



DIPLOMARBEIT

Titel der Diplomarbeit

Conserved pathways regulate Cellular Polarity –
The role of Trim2 in axon specification

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

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Wien, am 3. November 2008

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Abstract

Cellular polarity is a basic feature of diverse cell types in unicellular and multicellular organisms, ranging from bacterial cells and *Drosophila* neural progenitor cells (neuroblasts) to mammalian neurons. Despite the great diversity in the morphology of all the polarized cell types in different organisms, the establishment of cellular polarity is regulated by conserved pathways. For example, the Par complex (partitioning defective protein), consistent of the proteins Par3, Par6 and atypical Protein Kinase C (aPKC), plays an essential role in asymmetric cell division of *Drosophila* neuroblasts (Petronczky et al., 2001) and the establishment of polarity in mammalian neurons (Nishimura et al., 2004).

Recently, the Trim-protein Brain tumor (Brat) has been identified to be asymmetrically segregated in dividing neuroblasts of the *Drosophila* central nervous system (Betschinger et al., 2006). Brat is inherited by one of the two daughter cells and thereby it is specifying the fate of this daughter cell to become the ganglion mother cell (GMC), which divides only once more to differentiate into two post-mitotic neurons.

Interestingly, Brat has three mammalian homologues, which are members of the tripartite motif family (TRIM-NHL). Trim2, Trim3 and Trim32 show a similar protein structure as Brat. Strikingly, one of the three homologues – Trim32 – is shown to be involved in cell fate determination of mouse neural progenitor cells, due to its asymmetric segregation in one of the two daughter cells. As the Trim32-inheriting daughter cell generates differentiating progenitors, Trim32 retains the same function in the mouse as Brat in *Drosophila* (Betschinger et al., 2006 and Schwamborn et al., 2008). Therefore both proteins are part of conserved mechanisms, regulating cellular polarity.

This thesis is about Trim-NHL proteins and their role in neuronal polarity with a focus on one of the mammalian homologues of Brat, namely on Trim2. Compared to a dividing cell, a post-mitotic neuron shows a different morphology with a long axon and several short dendrites. Both compartments acquire specific characteristics that enable neurons to transmit electrical signals. Rather with gain- and loss of function in-vitro studies of Trim2 in cultured primary neurons from the developing mouse hippocampus, results indicate a role of Trim2 in the establishment of the axon. Furthermore hippocampal neurons of Trim2 knock-out

mice also show a defect in axon specification. This interesting phenotype leads to the question concerning the molecular mechanism underlying Trim2 function. As Trim-NHL proteins are shown to work as miRNA regulators in *Drosophila* and mouse (Neumueller et al., 2008; Schwamborn et al., 2008), it is likely that Trim2 is able to control brain-specific miRNAs, known to be involved in the establishment of neuronal polarity (Schratt et al., 2006).

Zusammenfassung

Zelluläre Polarität ist eine grundlegende Funktion der unterschiedlichsten Zelltypen. Einerseits sind einzellige Organismen, wie die Hefe, dazu in der Lage eine polarisierte Morphologie zu etablieren. Andererseits machen dies auch vielzellige Organismen in ihren verschiedenen Zelltypen – wie zum Beispiel im *Drosophila* Neuroblasten und im Neuron eines Säugetieres.

Obwohl die Zelltypen in den einzelnen Organismen große morphologische Unterschiede aufweisen, passiert der Aufbau der zellulären Polarität durch eine Reihe von konservierten Signalwegen. Ein wichtiger Signalkomplex, welcher diese Konserviertheit aufweist, ist der sogenannte „Par complex“ (partitioning defective), der aus den Proteinen Par3, Par6 und der atypical Protein Kinase C (aPKC) besteht, und eine besondere Rolle in der asymmetrischen Zellteilung eines *Drosophila* Neuroblasten (Petronczky et al., 2001) und in der Etablierung von neuronaler Polarität im Neuron eines Säugetiers (Nishimura et al., 2004) spielt.

Unlängst wurde gezeigt, dass dem Protein „Brain tumor“ (Brat), welches aufgrund seiner Proteinstruktur zur Familie der Trim (tripartite motif) Proteine zählt, eine Funktion in der asymmetrischen Teilung eines Neuroblasten, zueigen ist (Betschinger et al., 2006). Brat wird während der Zellteilung in eine der beiden Tochterzellen verteilt, welche sich daraufhin nur noch ein Mal teilt und sich in zwei Neuronen differenziert. Diese Tochterzelle (Ganglion Mother Cell, GMC) unterscheidet sich von der anderen, die weiterhin in der Lage ist, wie der Neuroblast, zu proliferieren.

Brat hat drei homologe Proteine im Säugetier. Trim2, Trim3 und Trim32 sind ebenfalls Mitglieder der Trim Protein Familie und weisen eine zusätzliche NHL-Domäne auf. Trim32 wurde bereits eine Rolle in der Zellteilung von Maus Vorläuferzellen im Nervensystem zugewiesen. Trim32 wird wie Brat in eine der beiden Tochterzellen verteilt und ändert dort das Schicksal dieser Zelle, die zu einer differenzierenden Zelle wird. Trim32 funktioniert in Maus also wie Brat in *Drosophila* (Betschinger et al., 2006 and Schwamborn et al., 2008). Diese Entdeckung zeigt ein weiteres Protein, welches konserviert zelluläre Polarität reguliert.

In dieser Diplomarbeit geht es um ein weiteres Mitglied der Trim-NHL Familie, nämlich um Trim2. Dies könnte aufgrund seiner Trim32-ähnlichen Proteinstruktur ebenfalls die konservierte Polarität-regulierende Funktion aufweisen. Die Wirkungsweise von Trim2 wurde in Neuronen untersucht. Ein Neuron zeigt eine eindeutig andere Morphologie als eine sich teilende Zelle. Ein Neuron hat ein Axon und mehrere kürzere Dendriten. Beide Kompartimente werden unterschiedliche Eigenschaften zugeteilt, um die Kommunikation mit anderen Zellen zu erleichtern.

Durch Überexpression und den Verlust des Proteins Trim2 (knock-down und knock-out) wurde in-vitro anhand von hippocampalen Neuronen gezeigt, dass Trim2 eine Rolle bei der Etablierung des Axons hat. Der Großteil der Neuronen, die nicht mehr in der Lage waren Trim2 zu exprimieren, schafften es nicht die Symmetrie durch Etablierung des Axons zu brechen.

Nachdem dieser Phänotyp gesehen wurde, wurde ebenfalls versucht den molekularen Mechanismus zu finden nach dem Trim2 funktioniert. Da Trim-NHL Proteine in Drosophila und Säugetieren kürzlich als Regulatoren von microRNAs (miRs) gezeigt wurden (Neumueller et al., 2008; Schwamborn et al., 2008), wurde versucht Trim2 eine regulatorische Funktion von Hirn-spezifischen miRs, welche bereits in der Etablierung von neuronaler Polarität nachgewiesen wurden (Schratt et al., 2006) zuzuweisen.

1. INTRODUCTION

1.1. Diversity in Cellular polarity

The definition of Cellular polarity is: asymmetry in cell shape and function, as well as in protein distribution (Nelson, 2003). The establishment of cellular polarity is a fundamental feature of all organisms, from single-cell organisms (bacteria, yeast) to multi-cellular invertebrates (*Drosophila*, *C. elegans*) and vertebrates (mammals). Each of them is reliant on the ability to form cellular asymmetry, to generate a great diversity of specialized cell types from single precursor cells. The final morphology of the different kinds of specialized cells is quite different and depends on its function in the particular organism (Figure 1a-e; Nelson, 2003).

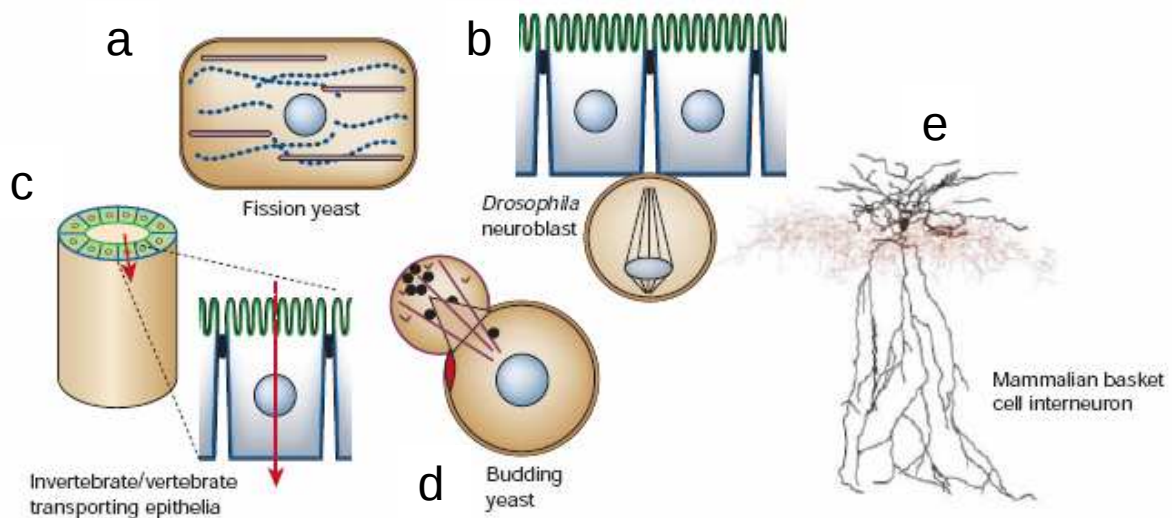


Figure 1: Diversity of polarized cell types and shapes (not to scale)

(a) In Fission yeast (*Schizosaccharomyces pombe*) actin (purple) and microtubules (blue) are distributed in a polarized manner in the long axis of the cell. (b) A dividing *Drosophila* neuroblast generates an apical neuroblast (stem cell) and a smaller ganglion mother cell (GMC). (c) Epithelial cells built up barriers to separate different biological compartments (apical membrane, green; basolateral membrane, blue). (d) The budding yeast (*Saccharomyces cerevisiae*) generates a daughter cell bud from the mother cell next to the previous site of cytokinesis (bud scar, red disc). (e) Neurons are highly polarized cells with a cell body, several shorter protrusions, called dendrites and a single long protrusion, called axon. (modified from Nelson, 2003)

On the one hand, a yeast cell generates cell polarity during its vegetative growth (Chant et al., 1996 and 1999). On the other hand, epithelial cells in multi-cellular organisms are necessary to built up barriers and distinct compartments to separate the inner cellular environment from the outer cellular environment (Figure 1c) (Cox et al., 1996 and Tepass et al., 2001). Furthermore, the asymmetric shape of a *Drosophila* neuroblast leads to two daughter cells with different fates after mitosis (Wodarz et al., 2005). *Drosophila* neuroblasts are specified in the ventral neuroectoderm (Figure 1b). Afterwards the neuroblasts delaminate from the epithelium and constitute the neural primordium between ectoderm and mesoderm (Figure 1b). The asymmetric cell division of a neuroblast produces a neuroblast and a smaller ganglion mother cell (GMC). The GMC divides once more and generates two post-mitotic neurons (Fuerstenberg et al., 1998; Matsuzaki, 2000 and Wodarz, 2005) (Figure 5). The asymmetric cell division enlarges the cellular diversity in a multi-cellular organism.

Moreover, post-mitotic neurons generate long processes to get over long distances to communicate with other cells and tissues (Figure 1e) (Dotti et al., 1988).

As the differences in the morphologies and functions of the polarized cell types show a great diversity, one would assume the same diversity in the mechanisms causing cellular polarity. But, strikingly, during the past years it became increasingly clear that the general mechanisms underlying cellular polarity in different processes and organisms are very well evolutionary conserved.

1.2. The machinery regulating cellular polarity

The conservation of the cell polarity machinery was shown in diverse organisms, but one of the best studied processes is the establishment of polarity in epithelial cells in *Drosophila* and *C.elegans* (Doe et al., 2001; Ohno, 2001). The plasma membrane of an epithelial cell is divided in two distinct regions. On the one hand, the membrane, which is in contact with a neighbouring cell, is called basolateral membrane. On the other hand the so called apical membrane is the free cell surface (Figure 1c). This apico-basal polarity of an epithelial cell is dependant on the establishment of the apical junctional complex (Figure 3; Nelson 2003 and Yamanaka et al., 2001), a protein complex consisting of membrane and scaffold proteins (Tepass et al., 2001 and Nelson, 2003). The proteins identified in the *Drosophila* apical junctional complex are: Cadherin/Catenins, Crumbs (Crb)/Stardust, Bazooka (Baz/Par3)/Par6/atypical protein kinase C (aPKC) and Lethal giant larva (Lgl)/Scribble (Scrib)/Discs large (Dlg) /LET-413 (Suzuki et al., 2001, Rolls et al., 2003 and Albertson et al., 2003).

The mentioned proteins are involved in the establishment and regulation of epithelial polarity. The initial signal is the Cadherin/Catenin-mediated cell-cell contact followed by the enrichment of the Par-complex proteins to the cadherin adherens junction. Adherens junctions separate the membrane in two mentioned parts - the part of the membrane above the adherens junction is the apical membrane; below the adherens junction is the basolateral membrane (Figure 1c and 2). After the recruitment of the Par proteins to the apical membrane, the protein complex Lgl/Scrib is positioned below the Par-complex proteins to block the spreading of the apical membrane by its function as an antagonist of the Par-proteins. Further regulation of Crb by Lgl/Scrib helps to maintain the basolateral membrane identity. As Crb/Stardust is located apical of the Par-complex it is also involved in the maintenance of the apical membrane identity (Figure 2) (Tepass et al., 1993 & 2001 and Nelson, 2003).

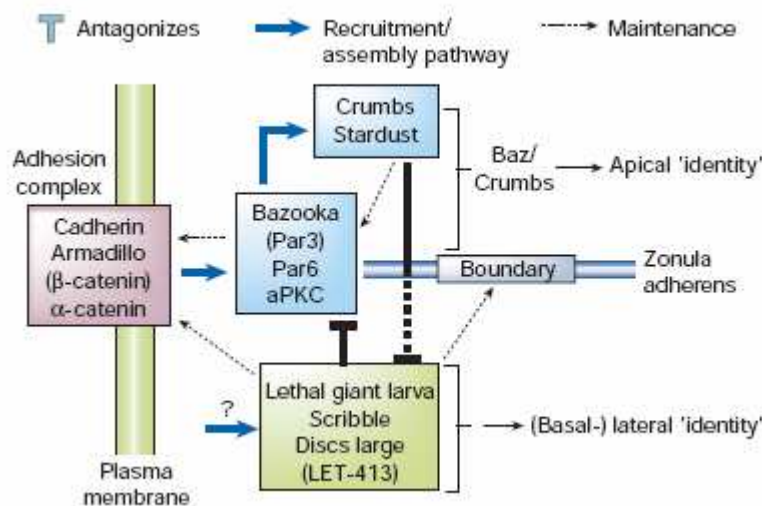


Figure 2: The apical junctional complex regulates membrane identity in epithelial cells

First cell-cell contact is formed via Cadherin/Catenin at the adherens junction (adhesion complex). The interaction of the Par-complex proteins (Baz(Par3)/Par6/aPKC) with Crumbs/Stardust regulates the identity of the apical membrane. The basal membrane is established and maintained by Lethal giant larva, Scribble and Discs large and their interaction with the Par-proteins as well as with Crumbs/Stardust. (Nelson, 2003)

Additionally to the Par polarity complex, the establishment and maintenance of epithelial polarity is reliant on the cytoskeleton of the cell, regulated by small GTPases (Rho, Cdc42, Rac1) (Fukata et al., 2001).

As the Par proteins have an essential role in the establishment of neuronal polarity, the following part describes their function.

1.3. The Par polarity complex

The Par complex (partitioning defective protein), consistent of Par3, Par6 and atypical Protein Kinase C (aPKC), was detected in asymmetric cell division in *C.elegans* embryogenesis (Etemad-Moghadam et al., 1995; Kemphues et al, 1988 and Watts et al., 1996). Strikingly, the polarity complex is highly conserved in the establishment of polarity in multi-cellular organisms. In *Drosophila* the Par-complex is found to regulate the establishment of the apical-basal axis in asymmetric cell division of neuroblasts (Petronczky et al., 2001) by influencing the localization of cell fate determinants, like Brat (Figure 5). It was shown that the

correct apical localization of Par6 and Par3 is dependant on the activity of aPKC (Hung & Kemphues, 1999 and Rolls et al., 2003). aPKC, as a kinase, phosphorylates Lgl in the apical region of a dividing neuroblast, causing inactivity of Lgl, which regulates the basal localization of Numb, Miranda and Prospero in its active form (Betschinger et al., 2003). Another function of the Par-complex in *Drosophila* affects the differentiation of oocytes (Huynh et al., 2001) and polarized epithelial cells during *Drosophila* embryogenesis (Hutterer et al., 2004). Moreover, the Par complex is responsible for the positioning and orientation of the mitotic spindle in dividing cells (Schaefer et al., 2000).

Furthermore, Par-proteins have an essential role in the protein distribution in divisions of mammalian neural stem cells (Costa et al., 2008). The function of the Par-complex as a polarity-establishing complex is dependant on its polarized subcellular distribution and is explained by the ability of Rho-GTPases (Cdc42 or Rac1) to activate the Par polarity complex. (Gotta et al., 2001; Johansson et al., 2000 and Lin et al., 2000). The activity of Rho-GTPases is regulated by guanine nucleotide exchange factors (GEFs), like STEF or Tiam1 (Rossman et al., 2005) and it modifies the actin cytoskeleton of a cell and stabilizes microtubules (Etienne-Manneville et al., 2003). Independent of the cell type and the organism the mutation of Par-proteins leads to a loss of polarity (Gotta et al., 2001 and Petronczki et al., 2001).

The Par-proteins are also essential in neuronal polarization, especially in the axonal outgrowth (Shi et al., 2003 and Nishimura 2004, 2005).

