http://dx.doi.org/10.1038/ncb0409-365

Siller, K.H., C. Cabernard, and C.Q. Doe. 2006. The NuMA-related Mud protein binds Pins and regulates spindle orientation in *Drosophila* neuroblasts. *Nat. Cell Biol.* 8:594–600. http://dx.doi.org/10.1038/ncb1412

Spana, E.P., and C.Q. Doe. 1995. The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development*. 121:3187–3195.

Speicher, S., L. García-Alonso, A. Carmena, M.D. Martín-Bermudo, S. de la Escalera, and F. Jiménez. 1998. Neurotactin functions in concert with other identified CAMs in growth cone guidance in *Drosophila*. *Neuron*. 20:221–233. http://dx.doi.org/10.1016/S0896-6273(00)80451-1

Speicher, S., A. Fischer, J. Knoblich, and A. Carmena. 2008. The PDZ protein Canoe regulates the asymmetric division of *Drosophila* neuroblasts and muscle progenitors. *Curr. Biol.* 18:831–837. http://dx.doi.org/10.1016/j.cub.2008.04.072

Tepass, U. 1996. Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*. *Dev. Biol.* 177:217–225. http://dx.doi.org/10.1006/dbio.1996.0157

Wang, H., S.R. Singh, Z. Zheng, S.W. Oh, X. Chen, K. Edwards, and S.X. Hou. 2006. Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the *Drosophila* testis. *Dev. Cell*. 10:117–126. http://dx.doi.org/10.1016/j.devcel.2005.11.004

Wirtz-Peitz, F., T. Nishimura, and J.A. Knoblich. 2008. Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. *Cell*. 135:161–173. http://dx.doi.org/10.1016/j.cell.2008.07.049

Wodarz, A., and W.B. Huttner. 2003. Asymmetric cell division during neurogenesis in *Drosophila* and vertebrates. *Mech. Dev.* 120:1297–1309. http://dx.doi.org/10.1016/j.mod.2003.06.003

Wodarz, A., A. Ramrath, U. Kuchinke, and E. Knust. 1999. Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature*. 402:544–547. http://dx.doi.org/10.1038/990128

Wodarz, A., A. Ramrath, A. Grimm, and E. Knust. 2000. *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.* 150:1361–1374. http://dx.doi.org/10.1083/jcb.150.6.1361

Yaffe, M.B., G.G. Leparc, J. Lai, T. Obata, S. Volinia, and L.C. Cantley. 2001. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat. Biotechnol.* 19:348–353. http://dx.doi.org/10.1038/86737

Yu, F., X. Morin, Y. Cai, X. Yang, and W. Chia. 2000. Analysis of partner of inscuteable, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in inscuteable apical localization. *Cell*. 100:399–409. http://dx.doi.org/10.1016/S0092-8674(00)80676-5

Focus Article

Cytoplasmic protein motility and polarized sorting during asymmetric cell division

Alyona Keder and Ana Carmena*

Cell polarity is inherent to the process of asymmetric cell division, which relies on the asymmetric distribution of multiple polarity proteins and cell-fate determinants in the cell cortex. The establishment and maintenance of cell polarity require the orchestration of numerous cellular processes. These include cytoplasmic movements, cytoskeleton dynamics, and different signaling events. Equally relevant is the plasma membrane composition, such as the lipid environment that endows particular membrane subdomains with specific characteristics. Sorting receptors and sorting determinants, including posttranslational modifications, also contribute to cell polarization. Together, all these mechanisms would be expected to have great relevance in the context of asymmetric cell division, an essential process in both physiological and pathological conditions.

How to cite this article:
WIREs Dev Biol 2013. doi: 10.1002/wdev.116

INTRODUCTION

Asymmetric cell division is an essential process during development and in cancer and stem cell biology. Tight control of this process ensures a proper cell proliferation–differentiation ratio and contributes to cell diversity generation.[1] In an asymmetric cell division, the mother cell divides to give rise to two different daughter cells, which undergo distinct genetic programs. Two main mechanisms underlie asymmetric cell divisions, extrinsic/non-autonomous or intrinsic/autonomous mechanisms. The former implies the existence of a niche that provides diffusible determinants to the stem cell, whose mitotic spindle is orientated perpendicularly to the surface of the niche. Only the daughter cell closest to the niche receives the signals required to retain a stem cell identity. Conversely, intrinsic mechanisms rely on the polarized distribution of different proteins in the mother stem cell, including cell-fate determinants. These determinants will segregate differentially into only one daughter cell, endowing this cell with specific properties. In turn, the asymmetric distribution of cell-fate determinants depends on multiple proteins (polarity proteins, small GTPases, protein kinases, etc.), the distribution of which is mostly polarized to specific membrane subdomains.[2] Moreover, once the stem cell has divided, the two distinct daughter cells make use of additional mechanisms to further reinforce their different cell fates. Thus, a coordinated compendium of events directs the intrinsic asymmetric protein localization both before and after stem cell division, involving asymmetric endocytosis or exocytosis, vesicular trafficking, and posttranslational modifications. The lipid environment may also exert an important influence on protein sorting to specific plasma membrane domains.

Drosophila melanogaster and *Caenorhabditis elegans* have served as excellent model systems in which to analyze the process of asymmetric cell division. In *Drosophila*, the neural stem cells of the central nervous system (CNS), the neuroblasts (NBs), as well as the sensory organ precursors (SOPs, also known as pI precursors) in the peripheral nervous system (PNS) have been studied extensively. Likewise, the one-cell stage *C. elegans* embryo has provided outstanding insight into this process. Unicellular organisms, such as the budding yeast *Saccharomyces cerevisiae*, have also shed light on

*Correspondence to: acarmena@umh.es

Instituto de Neurociencias CSIC/UMH, Sant Joan d'Alacant, Alicante, Spain

Conflict of interest: The authors have declared no conflicts of interest for this article.

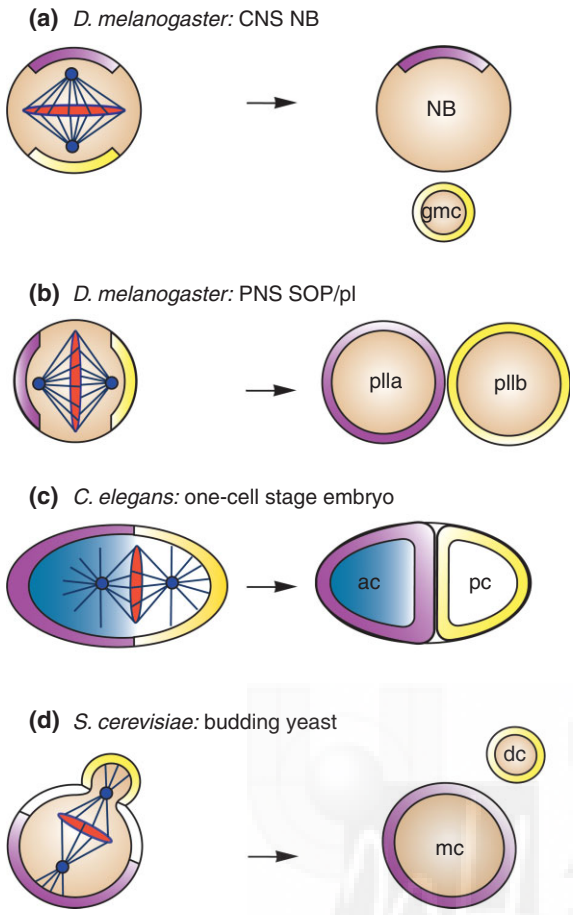


FIGURE 1 | Model systems in which to analyze asymmetric cell division. (a) *Drosophila melanogaster* central nervous system (CNS) neural stem cells (NBs). Apical proteins form a crescent (purple) at the apical pole of the NB, whereas cell-fate determinants form a basal crescent (yellow) during metaphase (DNA is shown in red). After division, cell-fate determinants segregate to the basal-most daughter cell, the ganglion mother cell (gmc). (b) *D. melanogaster* peripheral nervous system (PNS) progenitor cells [sensory organ precursors (SOP)s/pIs]. Anterior (purple) and posterior (yellow) proteins segregate asymmetrically during metaphase and end up in the pIIa or pIIb daughter cells after division, respectively. (c) *Caenorhabditis elegans* one-cell stage embryo. Anterior polarity proteins (purple), and posterior polarity proteins (yellow) accumulate asymmetrically at metaphase (DNA is shown in red). After division both types of proteins segregate differentially into the anterior (ac) or posterior (pc) daughter cells. (d) *Saccharomyces cerevisiae* budding yeast. Mother cell (mc) proteins (purple) and daughter cell (dc) determinants in the bud (yellow) localize asymmetrically at metaphase (DNA appears in red). After division, cell determinants segregate to the bud daughter cell.

