Stabilization of the Cortical Cytoskeleton by the Lipid Raft-Associated Protein Cap23

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1 INTRODUCTION

1.1 Summary

The mammalian nervous system is maybe the most complex achievement of evolution so far, consisting of multiple interconnected neuronal networks, which in order to adapt to new environmental circumstances has to be able to undergo different forms of plasticity, enabling the individual organism to create a memory of past events in order to better cope with future challenges.

One essential feature of neuronal plasticity is the ability to repair damaged neuronal connections. Axonal regeneration is a complex and not well understood mechanism, demanding big efforts of both academic and pharmaceutical research in order to understand the underlying mechanisms and to provide means for more efficient treatment of human patients affected by various neurodegenerative diseases or suffering from acute or chronic central nervous lesions. Though various different approaches are currently addressed in research (Liebscher et al., 2005; Blesch & Tuszynski, 2003; Natsume et al., 2003; Lu et al., 2004; Liu & Brady, 2004), the ability to cure lesioned nerves still seems to lie in far future.

The regulatory responses of neurons upon axon lesion have shown that distinct cellular programs seem to be elicited in order to maintain control over muscular activity and neuronal circuits. One such program is the upregulation of growth-associated proteins like Cap23 and Gap43, which play crucial roles in axon elongation and regeneration (Caroni, 1997; Carmichael et al, 2005).

Investigating the involvement of Cap23 in regulation of the actin cytoskeleton, we could show that the polarization of the actin cortex as well as of actin protrusions including dendritic spines is directly proportional to protein levels of Cap23. Lowering the amount of Cap23 at the cell surface not only increased the motile behavior of f-actin-based structures, but also affected the subsequent stabilization of these processes, indicating

that increased actin motility negatively affects the proper consolidation of actin-based processes.

Having established a striking phenotype of different aspects of the actin cytoskeleton, we then went on to ask whether other parts of the neuronal cytoskeleton were affected as well or if the observed effect was specific for the actin cytoskeleton. To this end, we analyzed the family of intermediate filament proteins, the largest group of the cytoskeleton. Strikingly, we found that the organization and assembly of IF and NF structures was severely affected in the absence of Cap23, pointing to a more general role for Cap23 in cytoskeletal regulation. Neurons with reduced levels of Cap23 showed a dramatic increase of IF-ring structures, indicating abnormal assembly and instability of axonal processes. In time-lapse experiments using GFP-tagged versions of IFs, it was obvious that proper docking of filaments was defect, as mutant filaments traveled over much longer distances inside axons with slightly increased velocities as compared to WT filaments.

We then focused on the motile behavior of another type of organelle, the mitochondrion, which regulated translocation was well known to be crucial for polarized process extension and consolidation of high-energy-demanding neuronal structures. Interestingly, the absence of Cap23 did not only reverse the ratio of stable versus motile mitochondria, but also caused an imbalance of bidirectional transport, especially at distal axonal locations.

This, together with the previous findings further strengthened the argument that Cap23 is involved in the local accumulation of structures involved in neuronal stabilization.

In summary, by combining different approaches we were able to define some important roles of Cap23 in various cell biological mechanisms of the neuron, especially regarding polarization of the neuronal cytoskeleton, stabilization of intermediate filament proteins, as well as mitochondrial docking.

1.2 Preface

Interest in the cytoskeleton-associated protein Cap23 was raised when studies directed towards a better understanding of axonal regeneration in spinal cord neurons demonstrated that upregulation of a small set of proteins like Cap23 and Gap43 not only correlates with axonal regeneration, but under certain experimental conditions triggered intrinsic regenerative responses without the need of otherwise essential co-stimuli (Bomze et al., 2001).

It was shown that Cap23 and Gap43 both are targeted to special lipid domains and play some role in regulation of the neuronal cytoskeleton (Laux et al., 2000). That cytoskeletal reorganization events play important roles in various aspects of neuronal morphology is well established, though how exactly this is achieved by different proteins remained elusive. We thus decided to take a closer look at Cap23, the less well-known modulator of axonal regeneration in order to better understand the processes involved in regulation of cytoskeletal plasticity and how they are mediated by Cap23.

This dissertation consists of a multi-faceted examination of the actin cytoskeleton, mitochondrial trafficking and intermediate filament stability in hippocampal neurons. Main interest was given to aberrations of cytoskeletal polarization as well as motility and organelle trafficking as they relate to processes regulated by the cortical cytoskeleton-associated protein Cap23.

The introduction broadly covers topics of lipid raft function, the importance of the neuronal actin cytoskeleton, mitochondrial trafficking and the involvement of intermediate filaments in cytoskeletal integrity. Mechanisms regulating plasticity and stability during neuronal development in respect to generation of a polarized cytoskeletal network and proper targeting of mitochondria and intermediate filaments are adressed.

Impaired cytoskeletal organization has critical consequences on neuronal health, can lead to reduced synaptic function or even loss, furthermore to axonal injury or degeneration and ultimately to neuronal death.

When one considers the importance of mitochondria in supporting cellular functions like energy supply at remote locations of the complex central nervous system, one can

appreciate the necessity for appropriate mitochondrial trafficking to maintain synaptic, neuritic and cellular health.

This thesis thus tries to cover several aspects of neuronal cell biology in order to better understand the multiple requirements for generating and maintaining a stable and healthy neuron.

1.3 Lipid rafts

1.3.1 Overview

The eukaryotic plasma membrane is a lipid bilayer and is involved in diverse biological processes, such as uptake of extracellular components (phago-/ endocytosis), regulated secretion of intracellular components (exocytosis, neutrotransmitter release) or cellular growth. It senses signals from both in- and outside and has to integrate them in order to respond properly. Lipid rafts are lipid microdomains which have a highly ordered lipid composition, but vary considerably in protein composition, dynamics and size.
By fusion of individual raft entities, which can be induced by different stimuli from the intracellular as well as the extracellular side, they can form larger platforms which serve specialized functions with characteristic signalling properties in a unique temporal and spatial manner.
Due to their small size (<10μm), direct visualization of lipid rafts has been technically challenging and their characterization has been mainly based on biochemical properties. Because of these difficulties to directly prove their existence *in vivo*, the lipid raft hypothesis has been controversial for a long time (Jacobson & Dietrich, 1999). Advance especially in the field of single-molecule trafficking has though recently helped to establish a firm basis for the existence and functional significance of lipid rafts (Douglass and Vale, 2005).

1.3.2 Raft Composition

Originally, lipid rafts were defined as membrane domains enriched in cholesterol and sphingolipids which can be isolated biochemically as detergent-resistant membrane fractions of low buoyant density (Simons & Ikonen, 1997). Since sphingolipids and phospholipids are enriched in saturated fatty acids, lipid rafts achieve a significantly

higher packing density than non-raft lipids, which mainly contain polyunsaturated fatty acid chains.
Proteins can associate with lipid rafts via different modifications, including GPI anchors or double acylation (palmitoylation, myristoylation). Phosphorylation or electrostatic interactions are further mechanisms to regulate raft association.

1.3.3 Biological Functions

Lipid rafts are involved in signal transduction, endocytosis, cell-cell interaction, pathogen entry, intracellular membrane trafficking and cell polarisation through cytoskeletal regulation.
The notion that rafts form membrane platforms that mediate signalling responses has been most extensively demonstrated in the immune synapse of T cells (Harder & Engelhardt, 2004; Balamuth et al., 2004; Manes & Viola, 2006). Pioneering work in lymphocytes has shown that the assembly of signalling modules critically depends on the functional compartmentalization of membrane lipids into ordered microdomains or lipid rafts. For example, upon interaction of T cells with an antigen-presenting cell (APC), lipid rafts and raft-associated proteins rapidly polarize and redistribute into confined domains at the cell-cell contact site (Hiltbold et al., 2003). This is essential for triggering the T-cell response, since pharmacological removal of raft components abolishes proper T-cell signalling.
Important signal transduction molecules like the src-family tyrosine kinases are located in lipid rafts (Harder et al., 1998), where they can specifically interact with and phosphorylate downstream effectors, and thus modulate signal cascades.

1.3.3.1 Cytoskeleton regulation and lipid rafts

Many of the molecular components regulating the actin cytoskeleton, cell motility, and adhesion are associated with rafts. These associations suggested that local accumulation

of rafts might mediate some of the spatial specificity in Rho-GTPase signalling to the actin cytoskeleton.

Polarization of rafts, and the ways in which rafts are involved in setting up cell polarity and polarized signalling are nicely illustrated in chemotactic cell migration. There, it has been shown that polarizing T-cells accumulate distinct raft components at their leading and trailing edge. Although initial signalling is raft-independent, the presence of intact rafts is necessary for sustained Rac- activation and actin polymerization (Pierini et al., 2003). Another interesting example of raft involvement in the generation of polarity is the growth cone, where raft integrity is specifically required for growth cone turning responses induced by different axonal guidance cues (Zheng et al, 2004).

Regulation of the cytoskeleton also plays a crucial role in the consolidation of rafts in T-cells (Meiri, 2005), showing the interdependence of raft- and cytoskeletal regulation.

1.3.3.2 Lipid Raft Involvement in Cell-Cell Contacts

Integrins are cell-surface receptors, which interact with neighboring cells and the extracellular matrix. There is evidence that integrin signaling couples sensing of the local environment to the local accumulation of rafts, as integrin activation leads to the accumulation of rafts at the cell surface (Grimmer at el., 2002). Furthermore, signaling events downstream of integrin signaling, which induce cell surface raft clustering, result in microtubule stabilization and cell polarization (Palazzo et al., 2004).

The most important cell-cell contact sites between neurons are synaptic contacts. Synapses among cultured neurons accumulate raft markers, and raft disruption leads to loss of synapses in vitro (Bruses et al., 2001; Hering et al., 2003). Synapse loss induced by raft disruption is partially prevented by treatments that stabilize the actin cytoskeleton, which suggests that rafts stabilize synapses by promoting linkages to the cortical cytoskeleton.

1.3.3.3 Lipid Raft Involvement in Membrane Trafficking

Local delivery of rafts at defined sites on the cell surface involves dedicated targeting mechanisms. The exocyst is an octameric complex involved in targeting proteins and vesicles to specified plasma membrane domains, thereby acting as a polarization cue. Recent evidence on how the exocyst is involved in synapse assembly and receptor trafficking points to extensive potential interactions between the exocyst complex and the lipid rafts in neurons (Sans et al., 2003; Riefler et al., 2003).

While exocytosis at specific sites is a means to add proteins and lipids to the plasma membrane, endocytosis is used for their removal from the surface. Raft components are selectively internalized through clathrin-independent / dynamin-dependent endocytosis under the control of Rho-GTPases (Lamaze et al., 2001). A specialized pathway involves association with caveolae, a stable plasmalemmal compartment where phosphorylation of the raft protein caveolin-1 is required to induce endocytosis (Le et al., 2002).

Endocytosis is also involved in the processing of amyloid-precursor protein (APP) by β-secretase, which both are thought to be associated with separate rafts and interact more efficiently upon entering endosomes (Ehehalt et al., 2003).

Many of the interactions mentioned above either take place exclusively in lipid rafts or lead to different signal quality or duration if triggered outside of rafts.

Hence, through regulation of the local environment of signaling platforms, lipid rafts achieve to sort out protein interactions by combining or separating components and thus regulating spatial and temporal signal quality.

1.3.4 Cap23

1.3.4.1 Overview

Cap23 (synonyms: Nap-22, BASP-1) is a major cortical cytoskeleton-associated and calmodulin binding protein, which localizes to cholesterol- and PI(4,5)P_2-rich domains at the inner leaflet of the plasma membrane. It is expressed in early development in a variety of tissues and later is restricted to certain brain regions like the hippocampus, cortex, hypothalamus, and the cerebellum. Recently also expression in immune cells and endothelial cells has been reported (Sprenger et al., 2006). Cap23 shows little secondary structure, but is functionally related to the growth-associated proteins Gap43 and MARCKS, which share a domain able to bind Ca^{2+}/calmodulin, PI(4,5)P_2, actin, and a PKC phosphorylation site.

Native Cap23 binds to cholesterol-rich domains and protects these during pharmacological cholesterol depletion, implying that lipid rafts can be stabilized by binding of Cap23 (Khan et al., 2003). Photobleaching experiments show that Cap23 displays low lateral mobility as compared to other raft components, which has been suggested to be a consequence of interactions between Cap23 and cholesterol. In addition to these protein-lipid interactions, Cap23 appears to drive PI(4,5)P_2-domain formation in an electrostatic manner (Epand et al., 2004). Especially the myristoylated N-terminus of Cap23 seems to promote the sequestering of lipids into lipid rafts in a cholesterol-dependent fashion.

Firm evidence shows that regulation of the actin cytoskeleton is tightly linked by PI(4,5)P_2-rich microdomains. PI(4,5)P_2 plays a central role in generating cytoskeletal responses by regulating proteins that control actin dynamics directly. Cap23, along with Gap43 and MARCKS, is a protein that influences the efficiency of raft-dependent signaling to the actin cytoskeleton, which regulates neurite outgrowth and synaptic plasticity (Laux et al., 2000). These proteins function by enhancing the accumulation and assembly of PI(4,5)P_2-rich plasmalemmal rafts in a Ca^{2+}/calmodulin- and PKC-regulated manner (Caroni, 2001). In consequence, clustered PI(4,5)P_2-containing rafts can sequester actin-binding proteins such as profilin, gelsolin, cofilin and ERM proteins.

These interactions are thought to stabilize rafts at the plasma membrane and to link them to the underlying cytoskeleton, thereby regulating actin dynamics at the cell surface, whereas actin disassembly and endocytosis mediates their dispersal or removal from the cell surface.

Cap23 plays an essential role in anatomical plasticity, especially during axonal regeneration following nerve lesion (see 1.2.4.2). Mice lacking Cap23 display several severe phenotypes among which there are: deficient stimulus-induced nerve sprouting at the adult neuromuscular junction, hindlimb ataxia and early lethality (Frey et al., 2000), whereas transgenic expression of Cap23 in motoneurons potentiates stimulus-induced nerve sprouting and induces substantial growth of synaptic structures (Caroni et al., 1997).

Taken together, these experiments identify Cap23 as an intrinsic determinant in synaptic plasticity.

1.3.4.2 Involvement of Cap23 in Nerve Regeneration

Axonal regeneration is a critical problem after brain insult, spinal cord injury and various neurodegenerative diseases of the central and peripheral nervous system. Though advance is being made in the field of promoting CNS axons to regrow after a lesion, e.g. by removal of growth inhibitory proteins like NogoA (Fouad et al., 2004) the molecular mechanisms and requirements for permissive and instructive nerve growth responses to damage are still largely enigmatic. Dorsal root ganglions (DRGs) are a valuable tool for studying regeneration. They possess a bifurcated axon, one branch projecting peripherally and the other extending through the dorsal root to the spinal cord. Damage to the peripheral branch of DRGs elicits a robust response on the mRNA level of growth-associated proteins like Cap23, Gap43 and Scg10 (Mason et al., 2002), and allows regeneration or this lesioned axonal branch.

Strikingly, if the central branch is damaged, the required gene expression response does not take place and regeneration can not occur, unless the peripheral branch has undergone a preconditioning lesion. Hence, there is a requirement for a signal elicited

from a peripherally lesioned axon in order to fully activate a regenerative response. Understanding the nature of this signal would greatly facilitate progress to develop new treatments for spinal-cord injured patients.

Studies performed on transgenic mice expressing either Cap23 or Gap43 have shed some light on the molecular mechanisms involved in axonal regeneration. Whereas DRGs from WT mice fail to regenerate a lesioned central axonal branch, regeneration was partially successful if these two proteins were overexpressed using double transgenic mice. Even though each of the two individual proteins displayed some axonal sprouting on their own, the significantly increased regenerative response in the majority of DRGs upon overexpression of Cap23 and Gap43 together showed that they function in a synergistic way (Bomze et al., 2001). Even more significant, the mere upregulation of Cap23 and Gap43 did not require a preconditioning lesion of the peripheral axonal branch in order to regenerate the central axon. In contrast to WT DRGs. neurons overexpressing Cap23 and Gap43 together therefore enabled axons to regenerate *in vivo*.

These experiments provide evidence that along with Gap43, Cap23 plays a pivotal role in mechanisms allowing lesioned axons to regenerate.