Neurons undergo several distinct morphological changes in a polarized fashion during their differentiation (see chapter 1.4. Neuronal Polarity – Axon and Dendrites in hippocampal neurons). In Stage 2 of the development of a neuron Par3 and Par6 are located at the tip of each neurite. More interestingly, both proteins are enriched at the tip of the single axon in a neuron in developmental Stage 3. The polarized expression pattern does not clarify the question – which signal initiates axonal outgrowth in one of the immature neurites; but it suggests a potential role of the Par-complex in the establishment of the axon (Nishimura et al., 2005). The Par proteins at the tip of the axon mediate signalling from Cdc42 to Rac1, which influences the dynamics of actin filaments at the tip of the axon to enhance rapid axon outgrowth. Binding of active Rac1 or Cdc42 to Par6/aPKC

enhances the activity of aPKC, that is necessary for the correct localization of the Par proteins (Etienne-Manneville et al., 2001 and Lin et al., 2000). The overexpression of Par3 and Par6 leads to loss of neuronal polarity. The neuron fails to specify the axon (Shi et al., 2003).

Additionally to the axon-specific localization of the Par-complex also the activity of PI3K (Phosphatidylinositol 3-Kinase) and its product PIP3 (Phosphoinositol-3,4,5-trisphosphat) is important for the establishment of an axon. Given that Rac1 can activate PI3K, this activation might form a feedback loop, which could sustain activity (Shi et al., 2003).

1.4. Neuronal Polarity – Axon and Dendrites in hippocampal neurons

Neurons are highly polarized cells. The development of a polarized morphology is an essential step in the development of neurons. They develop protrusions different in length and composition. Therefore neurons show a unique structure with three distinguishable domains: the cell body, one axon and several dendrites (Craig et al., 1994). The axon is defined as a long, Tau-1 positive neurite for transmission of electrical impulses from the cell body to target cells. The axon deals with outgoing (efferent) messages. Dendrites are MAP2-positive processes, shorter in length than the axon. Dendrites built up a network, the so called, dendritic tree to receive chemical messages from neighbouring cells via synapses (Dotti et al., 1988; definitions: www.MedicineNet.com).

Axonal outgrowth is the initial event of neuronal polarization in cultured hippocampal neurons, a well established model-system to study cellular asymmetry in post-mitotic, non-proliferating cells (Dotti et al., 1988 and Kaech et al., 2006). Cultured hippocampal neurons pass through five different stages during their development (Figure 4). The stages were determined by Dotti et al. 20 years ago (1987 and 1988).

Shortly after plating the neuron forms very short processes (stage 1) to attach to the surface, so called lamellipodia. In stage 2 the lamellipodia elongate and become immature neurites. The neurites are very dynamic in their extension and retraction. Next, in stage 3 one, and only one of these initially indistinguishable

minor processes starts to grow faster than the others and acquires axonal identity. In this stage, the so called symmetry breaking step, the polarized morphology of the neuron is established.

It was detected by Dotti et al. that cutting of the axon leads to the formation of a new axon by one of the remaining neurites (Dotti et al., 1987 and Goslin&Banker, 1989). So every single process maintains its ability to become the axon. Otherwise there would not be this active rescue system in all neurites. Additionally, it has been shown that the single immature neurites send inhibiting signals to the neighbouring neurites that influence the retraction or extension of one neurite. Simultaneously the neurite chosen to become the axon shows a self-promoting growth activity. Furthermore the neurites signal back to the cell body (Figure 3) (Arimura et al., 2007 and Bradke & Dotti, 2000).

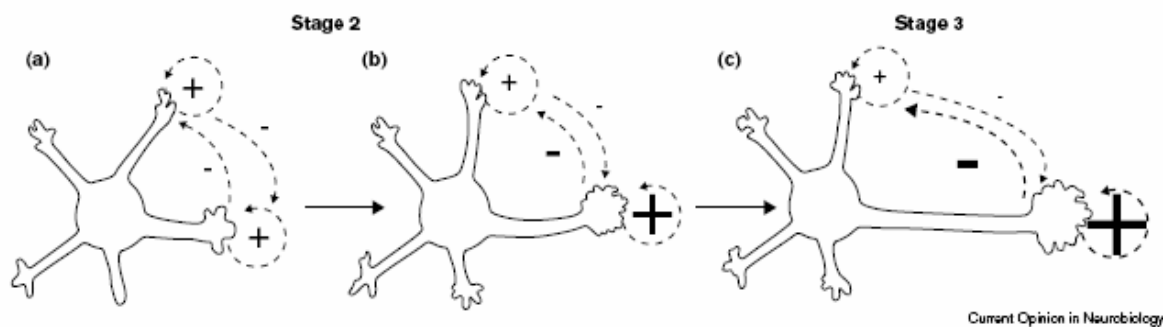


Figure 3: Axon or Dendrite? - Signalling defines the identity of neurites

Positive and negative feedback signalling is involved in the determination of the future axon. In a Stage 2 neuron every single immature neurite has the potential to become the axon. Every neurite sends inhibiting signals to the neighbouring neurite and promotes its own growth. In Stage 2 the cytoskeleton dynamics changes dramatically in one- and only one- neurite. Therefore the signalling of this single neurite becomes stronger and facilitates the elongation of this single neurite to acquire axonal identity. (modified from Bradke and Dotti, 2000)

After the specification and outgrowth of the axon the dendrites are specified in stage 4. In the last stage dendritic spines are formed. This is another example for the establishment of neuronal asymmetry. Spines are small extrusion on the dendrites that form the postsynaptic site of a synapse. Dendritic spines serve as a storage site for synaptic strength and help transmit electrical signals to the cell body of the neuron. Dendritic spines have a high content of Actin and Actin is critically involved in plasticity and synaptic activity (Kaeche et al., 2001).

The time needed to go through the 5 stages is dependant on the culture conditions (cell number, substrate, medium etc.). The usage of cultured hippocampal neurons is justified by the detection of Nakahira et al. They were able to show that neurons migrating from the ventricular zone to the hippocampus show the same morphology like cultured stage 2 neurons. (Nakahira et al., 2005)

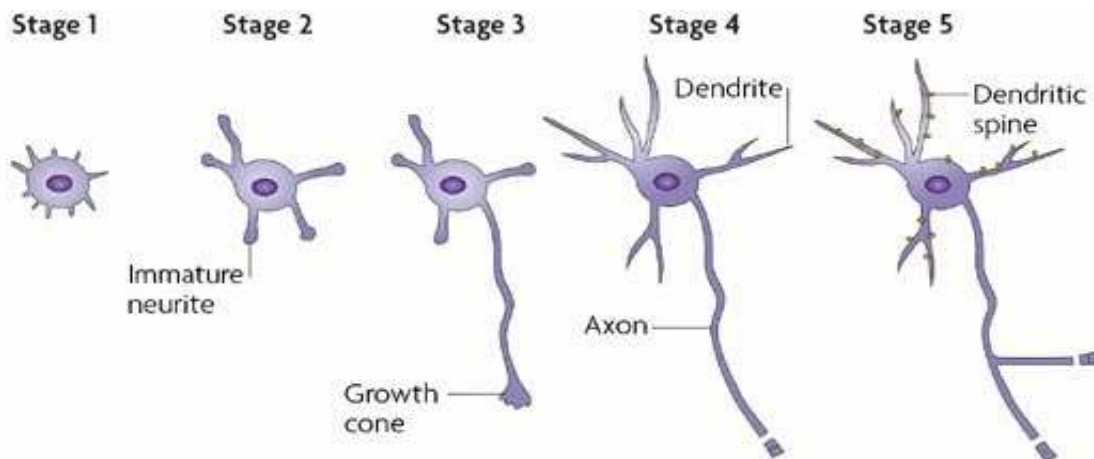


Figure 4: The development of asymmetry in hippocampal neurons

A neuron passes through 5 described developmental stages. From stage 2 to stage 3 the symmetry breaks down and one of the short immature neurites becomes the axon. Each neurite has a dynamic motility during neuronal polarization, and the polarization mechanism involves positive and negative feedback. Another asymmetric process is the development of dendritic spines in stage 5. (Arimura et al., 2007)

Although the process of the development of polarity in neurons is well studied and described, the molecular mechanisms causing asymmetry are not fully understood so far. The question how the distinct domains are generated and maintained remains to be elucidated.

1.5. Trim-NHL Proteins

The Trim (tripartite motif) proteins are multifunctional proteins with a conserved domain structure consisting of a RING finger with one or two B-boxes (zinc-binding motifs) and a leucine coiled-coil domain. Due to the combination of the mentioned domains the proteins are members of the so called RBCC or tripartite motif family. The structure of the proteins is found from plants to mammals (Saurin et al., 1996). Some Trim proteins also have an additional NHL-domain.

The family of TRIM proteins contains 68 members; only some of them are characterized (Reymond et al, 2001), but because of all the different structural motifs Trim proteins cover a broad spectrum of functions and features in diverse multicellular organisms. Just recently the RING-finger is shown to be involved in ubiquitinylation followed by degradation of other proteins (Niikura et al., 2003, Yang et al., 2003 and Joazeiro and Weissman, 2000). The other structural domains make Trim proteins to perfect binding partners for proteins. Trim proteins regulate stem cell proliferation and work as tumor suppressors. Furthermore, they can control microRNAs due to the connection with the RNase Argonaute-1 (Ago-1) (Neumueller et al., 2008 and Schwamborn et al., 2008).

One member of the Trim family is the protein Brain tumor (Brat). It is identified as cell fate determinant in *Drosophila* neuroblasts by Betschinger et al., 2006. As shown in Figure 5 Brat is localizing on the basal region at the opposite site of the Par complex in a dividing neuroblast. During the asymmetric division of the neuroblast Brat is segregated into one of the two daughter cells, where Brat inhibits dMyc on a posttranscriptional level. Therefore, this daughter cell divides only once more and starts to differentiate into post-mitotic neurons. The other daughter cell remains a self-renewing neuroblast and proliferates furthermore. In a brat mutant there is no generation of differentiating daughter cells. This leads to unregulated proliferation of neuroblasts and tumor formation (Betschinger et al., 2006; Figure 5).

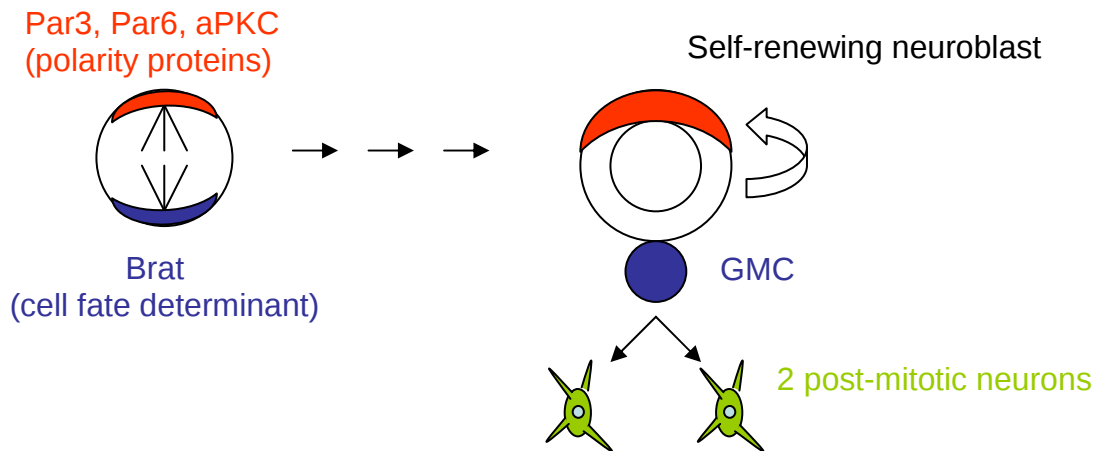


Figure 5: Asymmetric cell division of a Drosophila neuroblast

After the division of a neuroblast along its apical-basal axis, cell fate determinants (Brat) (Betschinger et al., 2006) and polarity proteins (Par3, Par6 and aPKC) are segregated asymmetrically into the daughter cells (Petronczky et al., 2001), and thereby generates two daughter cells with different fates – a larger apical cell with stem cell features and a smaller basal Ganglion Mother Cell (GMC) that inherits the cell fate determinant Brat and differentiates into two post-mitotic neurons (Fuerstenberg et al., 1998; Matsuzaki, 2000 and Wodarz, 2005).

1.5.1. Trim2 – function in neuronal polarity

Trim2 is a cytoplasmic RBCC-protein, containing the NHL-domain. It is a mammalian member of the Trim-NHL protein family, which shows a potential role in the establishment of cellular polarity due to its structural similarity to Brat. Trim2 shows a high expression level in the mouse cerebellum, the retina and the hippocampus, detected by β -Gal staining and in-situ hybridization (Martin Balastik; PhD thesis).

The tripartite motif proteins, Trim2 (NARF) and Trim3 (BERP) are closely related to each other. NARF show 67% identity to BERP, and, moreover, both proteins show the same length and structure. Therefore they have one common orthologue identified in molluscs, called L-Trim. L-Trim is involved in neurite outgrowth and regeneration in molluscs (van Diepen et al., 2005).

It is known that Trim2 and Trim3 are interaction partners of myosin V (Ohkawa et al., 2001; El-Husseini and Vincent, 1999), a myosin protein necessary for transport of vesicles to the peripheral region of neurons (Tabb et al., 1998; Molyneaux et al., 2000). Therefore, it is suggested to be involved in the maintenance of neural activity and it has been put forward as putative organizers of structural/neural plasticity. The level of the Trim2 protein increases in hippocampal and cerebellar neurons after pentylenetetrazol (PTZ)- and kainate (KA)-mediated seizure (Ohkawa et al., 2001). These observations indicate that Trim2 expression is enhanced by seizure-related neural activities and is involved in the phenomenon of neural plasticity. As neural plasticity, like long-term potentiation and long-term depression is described in connection with learning and memory (Bliss & Collingridge, 1993), Trim2 could be involved in this process.

Trim2 knock out mice have been created by Martin Balastik in 2008 via the gene trap method (evolved by enhancer trap method). The mutation is produced by insertion of a promoter-less vector into embryonic stem cell. In the stem cell the vector can integrate randomly to an exon, an intron or to a 5' UTR of an expressed gene. This integration disrupts the open reading frame of the trapped gene and results in a fusion product of the trapped gene and the known vector. The used vectors are promoter-less, but they contain a selection marker (neo gene) and as a reporter gene usually β -gal is used. Stem cells transcribing the vector are selected via application of a drug and they are used for generation of

chimeric blastocysts, chimeric mice and mutant mouse line. The trapped gene is detected by RACE-PCR with primers for the known vector sequence that is fused to the trapped gene after insertion. (all described in PhD thesis of M. Balastik, Göttingen, 2003).

Trim2 knock-out mice show severe abnormalities, like reduced weight and size. Additionally, they suffer from illnesses like ataxia and epileptic fits. The diseases get worse during the life-span. Recently, it has been shown that Trim2 binds to neurofilament light subunit (NF-L) and regulates NF-L ubiquitinylation via its RING-finger domain. Therefore it triggers neurodegeneration by its function as ubiquitin ligase (Balastik et al., 2008).

2. MATERIAL & METHODS

2.1. Cell and Mouse lines

N2a cells: murine neuroblastoma cells, cultured in DMEM-high glucose with supplements (DMEM-high glucose⁺⁺)

DMEM-high glucose⁺⁺:

- 10% FCS (Sigma)
- 1% L-Glutamine (Invitrogen)
- 1% penicillin/streptomycin (Invitrogen)

293T cells: HEK cells; human embryonic kidney cells; cultured in DMEM-high glucose⁺⁺

Hippocampal neurons: Hippocampal neurons were isolated at embryonic day 16.5 (E16.5). The neurons were grown on coverslips coated with Poly-Ornithin (Sigma, 1:10000 in 1x PBS; overnight) and Fibronectin (Sigma, 1:10000 in 1xPBS; 1hour) and cultured in Neurobasal media with supplements (NBM⁺⁺).

Neurobasal medium (NBM⁺⁺):

- 50 ml NBM (GIBCO™ Neurobasal™ Medium (1X) liquid, Invitrogen)
- 1 ml B27 supplement (GIBCO™ B-27 Supplement Minus AO (50X) liquid)
- 0.5 mM L-Glutamine (Invitrogen)
- 100 U/ml penicillin/streptomycin (Invitrogen)

For the isolation of hippocampal neurons two mouse lines were used. The C57BL/6J mouse line for analysis of wildtype neurons, gain of function (gof) and loss of function (lof) studies. Trim2 knock out mice were also a C57BL/6J mouse line. They were used to investigate the development of knock-out hippocampal neurons.

2.2. Isolation of hippocampal neurons

At E16.5 the brain of each mouse embryo was dissected to isolate the hippocampus. Single hippocampi were collected and washed three times with DMEM-high glucose medium without any supplements. Next, the hippocampi were incubated at 37°C in 5ml DMEM-high glucose, containing 5µl DNaseI recombinant, grade I (Roche) and small amount (tip of a spatula) of Papain (Sigma, 100MG) to decompose the tissue. The enzymatic reaction of the DNaseI and Papain was stopped after 15 minutes by washing the hippocampi with DMEM-high glucose⁺⁺.

Thereafter the tissue of the hippocampi was dissociated by pipetting with a blue tip followed by a yellow tip in 2ml of plating medium (DMEM-high glucose⁺⁺) and filled up with the plating medium to the desired end volume. 500µl of the cell suspension were pipetted on each coverslip, placed in a 24-well plate and coated with Poly-Ornithin and Fibronectin, and incubated over night at 37°C and 5% CO₂. The next day the plating medium was changed to NBM⁺⁺ to optimize the culturing conditions for neurons, incubated again at 37°C and 5% CO₂ for further development.

The method was published by Schwamborn, Li and Püschel (2006).

2.3. Immunostaining

To stain cultured hippocampal neurons the cells were fixed at different time points with 4% Paraformaldehyde (PFA; Sigma) + 15% Sucrose (Fluka) for 20 minutes at 4°C. Afterwards every working step was performed at room temperature (RT). After removing the PFA/Sucrose mixture from the cells by washing three times with 1xPBS, the neurons were permeabilized with 0,1% Triton X-100/ 0,1% Natrium-Citrate in 1xPBS for 3 minutes. The washing procedure was repeated and the cells were blocked with 10% FCS/ 0,01% Azid in 1xPBS for 1 hour. Then, the neurons were incubated with the primary antibody O/N. At the next day the unbound primary antibody was washed away with 1xPBS and the incubation with the secondary antibody was done for 90 minutes. After washing 3 times with 1xPBS and H₂O the samples were mounted with Aquamount (DAKO).