the cell machinery involved in protein motility and polarized sorting during asymmetric cell division, a highly conserved process (Figure 1). However, much less is known about the precise principles underlying asymmetric cell division in vertebrates. Nevertheless, as most of the proteins involved in regulating asymmetric cell division in flies and other model systems have orthologs in mammals, we would also expect there to be a significant degree of conservation in the mechanisms regulating asymmetric cell division.[1]

EXOCYTIC AND ENDOCYTIC VESICLE-BASED MOVEMENTS: THE SNARE COMPLEX AND RAB GTPASES

Polarized exocytosis mediates the delivery of intracellular proteins to particular domains of the plasma membrane for their subsequent secretion. Exocytic vesicle fusion in polarized epithelia is dependent on the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), whereby the vSNAREs on the vesicle membrane interact specifically with the tSNAREs at the cell membrane during vesicle fusion.[3] The apical and basolateral membranes express different tSNARES, including Syntaxin-3 and Syntaxin-4, respectively.[4] Another essential element for polarized exocytosis is the multiprotein complex known as the exocyst (or Sec6/8 complex). This complex was initially identified in yeast although it is highly conserved throughout evolution and it is comprised of eight proteins: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p.[5,6] Exocyst proteins tether exocytic vesicles to the plasma membrane where SNARE-mediated fusion takes place through the Rab GTPase Sec4 (Figure 2).

While both SNAREs and the exocyst are key elements in polarized vesicle trafficking, there is little evidence that they participate in protein polarization during asymmetric cell division. There is one example from the *Drosophila* PNS where primary SOP/pI precursors divide asymmetrically into a pIIa and pIIb daughter cells, which will divide again to ultimately give rise to the four cells that constitute the external sensory organ (Figure 3(a)).[7] In this process, Sec15 is required for the asymmetric distribution of the Notch ligand Delta (Dl) to a particular membrane domain of the pIIb cell that is in contact with its sibling pIIa, in which Notch is then strongly activated.[8] The Notch regulator Sanpodo (Spdo) also depends on Sec15 to reach the membrane of the pIIa cell (Figure 3(b)).[9] Hence, Sec15 appears to drive the directionality of Notch signaling and, consequently, the fate of the asymmetrically dividing cells. In addition, it was recently shown that other members of the exocyst also regulate different aspects of Notch signaling during asymmetric pI division, such as Exo84, Sec5, and Sec6.[10] However, *sec5* and *sec15* loss of function

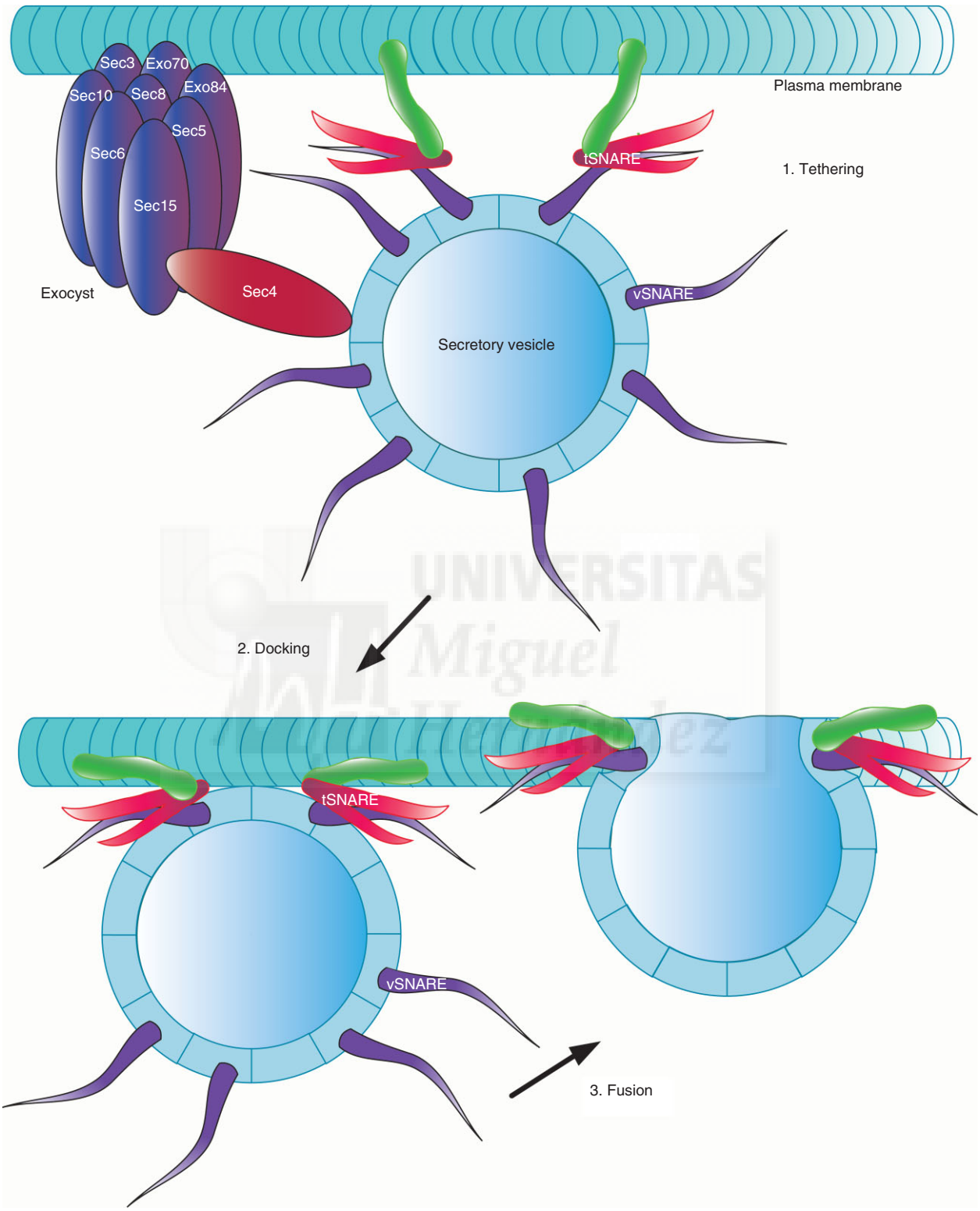


FIGURE 2 | Polarized exocytosis: the exocyst and the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex. (1) The exocyst complex tethers secretory vesicles to the plasma membrane through Sec4, where tSNARE proteins associated to the plasma membrane interact with vSNARE proteins present on the vesicles. (2) During the docking process, secretory vesicles are found in tight contact with the plasma membrane. (3) The vesicles finally fuse with the plasma membrane.