1.4 The Neuronal Actin Cytoskeleton

1.4.1 Overview

The neuronal cytoskeleton consists of actin, microtubules and intermediate filaments, three systems which have separate, but interrelated functions, which include longitudinal and radial neurite growth, synapse formation, endo- and exocytosis, vesicular transport and neurite stabilization. Many of these individual functions can be assigned to one cytoskeletal system, but in more and more mechanisms, it has become clear that a combination of cytoskeletal components is used to fulfil the diverse specialized needs of a given cell. This is valid for normal cellular processes as well as for pathological situations, where the amazing ability of a cell to adapt to altered internal or external conditions becomes obvious in generating a homeostatic response, utilizing cytoskeletal components which normally would not be involved under a certain setting.

1.4.2 Different Actin Structures and Their Biological Functions

Actin is an essential cellular protein, which exists in globular (g-actin) and filamentous (f-actin) form. F-actin is generated by ATP-dependent polymerization of monomers into longer cables. In the neuron, several morphologically distinct forms of polymerized actin exist:

1) Filopodia: short processes emanating from neurites, which in dendrites can mature to form dendritic spines, and in axons are sent out from the axonal shaft itself or from existing boutons in order to sense the environment for further growth of a sprout or synapse enlargement.

2) Lamellipodia: fan-like structures mostly at the growth cone, which are highly dynamic and motile and whose main function is to integrate extracellular signals (guidance cues) into polarized neurite extension for proper pathfinding.
3) Dendritic spines: specialized synaptic structures in dendrites of excitatory neurons, containing large amounts of f-actin (see 1.3.3).
4) Cortical actin: a subplasmalemmal actin meshwork of short filaments, which are highly branched and interconnected (see 1.3.6).

1.4.3 Dendritic Spines

Dendritic spines are the major sites of excitatory synapses in the brain and exist in various shapes and sizes. They can be thin, stubby or of a mushroom-like morphology. Mushroom spines are the most mature and complex postsynaptic structures and consist of a mushroom-shaped head which is separated from the shaft by a neck of variable length, thereby creating a compartmentalized region. This is necessary to prevent that calcium, entering the spine during synaptic activation, can leak into the shaft and thus to neighbouring synapses. This compartmentalization serves to keep information transfer synapse-specific and is relevant to synaptic plasticity, which is determined by the level of postsynaptic Ca^{2+} elevation.
Actin is located mainly below the postsynaptic density (PSD) of dendritic spines and serves to stabilize these fine structures, but also allows some form of motile behaviour besides acting as a scaffold for neurotransmitter receptors and adaptor proteins.

1.4.4 Functional Implications of Spine Morphology

The size of the spine head and the strength of the synapse seem to be correlated, presumably due to the higher levels of AMPA receptors in larger spines (Kasai et al., 2003). There is also evidence that smaller spines preferentially undergo long-term potentiation (LTP), whereas larger spines are more stable and display less plasticity

(Matsuzaki et al., 2004). Due to these observations it was proposed that thin spines might represent the more plastic spines and large mushroom spines might represent the more stable “memory” spines.

1.4.5 Plasticity of Dendritic Spines

Spines can change their shape on a small-scale basis, a phenomenon termed “morphing”. This describes a rapid movement of spine heads, the underlying mechanisms of which, despite many efforts, are not yet completely understood.

It is though widely accomplished that the regulated polymerization and/or depolymerization of actin underlies spine motility, growth and shape. Actin-disrupting drugs like latrunculin A or cytochalasin D completely abolish this motile behavior and, can lead to collapse of dendritic spines. Spine morphing is physiologically important, as experiments performed *in vitro* and *in vivo* have shown that anaesthetics like halothane, hypothermia or simply synaptic activation by AMPA or NMDA result in cessation of spine motility.

Spines can also undergo long-term changes in size, receptor number and synaptic strength. This phenomenon is called long term potentiation (LTP) and is widely believed to be a critical mechanism underlying memory storage. LTP is elicited by a series of events, among which the most critical are NMDA receptor-mediated Ca^{2+}-elevation, activation of Ca^{2+}/calmodulin dependent kinase II (CamKII) and in consequence phosphorylation of AMPA receptors, which ultimately leads to translocation and membrane insertion of these receptors at the PSD, where they contribute to synaptic strength. Induction of LTP also increases actin polymerization in spines (Okamoto et al., 2004). This might be mediated by the actin binding protein profilin II, which is recruited to spines by NMDA receptor activation, whereas blocking the spine targeting of profilin destabilizes spines (Ackermann et al., 2003).

1.4.6 Cortical Actin

Cortical actin consists of a subplasmalemmal actin meshwork of short filaments, which are highly branched and interconnected. This cortex can be organized in several layers, thus acting as a diffusion barrier for molecules at the interface between the cytoplasm and the plasma membrane. Its main function is thus to stabilize the neurite by forming a rigid cytoskeletal barrier below the plasma membrane and therefore also prevents uncontrolled diffusion between the cytoplasm and the membrane.

Besides being the driving force of motility, actin also plays a role in preventing actin-based outgrowth of processes. Focal application of latrunculin A to neurites destabilizes the actin cortex, which under normal conditions acts as a tight barrier, preventing process outgrowth. By this experimentally induced weakening of cortical actin, outgrowth of actin-rich protrusions is locally enabled. This is a nice example which illustrates how the same molecule, dependent on its state of polymerization, localization and regulation is able to perform multiple, partially opposite functions.

1.5 Intermediate Filaments

1.5.1 Overview and Classification

The largest group of cytoskeletal proteins are the intermediate filaments (IFs). They consist of five families, which share a similar protein structure made up of a central alpha-helical rod domain, flanked by a globular N-terminal head- and a C-terminal tail domain. Compared to microtubules (MTs) and actin filaments, IFs are rather flexible, and in contrast to MTs and actin filaments, which break when subjected to shear stress, are more viscoelastic. Neurons express mainly IFs of group III (peripherin, vimentin) and group IV (NFL, NFM, NFH, internexin).
Neurofilaments (NFs) differ from other IFs in that they possess long hypervariable C-terminal domains, which are involved in interaction with adjacent structures and can be highly phosphorylated, which regulates several aspects of their behaviour.

1.5.2 Function

Intermediate filaments play various important roles in neuronal development, which were not well understood for a long time due to redundancy of this system, which upon manipulating one of its components resulted in compensatory regulatory responses and hence making it difficult to assign unique functions to individual members of the IF family. However, approaches including the generation of multiple knockout mice have shed light on several functions of neuronal development, in which IFs are involved.
RNAi studies in PC12 cells have shown that peripherin regulates the initiation, extension, and maintenance of neurites (Helfand et al., 2003). Peripherin null mice display a substantial reduction in the number of unmyelinated sensory fibers, demonstrating a role in axonal elongation and cytoskeletal stabilization during development and regeneration. (Lariviere et al., 2002). Furthermore, mice carrying a mutant peripherin allele showed impairment of collateral sprouting of DRG axons into adjacent denervated skin, indicating

a critical role for intact IF in plasticity, specifically in compensatory nociceptive nerve sprouting (Belecki-Adams et al., 2003).

An important function of NFs is the promotion of radial axonal growth: after successful synapse formation, NF expression is markedly elevated, myelination begins and fully elongated axons increase in diameter up to tenfold. Since the speed of conductivity of a nerve impulse is directly proportional to axon caliber, NFs are thus involved in the speed of impulse conduction by regulating and maintaining the axon caliber. NFs are particularly abundant in large myelinated axons such as motoneurons, where conduction velocity is critical to normal function. A further implication for NFs in neurite extension was shown in Xenopus neuronal cultures, where disruption of NFM altered growth dynamics in a way that neurites spent a smaller fraction of time for active extension (Walker et al., 2001).

1.5.3 Expression and Assembly

In young neurons, IFs are expressed from early on and are successively replaced by NFs as the neuron matures (Shaw et al., 1985). When axons regenerate following a lesion, peripherin is upregulated, a mechanism believed to be important for neurite extension and consolidation.

NFs assemble head-to-tail, with their C-terminal tails not being in the coil, but forming sidearms, which extend from the filament and are involved in interactions with other NF polymers or other cytoskeletal structures like spectrin (Macioce et al., 1999). In contrast to group III IFs, which form homopolymers, NFs are obligatory heteropolymers. The characteristic long filament structures are generated by side-to-side as well as end-to-end fusion of IF particles, which generate short filaments, also termed “squiggles”, before forming longer and more stable filaments.

Besides the classical filamentous forms, ring-like structures have been observed to appear with age in hippocampal neurons (Shaw et al., 1985) as well in a variety of human neurodegenerative diseases.

1.5.4 Regulation

The main mechanism by which NFs are regulated besides changing protein levels is phosphorylation. NFs contain KSP domains, and can contain more than 100 phosphorylation sites; in fact, NFH is one of the most extensively phosphorylated neuronal proteins *in vivo*. Phosphorylation of NFs results in their dissociation from kinesin, thereby decreasing their transport speed. In *in vivo* experiments using antisense oligos, Cdk5 (Cyclin-dependent kinase 5) has been shown to be the major kinase for NFH correlated with neurite outgrowth, as reducing Cdk5 levels caused reduction of NFH phosphorylation and neurite outgrowth. Also CamKII (Ca^{2+}-calmodulin-dependent protein kinase II) has been shown to phosphorylate NFs (Hashimoto et al., 2000). Biological significance to NF phosphorylation is given by the fact that alteration of NFM and NFH tail phosphorylation is associated with the pathology seen in several neurodegenerative diseases, in which abnormally phosphorylated NFs occur in perikarya. These perikaryal structures are known to be a hallmark of disease, and several studies suggested a causal role for NF overexpression in the development of motor neuron disease (Cote et al., 1993; Xu et al, 1993; Lee et al., 1994; Collard et al, 1995).
The degradation of NFs is considered to happen within growth cones as well as at synapses, where NF dephosphorylation by protein phosphatase 2A precedes degradation (Gong et al., 2003).

1.5.5 Transport

NFs are transported along axons bidirectionally on microtubules via association with kinesin5a (Xia et al., 2003) and dynein. These movements have long been thought to be mediated by slow axonal transport, but it has recently become clear that NF movements consist of fast saltatory transport events, interrupted by long pauses, causing the net transport to appear slow. These pauses or docking events are not yet understood, but seem to be partially correlated with phosphorylation events.

Transport of IFs plays a role not only in cytoskeletal regulation, but also in signalling, as vimentin enables spatial translocation of pErk (extracellular signal-regulated protein kinase) by importins and dynein in lesioned nerve. This interaction is important in neuronal plasticity, since axonal regeneration seems inhibited or delayed in dorsal root ganglion neurons from vimentin null mice (Perlson et al., 2005).

1.5.6 Intermediate Filament Involvement In Disease

NFs have long been suspected to play a role in the pathogenesis of several types of neurodegenerative diseases including ALS (amyotrophic lateral sclerosis), Alzheimer's disease (AD), Parkinson disease (PD), diabetes and SMA (spinal muscular atrophy). It has clearly been shown that abnormal assembly and accumulation of NFs in motoneuron cell bodies and axons are commonly observed in these diseases (Lee et al., 1996). Detrimental effects of NF accumulation have further been shown in studies, where an increase of NFM or NFH did not only impair their transport but also interfered with dendritic arborization (Kong et al., 1998). Together with NFs, also peripherin was found in axonal inclusions in human ALS patients (Migheli et al., 1993). Evidence for a role of NFs in axonal regeneration *in vivo* derive from nerve crush studies, comparing regenerative responses thereafter (Zhu et al., 1997). The number of regenerating axons of NF-L -/- mice following crush injuries of sciatic and facial nerve was reduced to 25 and 5% as compared to WT mice, respectively. Though axonal sprouts were abundant in NFL null mice, maturation of regenerating fibers was occurring at a much slower rate.

Furthermore, numerous animal models provide clear evidence that altered protein levels or phosphorylation states of NFs are involved in various forms of axonal injury, neuronal degeneration and muscle atrophy (summarized in Liu et al., 2004).

1.6 Mitochondrial Function and Motility

1.6.1 Overview

Mitochondria are the primary cellular energy generators. They produce ATP via oxidative phosphorylation (aerobic respiration) or glycolysis (anaerobic respiration) in situations when oxygen is limited. Mitochondria are usually rod-like in appearance and are also important regulators of intracellular calcium homeostasis. Damaged mitochondria can promote apoptosis by production of harmful reactive oxygen species and are normally removed by delivery to autophagosomes.

1.6.2 Mitochondrial Docking and Distribution

Neuronal mitochondria need to be actively positioned to sites of local energy demand like growth cones (Povlishock, 1976) synapses and Nodes of Ranvier (Fabricius et al., 1993). Thus, axonal regions that have higher levels of activity have higher mitochondrial densities and/or activities. Therefore, mitochondria need to continuously adapt to changing cellular requirements and although mitochondria are known to undergo transitions between motile and stationary states, what exactly causes mitochondria to stop, has been unclear. Recently though, one docking signal has been identified. Mitochondria moving along axons treated with NGF-coated beads preferentially dock near sites where NGF signaling has been triggered by bead attachment (Morris and Hollenbeck, 1995). Interestingly, this docking is dependent on intact f-actin, since treatment with latrunculin B prevents mitochondrial docking, while bidirectional transport of mitochondria is unaltered. These results indicate that mitochondrial docking in neurons can be regulated by extracellular signals, and that this docking critically depends on interactions with the actin cytoskeleton.

Further candidates for mitochondrial docking are the IFs. Crossbridges of mitochondria to NFs (Leterrier et al., 1994) as well as to IFs (Mose-Larsen et al., 1982) have been

observed. IFs might also be involved in mitochondrial motility and distribution, perhaps by anchoring mitochondria in place during stationary periods (Toh et al., 1980; Reipert et al., 1999; Milner et al., 2000; Linden et al., 2001; Wagner et al., 2003).
Another physiological stimulus that can cause mitochondria to stop is NMDA receptor-dependent Ca^{2+}-influx, triggered by the neurotransmitter glutamate (Rintoul et al., 2003), which might be a signal targeting mitochondria to activated synapses and growth cones.

1.6.3 Mitochondrial Transport

Mitochondrial transport in neurons takes place in a bidirectional manner. Mitochondrial interactions with the kinesin motor proteins Kif1Bβ, Kif5B and dynein have been identified and mediate fast long-range transport along microtubules. When mitochondria encounter sites of high energy demand, this association has to be loosened and more subtle mechanisms seem to control mitochondrial distribution.
Several lines of evidence suggest that this mechanism is a switch to actin filament-based transport. In this light, myosin Va has been shown to mediate short-range movements of mitochondria along actin fibers. This actin-based transport serves as a local mechanism significantly improving the efficient targeting of mitochondria to sites of high energy demand. Even more, this dual transport system seems to contribute to the fine-tuning of mitochondrial localization, and is especially necessary in regions of the neuron where MTs are few or absent (such as axonal branch points and growth cones). It is also noteworthy that mitochondria move at significantly higher velocity on MT tracks than on actin filaments, but they also spend only half as much of their time moving on MTs as they do on actin filaments (Morris and Hollenbeck, 1995). This might be explained by the need for precise positioning of mitochondria, for which the actin-based transport system seems to be responsible.
Interestingly, the directionality of mitochondria, which recently have been found to contain $PI(4,5)P_2$, seems to be controlled by $PI(4,5)P_2$ clustering, induced by pleckstrin homology (PH)-domain binding. Overexpression of different PH domains alters the distribution of neuronal mitochondria in a way that plus-end-directed transport is elevated, while minus-

end-directed transport is decreased, without affecting motor properties or overall levels of motility (De Vos et al., 2003).

1.6.4 Mitochondrial Involvement in Disease

Mitochondrial dysfunction may cause motor neuron death by predisposing them to calcium-mediated excitotoxicity, by increasing generation of reactive oxygen species, and by initiating the intrinsic apoptotic pathway. In other neurodegenerative diseases like PD, AD, ALS or Huntington disease, mitochondrial dysfunction is increasingly being recognized as an important factor contributing to pathogenesis.

Superoxide dismutase (SOD) is a cytosolic protein, which in a mutant form can localize to some extent to mitochondria and can cause ALS in mice and humans (Liu et al., 2004). In a SOD1-based mouse model of ALS, the most obvious cellular abnormalities are the presence of membrane-bounded vacuoles in axons and dendrites, which appear to be derived from degenerating mitochondria (Wong et al., 1995). Detailed studies in these mouse models indicate that mitochondrial abnormalities begin prior to the clinical and pathological onset of the disease, suggesting that mitochondrial dysfunction may be causally involved in the pathogenesis of ALS.

There is also evidence that the balance between mitochondrial fusion and fission is important for proper functioning1the mitochondrial system, since disruption of this balance results in morphological alterations, including fragmentation and formation of ring structures (Dimmer et al., 2002, Shaw and Nunnari, 2002).

Mitochondria are thus regarded to play critical roles in a variety of neurodegenerative diseases.