To stain the HEK and N2a cells the same protocol with the following variances was used: For fixation of the cells cultured on a coverslip 4% PFA was used, permeabilization was carried out with 0,05% Triton-X100 and 3%BSA in 1xPBS served as blocking reagent. In the staining protocol for HEK and N2a cells the incubation time for the primary antibody was fixed with a minimum of 90 minutes at RT.

2.4. In-situ hybridization

The in-situ hybridization was carried out in a 24-well format. Four days after culturing the hippocampal neurons on coverslips were fixed with 4% PFA+ 15% Sucrose for 15 minutes. Next, the neurons were washed 3 times with 1xPBS at RT and acetylated for 10 minutes with acetylation solution (590µl DEPC treated water, 8ml triethanolamine (Fluka) and 1,05ml conc. HCl (Riedel) are mixed gently; 1,5ml acetic anhydride (Sigma) are added until it is dissolved and stirred gently for 10 additional minutes). Acetylation solution was removed to prehybridize the samples with 700µl hybridization buffer (50% formamide (Sigma), 5x 20xSSC, 5x 50xDenhardt's (Sigma), 200µg ml⁻¹ 20mg ml⁻¹ yeast tRNA (Sigma), 500µg ml⁻¹ 10mg ml⁻¹salmon sperm DNA (Sigma), 0,4g Roche Blocking reagent, filled up with Diethylpyrocarbonate (DEPC,Fluka)-treated water to desired endvolume) for 4-8 hours at RT. Afterwards, 500µl denaturing hybridization buffer (hybridization buffer containing 10% CHAPS (Sigma), 20% Tween (Fluka)) + 1µl Anti-digoxigenin antibody (DIG probe, Roche), incubated for 5 minutes at 80 °C, were added to each cover slip and incubated at 65°C O/N.

On the next day the slides were soaked in 5x SSC and in 0,2x SSC at 60 °C for 1 hour. Thereafter, the samples were incubated in B1 buffer (0,1M Tris pH 7,5/ 0,15M NaCl in 1 litre DEPC treated water) at RT for 10 minutes in the dark and blocked with B1+ 10% FCS for 1 hour, RT. After blocking the primary antibody anti-rabbit Trim2, diluted 1:200 in B1+1% FCS, was added to the slides. Additionally, the anti-DIG-Alkaline-Phosphatase antibody, diluted 1:2000 in B1+1% FCS, was put onto the neurons. All samples were incubated in a moist chamber O/N at 4°C.

On the next day the unbound primary antibody was removed by washing the samples three times in B1 buffer for 5 minutes. Then, the samples were incubated

with the secondary antibody anti-rabbit-488, diluted 1:1000 in B1+1% FCS, for 1 hour at RT. After this step the neurons were washed three times with B1 buffer again. Next, the slides were equilibrated in 10x Fast red buffer (1M Tris pH 8.2, DAKO) for 10 minutes at RT. Meanwhile, a tablet of Fast Red (DAKO) was added to 2 ml of the supplied buffer. The mixture was incubated in the dark for 8 minutes, followed by vortexing.

Each slide was placed on a few drops of filtered Fast Red solution (DAKO) and developed at RT in the dark until the red precipitate was formed. Depending on the levels of expression of the miRNA the time to develop can be between 30 minutes and 5 hours. After the red precipitate was visible, the reaction was stopped by washing three times in 1xPBT (Developer solution, 3.4ml 100mg ml⁻¹ NBT (Roche), 3.5ml 50 mg ml⁻¹ BCIP (Roche), 2.4ml 24mg ml⁻¹ levamisole (Sigma), 5ml of 10% Tween and 986 ml B3 buffer (0.1 M Tris pH 9.5/0.1 M NaCl/50 mM MgCl₂)) for 10 minutes and the slides were mounted with VECTASHIELD mounting medium.

The method was published by Obernosterer et al., in 2007.

2.5. Microscopy

All fluorescently labelled samples were analysed by confocal microscopy (Zeiss LSM 510 Meta/Axiovert) or fluorescence microscopy (Axioplan2 imaging/Coolsnap) and were attended by Adobe Photoshop CS3. Statistical analysis was done with Microsoft Excel.

2.6. Transfection

2.6.1. Transfection of HEK293T and N2a cells

For transfection of HEK293T (HEK) and N2a cells the FuGene transfection reagent (Roche), a multi-component lipid-based transfection reagent, was used. During transfection the reagent forms a complex with the DNA and transports it into the cell with high efficiency.

To transfect the HEK and N2a cells, cultured in one well of a 24-well plate, first of all 0,37µl FuGene reagent was diluted in 16,67µl transfection medium (OptiMEM; GIBCO) at RT for 5 minutes. Afterwards 125ng DNA were added to the diluted FuGene reagent dropwise and incubated for 15 minutes at RT.

The FuGene-DNA mix was pipetted onto the cell cultures in one well of a 24-well plate. Afterwards the tissue culture dish was placed into the controlled-atmosphere incubator (37°C, 5% CO₂) for 48 hours.

Another transfection reagent used for HEK and N2a cells was Lipofectamin2000 (LF2000; Invitrogen). This cationic liposome-mediated transfection shows higher transfection efficiency compared to the FuGene reagent.

For a Ø10cm tissue culture plate 800µl OptiMEM and 32µl LF2000 were mixed and incubated for 5 minutes at RT. In the meantime 800µl OptiMEM and 12,8µg DNA were prepared and added to the OptiMEM-LF2000 mixture, followed by incubation at RT for 20 minutes. Next, both mixtures were combined and pipetted onto the cells, which were incubated at 37°C/5% CO₂ again for 48 hours.

2.6.2. Transfection of hippocampal neurons with CaPO₄

The transfection of hippocampal neurons was performed in 24-well plates. To transfect the neurons cultured in 500µl NBM⁺⁺ in one well of the plate 1,5µl 2,5mM CaCl₂ were mixed with H₂O. The amount of H₂O is dependant on the concentration of the used DNA; the end volume of the mixture should be 15µl. After pipetting the CaCl₂ into H₂O 750ng of DNA were added to the H₂O-CaCl₂ mixture.

Next, BES buffer (pH 7,1, Sigma Aldrich) was added 1:1 to the mixture dropwise to guarantee a uniform precipitate. The whole reaction approach of 30µl was added to the neurons cultured in 500µl NBM⁺⁺ and incubated in a CO₂- free incubator at 37°C for at least 45 minutes.

After 45 minutes it was checked if there is a visible precipitate via light microscopy. If there was no precipitate, the plate was incubated again for further 15 minutes until the precipitate was formed. As the precipitate was visible, the neurons were washed two times with 1x HBSS (GIBCO). Finally transfected cells were incubated in 500µl NBM⁺⁺ at 37°C/ 5% CO₂ until further analysis.

The method was published by Goetze and Kiebler in 2004.

2.7. RNAi constructs

The RNAi constructs used for transfection of hippocampal neurons to silence Trim2 expression, were designed and tested by Jens Schwamborn.

As backbone the pSM2 vector was used to express a hairpin to silence the gene expression of Trim2. This hairpin is called short hairpin RNA (shRNA) is able to silence gene expression of a specific gene via RNA interference (RNAi).

The sequence of the hairpin to knock down Trim2 was:
TGCTGTTGACAGTGAGCGCGGTGTGGCCGTGGACTCAAATTAGTGAAGCCA
CAGATGTAATTTGAGTCCACGGCCACACCTTGCCTACTGCCTCGGA

2.8. Electrocompetent Pir1⁺ bacteria and Electrotransformation

To allow the replication of the pSM2-ShRNA vector, it has to be transformed into a Pir1⁺ E.coli strain. Pir1⁺ E.coli electrocompetent bacteria were generated for electrotransformation.

1µl of Pir1⁺ E.coli (Invitrogen) was added to 1ml of prewarmed LB-medium and incubated over night (O/N) at 37°C shaking. In a 1L Erlenmeyer bottle 100ml of LB-medium were inoculated with 1ml of the O/N culture and incubated at 37°C with vigorous shaking until the OD₆₀₀ of the culture reached 0,6. At this time point the bacteria were chilled on ice for 30 minutes. Next the bacteria were harvested in a centrifugation step for 15 minutes at 4000g, 4°C. The supernatant was

decanted immediately and the cell pellet was washed twice with 50ml of ice-cold 10% glycerol (10 minutes at 4000g, 4°C). The supernatant was discarded again immediately after centrifugation and the pellet was resuspended in 2,5ml ice-cold GYT (10% glycerol, 0,125% yeast extract and 0,25% tryptone). The bacterial cell suspension was stored in 200µl aliquots at -70°C.

Electrotransformation was performed using a Gene Pulser (BioRad). To 200µl thawed electrocompetent Pir1⁺ cells 5µl pSM2-ShRNA plasmid was pipetted and incubated on ice. After 10 minutes the bacteria-plasmid mixture was transferred into a Gene Pulser electroporation cuvette (BioRad). The conditions for electrotransformation were 25µF capacitance, 2,5kV and 200Ω resistance. After the pulse of electricity was performed the cells were transferred in 1ml of prewarmed LB medium to a falcon. Afterwards, the suspension was incubated at 37°C for 1 hour in a shaker. 100µl-200µl of the transformed cells were plated on LB-kanamycin/chloramphenicol plates and incubated at 37°C O/N.

2.9. Heat-Shock Transformation of E.coli

First the bacteria, stored on -70°C were thawed on ice gently. After the adding of 1-2µl (depending on the concentration) of the plasmid the mixture was incubated on ice for 30 minutes. Next, the heat-shock was performed by incubation of the bacteria-DNA mix on 42°C for 30 seconds. Afterwards the bacteria-DNA mixture was cooled down on ice for two minutes. 1ml of prewarmed LB-medium was added and the bacteria-DNA mix was incubated for 1 hour at 37°C with gentle shaking. Afterwards 100-200µl of the suspension was plated on LB-selection medium plates and grown O/N in a 37°C incubator.

2.10. Immunoprecipitation (IP)

To identify binding partners of a protein of interest an immunoprecipitation is used.

First of all HEK or N2a cells in a 10cm tissue culture plate were transferred in 10 ml 1x PBS in a 15ml falcon and centrifuged at 1250rpm for 15 minutes at 4°C.

After centrifugation the cell pellet was resuspended in 500µl lysisbuffer (500µl PMSF + 1ml Triton-X100 in 50ml 1xPBS) and transferred into a 1.5ml Eppendorf tube. The samples were incubated on the overhead-shaker (OHS) for 30 minutes at 4°C and centrifuged for 30 minutes at 13200rpm at 4°C. Next, the supernatant was transferred into a new 1.5ml Eppendorf tube. 20µl of the supernatant were taken aside as input control and mixed with 2x loading dye and stored at -70°C.

The remaining supernatant was mixed with 5µl of an antibody (anti-rabbit-EGFP, anti-rabbit-Trim2, anti-Ago-1) and incubated on the OHS for 4 hours at 4°C.

Afterwards, 30µl Protein A Agarose beads (Roche), washed three times with lysisbuffer, were pipetted into the reaction followed by O/N incubation at the OHS at 4°C.

The next day the samples were centrifuged for 5 minutes at 13200rpm at 4°C and the beads were washed three times with lysisbuffer. Every sample was frozen away at -70°C until usage in PCR or Western Blot.

2.11. cDNA synthesis

RNA is not appropriate for PCR or other procedures. Therefore it is necessary to transcribe RNA to DNA in a cDNA synthesis reaction.

10µl of Trim2 IP were mixed with 1µl of a random primer (Invitrogen). The mixture was incubated for 4 minutes at 70°C. Afterwards the reaction is cooled down on ice.

After cooling 19µl of a Master Mix (4µl 5x buffer, 1µl RNase Inhibitor, 2µl dNTP (100mM), 2µl SSII reverse transcriptase and 2µl 0,1µM DTT) were added to the reaction, followed by incubation of the reaction for 120 minutes on 42°C.

2.12. PCR

To detect a potential mRNA-Trim2 connection polymerase chain reaction (PCR) was carried out with primer pairs to amplify LIMK-1 and Cyclin E, two potential targets of miRNAs, that are regulated by Trim2. As a template the material of the Trim2-IP was used after cDNA synthesis (chapter 2.11.).

The used primer pairs (Sequence 5' to 3'):

LIMK-1 forward: AAT TCC ATC CAG GTT GGA GA

reverse: TTC CTT CAT CAC CAT CAC CTC

Cyclin E forward: ATC CCC ACC CCT AAC AAA GAA

reverse: ACC TTC TGC ATC AAC TCC AGT

2.13. Quantitative Real-Time PCR (q-RT-PCR)

The q-RT-PCR was performed to quantitatively measure the two different target microRNAs (miRs) of LIMK-1 and Cyclin E and its connection to Trim2. The protocol is divided into two parts-first a cDNA synthesis and amplification of the template by qPCR using a Real-Time Primer specific for the miR. In the second part the Real-Time PCR was carried out.

As template the Trim2-IP, described in 2.10., was used. As a control served an IP of a brain lysate performed with an anti-EGFP antibody (abcam). To detect the miRs two different approaches were done. For the miR-134, that targets LIMK-1, a TaqMan ® Gene Expression Assay (Applied Biosystems) was carried out. For miR-689, that targets Cyclin E, no TaqMan Assay was available, the Assay was cloned based on a method published by Schmittgen et al., 2008. First the Real-Time Primer (RT-Primer) was designed partially complementary to itself, that it can form a hairpin for usage in qPCR.

The sequence designed as RT-primer for miR-689 was (Sequence 5' to 3'): GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GAC CC

To amplify the starting material a master mix with 0,15µl dNTP Mix (100mM), 1µl reverse transcriptase, 1,5µl 10x buffer, 0,19µl RNase inhibitor and 4,16µl DEPC-treated water was prepared for 1 reaction. 7µl of this master mix were mixed with 5µl RNA (Trim2-IP). Furthermore 3µl 5x RT-primer (in case of miR-689 3µl of 1:10 dilution of RT-primer, 50nmol) were added.

The reverse transcription was done in a thermocycler with the following program:

30 minutes	16°C
30 minutes	42°C
5 minutes	85°C
∞	4°C

The qPCR served as template for the following RT-PCR. In the second part of the experiment 10µl of the TaqMan 2x Master Mix were mixed with 7,67µl H₂O for 1 reaction. To this 17,67µl master mix-water solution 1µl of the 20x TaqMan microRNA (for miR-689 0,3µl of the TaqMan probe were mixed with 0,3µl miRNA specific *forward and 0,3µl miRNA specific *reverse primer per reaction) assay mix and 1,33µl of the reverse transcription reaction were added. This 20µl reaction was transferred into a 96-well reaction plate. Every sample was done in triplicates.

As forward and reverse primers following sequences were used.

* miR-689 (Sequence 5' to 3') forward: CGT CC CGC TCG GCG

reverse: GTC GTA TCC AGT GCG AAT ACC T

The PCR conditions were

10 minutes	95°C	
15 seconds	95°C	} 45 times
60 seconds	60°C	

2.14. Antibodies

<u>Antibody</u>	<u>Dilution</u>	<u>Source</u>
anti-rabbit-Trim2	IF: 1:200 IP: 3µl	Jens Schwamborn
anti-mouse-LIMK-1	IF: 1:200	Santa Cruz Biotechnology, Inc
anti-rabbit-Ago-1	IP: 3-5µl	Abcam
anti-rabbit-CyclinE	IF: 1:100	Santa Cruz Biotechnology, Inc.
anti-mouse- αTubulin	IF: 1:1000	Sigma
anti-rabbit-EGFP	IP: 3-5µl	Abcam
anti-mouse-Tuj-1	IF: 1:400	Covance
anti-mouse-Tau-1	IF: 1:600	Chemicon International
anti-rabbit-Psd95	IF: 1:200	Chemicon International

Table 1: Antibodies used in the diploma thesis

2.15. Vector constructs

<u>Gene</u>	<u>Tag</u>	<u>Vector</u>	<u>Provided from</u>	<u>Source</u>
Trim2	GFP	pcDNA3	Jens Schwamborn	Invitrogen
EGFP	-	pEGFP-N1	Jens Schwamborn	Clontech
Trim2 shRNA	-	pSM2	Jens Schwamborn	Open Biosystems
pSM2	-	pSM2	Jens Schwamborn	Open Biosystems
CyclinE	-	pRC/CMV	S. Reed	Invitrogen
Cdk5	GFP	pEGFP-C1	P. Zelenka	Clontech
Cdk5 T33	GFP	pEGFP-C1	P. Zelenka	Clontech
Tubulin	Cherry	pcDNA3.1	Vic Small	Invitrogen

Table 2: Vectors used in the diploma thesis

3. RESULTS

3.1. Subcellular localization of Trim2 in cultured hippocampal neurons

As Trim2 is one of the mammalian homologues of the cell fate determinant Brat, it could be that Trim2 is involved in the establishment of cellular polarity in a neuron. Therefore, we decided to analyse the role of Trim2 in the development of the axon in hippocampal neurons, a well established system for analysis of neuronal polarity.

To investigate the role of Trim2 in axon formation one of the first interests was the analysis of the expression pattern of the protein in cultured mouse hippocampal neurons, isolated at E16.5. Therefore, in the first experiments the subcellular localization of Trim2 in the first three stages of neuronal development was tested. In the first two stages the neuron was stained with an anti-Trim2 antibody and an anti-Actin antibody. As Actin is a cytoskeletal component of a neuron, it was used to mark the whole cell. To detect the axon in stage 3 the marker Tau-1 was used, because of its characteristic expression level that increases from proximal to distal in the axon.

As shown in Figure 6 and 7 the expression of the protein Trim2 persisted in all three developmental stages. In the developmental stage 1 Trim2 was highly expressed throughout the whole cytoplasm of the cell body as well as in the lamellipodia of the neuron (upper panel, Figure 6). Compared to the Actin costaining in the image in the upper left corner there was some colocalization of Actin and Trim2 detectable. Moreover, Trim2 showed a dotted expression pattern in the cytoplasm of the cell body, the nucleus as well as in the lamellipodia.

This remarkable expression pattern did not change after the transition to the developmental stage 2 (lower panel, Figure 6). In a stage 2 neuron the protrusions are longer and called immature neurites. Trim2 was expressed in all of the immature neurites. In some neurons the protein started to leave the nucleus and it could be detected as a cytoplasmic protein, expressed in a dotted like pattern.