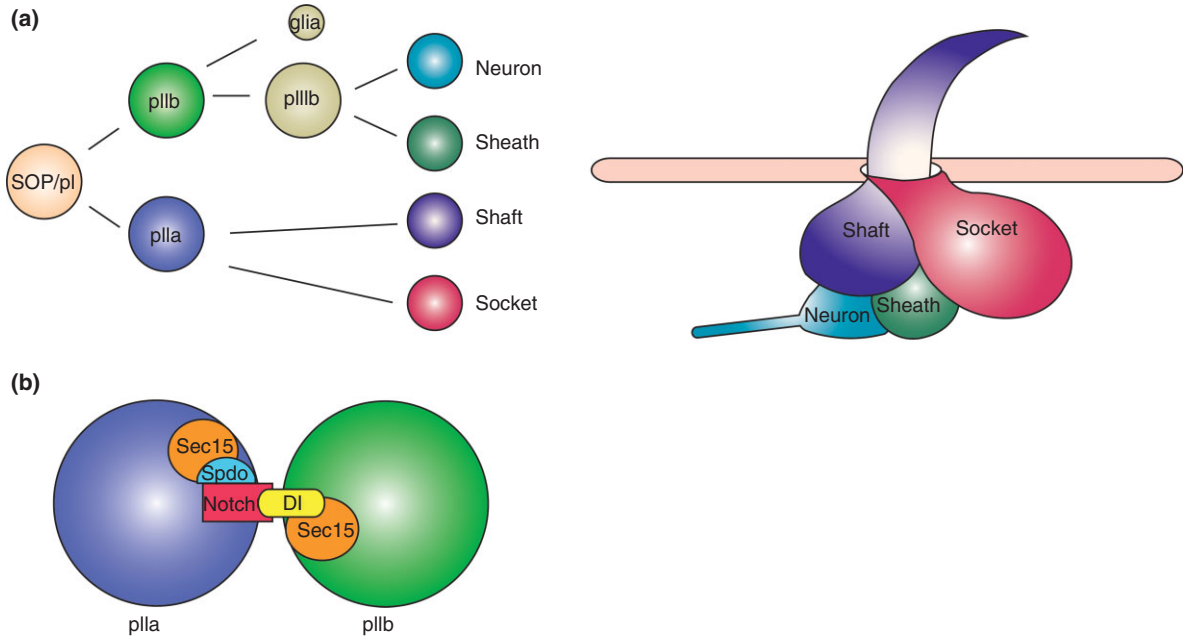


FIGURE 3 | Polarized exocytosis during asymmetric sensory organ precursors (SOP)/pI division. (a) *Drosophila melanogaster* SOPs divide asymmetrically to give rise to two daughter cells, pIIa and pIIb, which in turn divide asymmetrically to generate the four different cells that will form the sensory organ: a neuron, a sheath, a shaft, and a socket. (b) The exocyst protein Sec15 tethers the Notch ligand Delta (Dl) to a particular sub-membrane domain in the pIIb cell, while in the pIIa cell Sec15 helps the Notch regulator Sanpodo (Spdo) to reach the membrane.

does not affect the asymmetric distribution of the polarity protein Bazooka (Baz)/PAR-3 or that of the cell-fate determinant Numb in pI cells.[8,11] Similarly, mutations in the genes that encode the exocyst components Exo84, Sec5, Sec6, and Sec15 do not alter the apico-basal NB polarity in the *Drosophila* CNS.[12] Intriguingly, the exocyst is an effector of the Ral GTPase, which has been shown to regulate NB cortical polarity and spindle orientation during asymmetric NB division in the *Drosophila* embryonic CNS.[13]

Asymmetric endocytosis, the uptake of proteins into the cell at specific plasma membrane domains is also a key means of generating asymmetries within a cell. Many different proteins are involved in regulating asymmetric endocytosis, including Clathrin, Clathrin-adaptor proteins and Dynamin, and Rab GTPases (Figure 4). Rab GTPases are one of the five major branches of the Ras GTPase superfamily and their number varies between species. Rab proteins actively regulate vesicle trafficking along the cytoskeleton and the fusion of vesicles with target membranes.[14] They are associated to specific intracellular compartments; for example, Rab5 and Rab7 are associated to early and late endocytic vesicles, respectively, while Rab11 is present in the recycling endosomes. Rab proteins can recruit different effectors, such as components of the exocyst and motor proteins, including the kinesin and dynein microtubule motors. Indeed, the transport of Rab5-positive early endosomes is driven by kinesins and it is bidirectional along microtubules, whereas Rab7-positive late endosomes only move toward the minus end of the microtubules using cytoplasmic dynein motors. By contrast, Rab11-positive recycling endosomes use the exocyst member Sec15 to tether endosomes to the target membrane (Figure 4).[15]

In *Drosophila*, Rab proteins also participate in the asymmetric division of SOP/pI cells.[16] After pI division, Rab5- and Rab7-positive endosomes are symmetrically distributed into both daughter cells, whereas Rab11-positive recycling endosomes accumulate around the centrosome shortly after cytokinesis in pIIb, but not in pIIa cells.[17] The Ubiquitin-ligase Neuralized (Neur) is segregated asymmetrically to pIIb cell where it promotes the endocytosis of Dl.[18] Most of the endosomes that contain Dl are Rab11-positive and they favor Dl recycling in the pIIb cell.[17] Moreover, Numb and α-Adaptin promote the endocytosis of the Notch regulator Spdo in pIIb, further influencing the direction of Dl-Notch signaling, such that the pIIb becomes the ligand-presenting cell and pIIa the signal-receiving cell.

SMAD Anchor for Receptor Activation (SARA) endosomes also fulfill an important role in asymmetric endocytosis during pI asymmetric cell division.[16]