1.7. Goals of the Thesis

The broad aim of this study was to understand how Cap23 is involved in the regulation of various aspects of plasticity of the neuronal cytoskeleton. Thus in a first effort, we studied the effect of Cap23 on the actin cytoskeleton by comparing different developmental stages of hippocampal neurons cultured *in vitro*, of Cap23 null, heterozygous and transgenic mice.

The second aim was directed towards investigating the stabilization of another big part of the neuronal cytoskeleton, the intermediate filament system. We found that the integrity of this system critically depends on Cap23, which is necessary for proper assembly of NF structures and docking at defined sites along the axon.

The third aim of this study was the examination of mitochondrial docking. With the use of live imaging time-lapse experiments and retrospective immunocytochemistry, we could prove that the presence of Cap23 is important for the proper targeting of mitochondria towards the growth cone and to other sites in axons, part of which we identified as membrane varicosities containing the exocyst complex.

In summary, the goal of this PhD project was the elucidation of how Cap23 is involved in the regulation of lipid rafts at the cell surface, and how this would affect downstream mechanisms like the regulation of actin polymerization, stabilization of the neuronal cytoskeleton and proper organelle anchoring.

2 RESULTS

2.1 Morphological Aspects of the Actin Cytoskeleton

2.1.1 Dendritic Spine Length Decreases with Reduced Cap23 Levels

At the beginning of our quest to better understand the function of Cap23, we reasoned that since Cap23 is a cortical cytoskeleton-associated protein, altered protein levels thereof might precipitate in structural changes of the actin cytoskeleton. With the cytoskeleton being an essential determinant of neurite outgrowth and specifically axonal regeneration, in which Cap23 has been shown to be critically involved, actin was a good candidate to be involved in mechanisms downstream of Cap23, to affect the maturation and stability of growing neurites.

We thus prepared primary hippocampal neuron cultures from WT and Cap23 KO mice and analyzed them after different times of *in vitro* culture.

First, we analyzed dendritic spine morphology between WT and Cap23 KO neurons at various developmental stages. Dendrites were identified by their larger diameter, shorter length and characteristic filopodia-bearing processes, in contrast to axons, which had fewer filopodia and were longer and much thinner. Before the age of ca. 8-10 DIV, most actin-based protrusions are of immature morphology, being thin, simple in shape and elongated. Synaptogenesis starts around 7 DIV, peaking at around 14-18 DIV, when spines have adopted a more mature shape, often bearing a characteristic mushroom-like head at their tips. At around 3 weeks in culture, dendritic spines become either consolidated or eliminated.

At this time, mushroom-shaped spines have become the most frequent postsynaptic structures in the culture and neurons have adopted their final morphology.

We included immature spine-like protrusions of young neurons (4 DIV) in our analysis, as we were interested in the development of actin structures from early on.

At 4 DIV, dendritic protrusions extended for about 5um from the dendritic shaft, decreasing to an average of about 3.3um at 6 DIV (Fig. 1). In these young neurons we did not observe a difference in the length of protrusion in WT and Cap23 KO. In contrast, we

found a significant difference of spine length from 9DIV on, corresponding to the time of early synapse formation. Actin protrusions of KO dendrites of 9, 12, 17 and 23 DIV were consistently reduced by 41, 39, 49 and 42%, respectively (Fig. 2).

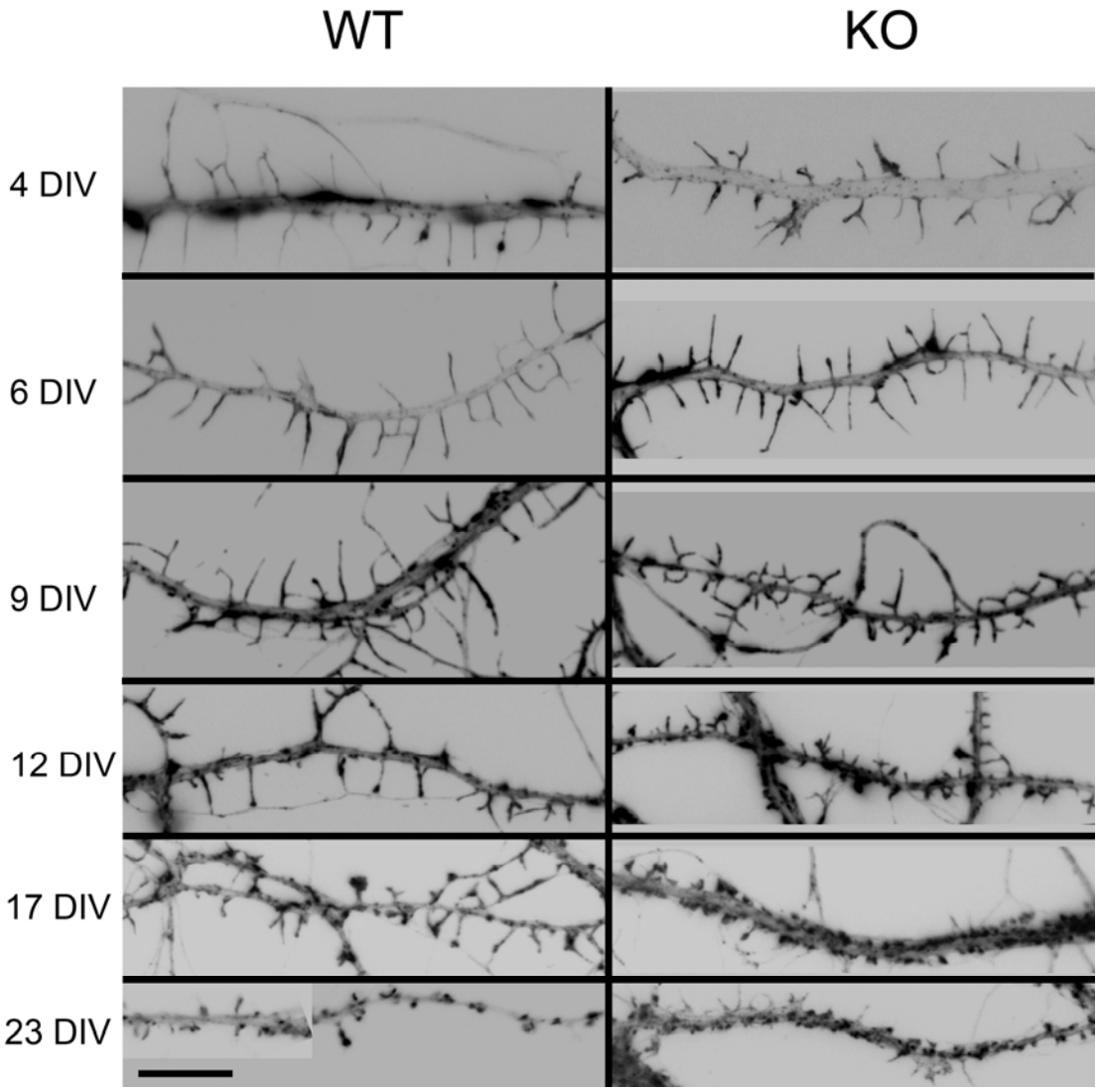

Figure 1. **F-actin-rich protrusions of hippocampal dendrites from WT and Cap23 KO mice.** WT and Cap23 KO hippocampal neurons were cultured for 4-23 days in vitro (DIV). The f-actin cytoskeleton was visualized by Alexa488-phalloidin. Scale bar=10μm.

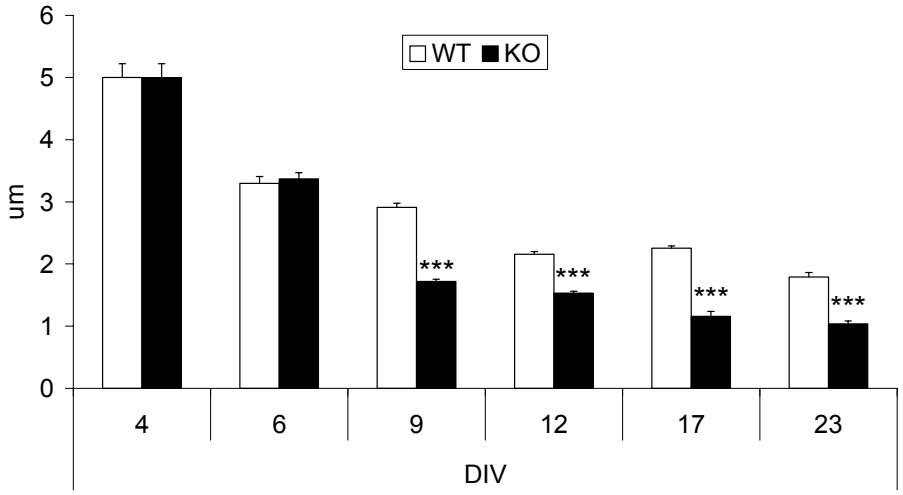

Figure 2. **Quantification of f-actin-rich protrusions in WT and Cap23 KO neurons.**
Spine length from neurons of 4 – 23 DIV of age was quantified. ***=p<0.001.

In addition, we observed that WT dendritic spines frequently displayed polarized accumulations of f-actin towards their tip from 12 DIV on, whereas this polarized distribution of the actin cytoskeleton was disturbed in the absence of Cap23 (Fig. 3). Furthermore, KO neurons had abnormally elevated contents of actin cytoskeletal elements, which accumulated with age throughout spines and inside dendritic shafts (Fig. 3).

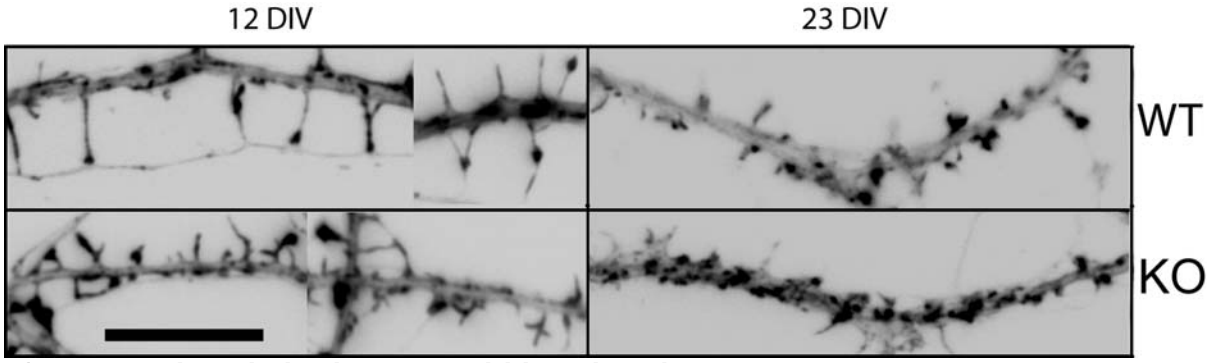

Figure 3. **Actin Polarization at Dendritic Spine Tips.**
WT and Cap23 KO hippocampal neurons were cultured for 12 and 23 DIV. The f-actin cytoskeleton was visualized by Alexa488-phalloidin. Scale bar=10μm.

This analysis revealed a striking difference in actin polarization and protrusion length, as well as an actin polarization defect in the absence of Cap23, indicating a role for Cap23 in

regulating actin-based process outgrowth. This phenotype might be triggered by reduced levels of clustered cholesterol (see Fig. 29) as a consequence of the absence of Cap23 at the plasma membrane. Since it is well established, that cholesterol and $PI(4,5)P_2$-enriched lipid raft domains at the cell surface can attract actin-binding proteins and by regulating those affect different aspects of actin polymerization, it is thus conceivable, that the phenotype we observed at the level of actin organization is an effect of Cap23-mediated lipid raft organization.

2.1.2 Dendritic Spine Length Increases with Augmented Cap23 Levels

In order to examine whether the effect of Cap23 on actin polarization and protrusion length directly correlated with levels of Cap23, we performed a similar analysis as described in 2.1.1, this time analyzing neurons of transgenic mice overexpressing *Thy1*-Cap23. Since this mouse line was bred in a different genetic background, WT mice from the same litter were used as controls.

Similar as for the absence of Cap23, overexpression of *Thy1*-Cap23 did not show a change in spine length before the onset of synaptogenesis (6 DIV; Fig. 4), whereas from 9 DIV on, the length of dendritic spines was consistently enhanced as compared to WT neurons. At 9, 10, 17 and 29 DIV, spines displayed an average increase in length by 19, 56, 60 and 63%, respectively (Fig. 5). This phenotype was accompanied by an increase of polarized f-actin accumulations at protrusion tips as well as in dendritic shafts, especially obvious at more mature stages of spinogenesis (29 DIV; Fig. 4).

Together with the results obtained from the analysis of spine length in neurons lacking Cap23 (2.1.1.), this indicated that levels of Cap23 play a role in the regulation of actin-based protrusions, especially dendritic spines. This might be a consequence of the ability of Cap23 to cluster cholesterol-rich lipid raft domains at polarized locations in dendrites (and axons), which are known to be critically involved in the regulation of actin polymerization. It is thus likely, that by increasing levels of Cap23 by transgenic overexpression, this aspect of Cap23 to induce highly organized lipid raft clusters at the plasma membrane leads to a further potentiation of signal transduction elicited at lipid rafts and targeted at the actin cytoskeleton.

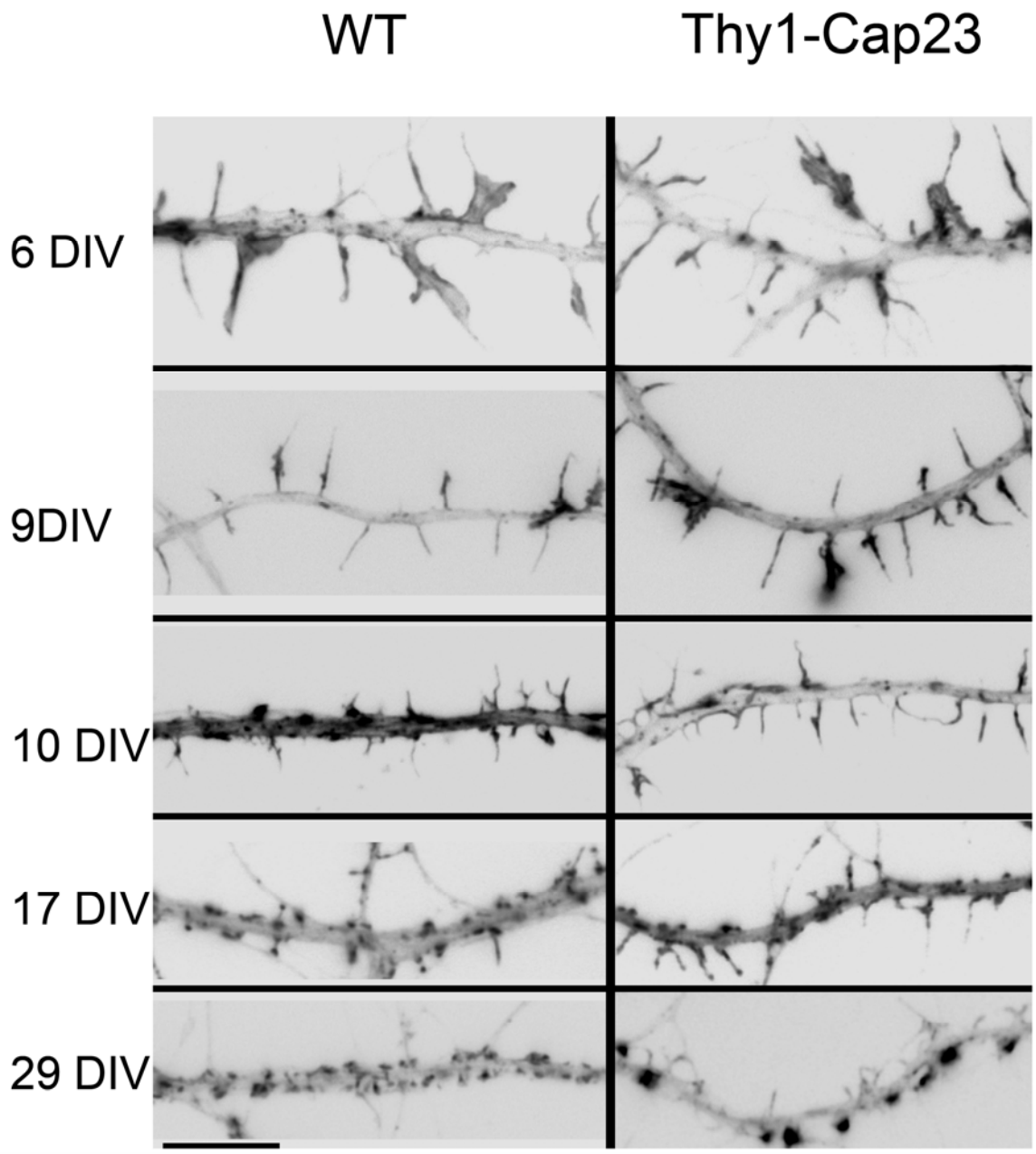

Figure 4. **F-actin-rich protrusions of hippocampal dendrites from WT and *Thy1*-Cap23 mice.**

WT and *Thy1*-Cap23 hippocampal neurons were cultured for 6-29 days in vitro (DIV). The f-actin cytoskeleton was visualized by Alexa488-phalloidin. Scale bar=10μm.