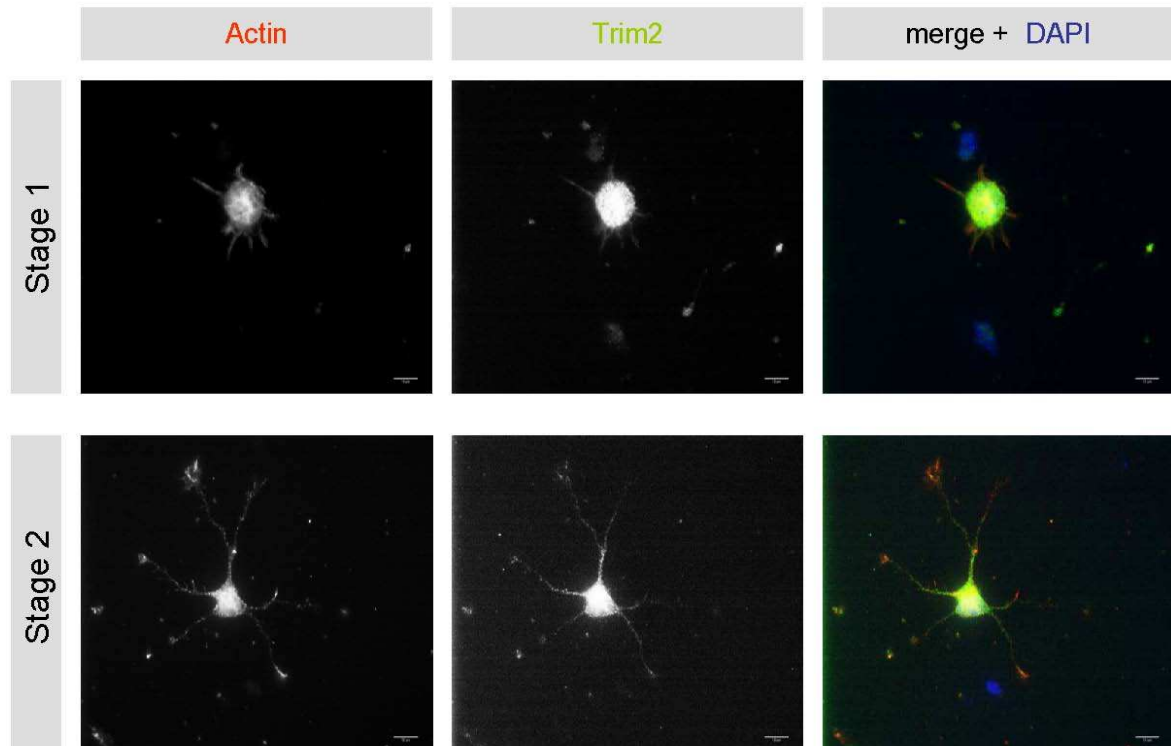


Figure 6: Stage 1 and Stage 2 mouse hippocampal neurons expressed Trim2

The hippocampal neurons were isolated at E16.5 and cultured for one day to analyse Stage 1. After one more day in vitro the majority of the cultured neurons was in Stage 2.

The neuron was detected with Actin in red. In Stage 1 Trim2 (green) was expressed in the whole neuron. In this stage the highest expression of Trim2 was in the nucleus. In Stage 2 the highest Trim2 expression was detectable in the cytoplasm of the cell body. But Trim2 was also measureable in the neurites and the nucleus (stained with DAPI in blue).

Figure 7 shows two different examples of a stage 3 neuron, with an axon, indicated with Tau-1, and several dendrites. Trim2 showed no accumulation in the axon like Tau-1, but it was expressed in the cytoplasm of the cell body, as well as in the axon and the dendrites in its dotted pattern. Strikingly, Trim2 expression was rarely detectable in the nucleus of a neuron in the developmental stage 3.

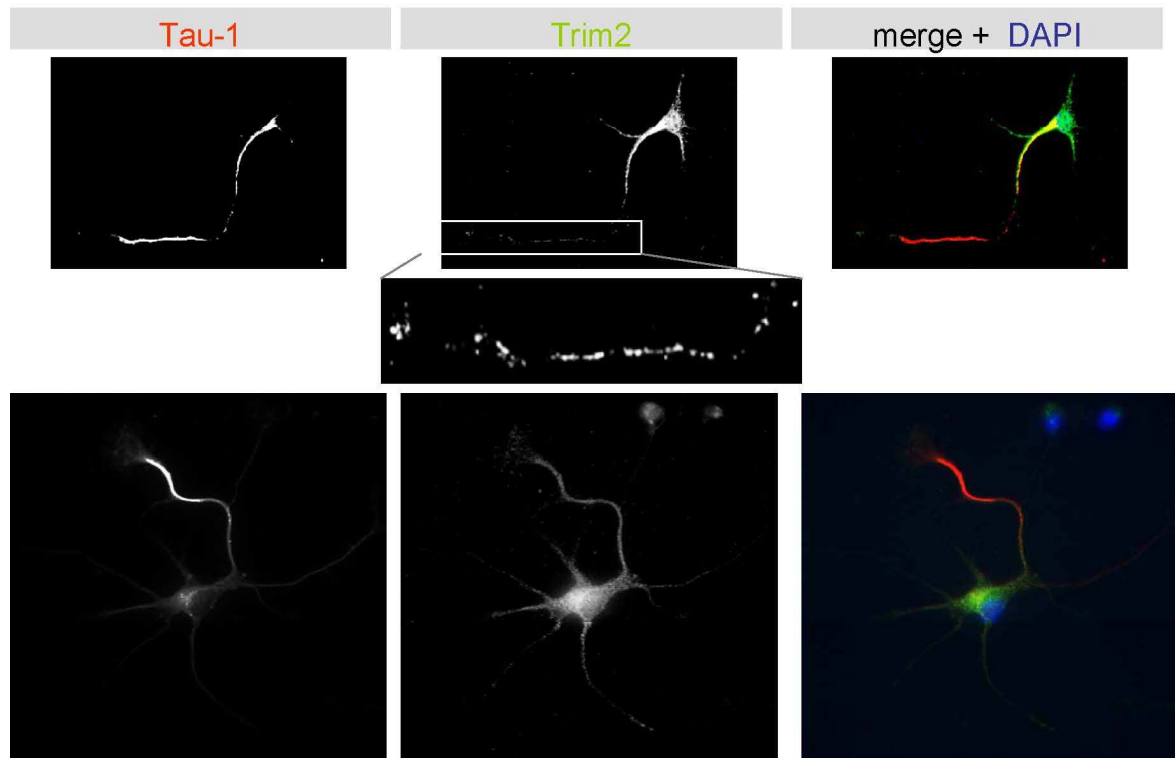


Figure 7: Stage 3 hippocampal neurons expressed Trim2

The hippocampal neurons were stained for Trim2 in green after three days in vitro to analyse the Trim2 expression pattern in developmental stage 3. The neurons were costained with the axonal marker Tau-1 in red and DAPI to detect the DNA in blue. In both panels the depicted neuron was polarized. The axon was indicated via the Tau-1 staining that was enriched from proximal to distal. Trim2 was expressed in the cell body, the axon and the neurites. Compared to Stage 1 Trim 2 was not enriched in the nucleus anymore.

The blow up showed the dotted expression pattern of Trim2 in the axon.

It seems to be the case that Trim2 leaves the nucleus beginning with developmental stage 2.

Furthermore, Trim2 showed no homogeneous distribution in the neuron, but several Trim2 particles were visible in all three developmental stages. As Trim2

was expressed in the neuron it could have a potential role in the development of neuronal polarity. Furthermore, the interesting dotted-like distribution in the cell suggests a role of Trim2 in hippocampal neurons, because other important factors like the transport protein Staufien (Kiebler et al., 1999 and Goetze et al., 2006), show a similar expression pattern as Trim2.

3.2. Downregulation of Trim2 in early developmental stages of cultured hippocampal neurons

To functionally analyse Trim2, the protein was knocked down specifically via an RNA interference (RNAi) (described in Material and Methods 2.7) approach at one day in vitro (DIV), where the neurons reside at stage 1. To investigate the potential role of Trim2 in the development of neuronal polarity, the transfected neurons were stained three days later with the axonal marker Tau-1. At this time point control neurons, just transfected with pSM2 and EGFP, were at the beginning of stage 3 and showed a polarized structure with an axon and several neurites/dendrites.

As shown in Figure 8 the RNAi transfected neuron depicted in the lower panel looks very different compared to the EGFP/pSM2 transfected control neuron shown in the upper panel. After silencing of Trim2 specifically the majority of the neurons failed to specify the axon. They stopped their development after the outgrowth of neurites; that means at stage 2.

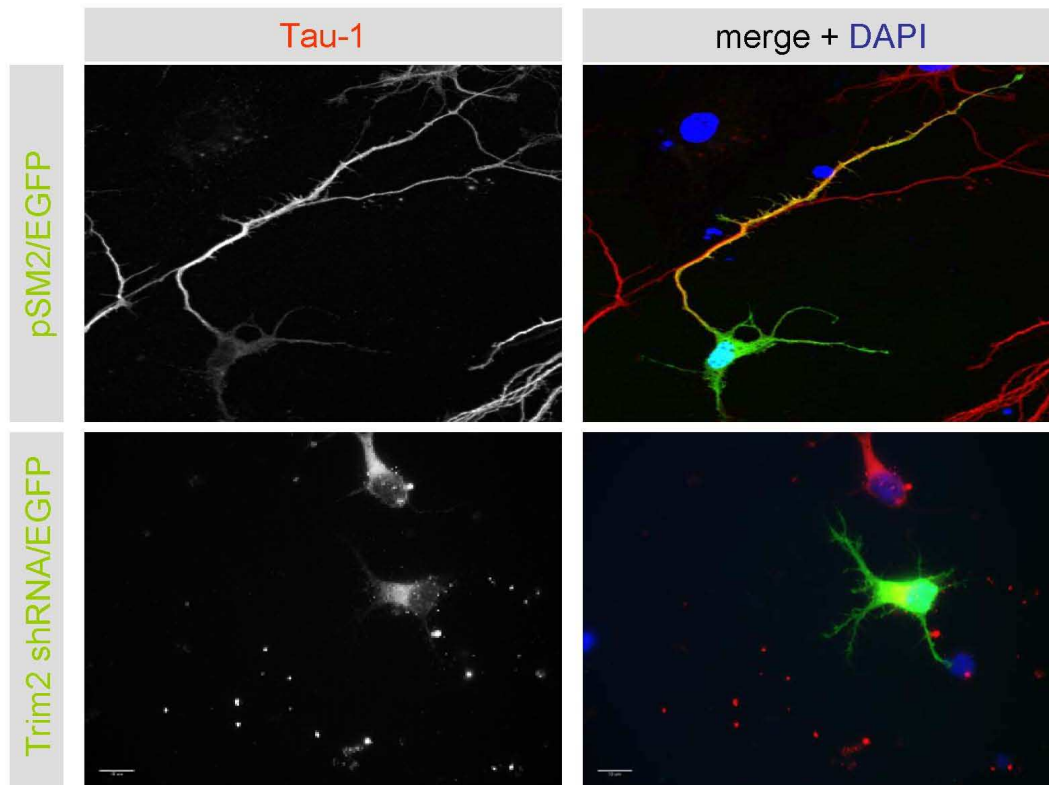


Figure 8: Knock-down of Trim2 in mouse hippocampal neurons

After one day in culture the hippocampal neurons were transfected with a vector containing pSM2 and a vector expressing a Trim2- specific shRNA (small hairpin RNA) to silence the expression of Trim2. In both cases the neurons were cotransfected with an expression vector for EGFP.

Three days later the culture was stained with Tau-1 in red to indicate the axon for further quantification and with DAPI to mark the nucleus of the neuron. After the knock-down of Trim2 the neuron illustrated in the lower panel showed a defect in the formation of the axon. In contrast, under the same conditions the transfected control neuron was polarized.

To quantify the number of transfected neurons, the population was divided in two distinct groups. Cells with an axon were scored as polarized; while cells with a defect in axon outgrowth were scored as unpolarized. To distinguish the polarized and the unpolarized neurons the axonal marker Tau-1 (left panel, Figure 8) was used. Furthermore the axon was defined by its structure and length.

In the control transfection 80% of the transfected neurons (n= 94) were polarized, but this percentage decreased to 40% after knock-down of Trim2 (n= 55). Because of the silencing of Trim2 the majority of the transfected neurons was unpolarized (see Figure 9).

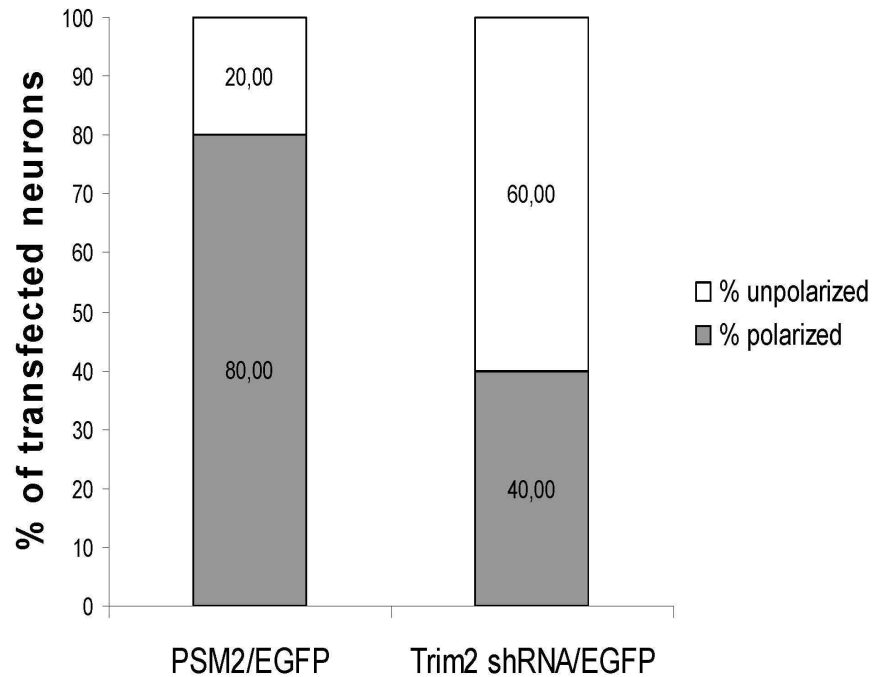


Figure 9: The silencing of Trim2 caused a decrease in the percentage of polarized neurons

The number of pSM2/EGFP and Trim shRNA transfected neurons was quantified and divided in two groups – polarized are neurons with an axon; unpolarized are neurons with neurites, but without an axon. The classification was done by using the axonal marker Tau-1. After the knock-down of Trim2 it was only half of the percentage of polarized neurons compared to the pSM2/EGFP transfection. (standard deviation EGFP/pSM2 = 5,21% ; Trim2 shRNA/EGFP = 4,69%)

This observation implicates that Trim2 plays a role in the establishment or specification of the axon; and furthermore in the establishment of neuronal polarity.

3.3. Hippocampal neurons from Trim2 knock-out mice

To confirm the phenotype of the RNAi experiment described before and to characterize the function of Trim2 more precisely, we obtained Trim2 knock-out mice (Balastik et al., 2008). The axonal outgrowth of cultured Trim2 knock-out hippocampal neurons was analysed after three days in vitro. Three days in culture is described as the earliest time point to investigate the establishment of an axon.

The neurons were immunostained with an antibody against the axonal marker Tau-1 and with a neuron specific beta III Tubulin antibody (Tuj-1) as a neuronal marker.

The single Trim2 knock-out neuron depicted as a representative example in the lower panel of Figure 10 appears remarkable different in comparison to the wildtype (wt) neuron in the upper panel.

The wt neuron was a normal developed and polarized neuron with an axon and several short neurites. In contrast the Trim2 knock-out neuron was not able to develop an axon.

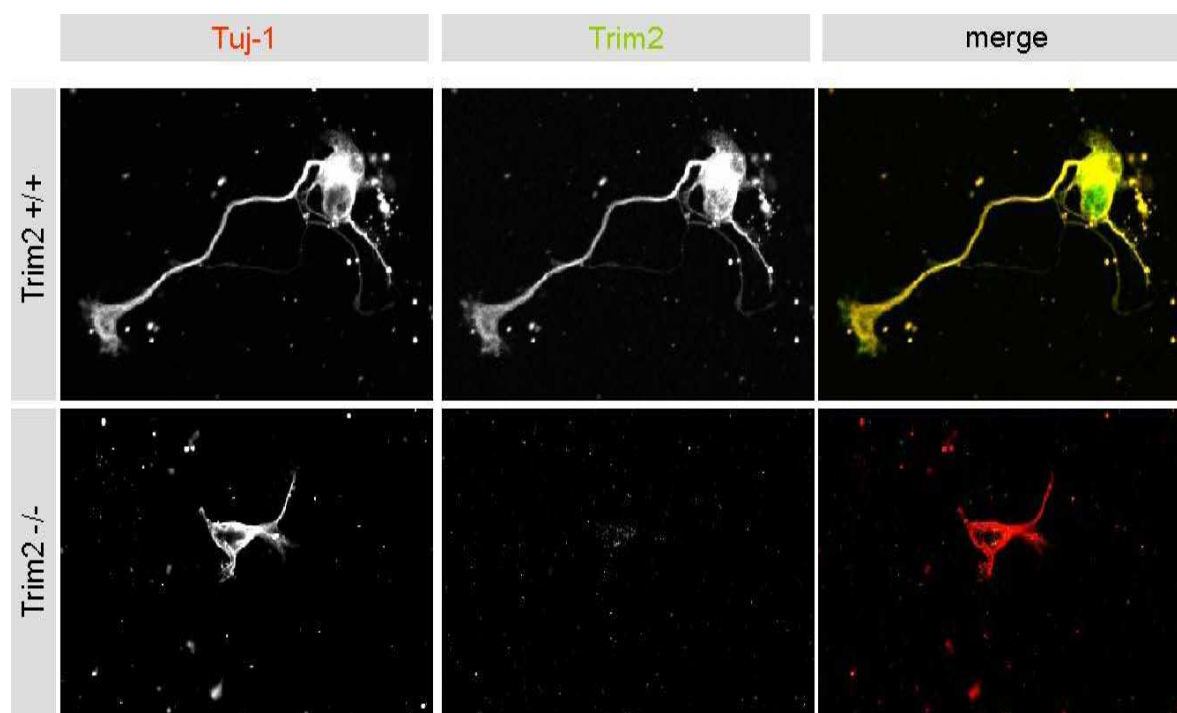


Figure 10: Trim2 knock-out hippocampal neurons failed to develop the axon

In the upper panel a wildtype neuron, stained with Trim2 in green and the neuronal marker Tuj-1 in red after three days in vitro, is depicted. It showed a polarized morphology. The lower panel shows the Trim2 knock-out hippocampal neuron cultured under same conditions and stained with the same marker at the same time point as the wildtype neuron. This neuron was able to form neurites, but not the axon.