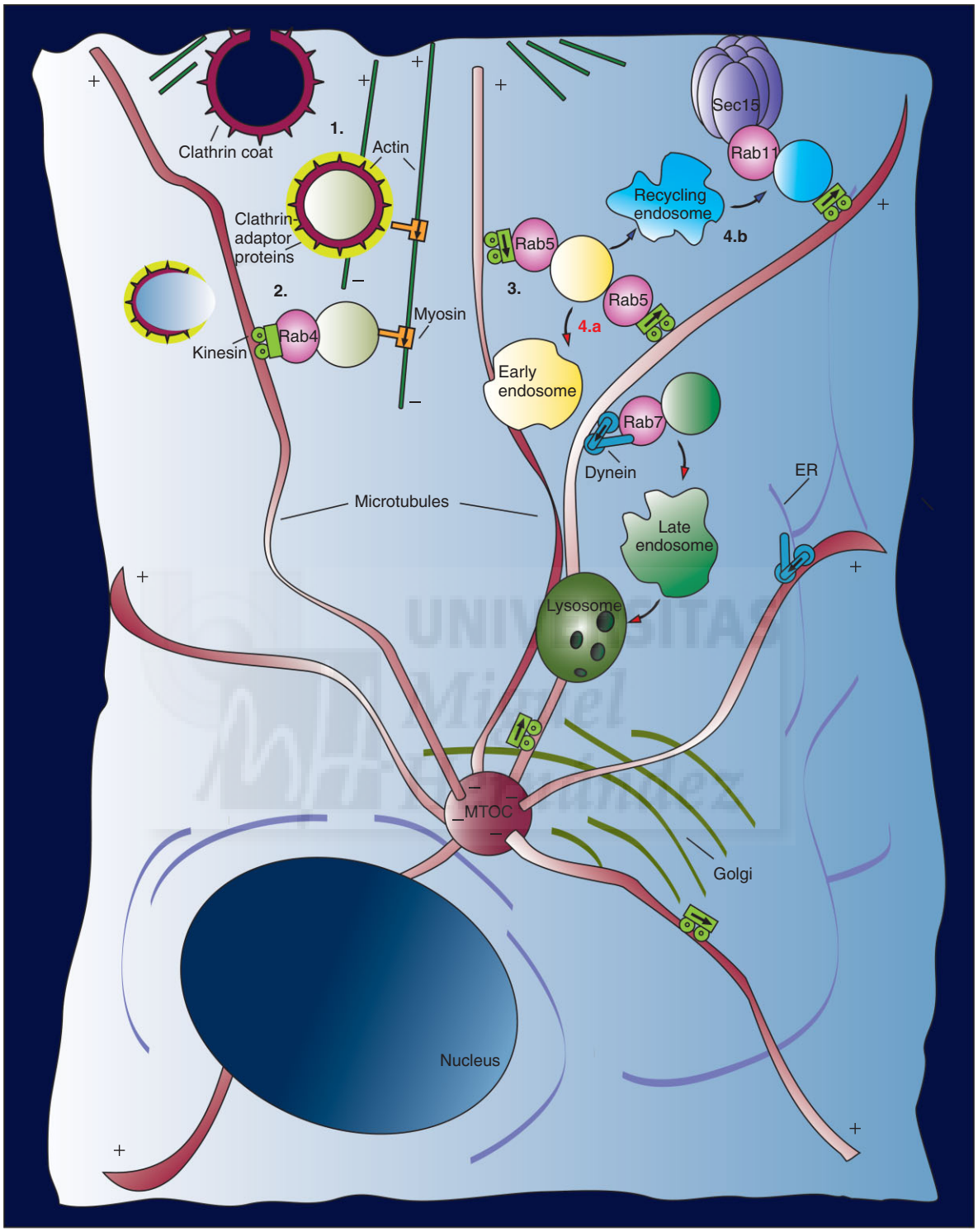


FIGURE 4 | Vesicle trafficking during endocytosis and exocytosis. (1) Clathrin-dependent endocytosis. Clathrin-coated vesicles and clathrin-adaptor proteins move along actin filaments using myosin motors. (2) After losing the clathrin coat, vesicles associate to Rab GTPases linked to kinesin motors to move along microtubules. (3) Rab5 GTPases associate with early endocytic vesicles to transport them using bidirectional kinesin motors. From here, there are two different pathways, the first of which (4a) involves the formation of early endosomes and subsequently late endosomes. Rab7 associates with the late endocytic vesicles to transport them using unidirectional dynein motors until the formation of late endosomes that will finally fuse with a lysosome. In the second pathway (4b) recycling endosomes are formed, which associate with Rab11 to transport them to the plasma membrane where Sec15 tethers the vesicle to the membrane. These vesicles will fuse with the plasma membrane and their components will be recycled.

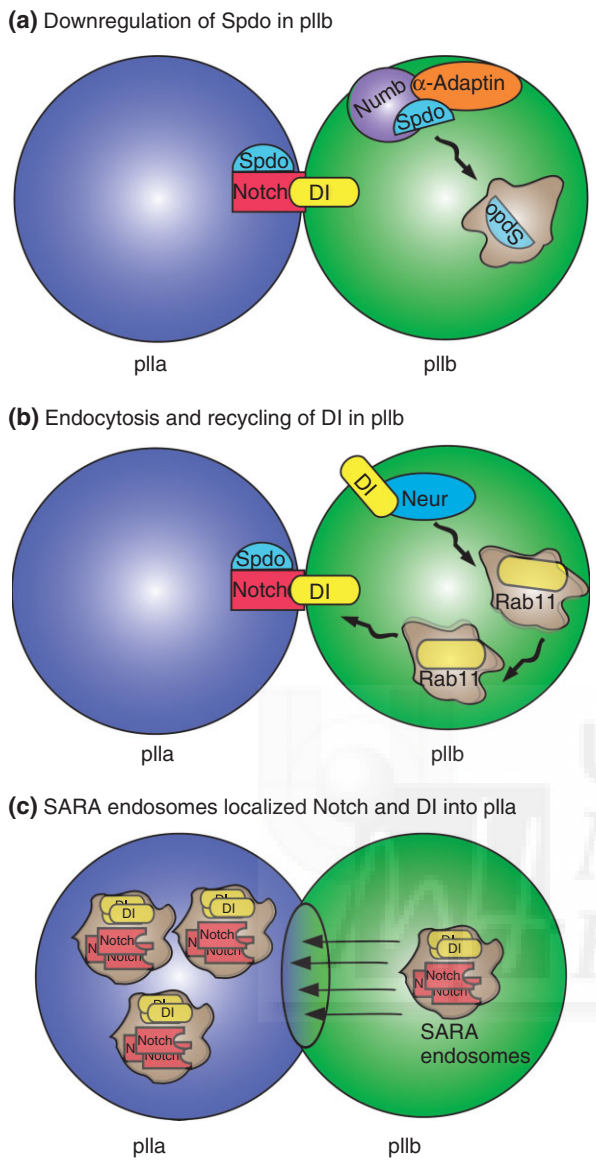


FIGURE 5 | Asymmetric endocytosis during asymmetric sensory organ precursors (SOP)/pI division. (a) Numb and α-Adaptin promote the endocytosis of the Notch regulator Sanpodo (Spdo) in pIIb. (b) Neur induces Delta (Dl) endocytosis and Rab11 drives the recycling of Dl in pIIb. (c) SMAD anchor for receptor activation (SARA) endosomes localize Notch and Dl to pIIa cells after division.

SARA endosomes are multivesicular endosomes (i.e., they contain intralumenal vesicles) that transport and asymmetrically segregate Dl/Notch to the pIIa cell, where Notch signaling will be activated.[19] It has been suggested that the asymmetric distribution of SARA endosomes does not depend on their particular cargo but rather on the time-window in which the cargo proteins have been internalized prior to cytokinesis. For example, only Dl molecules that are internalized more than 10 min before cytokinesis can be found in SARA-positive endosomes and, consequently, will end up in the signal-receiving pIIa cell. Otherwise (i.e., Dl molecules internalized less than ten minutes before cytokinesis), Dl will end up in early Rab5-positive endosomes that do not express SARA and hence, it will be symmetrically distributed to both daughter cells.[16] In conclusion, there are at least three different mechanisms underlying asymmetric endocytosis that causes pIIb cells to become signal-sending cells and pIIa signal-receiving cells: (1) Numb-dependent endocytosis and downregulation of the Notch effector Sanpodo (Spdo) in pIIb cells, (2) Neur-dependent endocytosis of Dl and Dl recycling by Rab11 endosomes in pIIb cells, and (3) SARA endosome-dependent localization of Notch/Dl to pIIa cells (Figure 5).[19] However, the loss of SARA, α-adaptin, Rab5, and Rab11 in *Drosophila* larval NBs does not produce any defects in polarity.[12] Moreover, the loss of Dynamin does not cause any effect in *Drosophila* NB polarity despite completely blocking endocytosis.[12]

Asymmetric endocytosis has been shown to play an important role in asymmetric cell division in other organisms. For example, early endosomes are asymmetrically distributed in *C. elegans* embryos.[20] More specifically, Dynamin DYN-1- and RAB-5-dependent endosomal trafficking can modulate the distribution of the Gβ heterotrimeric G-protein subunit GPB-1 in the one-cell stage *C. elegans* embryo. The Gβγ complex, formed by GPB-1 protein and the GPC-2 Gγ subunit, is an important negative regulator of force generators. This complex is situated at the cell membrane to promote the asymmetric positioning of the spindle during the asymmetric division of the one-cell stage embryo.[21] RAB-5 also participates in the asymmetric localization of the polarity protein PAR-6 through a mechanism dependent on DYN-1, as well as in the organization of the actin cytoskeleton through a DYN-1/endocytosis-independent mechanism, the latter causing specific defects in spindle positioning and cortical organization when depleting RAB-5 in the early *C. elegans* embryo.[22]