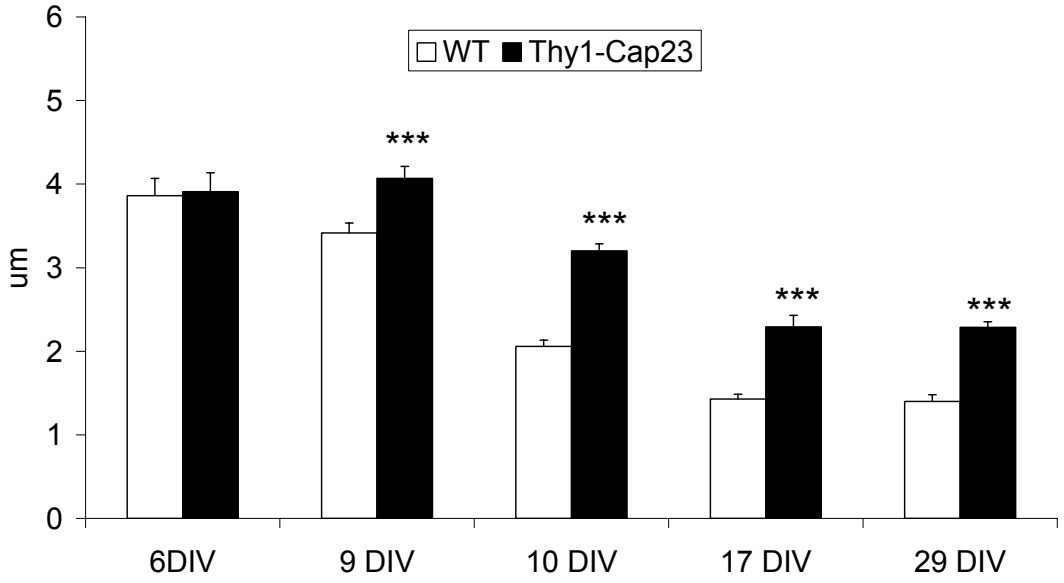

Figure 5. **Quantification of f-actin-rich protrusions (dendritic spines) in WT and *Thy1*-Cap23 neurons.**
Spine length was quantified using Metamorph 6.2r5. Error bars are s.e.m. student's t-test was performed ($p<0.001$ = ***).

2.1.3 Cortical Actin Stability Depends on Presence of Cap23

In addition to appearing as discrete clusters in axons and dendrites, Cap23 is decorating the entire plasma membrane at the inner leaflet. Therefore we reasoned that Cap23 might affect f-actin structures in a global manner in addition to having local effects on the cytoskeleton. Hence, we decided to have a closer look at the cortical actin cytoskeleton, which is situated directly below the plasma membrane, forming a rigid cortical meshwork.
Analyzing phalloidin stainings of hippocampal neurons cultured for 11 DIV revealed that WT dendritic shafts were very regular, allowing outgrowth of actin structures only locally at certain sites, whereas relatively amorphous actin protrusions with a wide range of lengths and shapes were observed to emanate from KO dendritic shafts. It appeared thus that in the absence of Cap23, unrestricted outgrowth of actin-based protrusions at multiple sites along dendritic shafts can occur.

In addition, it was obvious that the f-actin content was enhanced in the absence of Cap23 (Fig. 6), indicating that Cap23 seems to restrict actin polymerization at the plasma membrane.

From this additional phenotype we concluded that actin structures are affected by Cap23 in various ways: The dendritic actin cortex seems to be organized in a manner that dynamic actin polarization along the largest part of the plasma membrane is repressed, thereby preventing ectopic cortical actin dynamics, at the same time restricting actin polymerization to specified sites, where actin protrusions are meaningful to the neuronal morphology and connectivity in order to find contact sites to other cells in an efficient, regulated manner.

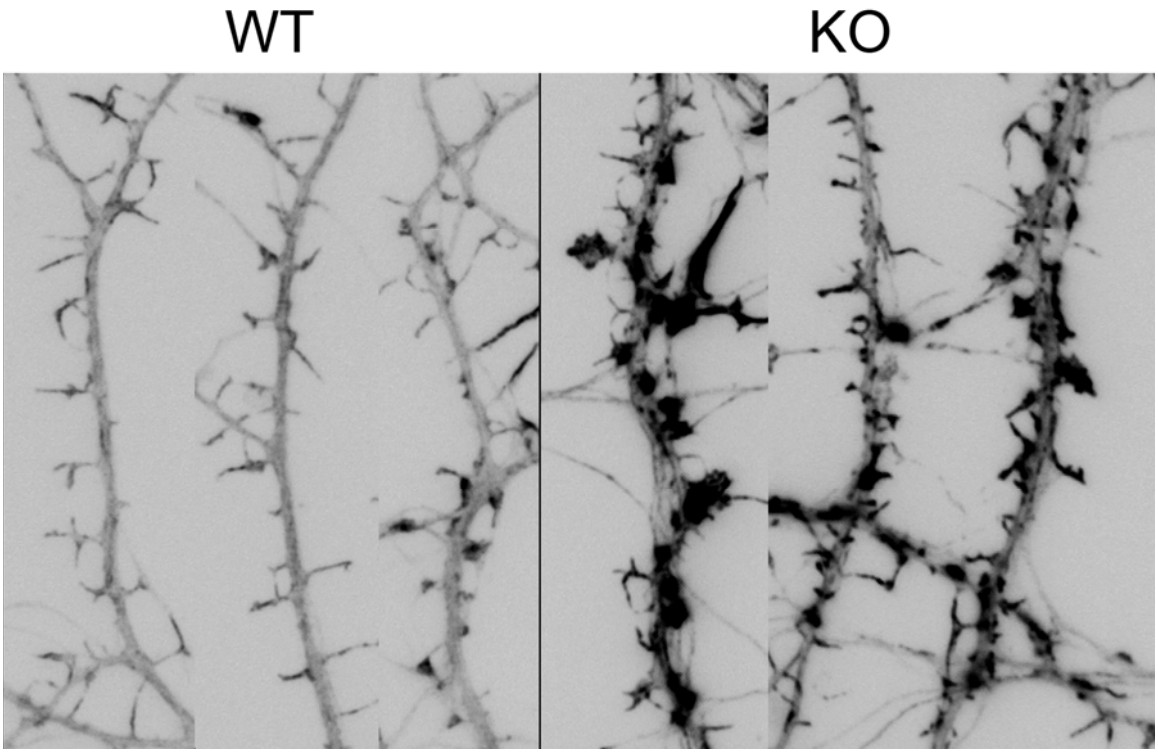

Figure 6. **Cortical actin is unstable in Cap23 KO neurons.**

WT and Cap23 KO hippocampal neurons were cultured for 11 DIV. The f-actin cytoskeleton was visualized with phalloidin-Alexa488. Scale bar=10μm.

2.1.4 Actin-based Motility is regulated by Levels of Cap23

As we had observed dramatic alterations of the actin cytoskeleton dependent on Cap23 protein levels in hippocampal neurons, we next wondered how the phenotypes that we had observed so far in fixed cells would translate into alterations of actin dynamics in living neurons. We therefore transfected hippocampal neurons of WT and Cap23 KO mice with a GFP-actin plasmid and observed the GFP signal in living neurons as early as 24h after transfection. Using a dedicated microscope setup, keeping the transfected neurons at 37°C and under constant 5%-CO_2 perfusion, we were able to perform time-lapse recordings, which provided further insight into the role of Cap23 in actin regulation.
Transfected neurons were imaged at different developmental stages, between 7-14 DIV. As expected, most GFP signal was detected in dendritic actin-rich filopodia and dendritic spines, and in axonal and dendritic growth cones. During the observation time, we observed relatively moderate motility of dendritic spines, which predominantly exhibited amorphous changes in shape, a phenomenon also termed “morphing”. The reasons and benefits for a neuron to use a lot of energy to keep its spines motile are not yet clearly understood, but morphing has been suggested to play a role in transient sampling of the surrounding neuropil and in synaptic input competition of spines having more than one presynaptic contact.
In sharp contrast to WT actin motility, which shape changes were very confined, actin motility in Cap23 KO neurons was severely altered. Actin protrusions were rapidly formed *de-novo,* they elongated, retracted or moved laterally over a much larger area than in WT neurons. Complex morphological changes including multiple branching of preexisting filopodia-like spines were frequently observed. In addition, time-lapse observation of GFP-actin structures revealed that growth cones (GCs) behaved differently between the genotypes. WT GCs were advancing slowly, sending out defined actin-rich filopodia-like structures from GCs, which had a characteristic lamellipodia-like structure. In contrast, KO GCs were much more actively protruding and retracting, with defined filament-like actin structures only rarely observed. In addition, whereas WT GCs achieved to “silence” the neurite directly distal to the GC, KO neurites failed to downregulate actin dynamics

distally to the GC. Instead, multiple actin protrusions emanated from the distal neuritic end, being filopodia- or lamellipodia-like in nature.

In a similar manner, large regions of the actin cortex of axons and dendrites, also more proximal to the cell body showed dramatically increased actin motility.

Taken together, these results strongly suggest that Cap23 is necessary for neurite stabilization at the level of the cell cortex and the underlying actin network, probably via organizing cholesterol-rich lipid raft domains at the cell surface, which are known to be critically involved in the regulation of actin polymerization in various ways.

2.2 Intermediate Filament Organization

2.2.1 Protein levels of IF class IV are Correlated with Protein Levels of Cap23

The neurofilament triplet proteins constitute the class IV subfamily of IFs, which unlike the members of the class III IFs co-assemble as obligate heteropolymeric structures consisting of NFL (neurofilament light chain; essential component) and one of the two other NF isoforms: NFM (neurofilament medium chain) and NFH (neurofilament heavy chain). They are involved in axonal maturation, contribute to cytoskeletal stabilization and directly regulate radial axonal growth and thus are indispensable components of the neuronal cytoskeleton.

Since we wondered whether the defect on the actin network might also affect other components of the cytoskeleton, we decided to analyze protein fractions of WT and Cap23 mutant mouse brains, in order to get further insight into the full scope of cytoskeletal instability in the absence of Cap23.

To this end, we fractionated brain proteins into lipid raft-fractions, non-raft fractions and whole brain homogenates (BH) and analyzed them separately by western blot and/or 2D-gel-electrophoresis, followed by coomassie- or silver staining and MALDI-TOF/MS sequencing.

Analysis of 2D-gels from WT and Cap23 mutant mice revealed that the group of neurofilament triplet proteins (NFL, NFM, and NFH) was consistently downregulated in the absence of Cap23 to approximately a third of WT protein levels (Fig. 7). Protein levels of heterozygous mice showed intermediate levels, ranging from 62 to 86%, which indicated that the levels of Cap23 protein are correlated with the levels of NF proteins, allowing us to speculate that there might be a close relationship between stability of NF proteins and the presence of Cap23.

When we had a closer look at NFL, which is an important NF component for the assembly of neurofilament structures, we realized that its downregulation in Cap23 KO mice was apparent over a wide range of developmental stages i.e. at P16, P28, P76 and even P123 (Fig. 8).

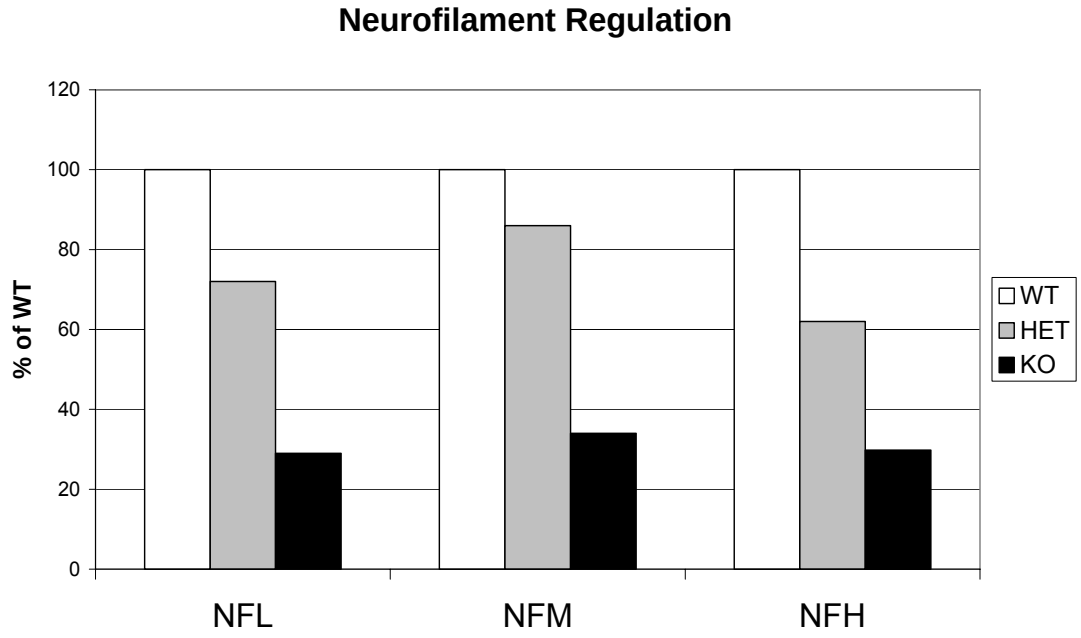

Figure 7. **Neurofilaments are downregulated in the absence of Cap23.**
Non-raft fractions of 28 day old WT, heterozygous (HET) and KO mice were analyzed by 2D-gel-electrophoresis, coomassie-staining and peptide sequencing analysis using MALDI-TOF/MS. Identified spots were quantified densitometrically.

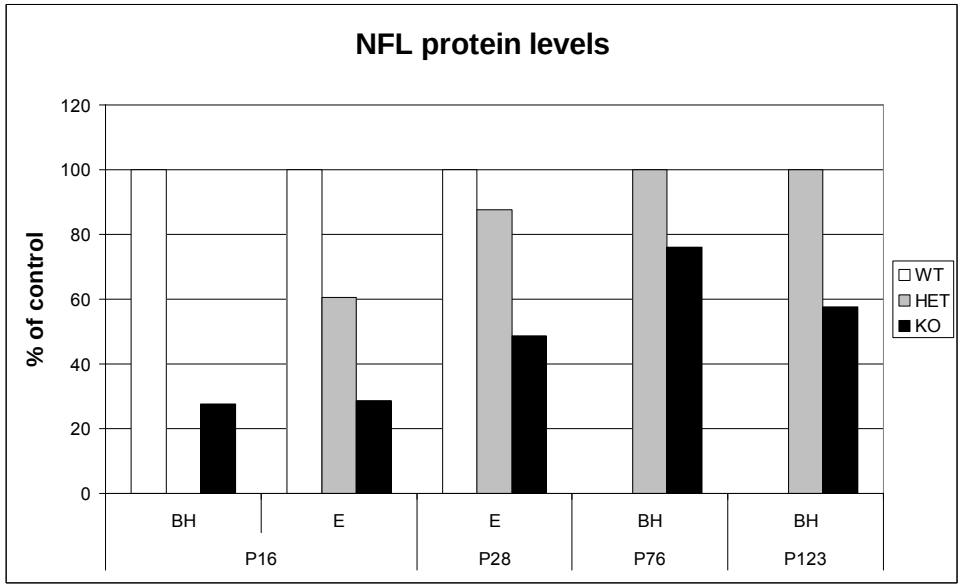

Figure 8. **NFL is downregulated at various ages in the absence of Cap23, and can be found at similar levels in non-rafts as in whole brain homogenates.**
Protein extracts of WT, heterozygous (HET) and KO mice were fractionated into lipid-raft and non-raft fractions. Non-raft fractions (E) and whole brain homogenates (BH) were probed by western analysis for NFL. Bands were scanned and densitometrically analyzed. Values presented are normalized to WT (100%) or HET (where WT mice of same age were unavailable

NF proteins, like most cytoskeletal proteins, were present only in the non-raft fraction, whereas lipid-raft fractions did not contain any NF protein. We also realized that if we compared protein contents of non-raft fractions to whole brain homogenates, we could detect almost the same protein levels (compare WT and KO levels at P16 of non-rafts (E) versus whole brain homogenate (BH), Fig. 8), indicating that the non-raft fraction probably contained the entire IF protein pool.