To quantify the number of axons and neurites per cell hippocampal neurons of three wildtype and three knock-out mice brains were analysed after three days in culture. The statistics in Figure 11 show the average of the values obtained with this analysis (n wt= 413 and n ko= 518).

The decrease in the number of axons per cell from 0,7 in the wt to 0,3 in the knock-out neurons was significant with a p-value of 0,005 (Figure 11). At the same time the number of neurites was unaffected (Figure 11).

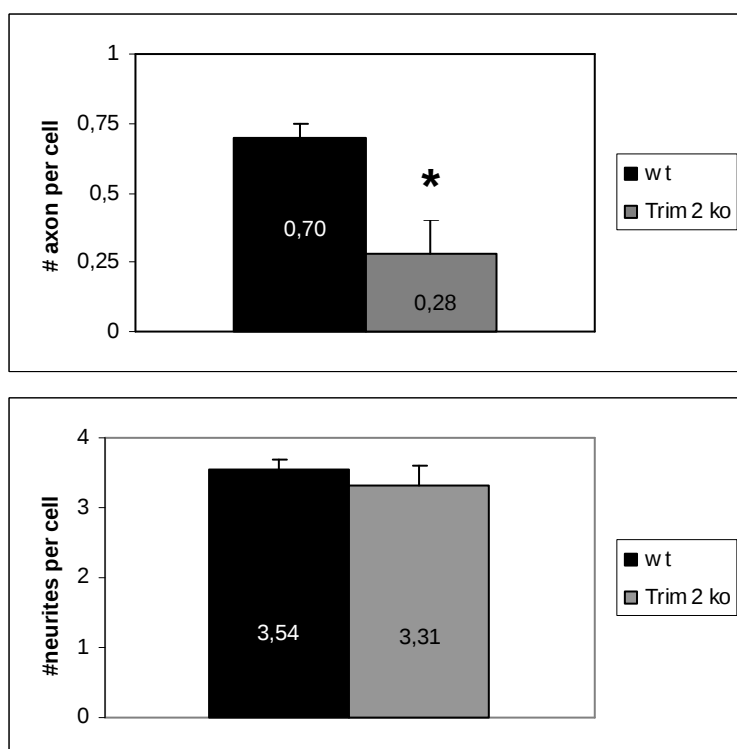


Figure 11: Quantification of the number of axons and neurites per cell

Hippocampal neurons of three wt and three knock-out mice brains were analysed after three days in culture. The decrease in the number of axons per cell was significant with a p-value of 0,005.

These results confirmed the detected role of Trim2 in the establishment of neuronal polarity in early stages of cultured hippocampal neurons.

In good agreement with the results obtained by knocking-down Trim2, these results show that Trim2 is necessary for neuronal polarization.

3.4. Overexpression of Trim2 in cultured hippocampal neurons

To check if Trim2 is not only necessary but also sufficient for the axonal outgrowth gain of function experiments were carried out. The neurons were transfected with a plasmid for EGFP-tagged Trim2. The vector construct was introduced into the neurons via the CaPO_4 -transfection at 1DIV. Three days later the neurons were immunostained with an antibody against Tau-1 (Figure12) to detect the axon.

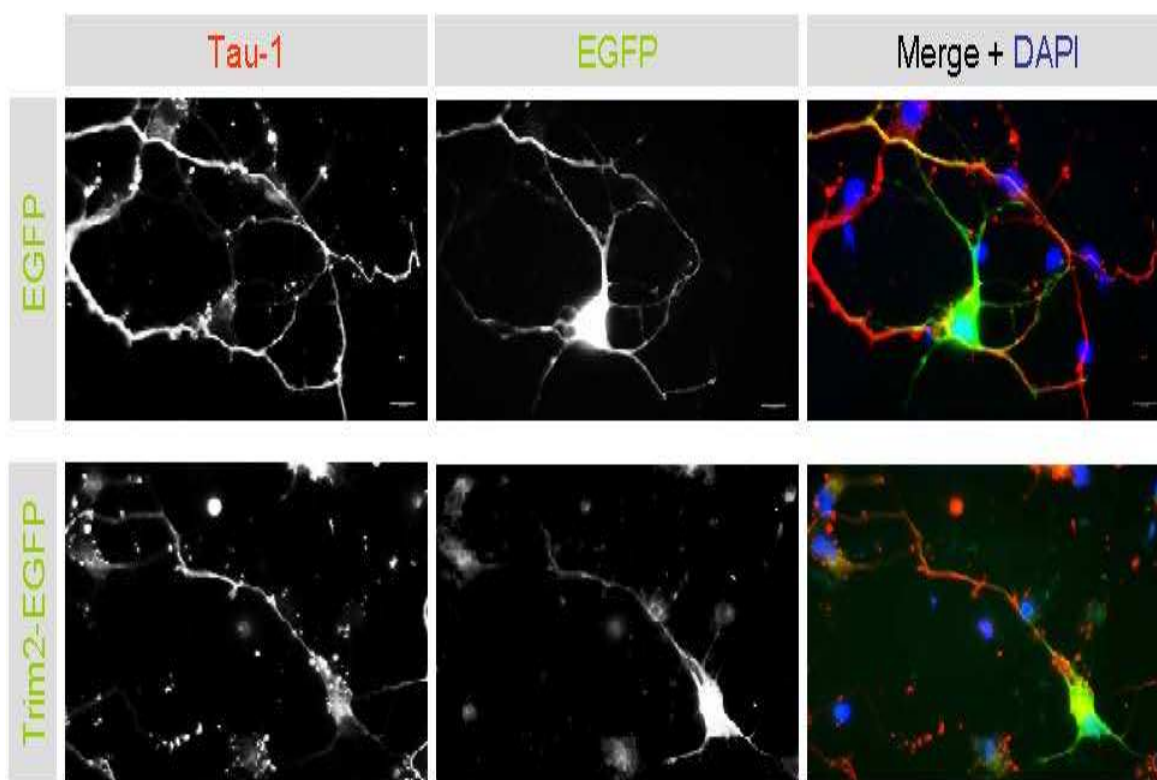


Figure 12: Overexpression of Trim2 in cultured mouse hippocampal neurons

The neurons were transfected at one day in vitro with the vector expressing EGFP as a control construct (upper panel) and a Trim2-EGFP fusion vector (lower panel). After four days in vitro the neurons were costained with the axonal marker Tau-1 in red and DAPI in blue.

Independent of the construct the majority of the transfected neurons was polarized.

The transfected neurons were polarized. They developed a single axon and several dendrites per cell. In the quantification the percentage of polarized neurons in the control EGFP transfection was 73,4% (n=94). Compared to the control the number of polarized neurons decreased to 57,14% in the overexpression of Trim2 (n= 42) (Figure 13).

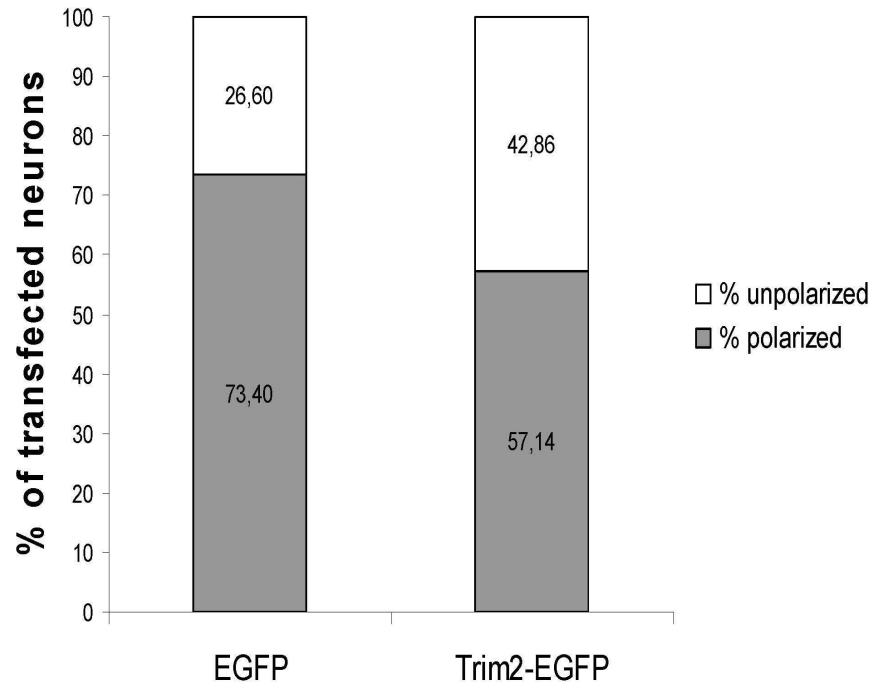


Figure 13: Quantification of transfected neurons after the overexpression of Trim2

The classification of unpolarized and polarized neurons was done via Tau-1 staining of the axon.

The decrease from 73,4% to 57,14% of polarized neurons after the overexpression of Trim2 from one day in vitro to four days in vitro was not significant.

(standard deviation EGFP = 4,58% ; Trim2/EGFP= 7,73%)

3. 5. Spine development

3.5.1. Subcellular localization of Trim2 in the developmental stage 5

As the development of dendritic spines is another formation of asymmetry in a neuron, it is of interest, if Trim2 is also expressed in later stages of neuronal development. The role of Trim2 in the early stage of neuronal polarity suggests a potential role of Trim2 in the development of dendritic spines.

To analyse this hypothesis the hippocampal neurons were stained after 15 days in vitro with an antibody against Trim2 and with a marker for spines – Psd95 (postsynaptic density). The postsynaptic density is located in the head of a spine. Trim2 was again expressed in the whole neuron (Figure 14a). But there was not enough co-localization of Trim2 and Psd-95 to make a strong statement concerning their connection. The blow up, shown in Figure 14b, displays the

dotted expression pattern of Trim2, seen in early stages of neuronal development. The protein was in the protrusions of the neuron with some accumulations and partial co-localization with Psd-95.

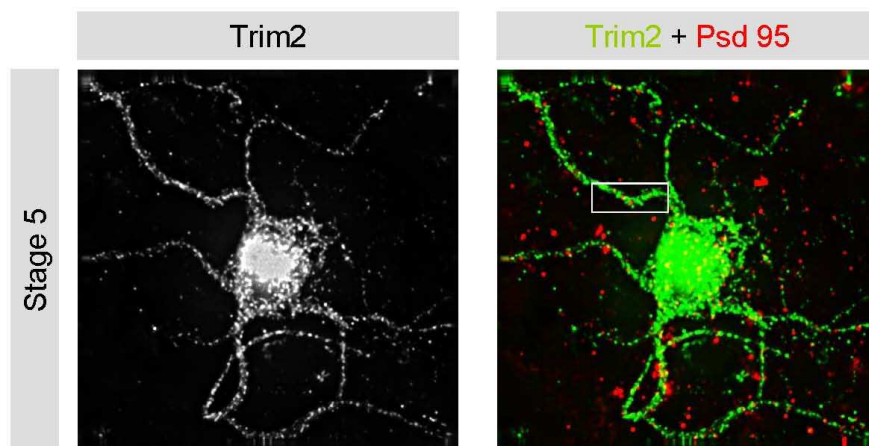


Figure 14a: Trim2 expression pattern in stage 5 hippocampal neurons

Trim2, stained in green, was expressed after 15 days in vitro in the mouse hippocampal neurons. The neurons were costained with Psd-95 in red to detect the postsynaptic density in a spine head.



Figure 14b: Colocalization of Spines and Trim2

The blow up of a part of a process in Figure 13a shows that Trim2 in green and the marker for spine heads, Psd95 in red, co-localized partially.

3.5.2. Loss of Trim2 in stage 5 hippocampal neurons

The results obtained showed the dotted-like expression pattern of Trim2 in Stage 5 of neuronal polarity. To investigate if Trim2 is involved in spine development, the first test was the knock-down of Trim2 via the same shRNA used to silence Trim2 in the early stages of neuronal development.

So, Trim2 was silenced at 15 days in vitro. Three days later the neurons were stained with the marker for the postsynaptic density in the spine head, Psd95. The Psd95 dots shown in Figure 15 indicate the dendritic spines (arrows).

Preliminary results showed that compared to the control transfection with an EGFP construct, the dendritic tree was not as complex after the silencing of Trim2. There was also less Psd95 detectable in the same sector.

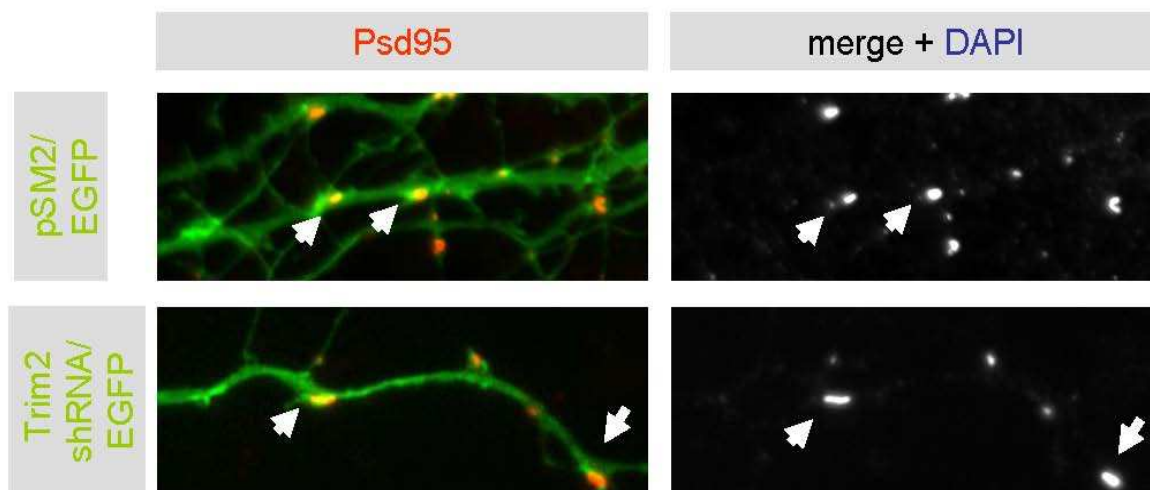


Figure 15: Silencing of Trim2 at stage 5 affected the dendritic tree

The hippocampal neurons were transfected with an EGFP construct or the construct to knock down Trim2 specifically at 15 days in vitro. Three days after the transfection they were stained with Psd95 depicted in red (left panel). The arrows indicate the Psd95 positive spine heads in the dendrites.

In the control transfection with the vector expressing EGFP the dendritic tree was more complex than after the silencing of Trim2. Furthermore the number of Psd95 molecules was lower after Trim2 knock down.

3.6. Molecular Mechanism

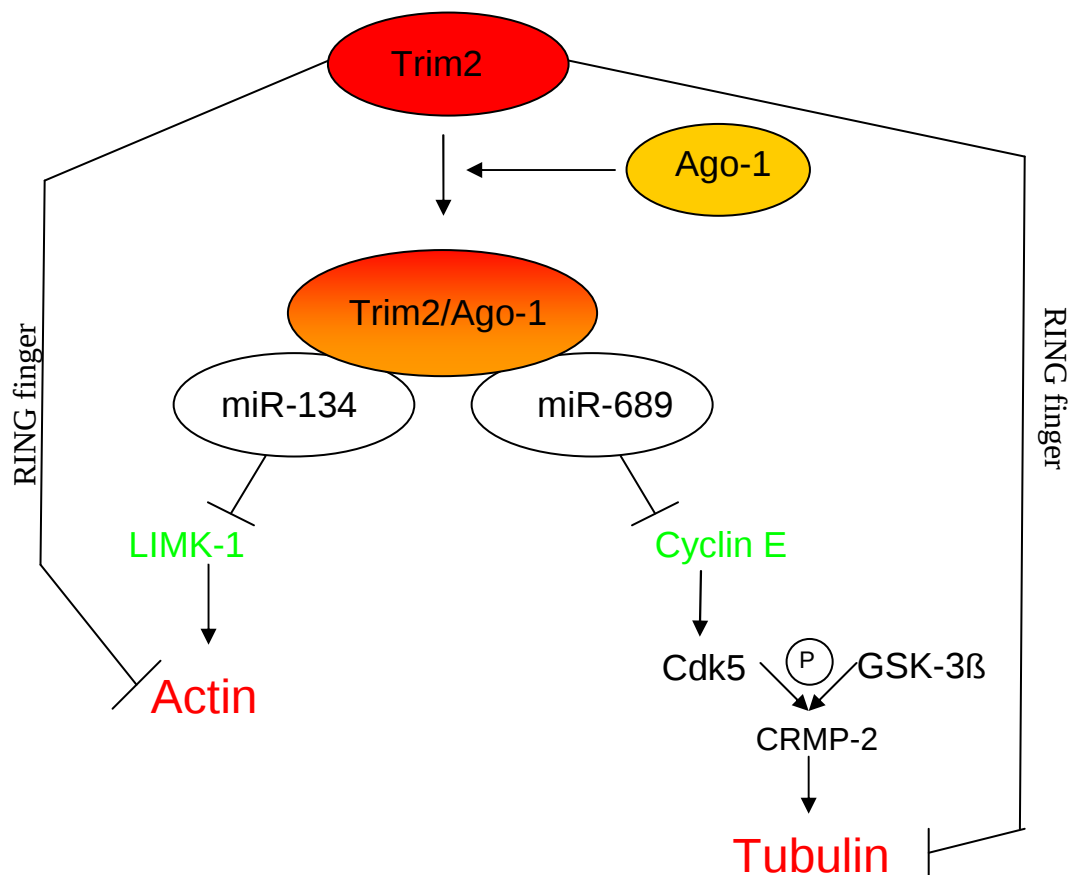


Figure 16: Hypothesis for the molecular Mechanism of Trim2

The hypothesis is based on work done in the lab. Jens Schwamborn was able to show the interaction of the Trim-NHL family member, Trim32, and Ago-1 (Schwamborn et al., 2008). This complex regulates miRNAs that at the end can control the rearrangement of the cytoskeleton of a neuron. The role of Trim32 as a miRNA regulator suggests the potential role of Trim2 in controlling miRNAs.