ACTIN FILAMENT-BASED MOVEMENTS USING MYOSIN MOTORS

Myosins are actin-dependent molecular motors that use the energy of ATP hydrolysis to move along actin filaments, these being polarized structures with 'fast growing' plus (or barbed) and minus (or pointed) ends. Most myosins move toward the actin minus ends. Unlike microtubules, which facilitate more long-range organelle transport, actin filaments are responsible for

short-range organelle transport. In many different cell types actin filaments form a meshwork along which endocytotic and exocytotic vesicles can move when anchored to myosins.[23]

The asymmetric distribution of cell-fate determinants in the progeny of a dividing cell is critical in asymmetric cell division. The adaptor protein Miranda (Mira) in *Drosophila* fulfills an essential role in this process, asymmetrically locating the cell-fate determinants Prospero (Pros) and Brain tumor (Brat) to the basal pole of NBs in the CNS.[24–27] In turn, two myosins are important in regulating the localization of Mira, Myosin II (called Zipper in *Drosophila)* and Myosin VI (Jaguar), with Mira binding to both Myosin II and VI (Figure 6).[28–30] Time-lapse confocal microscopy combined with fluorescence recovery after photobleaching (FRAP) studies in embryonic NBs have shown that an inactive Myosin II retains Mira in an apical crescent at interphase. At prophase, Myosin II is activated by the aPKC-mediated phosphorylation of Lgl, excluding Mira from the cortical apical domain and driving its relocation to the cytoplasm where Mira reaches the basal pole by passive diffusion. Finally, at metaphase Myosin VI is located in the basal domain of the NB and is essential to deliver Mira to an anchor at the basal cortex, where Mira retains Pros and Brat.[29] More recent live-imaging studies have described a very different distribution of Myosin II during NB division.[31,32] It is important to note that that these studies have been done in *Drosophila* larval NBs, whose asymmetric division displays some differences with the embryonic asymmetric NB division. In the PNS, the Myosin II Zipper also plays a role in Notch signaling during asymmetric SOP/pI division.[10]

The dynamic movement of other adaptor proteins, such as Partner of Numb (Pon) in NBs, also depends on Myosin II but it is independent of Myosin VI.[29] However, the distribution of Pon and Numb during asymmetric SOP/pI precursor division in the PNS does not seem to depend on myosin transport. FRAP experiments show that Numb and Pon rapidly exchange between the cytoplasm and the cell cortex, and that Lgl is responsible for generating high-affinity binding sites for the basal localization of these determinants during mitosis.[33] Nevertheless, the existence of myosin-dependent or myosin-independent vesicle trafficking of Pon or Numb transmembrane receptors at the cell cortex, such as the Numb-interacting protein called Nip, cannot be excluded.[34]

The cortical actomyosin cytoskeleton participates in the asymmetric distribution of cell-fate determinants or polarity proteins in the *C. elegans* embryo. Once activated by PAR proteins, this actomyosin cytoskeleton generates motile ruffles throughout the cortex that lead to the transport of cytoplasmic determinants to the anterior part of the embryo, such as PAR-3, PAR-6, and PKC-3, as well as to the movement of P granules of ribonucleoproteins (RNPs) to the posterior domain where the PAR-2 and PAR-1 proteins are found. This segregation of PAR proteins is fundamental for the partitioning of the cytoplasm during asymmetric cleavage of the *C. elegans* one-cell embryo.[35] Two small

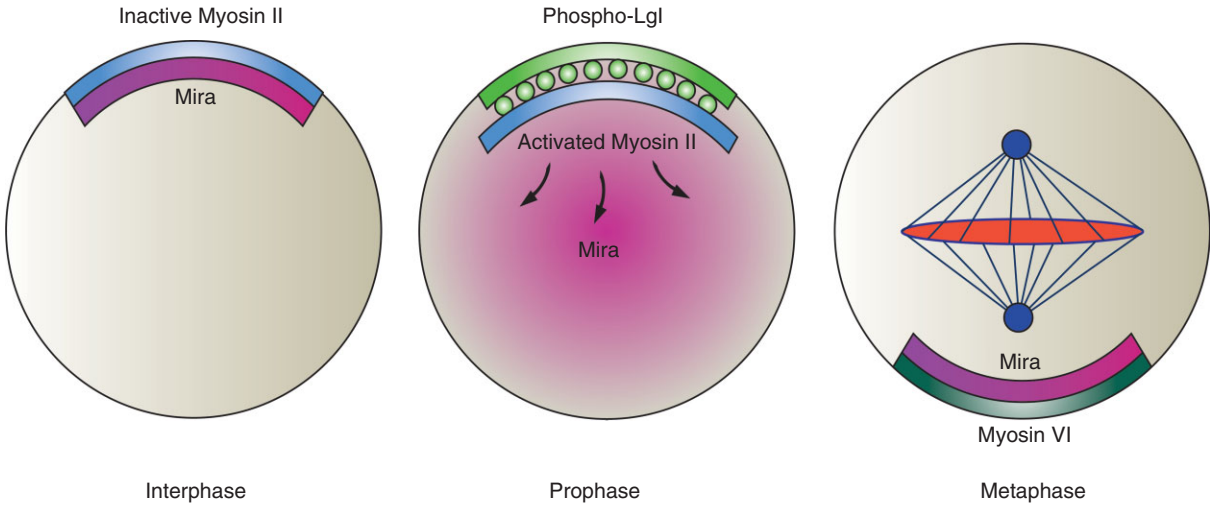


FIGURE 6 | Mira localization during asymmetric cell division is Myosin II- and Myosin VI-dependent. At interphase, Mira forms an apical crescent in contact with inactive Myosin II. Later, at prophase, phosphorylation of Lgl activates Myosin II and Mira is excluded from the cortex and diffuses into the cytoplasm. At metaphase, Mira forms a basal crescent in contact with Myosin VI.