When we analyzed mice overexpressing *Thy1*-Cap23, we determined that total levels of Cap23 were elevated by 114%, consisting of a slight downregulation of the endogenous Cap23, and 71% of total Cap23 in these mice deriving from the overexpressed chickCap23 under the *Thy1*-promoter (Fig. 9). Thus, transgenic *Thy1*-Cap23 mice contain more than twofold elevated protein levels of Cap23.

Strikingly, we found that NFL levels are not only downregulated in mice containing reduced levels of Cap23 protein, but that NFL protein levels in non-raft fractions of *Thy1*-Cap23 mice were elevated by 75% (Fig. 10), compared to WT levels. We interpreted these findings as a further proof that protein levels of Cap23 directly affect NF protein levels.

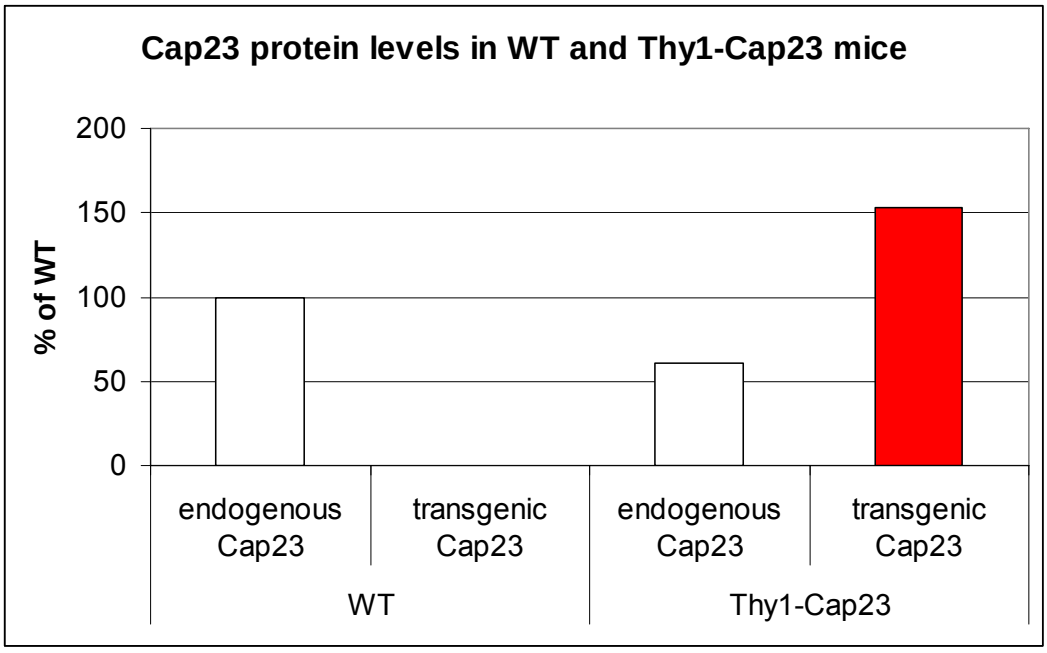

Figure 9. **Cap23 protein levels are elevated in mice overexpressing transgenic Thy1-Cap23.** Raft fractions of ca. 1 month old WT and Thy1-Cap23 transgenic mice were analyzed by 2D-gel-electrophoresis, coomassie-staining and peptide sequencing analysis using MALDI-TOF/MS. Identified spots containing Cap23 were quantified densitometrically. Values presented are normalized to WT (100%).

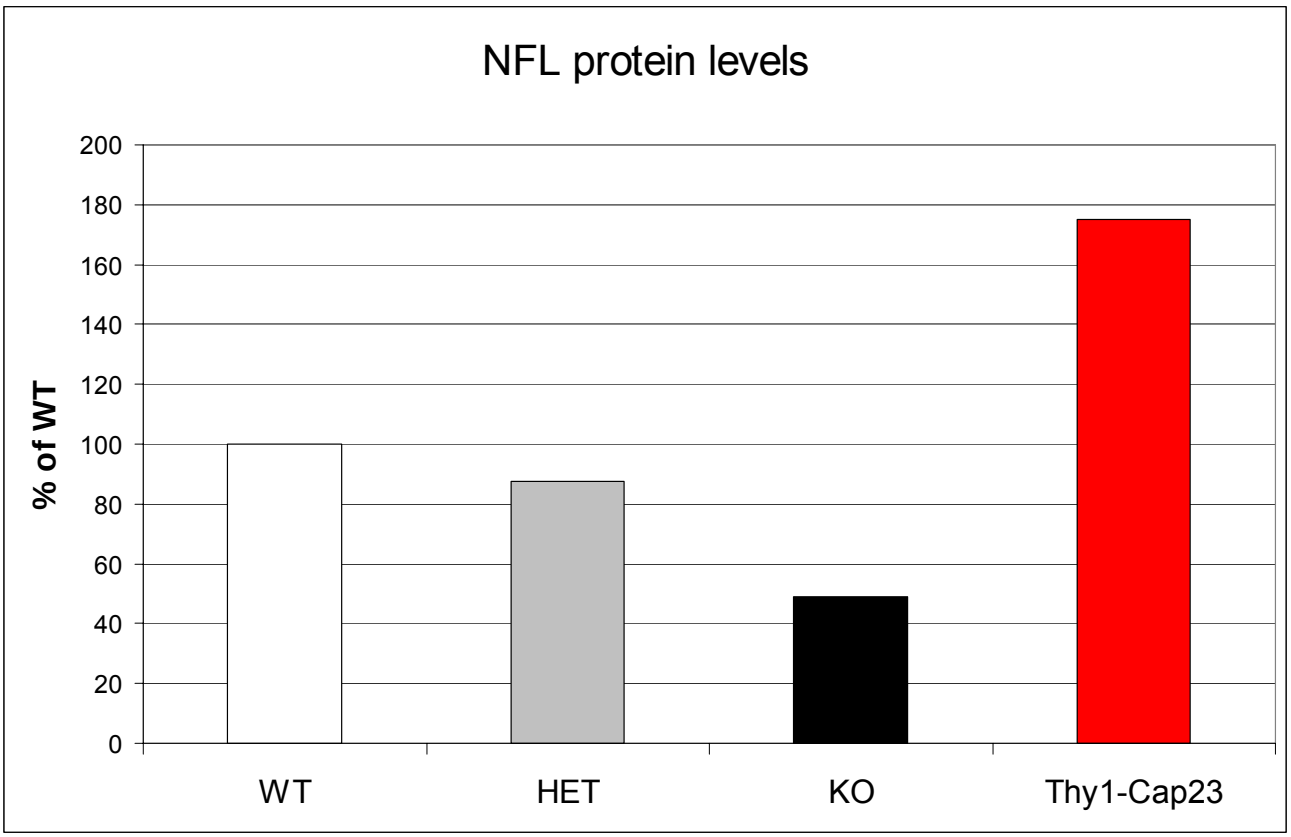

Figure 10. **NFL protein levels correlate with Cap23 protein levels as analyzed in Cap23 mutant mice.**
Non-raft fractions of ca. 1 month old WT, heterozygous (HET) KO and Thy1-Cap23 transgenic mice were analyzed by 2D-gel-electrophoresis, coomassie-staining and peptide sequencing analysis using MALDI-TOF/MS. Identified spots containing NFL were quantified densitometrically. Values presented are normalized to WT (100%).

2.2.2 Morphology and Assembly Differences Related To Protein Levels of Cap23

Having demonstrated that total NF protein levels correlate with Cap23 protein levels, we next wondered how this reduction of protein content would translate into morphology of neurofilaments in intact neurons. We thus performed immunocytochemistry experiments in cultured hippocampal neurons from WT and Cap23 KO mice.

Using an antibody directed against an NFL epitope, we labeled methanol-fixed neurons and found striking alterations in structures immunoreactive for NFH. Whereas in WT neurons, NFH labeled typical filamentous structures of varying length depending on the maturation state of the cell, Cap23 KO neurons displayed striking accumulations of lariat-

and ring-like structures from early developmental stages on (4 DIV), which were increased in frequency ca. 7.6-fold (Fig. 13). These ring structures were present inside axons, varying in size between ca. 1 and 3 μm (Fig. 11).

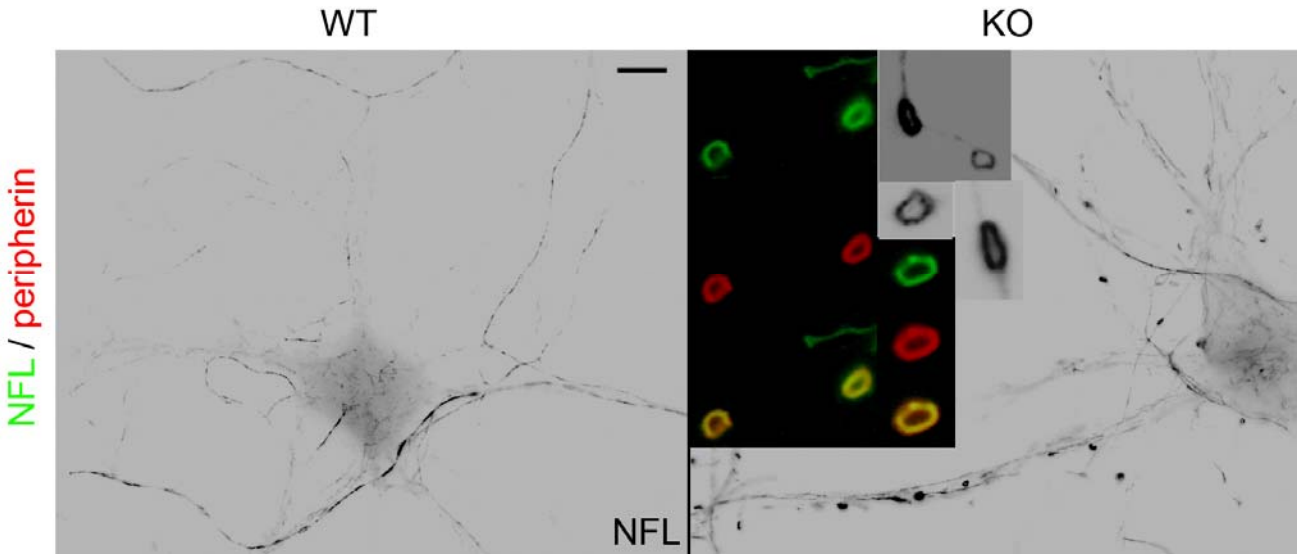

Figure 11. **IFs accumulate in ring-like structures.**
Hippocampal neurons were cultured for 8 DIV and stained with antibodies against NFL and peripherin. WT neurons contain mostly filamentous structures, while KO axons contain mostly ring-like structures. Scale bar=10μm.

Further investigation using antibodies directed against other members of the IF family revealed that these ring structures were immunoreactive for all members of the IF family III (peripherin, vimentin, internexin) as well as IF family IV (neurofilaments) (example showing peripherin and NFL as one representative for each family in Fig. 11). Similar IF structures had already been reported by a plethora of studies to appear in a variety of neurodegenerative diseases as well as in aged neurons of humans and laboratory animals (see introduction). We therefore reasoned that the appearance of these structures might represent the presence of a pathological instability of axons, as has been demonstrated for axonal degeneration. Furthermore it was apparent, that these ring structures also appear in WT neurons, though only at prolonged times of culturing (not shown), which is in line with pathological accumulation of these spherical IF-structures in aged human cortical neurons. This indicated that the appearance of IF-positive spheroids

is not exclusively related to disease, but rather to cytoskeletal instability, which can occur during the terminal stages of a neuron's lifetime and can be accelerated by various neurodegenerative processes.

These results confirmed our data obtained from our proteomic analyses, by showing a pronounced defect in IF stability and morphology.

Interestingly though, it seemed that also the filamentous forms of NF was changed in the absence of Cap23. WT neurons displayed characteristic elongated filaments (Fig.12), which matured with time from shorter, individual fragments into longer intermediate filament-networks, which has been shown to be part of the neuronal cytoskeleton maturation process (Shaw et al., 1985). In contrast, Cap23 KO neurons contained shorter filaments even at older age, pointing towards a defect in the maturation process of IFs. In addition, it seemed that some Cap23 KO neurons contained increased, rather then decreased levels of IF proteins (Fig.12). However, those filaments were more diffuse, partially longer and thinner.

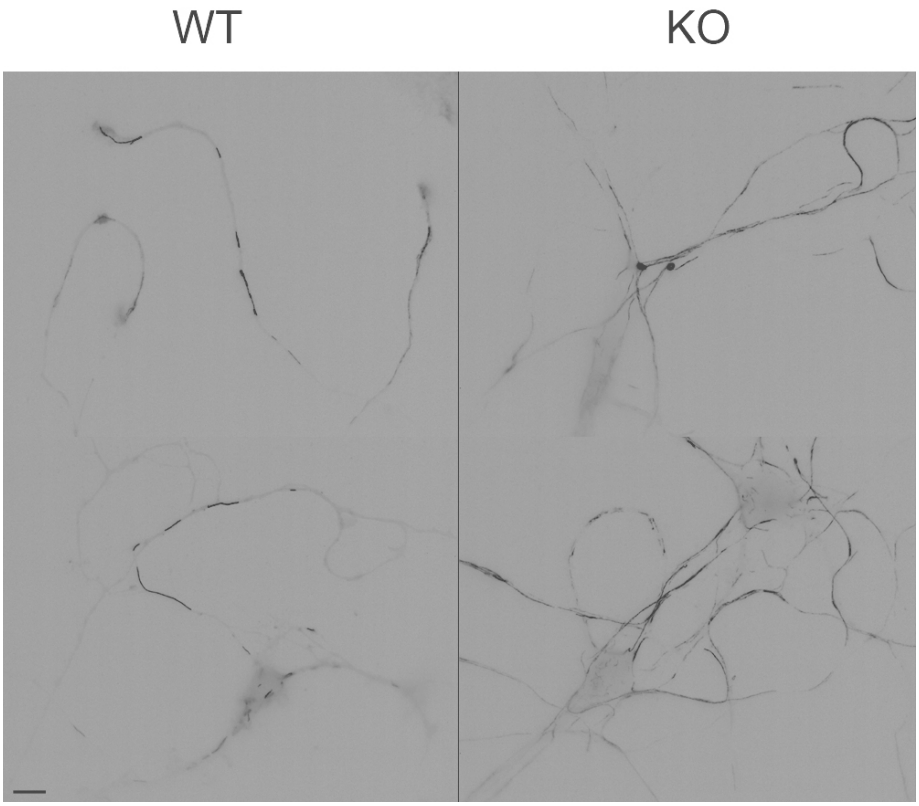

Figure 12. **Internexin morphology is altered in Cap23 KO neurons.**
Hippocampal neurons were cultured for 4 DIV and stained with a monoclonal internexin antibody. WT neurons contain clearly defined filamentous structures, while filaments in KO axons are more diffuse, partially longer and thinner and contain several ring-like structures. Scale bar=10μm.

At a first glance, this seems to be somehow in contrast to the downregulation of IFs we had observed on a proteomic level (2.2.1) but can be interpreted in the following way: Protein analysis was performed using whole brain tissue or fractions thereof as starting material, which includes all neurons of the brain, in contrast to only hippocampal neurons being analyzed by *in vitro* cell culture in immunocytochemistry experiments. We therefore cannot rule out, that hippocampal neurons behave in a specific manner, different from other neuronal subtypes. It is definitely conceivable, that neurons which cytoskeletal integrity is disturbed, would aim at promoting their cytoskeletal stability by compensatory regulation. In fact, the IF families III and IV have been shown in a variety of studies to be able to react to a broad range of interference with the cytoskeleton by up- or downregulation of individual IF components on a protein level.

It is thus likely, that Cap23 KO hippocampal neurons, which cytoskeletal integrity on the level of f-actin is disturbed, use upregulation of IF components as a mechanism to compensate for an unstable axonal cytoskeleton.