Experiments on another member of Trim-NHL proteins - Trim32 - done by Jens Schwamborn provided the result that Trim32 binds to the RNase Argonaute-1 (Ago1) via its NHL domain in mouse neural stem cells (Schwamborn et al., 2008). Strikingly, the similarity of the protein structure of Trim32 and Trim2 suggests the possibility that Trim2 is able to interact with Ago-1 and shows the same ability as Trim32 to regulate miRNAs (see Figure 16). The binding of proteins to Argonaute-1 is known to be conserved and essential for microRNA (miR) mediated down-regulation of gene expression.

Amongst others, two interesting miRNAs were found in a complex with Trim32. The first miRNA, miR-689, was predicted as a target of Cyclin E by Jens Schwamborn. Cyclin E could be part of the regulation pathway of Tubulin via the priming kinase Cdk5. The other interesting miR found in complex with Trim32 is miR-134. The known target of the miR-134 is LIMK-1 (Schratt et al., 2006).

Both targets regulate the rearrangement of the cytoskeleton of the neuron. This rearrangement is one of the most crucial steps for axonal outgrowth in a neuron. Cyclin E is able to activate Cdk5 (Matsunaga et al., 2000). Cdk5 phosphorylates the collapsin response mediator protein-2 (CRMP-2). The tubulin associated protein CRMP-2 becomes inactive due to a second phosphorylation by GSK-3 β (Yoshimura et al., 2005). In its inactive form CRMP-2 is not able to stabilize microtubules any longer. Therefore the formation of the axon is suppressed (Fukata et al., 2002).

The other pathway targets LIMK-1. This kinase regulates the stability of Actin in the future axon (Watabe-Uchida et al., 2006 and Endo et al., 2007).

3.6.1. miRNA-689 and its target Cyclin E

The protein Cyclin E is expressed in post-mitotic neurons of the central nervous system, where it interacts with Cdk5, the priming kinase of the GSK-3 β /CRMP-2 pathway (Matsunaga et al, 2000 and Yoshimura et al., 2005). Overexpression of CRMP-2, which is accumulated in the distal part of the axon, induces the formation of multiple axons (Inagaki et al., 2001). CRMP-2 associates with tubulin dimers, numb and kinesin-1, and so, might regulate the fate of axons and dendrites through a variety of mechanisms (Fukata et al., 2002).

In this hypothesized pathway the Trim2 – Ago-1 complex recruits the miR689 that regulates the expression of Cyclin E. Cyclin E activates the kinase Cdk5 that phosphorylates CRMP-2, like GSK-3 β (Yoshimura et al., 2005). The phosphorylation level of the microtubules-associated protein CRMP-2 increases, and thereby destabilizes microtubules. In case of Trim2 knock-down or knock-out the regulation of Cyclin E by miR-689 is missing, and the neuron fails to specify the axon due to microtubule destabilization.

3.6.1.1. Cyclin E – subcellular localization

To clarify if Cyclin E could be involved in the Trim2 – Cyclin E – Cdk5 pathway, the subcellular localization of Cyclin E in hippocampal neurons was tested via immunostaining.

In Figure 17 and 18 the expression pattern of Cyclin E in the stages 1-3 of neuronal polarization is shown. Cyclin E was expressed in hippocampal neurons in all 3 developmental stages. In the first two stages the neurons were co-stained with Tubulin to detect a potential co-localization of Cyclin E with α -Tubulin (Figure 17). The co-localization could suggest a regulation of Tubulin by Cyclin E.

The results depicted in Figure 17 show that Cyclin E is expressed in Stage 1 and Stage 2 of neuronal development. In both stages Cyclin E was expressed throughout the whole neuron with some co-localization to α -Tubulin.

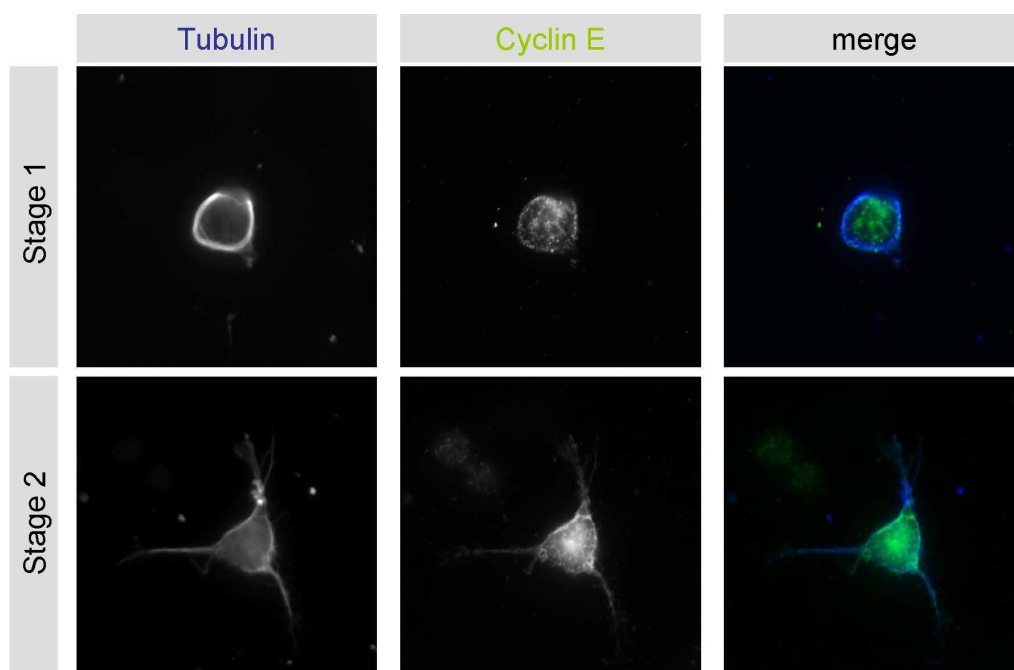


Figure 17: Cyclin E expression in Stage 1 (upper panel) and Stage 2 (lower panel) mouse hippocampal neurons

Neurons were stained with a marker for α -tubulin (shown in blue) and Cyclin E (shown in green) after one (Stage 1) and after two days (Stage 2) in vitro. Both proteins were expressed in both stages throughout the whole neuron. Cyclin E showed co-localization with Tubulin in both stages.

In the third stage the co-staining was done with Tau-1 to investigate a potential co-localization of Cyclin E (Figure 18).

The staining showed that Cyclin E shows a low expression level in the whole length of the axon. But it was expressed at the tip of the axon in nearly every Stage 3 neuron. Furthermore Cyclin E was also located in the cytoplasm of the cell body.

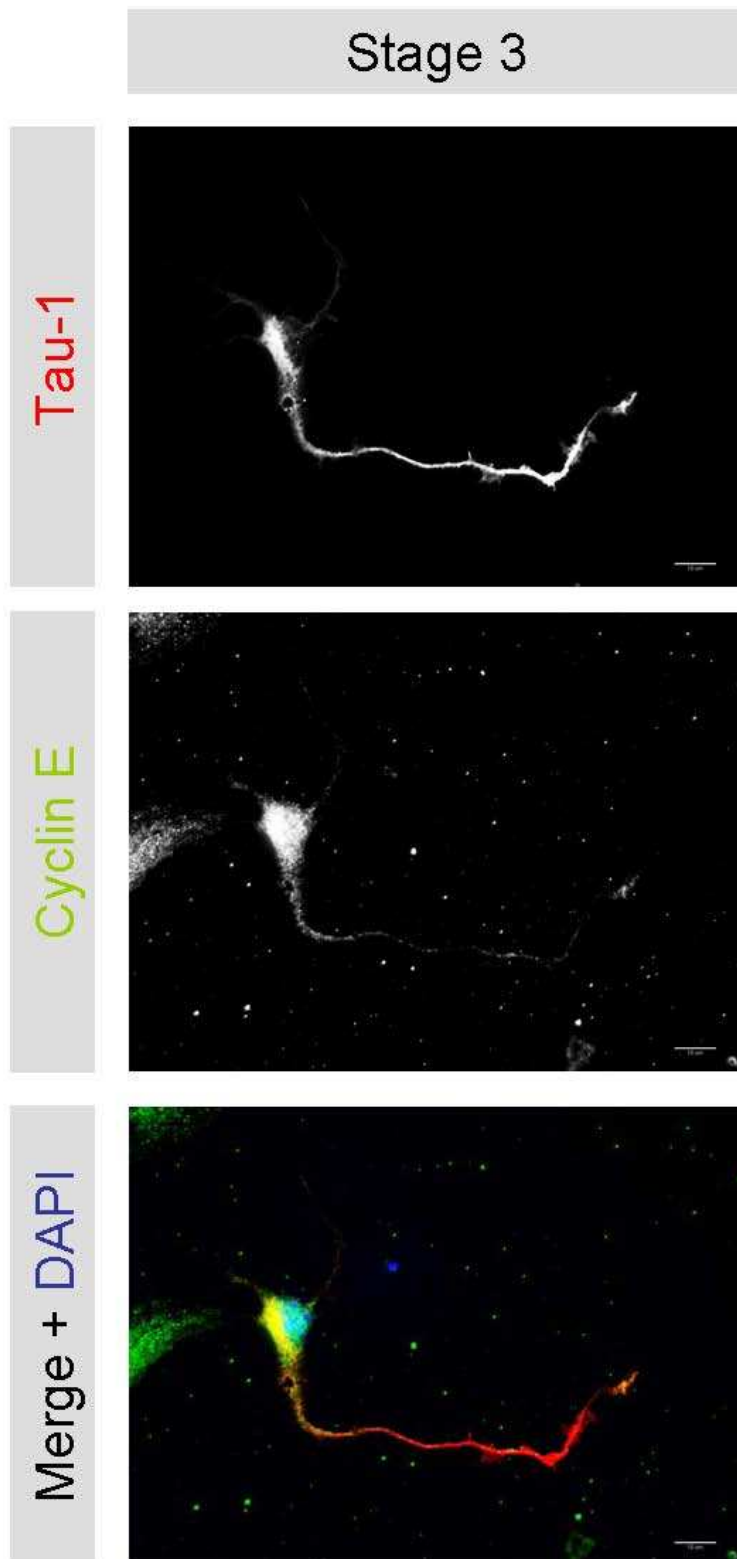


Figure 18: Cyclin E expression in mouse hippocampal neurons in Stage 3

The hippocampal neurons were isolated and cultured for four days to analyse the Cyclin E expression and its connection to axon outgrowth.

In the developmental stage 3 the axon was formed (Tau-1 staining in red) and Cyclin E (green) was expressed in the cell body of the neuron and the tip of the axon.

3.6.1.2. Cyclin E - gain of function

After showing that Cyclin E was indeed expressed at the right time and place in hippocampal neurons, it was expected that the gain of function of Cyclin E generates the same phenotype as the Trim2 knock-out or knock-down.

If Trim2 is not expressed in the neuron, the Trim2 – Ago-1 complex could not be formed. That causes that the miR-689 is not recruited and is not able to control the Cyclin E expression in the neuron. Therefore, the Cyclin E level increases without regulation and the following cascade via Cdk5, GSK-3 β and CRMP-2 causes microtubule disassembly.

However, as shown in Figure 19 and 20 overexpression of Cyclin E at early stages of development was not affecting axonal specification. The Cyclin E transfected neurons (n= 47) established the axon perfectly like control neurons that were transfected with an expression vector for EGFP (n= 60).

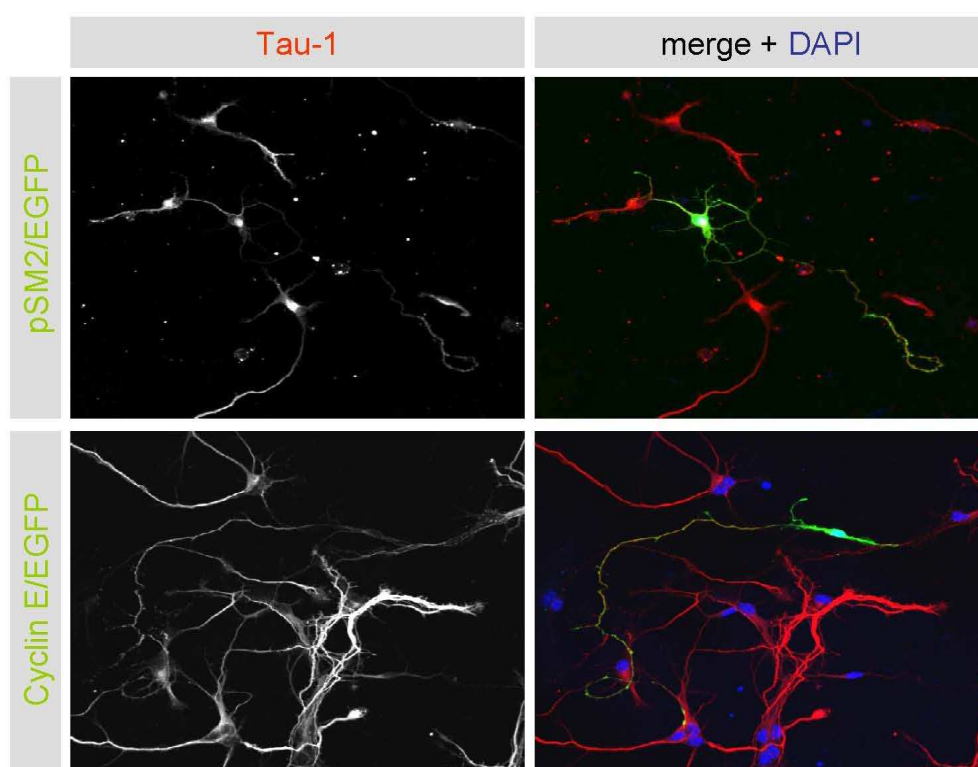


Figure 19: Overexpression of Cyclin E in mouse hippocampal neurons did not influence axonal specification

The neurons were transfected after one day in vitro. Three days later they were stained with the axonal marker Tau-1 (red).

Upper panel: The EGFP control transfection generated a polarized neuron.

Lower panel: The overexpression of Cyclin E caused the same polarized phenotype.

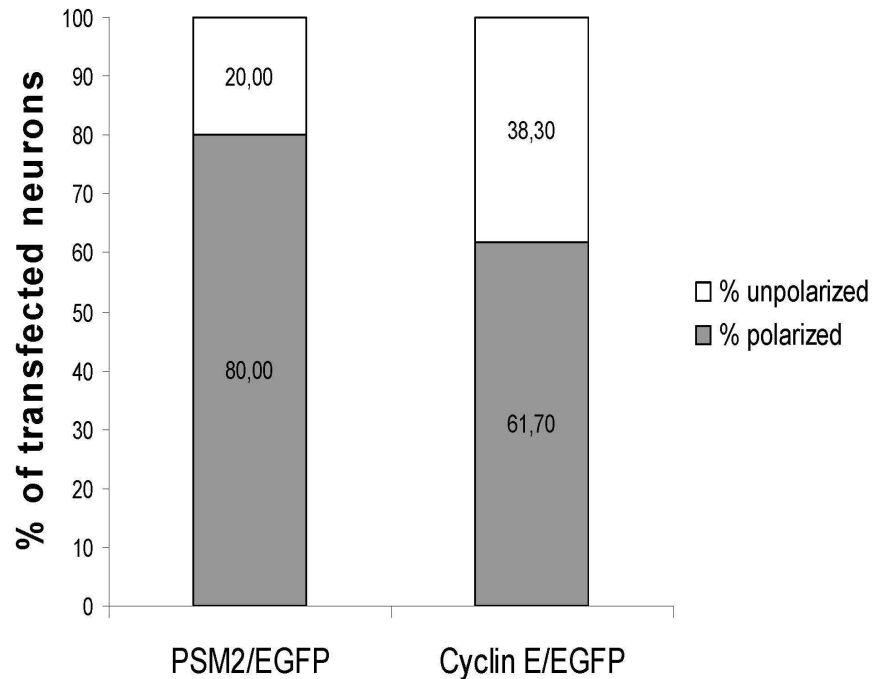


Figure 20: Quantification of Cyclin E transfected neurons.

Isolated hippocampal neurons were cultured for one day. Afterwards they were transfected with a vector construct expressing Cyclin E or pSM2 to control the experiment. In both cases the vector expressing EGFP was cotransfected. After three days incubation the transfected neurons were quantified. 80 % pSM2/EGFP transfected cells were polarized, whereas 61.7 % of CyclinE/EGFP transfected cells showed the polarized phenotype with an axon and several dendrites. The majority of the transfected neurons was able to specify the polarized morphology with an axon and several dendrites.

(standard deviation pSM2/EGFP = 5,21% ; Cyclin E/EGFP= 7,17%)

3.6.1.3. Cdk5 – gain of function

Although the expression of Cyclin E did not influence neuronal polarization, a potential function of the Cyclin E binding partner, and GSK-3 β priming kinase, Cdk5 was investigated.

The overexpression of Cdk5 and its dominant negative form should cause the block of axon formation too due to increased phosphorylation of its target CRMP-2 (collapsin response mediator protein-2). After the transfection of either an expression vector of EGFP, a Cdk5-tagged EGFP or a dominant negative form of Cdk5 (dn Cdk5) the neurons were analysed at four days in vitro. In Figure 21 it is

shown that the expression of both constructs, the wt Cdk5 and the dn Cdk5, was not interfering with normal neuronal polarization.

Furthermore the statistics in Figure 22 confirms the phenotype seen after the overexpression of a Cdk5 construct (n= 82) and its dominant negative form Cdk5 T33 (n= 48). There was no significant difference in the percentage of polarized and unpolarized neurons compared to the EGFP control transfection (n= 94).