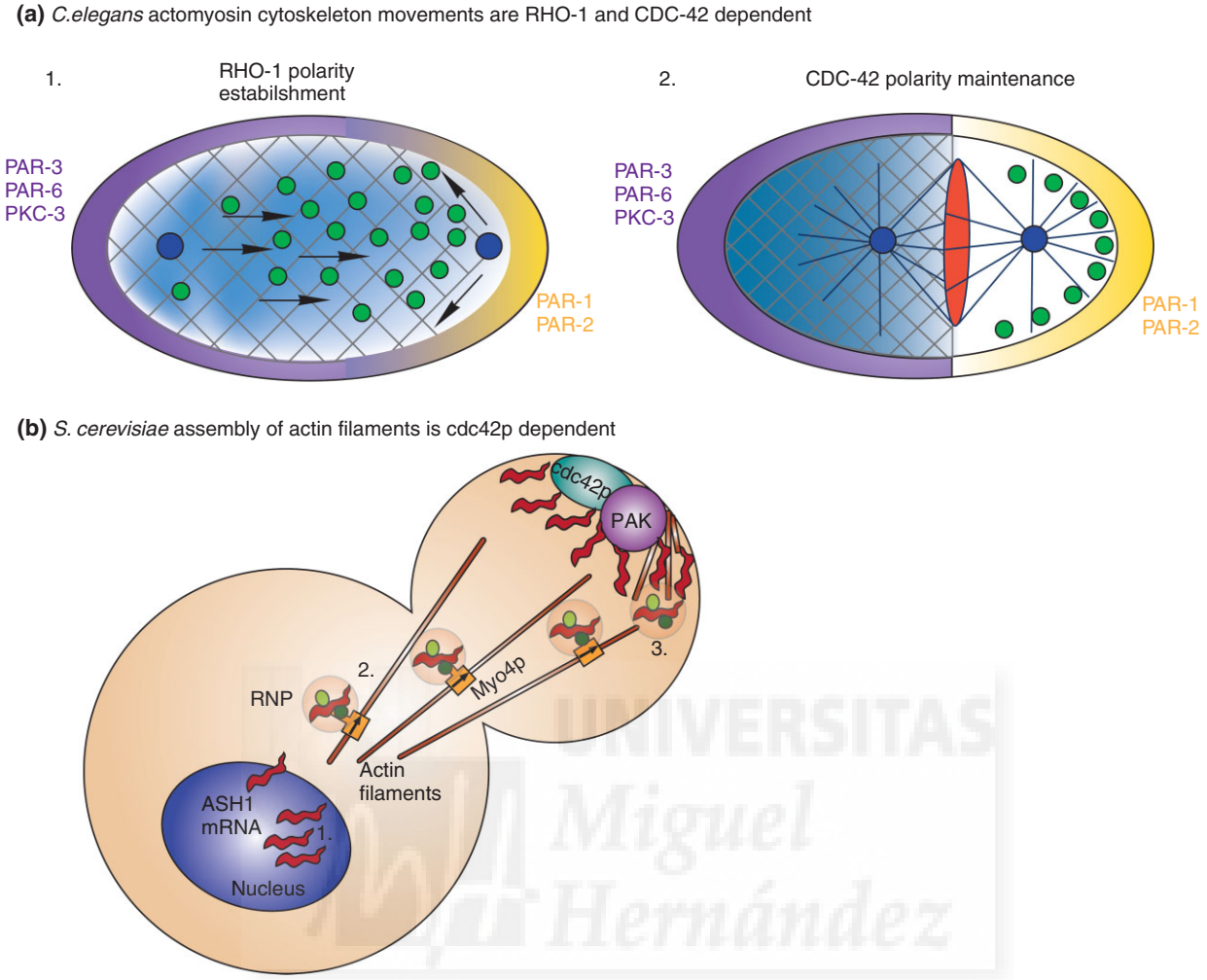


FIGURE 7 | The actomyosin cytoskeleton induces an asymmetric distribution of cell-fate determinants or polarity proteins in *Caenorhabditis elegans*. (a) RHO-1 GTPase induces a cortical flow that promotes the displacement of P granules (green) and the actomyosin cytoskeleton to the posterior and to the anterior pole of the embryo, respectively. As a result, PAR proteins localize asymmetrically, with PAR-1 and PAR-2 proteins accumulating in the posterior part of the embryo, whereas PAR-3, PAR-6, and PKC-3 form a complex in the anterior domain (1). The CDC-42 GTPase maintains the asymmetric distribution of the PAR protein at later stages (2). (b) In *Saccharomyces cerevisiae*, the complex of cdc42p with PAK effectors drives the assembly of actin filaments in the growing bud. ASH1 mRNA (red) is transcribed in the nucleus (1) and it is then packed into RNP complexes that contain myosin-binding proteins (green), which in turn bind to the myosin Myo4p motor (orange). Myo4p transports the RNPs along actin filaments toward the growing bud (2). At the membrane of the bud tip, the RNPs dissociate liberating ASH1 mRNAs at the cell membrane. These transcripts will later be translated into Ash1p protein, which will repress the mating-type switching in this cell (3).

GTPases, RHO-1 and CDC-42, are key regulators at this stage. RHO-1 is necessary for the cortical contractility previously mentioned during the 'establishment phase', and CDC-42 is needed to maintain the polarized distribution of PAR proteins after its initial establishment ('maintenance phase'). Both RHO-1 and CDC-42 are required independently for the polarized distribution of the non-muscle myosin NMY-2 at the cell cortex and, in turn, this polarizes the distribution of CDC-42 in a positive feedback loop during the maintenance phase (Figure 7(a)).[36,37]

The role of Cdc42 in segregating cortical determinants through its influence on the actomyosin cytoskeleton is highly conserved in eukaryotes. For example, in the yeast *S. cerevisiae*, Cdc42p organizes the assembly of actin cables in the particular orientation required for the polarized transport of secretory vesicles. Cdc42p acts through p21-activated kinases (PAKs) effectors, which in turn act on the class I myosins Myo3p and Myo5p that organize the cytoskeleton.[38] The asymmetric segregation of specific mRNAs packed into RNPs in budding yeast is also actomyosin-dependent. These RNP particles consist

of at least one myosin motor, the particular mRNA and an RNA-binding protein. For example, the ASH1 mRNA that regulates mating-type switching of yeast co-localizes with the type V unconventional myosin homolog Myo4p, which transports this mRNA to the daughter cell where it is anchored in the bud tip. Ash1p represses the mating-type switching in this cell whereas the switch does take place in the mother cell (Figure 7(b)).[39] However, it was recently shown that aggregates of damaged proteins that are asymmetrically segregated to the mother cell and cleared from the bud, do not rely on retrograde transport along actin filaments. Although a role for the actin cytoskeleton in the segregation of these aggregates cannot be ruled out, live imaging reveals random walking and diffusion of these damaged proteins without any particular directional bias.[40]

MICROTUBULE-BASED MOVEMENTS USING DYNEIN AND KINESIN MOTOR COMPLEXES

Transport along microtubules is also crucial for intracellular trafficking and membrane dynamics, and hence, to establish the asymmetric distribution of cell determinants. Microtubules form a polarized cytoskeleton in which the microtubule plus ends grow dynamically from a microtubule-organizing centre (MTOC), to which the minus ends are attached. While myosins are actin-dependent motor proteins, kinesins and dyneins are microtubule-dependent motor protein. The kinesins and dyneins protein superfamilies differ in certain respects. For example, while all kinesins (up to 45 members) share a common motor domain but diverge in their cargo-binding tail domains, there is only one form of cytoplasmic dynein that is capable of transporting many different cargos. In addition, dynein requires a dynactin complex to reinforce cargo binding and boost motor processivity. Most kinesins are involved in peripheral transport from the Golgi to the plasma membrane. By contrast, cytoplasmic dynein is the main motor for the transport of vesicles and organelles along microtubules to the center of the cell.[15]

Microtubules can induce cortical polarity in many different tissues and in different organisms.[41] During *Drosophila* development the Egalitarian (Egl)/Bicaudal-D (BicD)/dynein motor complex is used to transport mRNA transcripts toward the minus ends of microtubules. For example, during asymmetric NB division, *inscutable (insc)* mRNA forms a complex with Egl/BicD at the apical pole of NBs during interphase. This distribution of *insc* mRNA is disrupted in *egl* and in *bicD* mutants, as well as upon downregulation of the dynein complex. However, most NBs display normal apical enrichment of the Insc protein suggesting that independent mechanisms are used to target Insc protein to the apical pole.[42] In addition, Insc and the RNA-binding protein Staufen interact and colocalize with *pros* mRNA at the apical cortex of interphase NBs. Staufen acts downstream of Insc to target *pros* mRNA to the basal cortex in a microtubule-dependent manner.[43] As mentioned above, in other cell types (e.g., *Drosophila* oocytes) and in other organisms (e.g., *C. elegans*) microtubules display a central role in polarizing proteins to the cell cortex and in their asymmetric distribution into daughter cells after cell division. As this issue was comprehensively dealt with in a very recent and excellent review, we will not enter into further detail here.[44]