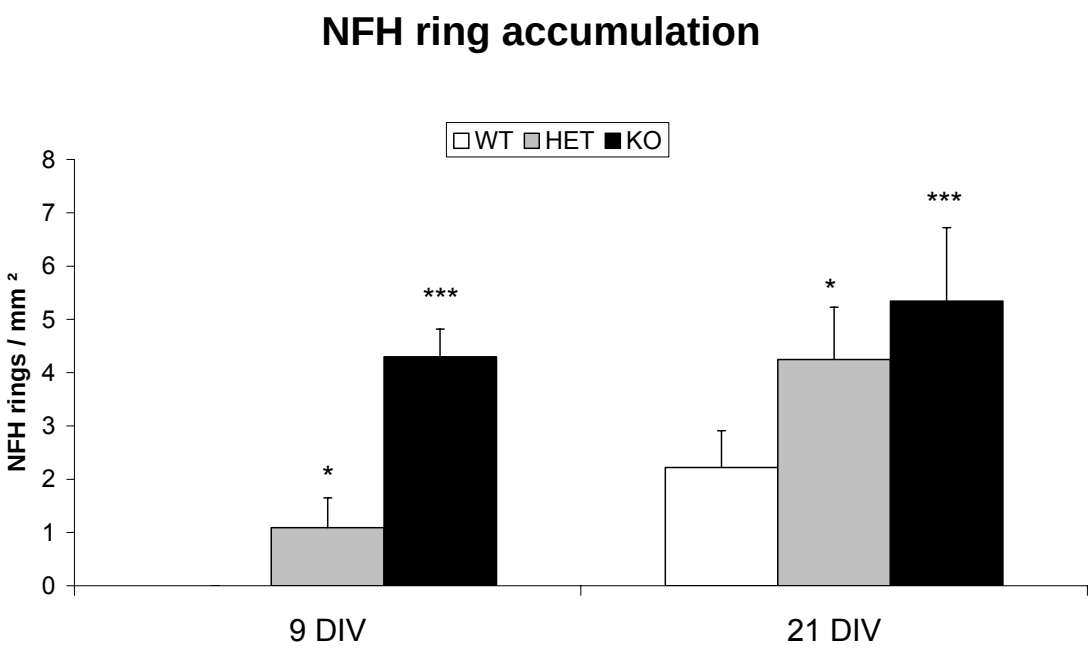

Figure 13. **Ring-like structures are increased in Cap23 KO neurons.**
Hippocampal neurons were cultured for 9-21 DIV and then stained for NFH. Rings were counted and normalized to area (mm^2).

2.2.3 Involvement of the Actin Cytoskeleton in IF Stability

We wondered whether the f-actin phenotype we had observed was related to the alterations in IF integrity and thus decided to perform analyses, in which we pharmacologically interfered with the integrity of the f-actin cytoskeleton and consecutively assessed the state of the intermediate filament cytoskeleton.

To this end, we cultured WT and Cap23 KO hippocampal neurons in the presence or absence of low doses of CytochalasinD, which at higher doses rapidly depolymerizes f-actin. CytochalasinD was added 2 days after neuronal cultures were set out and replenished at every medium change (every 3 to 4 days). After 16 days of total culturing, neurons were stained for NFM (Fig. 14) or peripherin (Fig. 15) and cytoskeletal morphology was analyzed.

While non-treated) WT neurons displayed fine filamentous NFM structures with only occasional rings appearing, 2 weeks of treatment with cytochalasinD resulted in a more pronounced NFM immunoreactivity, obvious in long filaments, as well as in the appearance of numerous lariat- and ring structures. These rings were already present in untreated KO neurons, while weakening the actin cortex with cytochalasinD further emphasized this phenotype. The number as well as the staining intensity of rings was increased, concomitantly with an increased frequency of filamentous structures.

The observed upregulation of NFM was not specific to this IF component, but rather seemed to be part of a more general process involving various intermediate filaments in cellular responses to cytoskeletal destabilization. Also peripherin, a type III IF component showed a similar upregulation into more pronounced filamentous structures and increased frequency of ring structures upon treatment with cytochalasinD (Fig. 15).

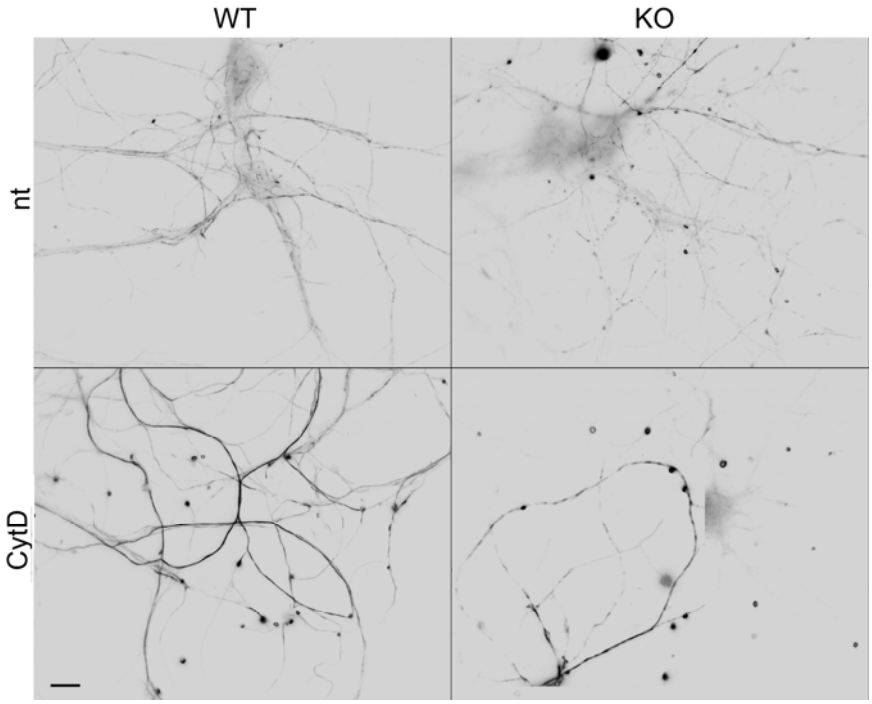

Figure 14. **Softening the Actin Cortex by CytochalasinD affects NFM Structure.** Hippocampal neurons were treated from 2 DIV on in the presence of cytochalasinD (100nM) and then cultured for 2 more weeks. Neurons were then stained for NFM. Scale bar=10μm.

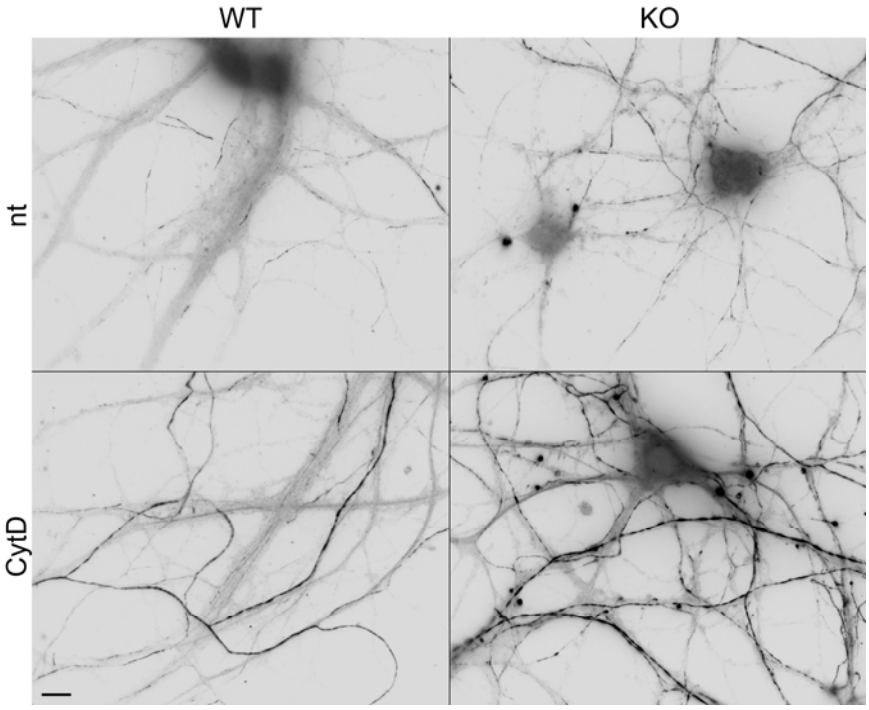

Figure 15. **Ring Softening the Actin Cortex by CytochalasinD affects Peripherin Structure.** Hippocampal neurons were untreated (nt) or treated from 2 DIV on in the presence of cytochalasinD (10nM) and then cultured for 2 more weeks. Neurons were then stained for peripherin. Scale bar=10μm.

The increase of filamentous structures upon chronic depolymerization of f-actin by cytochalasinD which occurred in WT and in Cap23 KO neurons likewise thus seems to be a cellular response to destabilization of the actin cytoskeleton and is likely to be a compensatory homeostatic mechanism aiming at restoration of cytoskeletal integrity. The fact that the phenotype in Cap23 KO neurons can be further potentiated by this treatment indicates that the actin cytoskeleton in the absence of Cap23 is only partially defect and can still be further affected by drugs like cytochalasinD. The observation that disruption of the actin cytoskeleton by cytochalasinD increases the number of ring-like structures immunopositive for NFM demonstrates, that an intact actin cytoskeleton is necessary for proper assembly and morphology of neurofilament structures. We could therefore demonstrate that pharmacological interference with the actin cytoskeleton partially mimics the absence of Cap23; further substantiating the notion that Cap23 is involved in stabilization of the f-actin cytoskeleton.

2.2.4 Dynamics of the Intermediate Filament System

In the previous experiments we had obtained solid evidence for an effect of Cap23 on the IF system organization. We then wondered how the various observed structures and morphologies of intermediate filaments evolved and whether they would be dynamic in WT and Cap23 KO neurons and thus decided to perform time-lapse recordings using GFP-coupled IF protein constructs.

Cultured hippocampal neurons were transfected with either NFM-GFP or vimentin-GFP and imaged 8-16 h after transfection, since it is known that prolonged overexpression (>24 h) of IFs in neurons can have toxic effects.

Our first observations showed that indeed, NFM filament dynamics could readily be observed inside axons. In WT neurons, 70% of total observed filaments were non-motile, while the remaining 30% showed longitudinal motility (Fig. 16) of variable degree. In contrast, this ratio was inversed in Cap23 KO neurons, which contained only 33% stable filaments, whereas 67% were motile.

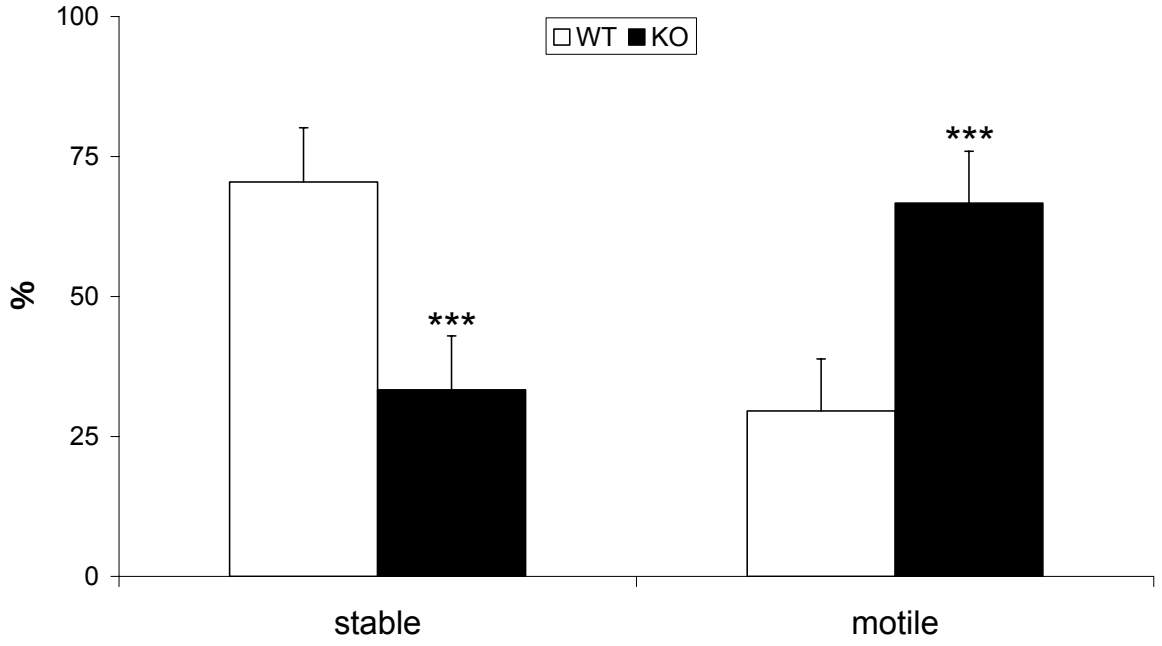

Figure 16. **Distribution of Stable versus Motile Filaments in NFM-GFP Transfected Neurons.** Hippocampal neurons were transfected with NFM-GFP at 9.5 DIV and imaged at 10DIV. Time-lapse recordings were performed and observed NFM-GFP filaments were classified as stable or motile. ***=p<0.001

As we realized that motile filaments seemed to be shorter in respect to non-motile filaments, we quantified the lengths of both classes and could demonstrate, that motile filaments in WT and Cap23 KO were unaltered regarding their length, with average values of 4 um and 3.7 um, respectively (Fig. 17). Interestingly though, stable filaments in Cap23 KO axons were significantly shorter than WT stable filaments (Fig. 17).
Neurofilaments assemble into longer filament structures by fusion at their ends, but whether fusion takes place preferentially during non-motile phases or during motile events, has not been described in the literature. The data presented here suggest, that fusion into longer filament networks might need a certain degree of neurofilament docking through immobilization at certain sites.

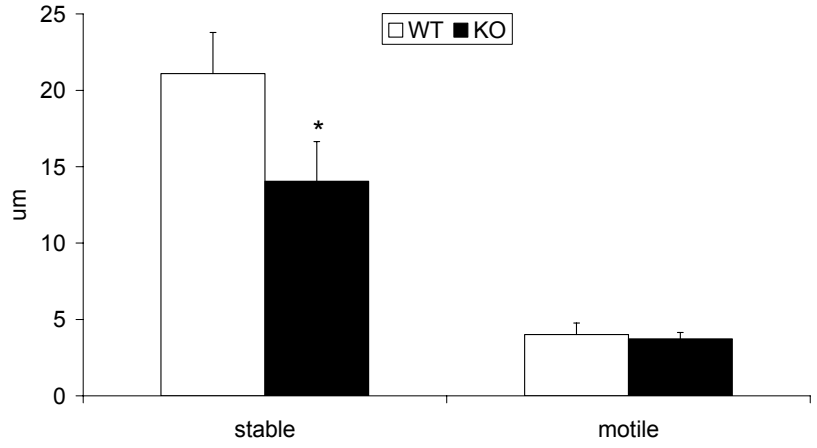

Figure 17. **Length of Stable versus Motile Filaments in NFM-GFP Transfected Neurons.** Hippocampal neurons were transfected with NFM-GFP at 9.5 DIV and imaged at 10DIV. Time-lapse recordings were performed and stable or motile NFM-GFP filament length was measured. *=p<0.05

During time-lapse recordings, we were able to observe some events in WT axons, where fusion of a stable filament with a previously motile filament was observed, resulting in the formation of a longer filament which then remained non-motile over its entire length. The decreased length of stable filaments in the absence of Cap23 could thus be explained by a reduction of neurofilament docking along the axon. The length of neurofilaments may also directly be related to their motile behavior, since it has been demonstrated that especially NFM and NFH can undergo interactions through their C-terminal side chains with cytoskeletal structures like e.g. spectrin or other unidentified cytoskeletal

components. It follows thus that longer filaments, through their increased number of side chains can form stronger links to other cytoskeletal structures and thus are more readily immobilized. If NF fusion by the decreased docking ability of individual filaments was compromised, e.g. by the absence of Cap23, resulting filament structures would be expected to be shorter and remain more motile.

We also compared the velocities of individual translocation events of NFM-GFP filaments and observed a small tendency towards faster velocities in the absence of Cap23 (Fig. 18). A possible explanation for this effect is that filaments might be able to interact with other organelles during their translocation phase, which could result in decreased transport velocity. Evidence for this was obtained during time-lapse recordings performed after double labeling of NFM filaments with GFP and mitochondria with Mitotracker. In such experiments, we could frequently observe that both organelles were moving in parallel, also in a bidirectional or oscillating manner.

It is likely that due to the reduced ability of Cap23 KO filaments to interact with neighboring cytoskeletal structures, translocation of KO filaments occurs in an un-retarded fashion, leading to increased velocities.