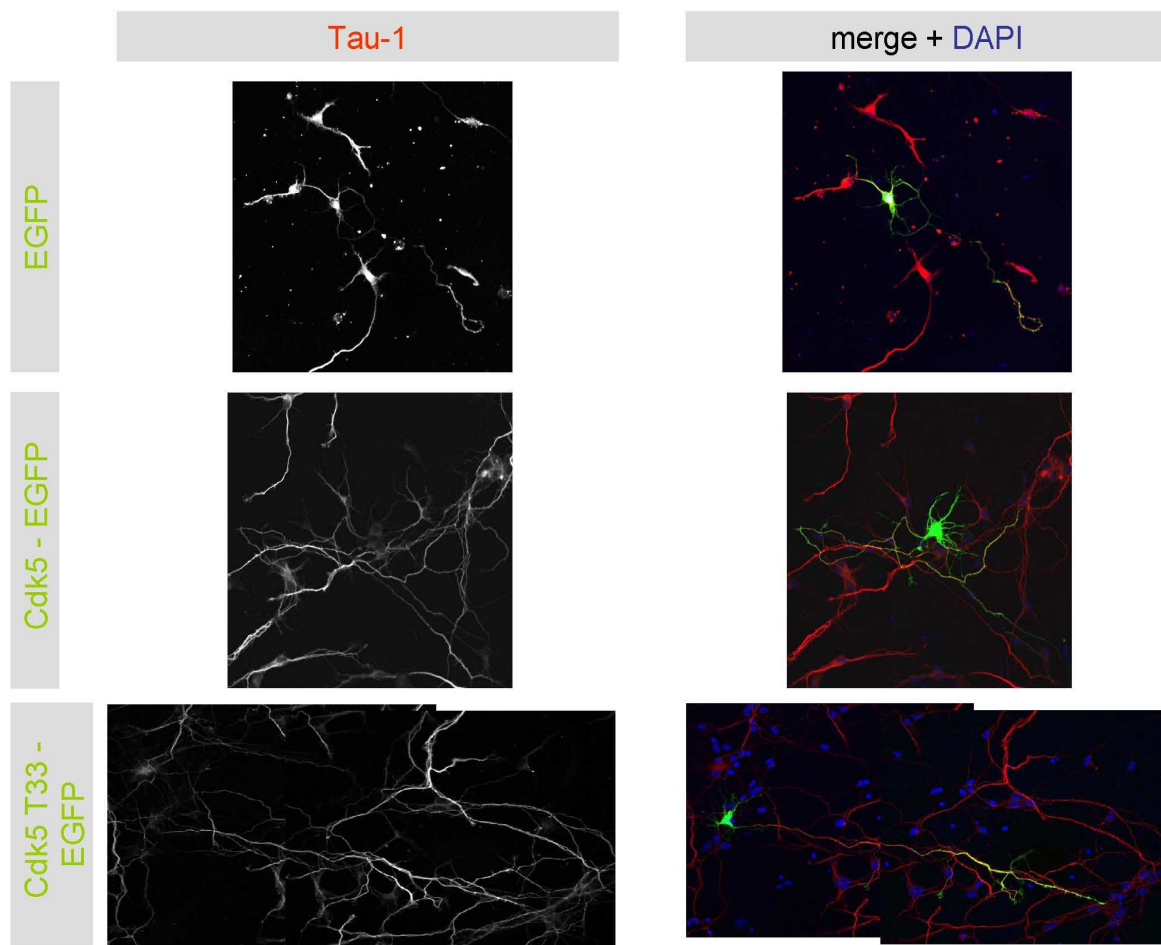


Figure 21: Overexpression of Cdk5 and Cdk5 T33 (a dominant negative mutant) did not affect neuronal polarization.

The neurons were transfected after one day in vitro with constructs expressing Cdk5 (middle panel) and the dominant negative mutant, Cdk5 T33 (lower panel). Three days later they were stained with the axonal marker Tau-1 in red and DAPI in blue.

The upper panel shows the EGFP transfected control neuron.

All three transfections caused the same phenotype – the majority of the neurons was able to form an axon.

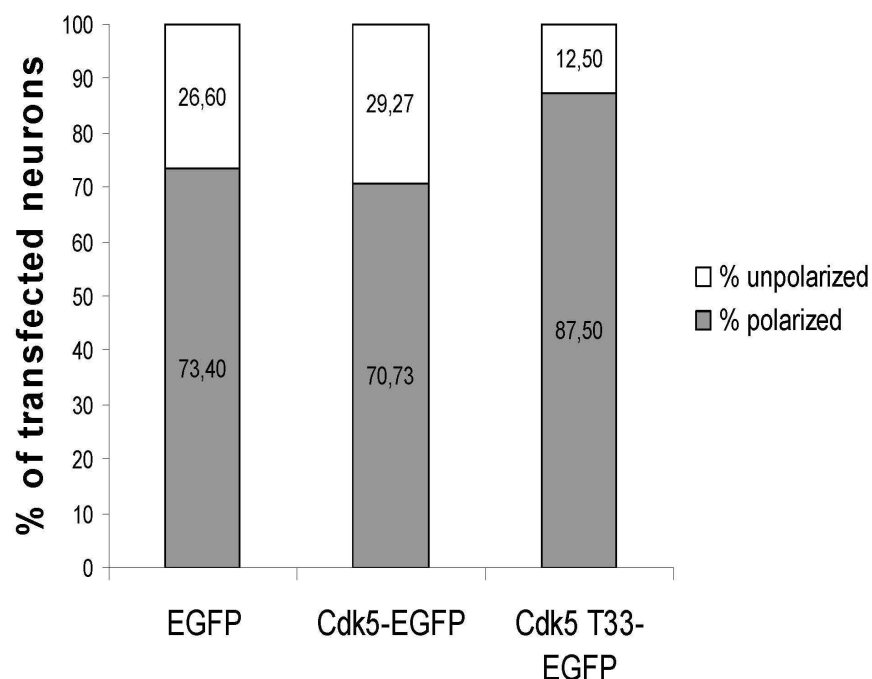


Figure 22: Quantification of control (EGFP), Cdk5 and Cdk5 T33 transfected neurons

After cultivation the hippocampal neurons they were transfected with a vector construct expressing Cdk5-EGFP or its dominant negative form-Cdk T33-EGFP. As a control the vector expressing EGFP was transfected. After three days the transfected neurons were quantified. Compared to the control transfection the percentages of polarized neurons vary from 70% to 90%. The majority of the transfected neurons was able to form an axon and several dendrites.

(standard deviation EGFP = 4,58% ; Cdk5-EGFP= 5,06% ; Cdk5 T33-EGFP = 4,82%)

3.6.1.4. miR-689 in the Trim2 – Ago1 complex

To test the possibility that the miR689 regulates Cyclin E expression and is a component of the Trim2-Ago-1 complex, the protein Trim2 was immunoprecipitated from embryonic brain lysates. As controls EGFP and Ago-1 were immunoprecipitated. The three different IPs were used as templates for Real Time PCRs with specific primers for the miR-689 designed based on a protocol published by Schmittgen et al., 2008. (described in Material and Methods; 2.13. Quantitative Real-Time PCR (q-RT-PCR))

In repeated experiments the miR-689 could not be detected in any of the IPs.

3.6.2. miR-134 and its target LIM Kinase-1 (LIMK-1)

3.6.2.1. LIMK-1 – subcellular localization

The miR-134 and its target LIMK-1 were shown to be involved in Actin regulation by Schratt et al (2006). The stability of Actin is regulated by LIMK-1 directly (Watabe-Uchida et al., 2006 and Endo et al., 2007).

If the Trim2 – Ago-1 complex recruits the miR-134 for the regulation of Actin, this branch of the hypothesis was checked via costaining of LIMK-1 and Trim2. In Figure 23b it is depicted that both proteins were expressed in the axon of a stage 3 neuron with partial overlap, whereas LIMK-1 was not expressed in the neurites of a stage 2 neuron (Figure 23a). At this stage the protein LIMK-1 was expressed in the cytoplasm of the cell body, but could not be found anywhere else.

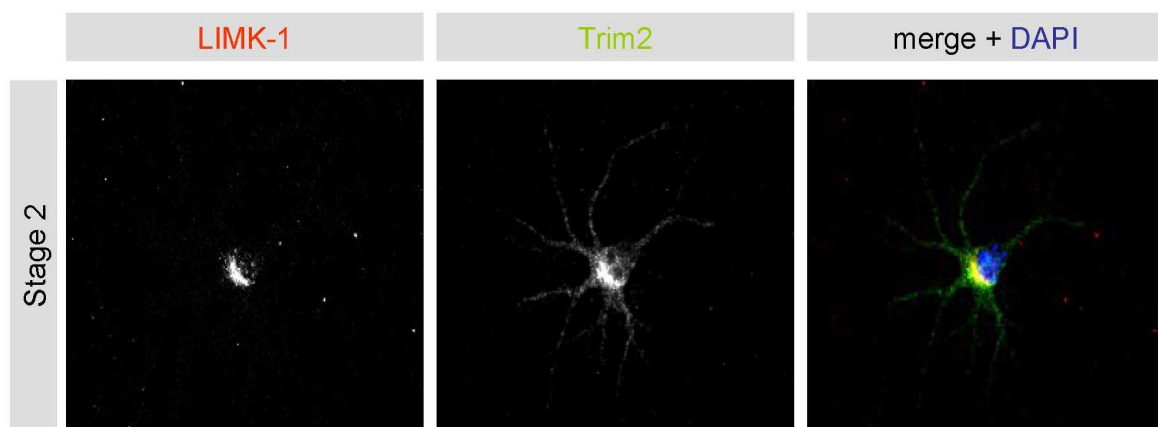


Figure 23a: LIMK-1 expression in Stage 2 of neuronal development

In a stage 2 neuron (after 3 days of cultivation) the protein Trim 2 (green) was expressed in the whole neuron. In contrast LIMK-1 (red) was expressed in the cytoplasm of the cell body, but not in the neurites.

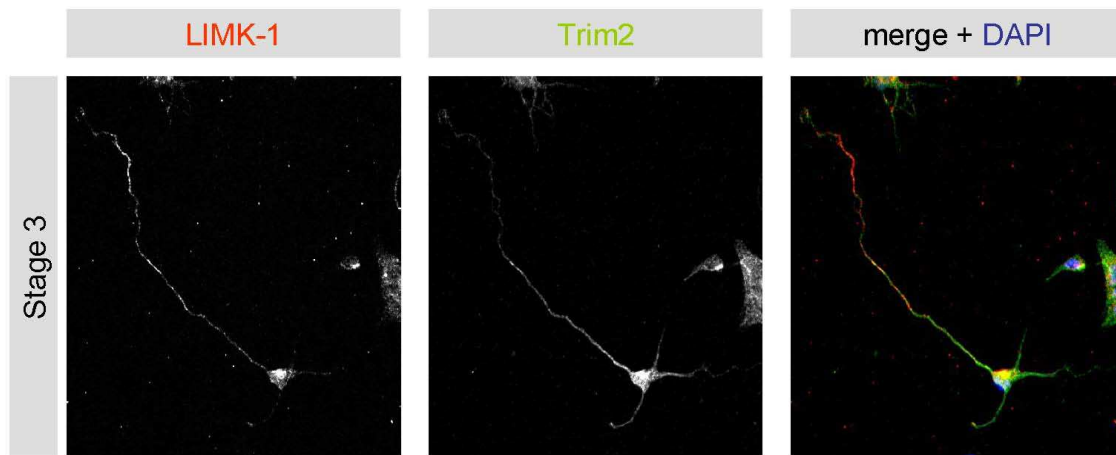


Figure 23b: LIMK-1 was expressed in neurons in developmental Stage 3

After four days of cultivation the neuron was in the developmental stage 3 and showed a polarized morphology with an axon and several shorter neurites.

LIMK-1 was expressed in the cell body and the axon, but not in the minor neurites at this time point.

3.6.2.2. LIMK-1 in a complex with Trim2

Based on previous findings that miR-134 controls LIMK-1 expression, the mRNA of LIMK-1 should be in the Trim2 – Ago-1 complex for regulation. Therefore the presence of LIMK-1 mRNA in the Trim2 – Ago-1 complex was investigated. As template the Trim2 IP of brain lysates was used after cDNA synthesis for LIMK-1. As a control the same procedure was done with a total RNA sample and an EGFP control IP (Figure 24). The cDNA of LIMK-1 was amplified via PCR using primers specific for LIMK-1 (described in Material and Methods; 2.12. PCR).



Figure 24: LIMK-1 was detectable in a Trim2 IP

The protein LIMK-1 was interacting with the protein Trim2 in whole brain lysates at E16.5. As can be seen in lane 2 of an agarose gel(1%)LIMK-1 was expressed in the sample of the Trim2 IP. EGFP was used as a negative control whereas LIMK-1 amplified from total brain lysate served as a positive control.

3.6.2.3. miR-134 in complex with Trim2

LIMK-1 is regulated by miR-134 (Schratt et al., 2006). Furthermore the miR-134 is detected in the Trim32 – Ago-1 IP. As Trim32 is closely related to Trim2 the next question was if the miR-134 is in a complex with Trim2 and Ago-1. This was tested via Real Time PCR of a Trim2 IP template.

The Real Time PCR showed an 1,8-fold increase of miR-134 in the Trim2 IP compared to the EGFP IP (Figure 25).

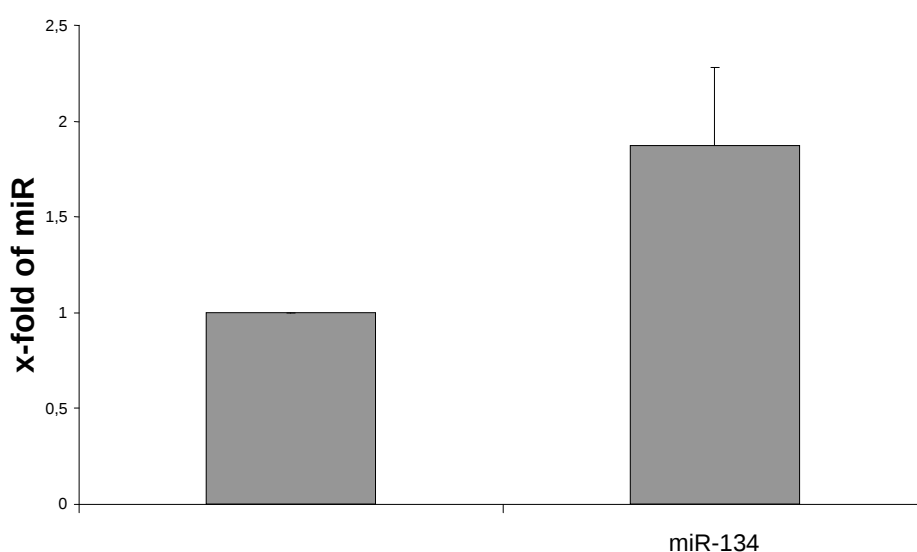


Figure 25: RT-PCR detected miR-134 in the Trim2 IP

Quantitative Real Time PCR was used to amplify the miRNA-134. The template to detect miR-134 was a Trim2 Immunoprecipitation of whole brain lysates. The miR-134 was enriched in the Trim2 IP.

3.6.2.4 miR-134 in hippocampal neurons

To investigate the subcellular localization of miR-134 in hippocampal neurons in-situ hybridization was used. Due to the treatment necessary for the in the in-situ hybridization (see Material and Methods) the Trim2 antigen was destroyed. Therefore a specific Trim2 signal was not detectable (Figure 26).

Nevertheless it was possible to detect miR-134 in the hippocampal neurons (blow-up, Figure 26).

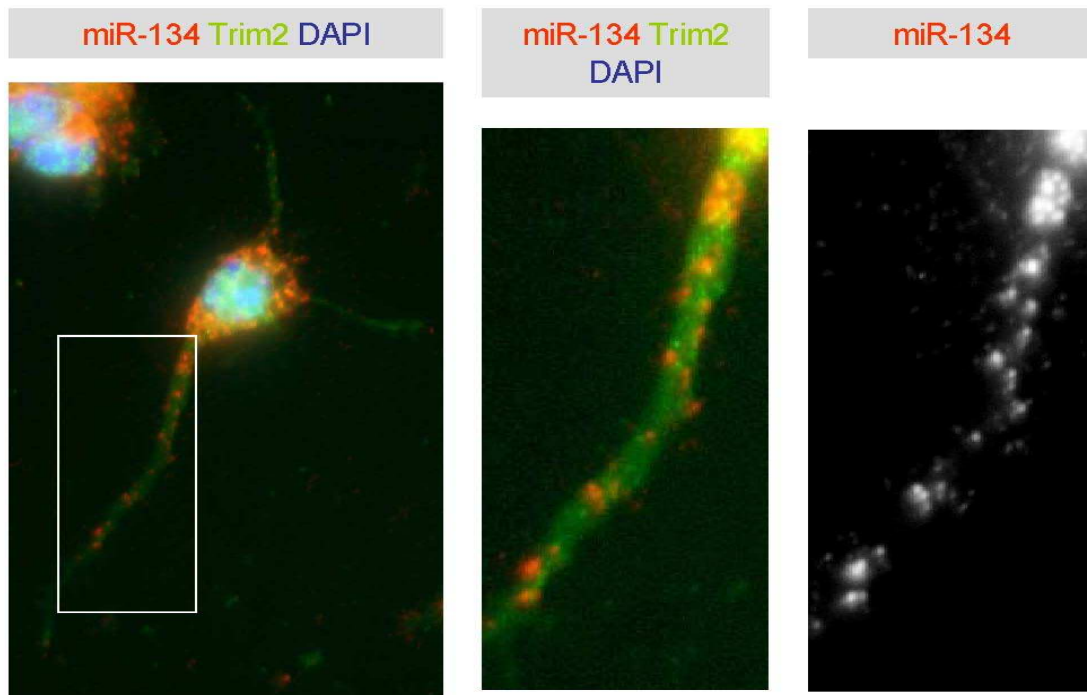


Figure 26: miR-134 was expressed in neurites of mouse hippocampal neurons

The neurons were cultured for four days. Afterwards the miR-134 (red) was detected via in-situ hybridization. Trim2 was stained and is shown in green. The blow up of a part of a neurite shows that miR-134 was expressed in neurons at this time point.

3.6.3. Tubulin

The three important processes to establish neuronal polarity are rearrangement of Actin, followed by controlled organization of microtubules and finally vesicular transport along microtubules to the tip of the neurites.