THE INFLUENCE OF POSTTRANSLATIONAL MODIFICATIONS AND THE LIPID ENVIRONMENT ON POLARIZED SORTING

The asymmetric location of proteins to specific plasma membrane domains in epithelial cells ultimately relies on particular sorting determinants, posttranslational modifications present in these proteins, as well as on the characteristics of those plasma membrane domains and their specific protein and lipid composition.[45] In the case of membrane proteins, sorting determinants can be present in the intracellular, extracellular, or membrane anchor domain. For example, the presence of PDZ-binding motifs in the cytoplasmic domain or glycosylphosphatidylinositol (GPI) in the anchor domain sorts proteins to the apical domain of epithelial cells.[45] Indeed, many proteins involved in asymmetric cell division contain PDZ domains that contribute to their clustering in particular submembrane domains.[2] Phosphorylation or dephosphorylation events also fulfill an important role in localizing proteins to specific submembrane domains. Indeed, during the process of asymmetric cell division, the polarized localization of many cell-fate determinants is regulated in this way.[46] For example, in *Drosophila* SOPs and in mammalian epithelial cells, the phosphorylation of Numb by aPKC promotes its asymmetric distribution in both cell types.[47] In addition, Partner of Numb (Pon) is phosphorylated by the Polo kinase in *Drosophila* NBs, this event

being crucial for the basal location of both Pon and Numb in NBs.[48] The Aurora-A (Aur-A) kinase is also required for the asymmetric distribution of Numb in both the *Drosophila* PNS and in the CNS. In the CNS, Aur-A phosphorylates the polarity protein Par-6 at the apical pole of NBs and phosphorylated Par-6 in turn activates aPKC. As a result, aPKC phosphorylates Numb releasing it from the apical cortex.[49–51]

Different polarity proteins that are involved in asymmetric cell division also use other sorting determinants for their anchoring to the plasma membrane. One example is the GTPase Cdc42, a protein with a well-conserved role in asymmetric cell division that attaches to the membrane after prenylation.[52] Palmitoylation and myristoylation are other posttranslational modifications known to be relevant for the membrane localization of proteins.[53] For example, the subunit Gα that forms a complex with Pins/AGS3/GPR to regulate crucial events during asymmetric cell division in different organisms is anchored to the membrane after covalent attachment of myristate and/or palmitate lipids to its N-terminus.[54] Also, in *Drosophila,* the Numb isoform A and the Neur isoforms C and D, as well as two Neur-like homologs in mice, are capable of undergoing N-terminal myristoylation both *in vitro* and *in vivo*. However, the elimination of the myristate anchor does not affect the asymmetric localization of Numb in NBs.[53] In fact, all these lipid modifications (e.g., prenylation, palmitoylation, or myristoylation) do not necessarily alter the asymmetry of polarity proteins but they do affect their subcellular location (i.e., membrane vs cytoplasmic accumulation). In addition, it is likely that at least partially redundant modifications exist that reinforce each other to assure the correct asymmetric distribution of polarity proteins at the membrane.

Despite the relevance of sorting determinants, not much is known about the sorting receptors that interact with them and the protein–protein interactions that contribute in this way to cell polarization. At the same time, there is increasing evidence of the relevance of the lipid environment and of lipid-protein interactions during polarized sorting. In this regard, lipid rafts, membrane regions enriched in cholesterol and sphingolipids, have been considered to be potential generators of polarized domains in membranes.[45] In addition, phosphoinositides, phosphorylated intermediates of phosphatidylinositol (PI) that represent a minor component of phospholipid bilayers, seem to play an important role in membrane dynamics and in modulating vesicular trafficking.[55] In fact, it was recently shown that the binding of plasma membrane phosphoinositides to the C-terminal region of the polarity protein Baz/PAR-3 is necessary and sufficient for Baz membrane targeting during asymmetric NB division.[56]

CONCLUSION

The polarized distribution of different proteins, including cell-fate determinants, is an intrinsic landmark that underpins the process of asymmetric cell division. The cellular machinery must orchestrate protein motility and sorting into specific membrane subdomains in a highly coordinated manner, and live-imaging techniques have become an essential tool to analyze the processes that regulate these events. These processes include asymmetric endocytosis and exocytosis and the concomitant vesicular trafficking along cytoskeletal elements. Likewise, sorting determinants, different posttranslational modifications and the membrane lipid environment are all critical in driving the asymmetric distribution of many proteins incorporated into intricate protein complexes to ensure correct asymmetric cell division.

ACKNOWLEDGMENT

The work in our laboratory is supported by grants from the Spanish Government BFU2009–08833 and CONSOLIDER-INGENIO 2010 CSD2007-00023.

REFERENCES

1. Knoblich JA. Mechanisms of asymmetric stem cell division. *Cell* 2008, 132:583–597.

2. Carmena A. Signaling networks during development: the case of asymmetric cell division in the *Drosophila* nervous system. *Dev Biol* 2008, 321:1–17.

3. Jahn R, Scheller RH. SNAREs—engines for membrane fusion. *Nat Rev Mol Cell Biol* 2006, 7:631–643.

4. Low SH, Vasanji A, Nanduri J, He M, Sharma N, Koo M, Drazba J, Weimbs T. Syntaxins 3 and 4 are concentrated in separate clusters on the plasma membrane

before the establishment of cell polarity. *Mol Biol Cell* 2006, 17:977–989.