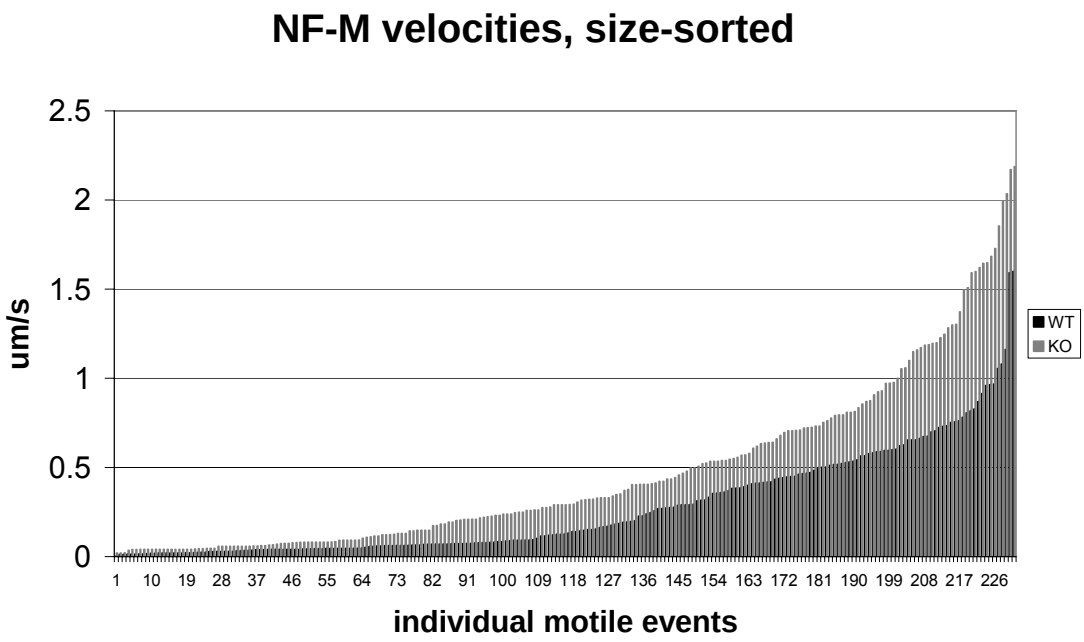

Figure 18. **Velocities of Motile Filaments in NFM-GFP Transfected Neurons.**
Hippocampal neurons were transfected with NFM-GFP at 9.5 DIV and imaged at 10DIV. Time-lapse recordings were performed and velocities of 231 motile NFM-GFP events each for WT and Cap23 KO were measured. Values are depicted size-sorted, for better visualization.

2.2.4.1 Interactions between Vimentin and Mitochondria

In time-lapse recordings of neurons transfected with vimentin-GFP as well as Mitotracker-labeled mitochondria, we could observe that as motile mitochondria were passing stable GFP-labeled vimentin filaments, their movement was markedly retarded, strongly indicating an interaction between mitochondria and vimentin filaments or IF-associated structures.

Vimentin-GFP rings were observed in axons of Thy1-Cap23 mice, which suddenly opened to become short filaments and underwent translocation along the axon.

In another example, a short and thick initially stable filament suddenly separated from an obviously associated “mother”-filament, became motile and fused with the end of a different stable filament. Shortly after this fusion event a clear ring structure formed and disappeared again, after which the filament stayed non-motile for the rest of the observation period.

Another example showed a pre-existing ring to transform into a short filament, thereby gaining motility. This might indicate that IF-rings somehow serve as intermediates for fusion of individual filaments. The overabundance in neurons lacking Cap23 could therefore represent a fraction of filaments which are unsuccessful in fusing with other filament units.

2.3 Mitochondrial Motility versus Stability

2.3.1 Motility and Stability of Axonal Mitochondria

Having observed that organelles like IFs display defects in docking, assembly and motility in the absence of Cap23, we wondered whether this effect would be specific to the IF family of proteins or whether other organelles were affected as well. We thus decided to analyze mitochondria, whose function in supplying selected cellular compartments with energy by ATP production is well established, and especially important in neurons, which are extremely polarized cells with processes that can extend for up to 1 meter from the cell body and therefore depend on a tightly regulated mechanism to ensure proper targeting of mitochondria to remote cellular locations.

Studies of mitochondrial motility in cultured neurons have shown, that mitochondrial density in axons is lower compared to dendrites, and that the fraction of motile axonal versus dendritic mitochondria is increased by more than 4-fold (Chang et al., 2006). Thus, individual mitochondria are easier to follow over time in axons. We thus focused our analysis on axons and observed various striking differences in mitochondrial structure, anchorage and motility.

As we had observed a change in the distribution of stable versus motile NFs in previous experiments, we performed a similar analysis of mitochondria in axons of WT and KO neurons using time-lapse recordings. Strikingly, we found that the fraction of motile mitochondria in Cap23 KO neurons was more than doubled (Fig. 19). In WT, most mitochondria (79%) were resting for the observed time period, 10% of the total mitochondrial pool showed oscillating behavior (bidirectional movements being centered on the same location, <10μm translocation) and 11% displayed significant translocation events (unidirectional, >10μm translocation; Fig. 19). These values are consistent with earlier studies, reporting 5-20% of the total mitochondrial pool to be motile in cultured hippocampal neurons (Overly et al., 1996).

In sharp contrast, only 45% of KO mitochondria were stable, 33% showed oscillation behavior and 23% were actively translocating, representing 56, 313 and 215% compared to WT, respectively.

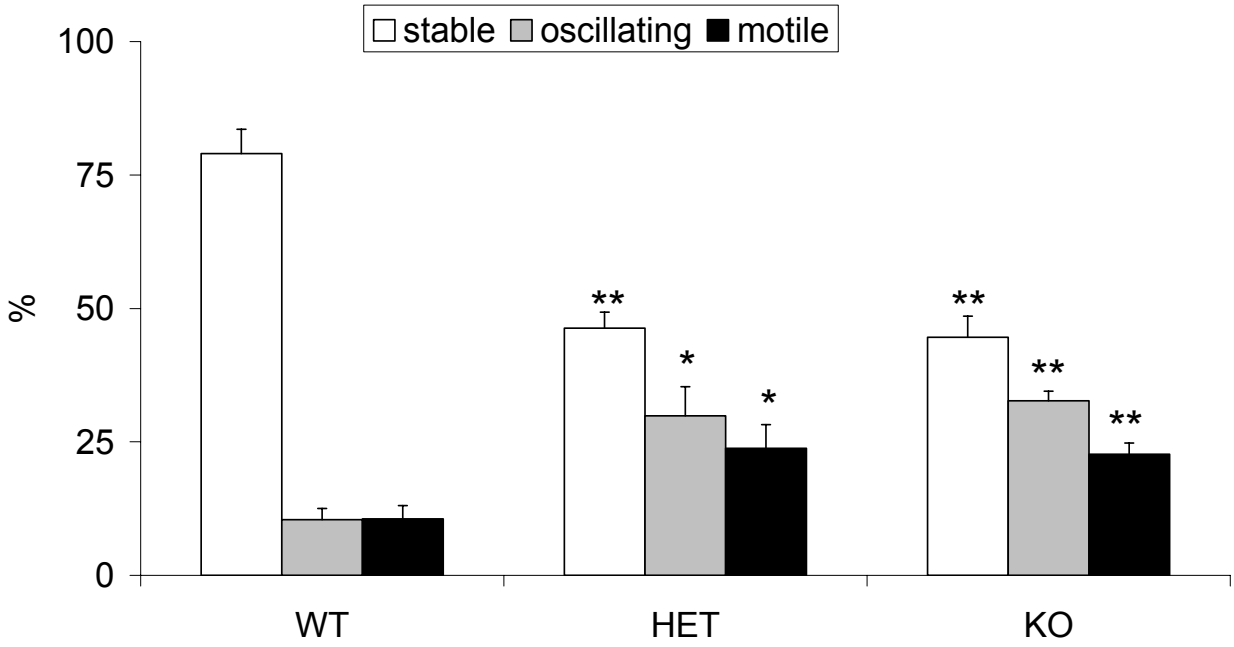

Figure 19. **Stability of mitochondria at 10 DIV.**
5 min time-lapse movies were analyzed by grouping mitochondria as being stable (no movement), oscillating (bidirectional motility <10μm) or motile (unidirectional translocation >10μm). Total mitochondria counted: WT (n=295), KO (n=236), HET (n=270). **=p<0.01; *=p<0.05.

On top of this, we realized that those mitochondrial translocation events of >10μm in the Cap23 KO were not only much more frequent than in WT (2.15x more, Fig. 19), but were usually also occurring over longer distances, in contrast to WT translocation events, which were shorter and more frequently interrupted. To this end, we measured the excursion lengths of individual mitochondria translocating >10μm and normalized the obtained values against the total length of the axons analyzed. This analysis revealed that the distance of uninterrupted excursions of mitochondria in Cap23 KO axons was approximately 4 times longer compared with mitochondria in WT axons (Fig. 20).
This suggested that the longer distances covered by KO mitochondria were a consequence of reduced interactions (“docking”) with stable structures along the axon.

This docking phenomenon has already been described in a recent study of axonal mitochondrial motility in cultured chick sympathetic neurons, where beads coupled to nerve growth factor (NGF) were able to trigger immobilization of mitochondria near contacts sites of axons to these beads (Chada & Hollenbeck, 2004). This study further showed that by interfering with f-actin by latrunculin B, mitochondria no longer are able to accumulate in the region of NGF stimulation, suggesting that upon certain stimuli, axonal mitochondria undergo docking interactions with the actin cytoskeleton.
In combination with our findings, this suggests that interactions between mitochondria and the cytoskeleton can retard and/or interrupt mitochondrial movements, indicating a close relationship between mitochondrial docking and cytoskeletal stability.

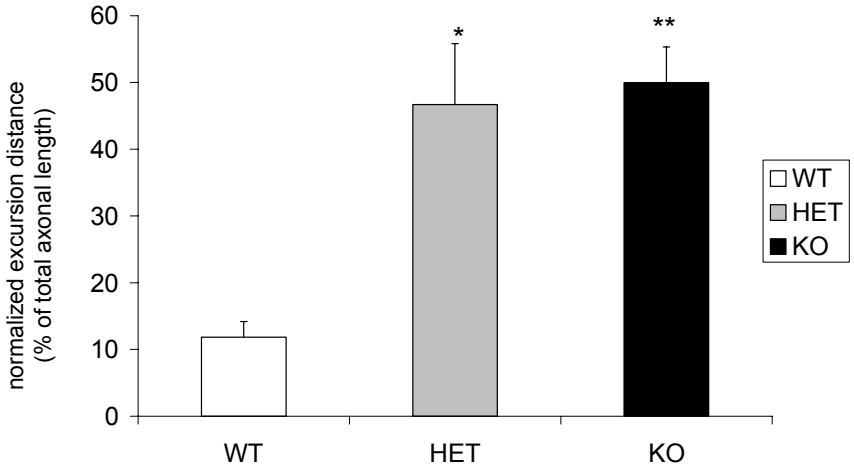

Figure 20. **Distance of mitochondrial excursions at 10 DIV.**
5 min time-lapse movies were analyzed by measuring mitochondrial translocation length. Values were normalized to total axonal length observed for each genotype. **=p<0.01¸*=p<0.05.

2.3.2 Mitochondrial Oscillation

Oscillatory movements of mitochondria have not been described so far, and according to our observations in WT neurons, account for only a minor part of total motile events (10%, Fig. 20). The fact that we observed a more than threefold higher fraction of mitochondria oscillating in KO neurons (Fig. 19) prompted us to deepen this analysis. We therefore analyzed this phenomenon by measuring the mitochondrial displacement between

consecutive time points of 5 min time-lapse recordings of individual mitochondria in WT versus KO. Strikingly, this analysis showed that in 10 and 15 DIV Cap23 KO axons, mitochondrial displacement was increased by 69 and 75%, respectively, compared to WT (Fig. 21).

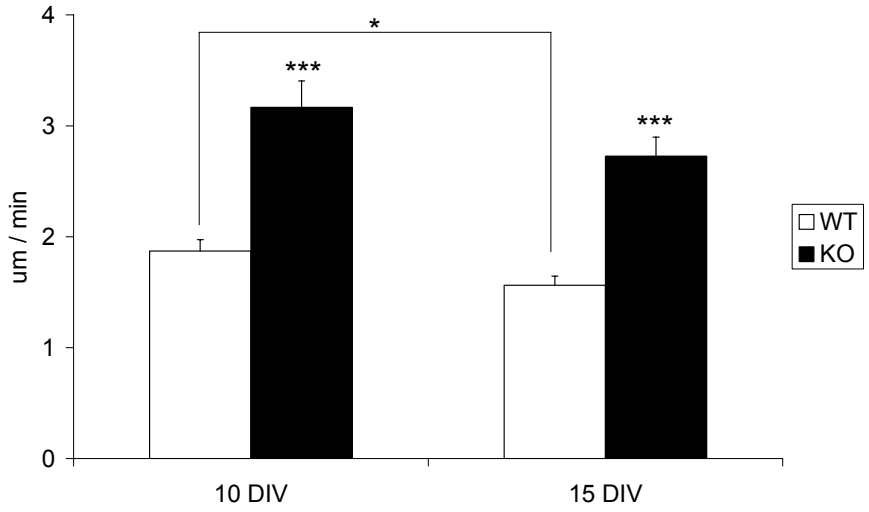

Figure 21. **Displacement of mitochondria during oscillation.**
Degrees of oscillation were analyzed by measuring the mitochondrial displacement between consecutive time points of 5 min time-lapse recordings of individual mitochondria at 10 and 15 DIV. ***=p<0.001¸*=p<0.05.

Interestingly, there was a small but significant decrease seen in the oscillation values of WT between 10 and 15 DIV. This might indicate that at more mature stages of the neuron, mitochondria become progressively more stably docked at specified sites along the axon as e.g. at branching points or synapses.

In line with this suggestion, recent studies have shown that during synapse maturation mitochondria preferentially localize at presynaptic sites (Chang et al., 2006). Furthermore, other neuronal stabilization processes occur during this time as e.g. consolidation of dendritic spines or maturation of the intermediate filament network (Shaw et al., 1985). As synaptic sites mature, they also require long-term support of energy, e.g. for synaptic potentiation or mobilization of reserve pool vesicles, and thus are proposed to act as stable “docking sites” for mitochondria (Tang and Zucker, 1997; Verstreken et al., 2005). Synaptic silencing by TTX induces a remarkable increase in mitochondrial motility, though no changes in the fraction of mitochondria that dock at synaptic or nonsynaptic sites were observed (Chang et al., 2006). It was therefore suggested, that inactive synapses still

serve as targets for mitochondrial localization, indicating that structural rather than activity-dependent mechanisms may cause mitochondria to stop at synaptic sites (Chang et al., 2006).

The subtle decrease in KO oscillation between 10 and 15 DIV was not statistically significant, indicating that a potential stabilization effect appearing in WT neurons as they mature, is absent in Cap23 KO.

2.3.3 Direction and Velocity of Transport

A further interesting result was obtained by comparing polarized mitochondrial transport events and their velocities between WT and KO. To make statements about the directionality of transport, we analyzed slightly younger axons (7DIV), due to the fact that at this age, axonal density in the culture is still moderate, and individual, non-overlapping axons can be easily distinguished.

The vast majority of translocation events in the WT was directed towards the growth cone (anterograde, see green lines in Fig. 22) and relatively slow (<0.1 um/s; Fig. 23), whereas in KO axons, anterograde movements were much faster, peaking around 0.4 um/s (Fig. 23). In addition, while retrograde transport events were rare in WT, they were not only very frequent in KO, but also occurred at relatively high velocities with values of up to >3um/s. Furthermore it was obvious, that KO GCs contained much less mitochondria than WT GCs (Fig. 22). This can easily be imagined to result in considerable alterations of growth cone energy supply and thus neurite extension, since especially in neurites of this age, a large part of neuronal ATP is consumed for growth processes, mainly taking place at growth cones.

Furthermore, a comparison of total motile events of mitochondria demonstrated that, normalized to total axonal length observed, there were approximately twice as many motile events in KO axons (Fig. 24) as compared to WT. While the balance between anterograde and retrograde transport events were not altered in the KO, there was a small but significant imbalance towards anterograde translocation events in the WT. This

is expected for actively elongating neurites, which need to accommodate increasing numbers of mitochondria in distal regions of neurites, especially in the GC, as they grow. Importantly, this tendency of mitochondria to be transported towards the GC was absent in KO axons (Fig. 24), resulting in a marked reduction of mitochondria in KO GCs (Fig. 22). The need to maintain a constant density of mitochondria in growing neurites requires anterograde transport events to occur more frequently than retrograde ones. As we found antero- and retrograde translocation events to occur at similar frequencies in Cap23 KO axons, it follows that mitochondria in the absence of Cap23 cannot be properly retained at distal axonal locations, which is exactly what we observed (Fig. 22).