The RING finger motif of Trim32 is known to regulate the Actin cytoskeleton by ubiquitinylation (Joazeiro and Weissman, 2000). As Trim2 contains a RING finger domain too, it could regulate the Actin or Microtubule cytoskeleton of the neuron in a direct manner.

To test the possible direct regulation of Actin via the RING finger of Trim2, the protein was overexpressed in HEK (293T) cells. Compared to the EGFP control transfection, no effect was detectable on the Actin of the cells, but surprisingly Trim2 was located at the mitotic spindle (Figure 27).

As this overexpression resulted in an attachment of Trim2 to the mitotic spindle (Figure 27), a second experiment was done to check the interaction of Trim2 and

α -Tubulin. Therefore a vector construct for α -Tubulin was co-transfected with Trim2.

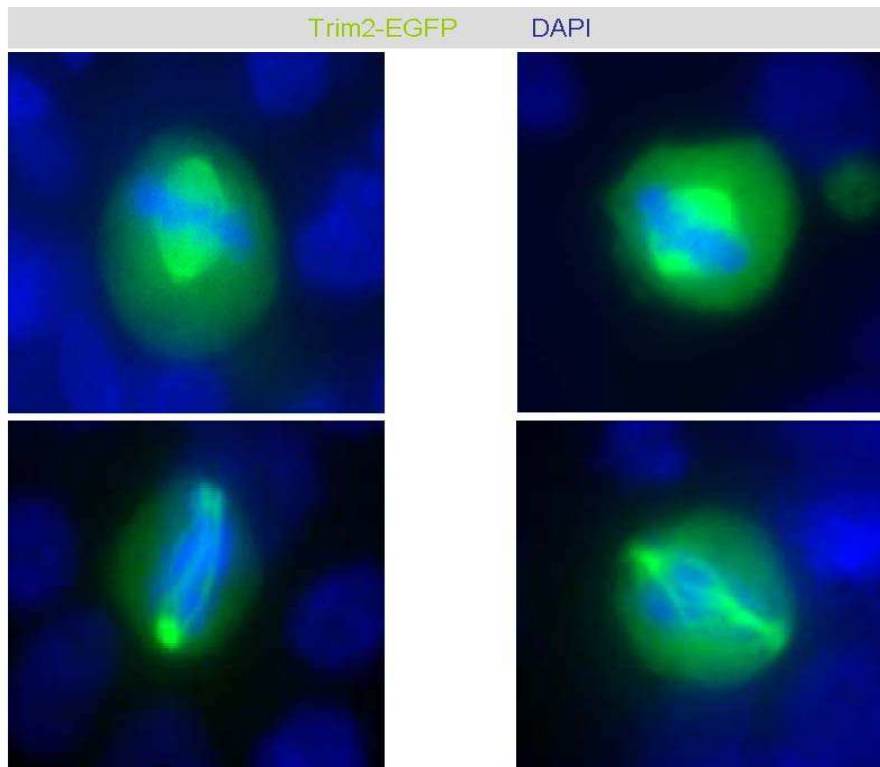


Figure 27: Overexpression of Trim2 in HEK (293T) cells

The Trim2-EGFP construct was expressed in HEK cells for 48 hours and during that time it accumulated at the mitotic spindle. Four different examples of transfected cells costained with DAPI were shown.

As depicted in Figure 28 also the overexpression of α -Tubulin led to the result that Trim2 accumulated at the mitotic spindle, marked with Cherry-Tubulin, in 293T cells.

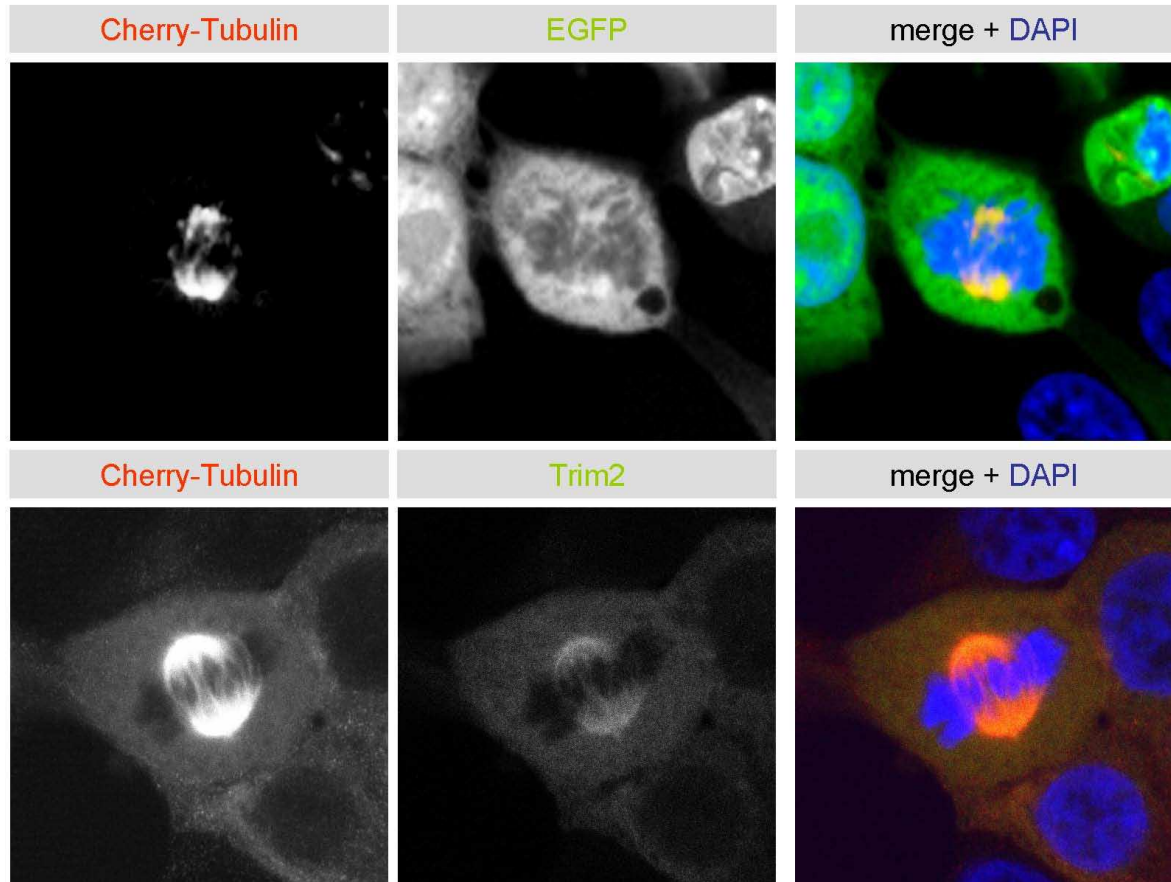


Figure 28: Cotransfection of Trim2-EGFP and Cherry-Tubulin in HEK cells

For further investigation of Trim2 and its connection to microtubules HEK cells were cotransfected with the vector expressing the fusion protein Trim2-EGFP and an expressionvector for Cherry-Tubulin (red). Both fusionproteins were expressed in the transfected cells and both of them co-localize at the mitotic spindle.

4. DISCUSSION

4.1. Trim2 in mouse hippocampal neurons

To discover the role of Trim2 that is one mammalian homologue of Brat during the development of a polarized morphology in hippocampal neurons, first the Trim2 expression pattern was analysed in the different stages of neuronal differentiation. Trim2 was expressed throughout the whole neuron in a dotted-like pattern. There was no enrichment of Trim2 in one of the different developmental stages in one of the distinct compartments detectable. As Trim2 was expressed homogenous throughout the whole neuron, it could not be the initial factor for the determination of the future axon, but it could be a matter of activity that makes Trim2 to a potential regulator involved in the establishment of a polarized morphology.

Moreover, the overexpression of Trim2 did not cause a multipolarization; that means a neuron with more than one axon. This phenotype is observed for other proteins, like CRMP-2 (Inagaki et al., 2001) or signalling molecules like Rap1B (Schwamborn et al., 2004). Nevertheless, there is the possibility that a difference in the length or stability of the axon compared to the control transfected neurons exists. It could be that Trim2 has a stabilizing role in the axonal development because of the described interaction with myosin Va, that is an important motorprotein for transport functions in neurons (Ohkawa et al., 2001).

More interestingly, the knock-down and knock-out experiments performed on hippocampal neurons, suggested a potential role of Trim2 in axonal outgrowth and specification. After blocking the expression of the protein the majority of the neurons, analysed after 4 days in vitro, fail to specify the axon and they stop their development at stage 2 after the outgrowth of neurites.

The question that remains is if this phenotype is caused by a block or by a delay in the development of neurons. This could be done by further analysis of cultured hippocampal neurons after 6 days in vitro. If the neuron is able to form an axon it will be visible at this time point.

4.2. The mechanism underlying Trim2 function

As the phenotype in the Trim2 knock-out and knock-down analysis of hippocampal neurons is very interesting, there must be relationship between Trim2 and the outgrowth of the axon.

Based on work done in the lab and on other published data a hypothesis (scheme can be seen in 3.6. Molecular Mechanism, Figure 16) was built up.

The starting point of the hypothesis is the Trim2 – Ago1 connection. Due to this interaction the Trim2 – Ago1 complex could recruit miRNAs like miR-134 and miR-689. miR-134 is described in spine development as a regulator of the Actin cytoskeleton of the neuron. This regulation happens via its target LIMK-1. (Schratt et al., 2006)

The miR-689 was enriched in the Trim32 – Ago1 complex. Therefore it is possible that this miR is also part of the Trim2 – Ago1 binding. The miR-689 is predicted to control the expression of Cyclin E, which is shown to be part of the microtubule regulating machinery.

The points supporting the hypothesis are:

1. Trim32, another member of the Trim-NHL family, interacts with the RNase Ago-1 via the NHL domain (Schwamborn et al., 2008); also Trim2 contains the NHL-domain to form a complex with Ago-1
2. The Trim32-Ago-1 complex recruits, amongst others, the miR-689 and miR-134; by work done in the lab miR-689 is predicted to target Cyclin E; it was published that miR-134 regulates the expression of LIMK-1 (Schratt et al., 2006)
3. Both targets regulate the rearrangement of the cytoskeleton of the neuron. LIMK-1 is expressed in the axon to destabilize Actin (Watabe-Uchida et al., 2006 and Edno et al., 2007). Cyclin E activates Cdk5 (Matsunaga et al., 2000). Cdk5 is the priming kinase for GSK-3 β . Both kinases phosphorylate the collapsin response mediator protein-2 (CRMP-2) – a tubulin associated protein that becomes inactive by phosphorylation (Fukata et al., 2002 and Yoshimura et al., 2005).

4.2.1. miR-689 and its target Cyclin E

In this pathway the miR-689 is recruited to the Trim2 – Ago-1 complex to regulate the expression of CyclinE. CyclinE activates Cdk5, the priming kinase of CRMP-2. After this phosphorylation GSK-3 β can phosphorylate CRMP-2 a second time to inactivate CRMP-2. CRMP-2 associates with tubulin, numb and kinesin1, and might regulate the fate of axons and dendrites through a variety of mechanisms (Fukata et al., 2002). A phosphorylated CRMP-2 is not able to act as binding partner for microtubule-associated proteins anymore and therefore the neuron fails to establish the axon. The inhibition of GSK-3 β by Akt kinase promotes axonal outgrowth, because the level of phosphorylation of microtubule-associated proteins is decreased; followed by microtubule stabilization. So, phosphorylation of the single components in the pathway is regulating axonal outgrowth (Jiang et al., 2005, Yoshimura et al., 2005,2006 and Garrido et al., 2007).

In the Trim2 knock-down and knock-out analysis the majority of the neurons are not able to establish the axon. That means, if Trim2 is missing no Ago-1 complex could be formed. Therefore, also the miRNA-689 cannot be recruited to the complex to regulate the expression level of Cyclin E. In the Trim2 knock-out or knock-down it is suggested that due to high levels of Cyclin E, Cdk5 is active to arrange CRMP-2 for the second phosphorylation by GSK-3 β . So, in this pathway CRMP-2 must be in its inactive phosphorylated form. No axon is established, because of the low binding activity of inactive CRMP-2 to tubulin.

To prove this suggestion, it is expected that the Cyclin E overexpression shows the same phenotype like the Trim2 downregulation, that causes the uncontrolled expression of Cyclin E.

Data shown in this thesis disprove the hypothesis. The overexpression of Cyclin E and additionally the overexpression of Cdk5 do not cause a difference in the formation of the axon compared to the control transfection.

Therefore, the Trim2 – Ago-1 – Cyclin E – Tubulin connection could not be verified.

But, it is of great interest to test the overexpression of the diverse components in Trim knock-out neurons too. This could be more revealing, because maybe not only one component is important to establish the whole pathway. It is possible

that the activity of GSK-3 β , regulated by Akt, is additionally controlled by another mechanism.

4.2.2. miR-134 and its target LIMK-1

The second pathway, via miR-134 and LIMK-1, is also based on the connection of Trim2 and Ago-1. If Trim2 is not expressed in the neurons, there is no Ago-1 complex that can recruit the miR-134. That means, that there is no regulation of the expression of LIMK-1. The high levels of LIMK-1 are not able to control the cytoskeleton of the neuron correctly and no axon is formed.

As the miR-134 is known to play an essential role in the spine development by regulating the Actin in a neuron (Arber et al., 1998 and Schratt et al., 2006), the connection to Trim2 is more promising than to Cyclin E.

The cytoskeleton of each neurite is highly dynamic during neuronal polarization, but only the Actin of the future axon has to become more instable (Watabe-Uchida et al., 2006). This instability can be caused by LIMK-1, which is highly expressed in growth cones of neurites, to regulate the Actin filament assembly in the future axon (Endo et al., 2007).

Strikingly, the expression of LIMK-1 started at developmental stage3 in hippocampal neurons and an 1,8-fold enrichment of the miR-134 was detected in a Trim2 IP by doing Real Time PCR. This results suggests that the signalling cascade starting with the regulation of miR-134 by the Trim2-Ago-1 complex could be verified. But, it is not clear if the moderate increase of 1,8-fold of miR-134 is able to control LIMK-1 and whole cytoskeleton of the neuron.

The problem could be that there is a limited amount of Trim2 in the IP. Furthermore it is possible that the interaction of Trim2 and miR-134 is limited to a specific time window. In principle the interaction is needed between the developmental stage 2 and 3. In an IP of the whole brain it is not possible to distinguish neurons from other cell types. One could also not separate the single developmental stages of the different types of neurons in the brain.

It would be good to test the interaction in cultured hippocampal neurons to have reproducible conditions, but in the culture there is not enough of Trim2 to work with as a template.

4.2.3. Direct regulation via the RING-finger

As a third pathway the direct interaction of the RING-finger of Trim2 and the cytoskeleton was tested. It is possible that Trim2 regulates the cytoskeleton via its RING-finger domain, which is shown to be part of the ubiquitinylation complex (Niikura et al., 2003 and Yang et al., 2003).

So, Trim2 was overexpressed in HEK293T cells to analyse the potential role of the RING-finger in regulating the cytoskeleton of the neuron.

The overexpression of Trim2 did not cause any changes in the cytoskeleton of the neuron, but strikingly the high levels of Trim2 in HEK cells showed an accumulation at the mitotic spindle that is built up by microtubules.

This result suggests that Trim2 is not responsible for degradation of the Actin or tubulin in a neuron, but leads to the hypothesis that there is a connection to microtubule filaments.

In this case Trim2 potentially has a stabilizing role by binding to myosin V, that is involved in the axonal transport. The directional transport of axonal molecules has essential roles in the initial events of neuronal polarization. Therefore, microtubule-based molecular transport might be a basic way of specifying axonal or dendritic fate. If the Trim2 expression is blocked by RNA interference the transport could be stopped as well, and the neuron is not able to form an axon anymore.

As a second function Trim2 degrades Neurofilament protein and regulates neurodegeneration (Balastik et al., 2008). The phosphorylation of Neurofilament regulates axonal transport and provides the stability of mature axons (Shea et al., 2008). Therefore, it is possible that after the knock-down or knock-out of Trim2 Neurofilament accumulates in the neuron and destroys the development of neuronal polarity. This could be another reason why the axonal outgrowth is suppressed after Trim2 loss of function.

One could also speculate about the role of Trim2 in mitotic cells due to its localization at the mitotic spindle. As evolutionary conserved mechanisms regulate the establishment cellular polarity by influencing the mitotic spindle, Trim2 could also have a function in asymmetric cell division.

4.3. Spines

The establishment of dendritic spines is the last step in the development of mouse hippocampal neurons. A spine is a small extrusion on a dendrite and it forms half of a synapse. It is built up with a spine head and a spine neck. Especially, many signalling molecules and proteins are accumulated in the spine head. As spines change their structure during their development (Tada&Sheng, 2006) it is not easy to analyse the effect of Trim2 on spine. But Trim2 could also have a function in the establishment of dendritic spines. With the results obtained during the diploma thesis it was shown that Trim2 is expressed in the developmental stage 5 too. After the knock-down of Trim2 at the time point where dendritic spines are formed, the dendritic tree is much simpler and there are not as much Psd95 spots in a dendrite.

Because of its expression pattern and the loss of function data, it is possible that Trim2 is involved in spine development. It is likely that Trim2 regulates the miR-134 and its target LIMK-1 that are shown to be necessary in the establishment of spines. (Schratt et al., 2006)

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Acknowledgments

First of all I want to thank Jürgen Knoblich for all his support, discussions, ideas and especially for giving me the opportunity to do my diploma thesis in his lab.

Furthermore, I want to thank Jens Schwamborn for his supervision. He guided me through 12 months of my diploma thesis and helped with words and deeds.

I have to thank both of you for placing the basic module of my scientific career. It was an instructive and exciting part of my life.

Many thanks go to all the Knoblich group members for a great working atmosphere.

Moreover, I want to thank my family, especially my parents – not only for financial support, but also in fact to believe in me. Thank you for all the faith in me to make my own decisions and at the same time for giving me the opportunity to come home whenever I want.

Thank you for backing me all my way!

Next I want to thank all my friends for 1000 of things – believe me, there is not enough space to list everything, but to make it short – thank you for listen to everything from peanuts to problems that one can not discuss with parents. ;-)

Thank you for balancing my lab life!