5. He B, Guo W. The exocyst complex in polarized exocytosis. *Curr Opin Cell Biol* 2009, 21:537–542.
6. TerBush DR, Maurice T, Roth D, Novick P. The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J* 1996, 15:6483–6494.
7. Roegiers F, Younger-Shepherd S, Jan LY, Jan YN. Two types of asymmetric divisions in the *Drosophila* sensory organ precursor cell lineage. *Nat Cell Biol* 2001, 3:58–67.
8. Jafar-Nejad H, Andrews HK, Acar M, Bayat V, Wirtz-Peitz F, Mehta SQ, Knoblich JA, Bellen HJ. Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of *Drosophila* sensory organ precursors. *Dev Cell* 2005, 9:351–363.
9. Tong X, Zitserman D, Serebriiskii I, Andrake M, Dunbrack R, Roegiers F. Numb independently antagonizes Sanpodo membrane targeting and Notch signaling in *Drosophila* sensory organ precursor cells. *Mol Biol Cell* 2010, 21:802–810.
10. Bras SL, Rondanino C, Kriegel-Taki G, Dussert A, Borgne RL. Genetic identification of intracellular trafficking regulators involved in Notch-dependent binary cell fate acquisition following asymmetric cell division. *J Cell Sci* 2012, 125:4886–4901.
11. Balakireva M, Rosse C, Langevin J, Chien YC, Gho M, Gonzy-Treboul G, Voegeling-Lemaire S, Aresta S, Lepesant JA, Bellaiche Y, et al. The Ral/exocyst effector complex counters c-Jun N-terminal kinase-dependent apoptosis in *Drosophila melanogaster*. *Mol Cell Biol* 2006, 26:8953–8963.
12. Halbsgut N, Linnemannstons K, Zimmermann LI, Wodarz A. Apical-basal polarity in *Drosophila* neuroblasts is independent of vesicular trafficking. *Mol Biol Cell* 2011, 22:4373–4379.
13. Carmena A, Makarova A, Speicher S. The Rap1-Rgl-Ral signaling network regulates neuroblast cortical polarity and spindle orientation. *J Cell Biol* 2011, 195:553–562.
14. Mizuno-Yamasaki E, Rivera-Molina F, Novick P. GTPase networks in membrane traffic. *Annu Rev Biochem* 2012, 81:637–659.
15. Caviston JP, Holzbaur EL. Microtubule motors at the intersection of trafficking and transport. *Trends Cell Biol* 2006, 16:530–537.
16. Furthauer M, Gonzalez-Gaitan M. Endocytic regulation of notch signalling during development. *Traffic* 2009, 10:792–802.
17. Emery G, Hutterer A, Berdnik D, Mayer B, Wirtz-Peitz F, Gaitan MG, Knoblich JA. Asymmetric Rab 11 endosomes regulate δ recycling and specify cell fate in the *Drosophila* nervous system. *Cell* 2005, 122:763–773.
18. Le Borgne R, Schweisguth F. Unequal segregation of neuralized biases notch activation during asymmetric cell division. *Dev Cell* 2003, 5:139–148.
19. Coumailleau F, Furthauer M, Knoblich JA, Gonzalez-Gaitan M. Directional Delta and Notch trafficking in Sara endosomes during asymmetric cell division. *Nature* 2009, 458:1051–1055.
20. Andrews R, Ahringer J. Asymmetry of early endosome distribution in *C. elegans* embryos. *PLoS One* 2007, 2:e493.
21. Thyagarajan K, Afshar K, Gonczy P. Polarity mediates asymmetric trafficking of the Gβ heterotrimeric G-protein subunit GPB-1 in *C. elegans* embryos. *Development* 2011, 138:2773–2782.
22. Hyenne V, Tremblay-Boudreault T, Velmurugan R, Grant BD, Loerke D, Labbe JC. RAB-5 controls the cortical organization and dynamics of PAR proteins to maintain *C. elegans* early embryonic polarity. *PLoS One* 2012, 7:e35286.
23. Krendel M, Mooseker MS. Myosins: tails (and heads) of functional diversity. *Physiology* 2005, 20:239–251.
24. Betschinger J, Mechtler K, Knoblich JA. Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. *Cell* 2006, 124:1241–1253.
25. Ikeshima-Kataoka H, Skeath JB, Nabeshima Y, Doe CQ, Matsuzaki F. Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions. *Nature* 1997, 390:625–629.
26. Lee CY, Wilkinson BD, Siegrist SE, Wharton RP, Doe CQ. Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. *Dev Cell* 2006, 10:441–449.
27. Shen CP, Jan LY, Jan YN. Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. *Cell* 1997, 90:449–458.
28. Petritsch C, Tavosanis G, Turck CW, Jan LY, Jan YN. The *Drosophila* myosin VI Jaguar is required for basal protein targeting and correct spindle orientation in mitotic neuroblasts. *Dev Cell* 2003, 4:273–281.
29. Erben V, Waldhuber M, Langer D, Fetka I, Jansen RP, Petritsch C. Asymmetric localization of the adaptor protein Miranda in neuroblasts is achieved by diffusion and sequential interaction of Myosin II and VI. *J Cell Sci* 2008, 121:1403–1414.
30. Barros CS, Phelps CB, Brand AH. *Drosophila* nonmuscle myosin II promotes the asymmetric segregation of cell fate determinants by cortical exclusion rather than active transport. *Dev Cell* 2003, 5:829–840.
31. Cabernard C, Prehoda KE, Doe CQ. A spindle-independent cleavage furrow positioning pathway. *Nature* 2010, 467:91–94.
32. Connell M, Cabernard C, Ricketson D, Doe CQ, Prehoda KE. Asymmetric cortical extension shifts cleavage furrow position in *Drosophila* neuroblasts. *Mol Biol Cell* 2011, 22:4220–4226.

33. Mayer B, Emery G, Berdnik D, Wirtz-Peitz F, Knoblich JA. Quantitative analysis of protein dynamics during asymmetric cell division. *Curr Biol* 2005, 15:1847–1854.
34. Qin H, Percival-Smith A, Li C, Jia CY, Gloor G, Li SS. A novel transmembrane protein recruits numb to the plasma membrane during asymmetric cell division. *J Biol Chem* 2004, 279:11304–11312.
35. Munro E, Nance J, Priess JR. Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early C. elegans embryo. *Dev Cell* 2004, 7:413–424.
36. Cheeks RJ, Canman JC, Gabriel WN, Meyer N, Strome S, Goldstein B. C. *elegans* PAR proteins function by mobilizing and stabilizing asymmetrically localized protein complexes. *Curr Biol* 2004, 14:851–862.
37. Kumfer KT, Cook SJ, Squirrell JM, Eliceiri KW, Peel N, O'Connell KF, White JG. CGEF-1 and CHIN-1 regulate CDC-42 activity during asymmetric division in the *Caenorhabditis elegans* embryo. *Mol Biol Cell* 2010, 21:266–277.
38. Pruyne D, Bretscher A. Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J Cell Sci* 2000, 113 (Pt 3):365–375.
39. Chartrand P, Singer RH, Long RM. RNP localization and transport in yeast. *Annu Rev Cell Dev Biol* 2001, 17:297–310.
40. Zhou C, Slaughter BD, Unruh JR, Eldakak A, Rubinstein B, Li R. Motility and segregation of Hsp104-associated protein aggregates in budding yeast. *Cell* 2011, 147:1186–1196.
41. Moore JK, Cooper JA. Coordinating mitosis with cell polarity: molecular motors at the cell cortex. *Semin Cell Dev Biol* 2010, 21:283–289.
42. Hughes JR, Bullock SL, Ish-Horowicz D. Inscuteable mRNA localization is dynein-dependent and regulates apicobasal polarity and spindle length in *Drosophila* neuroblasts. *Curr Biol* 2004, 14:1950–1956.
43. Li P, Yang X, Wasser M, Cai Y, Chia W. Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during *Drosophila* neuroblast cell divisions. *Cell* 1997, 90:437–447.
44. Sugioka K, Sawa H. Formation and functions of asymmetric microtubule organization in polarized cells. *Curr Opin Cell Biol* 2012, 24:517–525.
45. Schuck S, Simons K. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. *J Cell Sci* 2004, 117:5955–5964.
46. Sousa-Nunes R, Somers WG. Phosphorylation and dephosphorylation events allow for rapid segregation of fate determinants during *Drosophila* neuroblast asymmetric divisions. *Commun Integr Biol* 2010, 3:46–49.
47. Smith CA, Lau KM, Rahmani Z, Dho SE, Brothers G, She YM, Berry DM, Bonneil E, Thibault P, Schweisguth F, et al. aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. *EMBO J* 2007, 26:468–480.
48. Wang H, Ouyang Y, Somers WG, Chia W, Lu B. Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. *Nature* 2007, 449:96–100.
49. Lee CY, Andersen RO, Cabernard C, Manning L, Tran KD, Lanskey MJ, Bashirullah A, Doe CQ. *Drosophila* Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. *Genes Dev* 2006, 20:3464–3474.
50. Wang H, Somers GW, Bashirullah A, Heberlein U, Yu F, Chia W. Aurora-A acts as a tumor suppressor and regulates self-renewal of *Drosophila* neuroblasts. *Genes Dev* 2006, 20:3453–3463.
51. Wirtz-Peitz F, Nishimura T, Knoblich JA. Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. *Cell* 2008, 135:161–173.
52. Johnson DI. Cdc42: an essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol Mol Biol Rev* 1999, 63:54–105.
53. Benetka W, Mehlmer N, Maurer-Stroh S, Sammer M, Koranda M, Neumuller R, Betschinger J, Knoblich JA, Teige M, Eisenhaber F. Experimental testing of predicted myristoylation targets involved in asymmetric cell division and calcium-dependent signalling. *Cell Cycle* 2008, 7:3709–3719.
54. Wedegaertner PB, Wilson PT, Bourne HR. Lipid modifications of trimeric G proteins. *J Biol Chem* 1995, 270:503–506.
55. Mayinger P. Phosphoinositides and vesicular membrane traffic. *Biochim Biophys Acta* 2012, 1821:1104–1113.
56. Krahn MP, Klopfenstein DR, Fischer N, Wodarz A. Membrane targeting of Bazooka/PAR-3 is mediated by direct binding to phosphoinositide lipids. *Curr Biol* 2010, 20:636–642.