Defect mitochondria are known to be retrogradely transported out of distal axonal regions and degraded in the cell body, which represents a mechanism to recycle inefficient organelles. Based on our findings of increased mitochondrial retrograde translocation, it seems likely that due to improper docking, the large fraction of retrogradely transported mitochondria in Cap23 KO axons ends up in the same pathway and is targeted towards the cell body for degradation.

Therefore, it seems likely that these frequent retrogradely transported mitochondria in the KO (Fig. 22, 23) represent a fraction of organelles with reduced ability to interact with neighboring structures like the actin cytoskeleton and/or intermediate filaments.

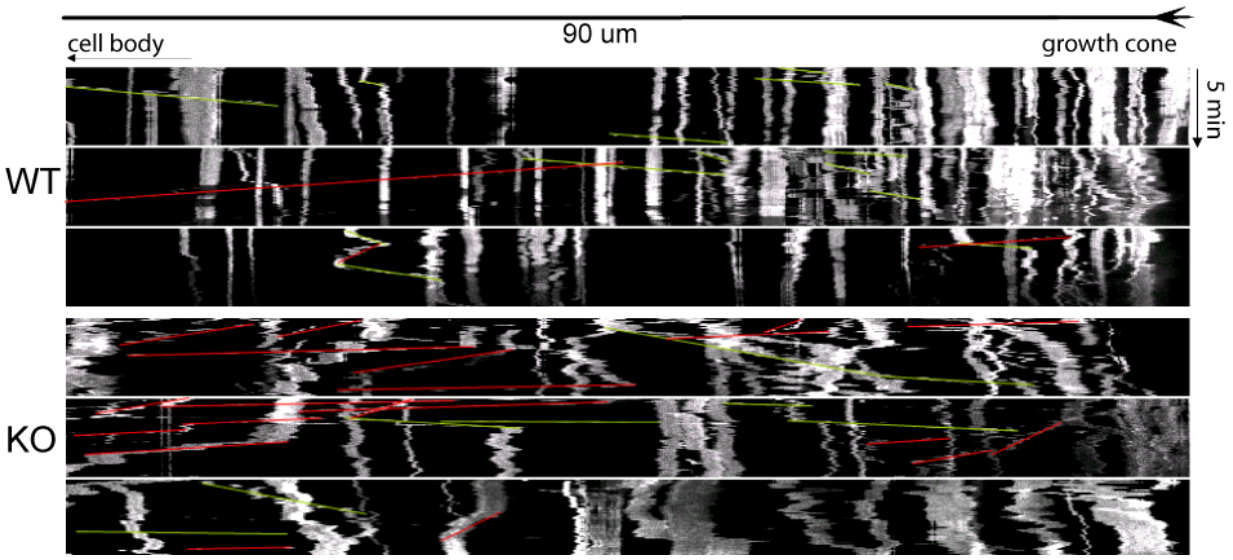

Figure 22. **Distribution of bidirectional mitochondrial motility.**
Mitochondrial motility was analyzed by time-lapse recordings in distal axons of 7 DIV neurons. Kymographs were produced using MetaMorph software by manually drawing lines over distal axonal segments, which were converted into lines (x axis). Each line represents a single time point with 61 time points comprising 1 kymograph of 5 min total (y axis). The distal 90μm of 3 WT and KO axons are depicted. The growth cone is included on the right side (indicated), the cell body is in direction of the left side (indicated; arrow). Anterograde and retrograde motile events are highlighted in green and red, respectively.

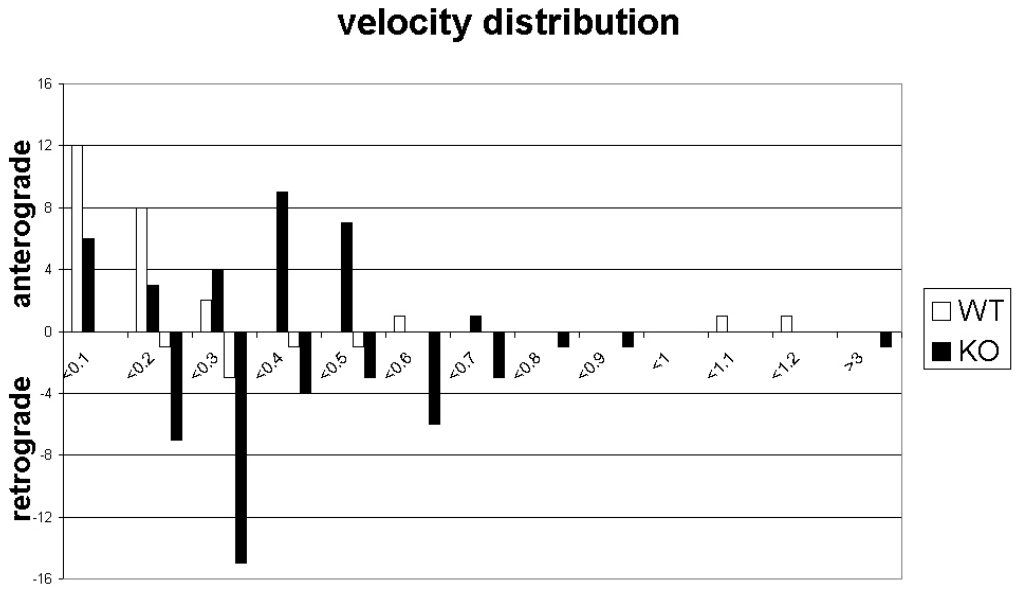

Figure 23. **Velocity distribution of motile mitochondrial events.**
Anterograde and retrograde translocation events as observed by time-lapse imaging of Mitotracker-labeled mitochondria were measured in the distal 90μm of axons including the growth cone.

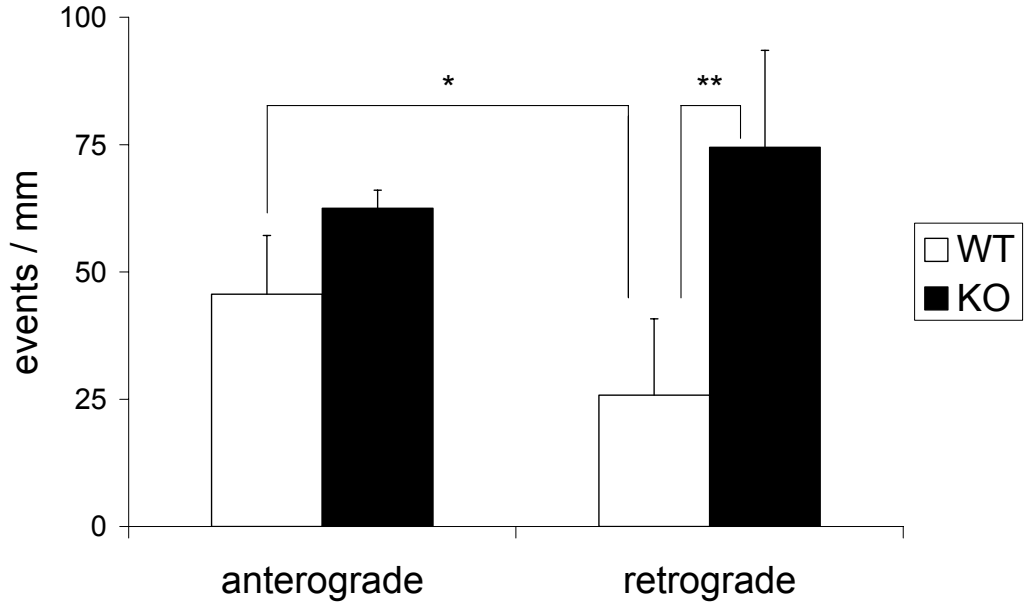

Figure 24. **Distribution of bidirectional mitochondrial motility.**
Anterograde and retrograde translocation events as observed by time-lapse imaging of Mitotracker-labeled mitochondria in WT and Cap23 KO axons were measured in the distal 90μm of axons including the growth cone. *=p<0.05; **=p<0.01

2.3.4 Mitochondrial Docking Near Cap23 Accumulations

To better understand the mitochondrial docking phenomenon and in order to reveal whether the localization of Cap23 would be involved in mediating this “stop”-signal, we decided to study mitochondrial motility followed by identification of Cap23 clusters by retrospective immunocytochemistry. To this end, we performed labeling of mitochondria in living neurons, imaged mitochondrial motility during observation times of 5 min, followed by fixation and staining of Cap23. At the end of the staining procedure, we managed to find back the exactly same regions which we had imaged before fixation and thus could correlate regions of Cap23 accumulation with events of mitochondrial motility and/or docking.

This analysis revealed an unprecedented correlation between stable mitochondria and Cap23 accumulation. A strikingly high fraction of mitochondria which were stable during the observed period could be located in the close vicinity of Cap23 clusters (Fig. 25a). While often the colocalization was directly overlapping, we also frequently observed that mitochondria were situated close to Cap23 clusters, though not being in direct contact with the Cap23 cluster. We cannot rule out the possibility that Cap23 had undergone some motility during the imaging period, and that in fact the real colocalization could therefore be higher or lower as our correlation showed. Since we were not able to obtain any construct which would enable us to follow Cap23 localization in living neurons (neither GFP-fusion constructs nor FLASH-labeling of Cap23 turned out to retain the original localization of Cap23), we were limited to this procedure, by which we could only assess the localization of Cap23 at the end of live-imaging sessions using fixation and immunostaining. The fact, that the colocalization of stable mitochondria with Cap23 clusters was very pronounced made it seem unlikely to us, that Cap23 clusters would display elevated degrees of motility. This is supported by a study demonstrating that lateral mobility of Cap23 clusters in artificial lipid bilayers is much lower than that of other raft components in model membranes (Khan et al., 2003).

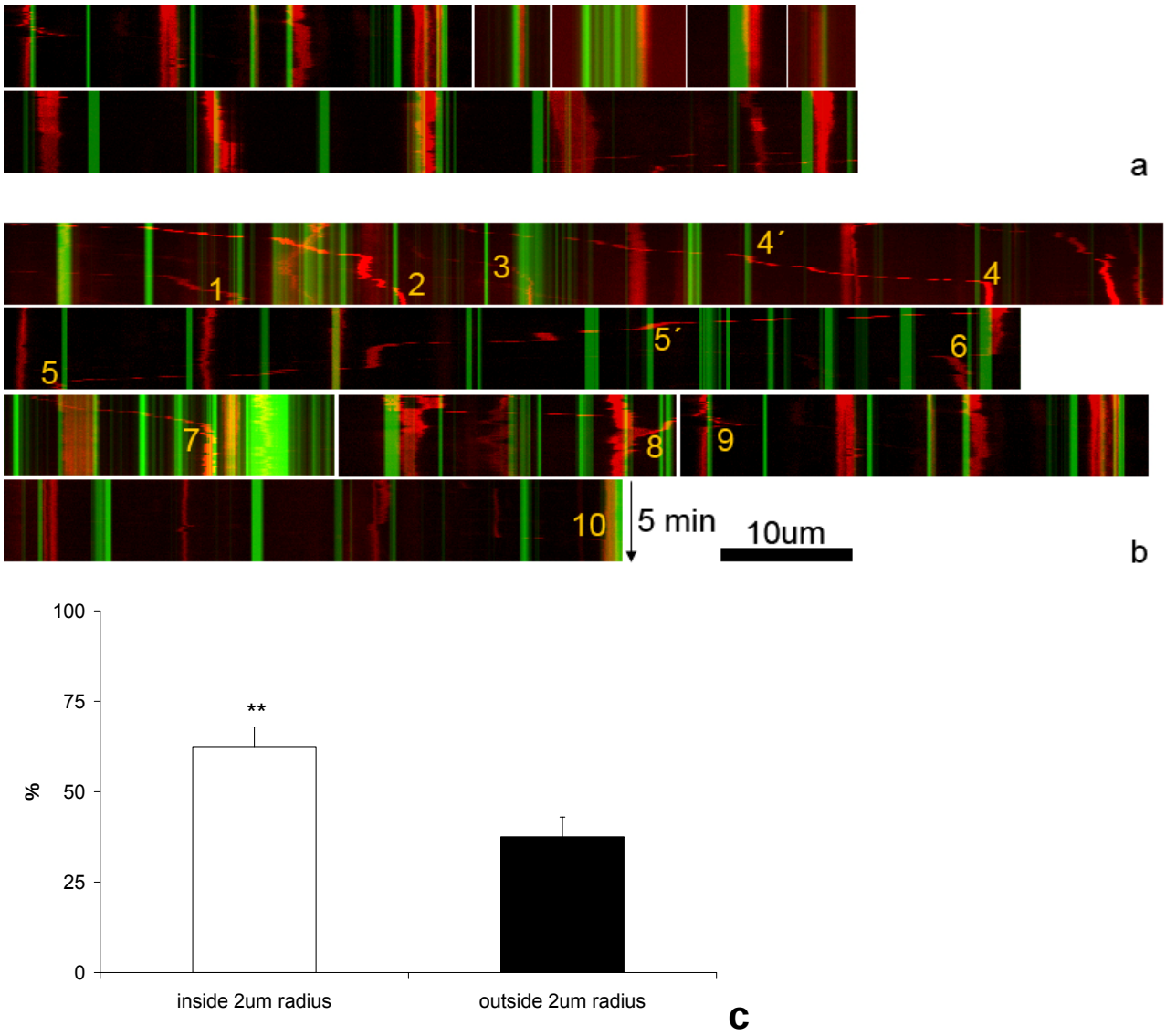

Figure 25. **Mitochondrial docking at Cap23 clusters.**
Following time-lapse recordings of Mitotracker-labeled axons in 9 DIV WT neurons, cells were fixed and stained for Cap23. Imaged regions were re-identified and time-lapse movies were overlaid with Cap23 stained clusters. Mitochondria are shown in red and Cap23 in green.

a) examples of stable mitochondria being located near Cap23 clusters
b) examples of motile mitochondria undergoing docking events, often at or close to Cap23 clusters, as indicated by numbers
c) quantification of mitochondria, which are in close vicinity (<2μm radius) of Cap23 clusters. A total of 183 mitochondria were counted from 5 individual neurons. **=p<0.01

Even more striking was our finding that mitochondria which underwent docking after periods of translocation displayed a high tendency to dock near Cap23 clusters (Fig. 25b). Again, docking occurred either exactly at Cap23 clusters or in a very close region, usually less than 1μm from the Cap23 cluster. Ten individual mitochondria undergoing docking

events are illustrated in Fig25b. Event #4 shows a single mitochondrion traveling for >50um, passing several Cap23 clusters and finally settling close to a Cap23 cluster, after which it remained stably docked for the rest of the observation period. Interestingly, this mitochondrion showed a significant pause directly below another Cap23 cluster (4'), before moving on and reaching its final destination. A similar event is illustrated for #5, which at #5' paused close to a Cap23 cluster for ca. 30 seconds. In general, all mitochondria which were docked at a Cap23 cluster, remained at the same cluster stably docked for the whole imaging period, with only one exception, which left one cluster, just to dock at a neighboring one again (#6). #9 shows an example of a stable mitochondrion which transiently loses its attachment to a Cap23 cluster, but after 45 seconds re-attaches to the very same location as before.

Quantification of the stable mitochondrial fraction in the vicinity of Cap23 clusters showed that significantly more stable mitochondria (62.5% of total stable mitochondria) were located inside a small radius of <2μm of a Cap23 cluster rather than appearing in regions where no Cap23 clustering could be observed (Fig. 25c). This indicates that Cap23 plays a major role in promoting immobilization of mitochondria, though other mechanisms must exist in parallel, as 37.5% of stable mitochondria were observed to be docked in the absence of Cap23 clusters.

These data provide clear evidence that accumulations of Cap23 serve as attraction points for mitochondria, either residing close to Cap23 clusters for extended periods of time, transiently pausing during translocation or terminating their translocation at these clusters. The fact, that association of mitochondria often seems to take place in a very confined region around Cap23, if not exactly at these clusters, indicates that Cap23 does not necessarily interact with mitochondria directly, but probably provides a scaffold together with other proteins to mediate direct binding at these sites. These other components are likely to be cytoskeletal components or at least associated with the cytoskeleton, since studies interfering with f-actin in axons have previously demonstrated to result in reduced docking of mitochondria (Chada & Hollenbeck, 2004).

2.3.5 Structural Aberrations of Mitochondria

When we next examined the structure of mitochondria in WT and Cap23 KO cultures, we found a pronounced difference regarding mitochondrial length. In the absence of Cap23, mitochondria were longer by 52, 82 and 66% in 5, 10 and 15 DIV axons, respectively (Fig. 26, see also Fig. 22). However, this phenotype was not yet apparent in mitochondria of neurons cultured for 2 DIV, which showed similar lengths in both WT and KO, indicating that while initially, mitochondria in KO neurons are normal in length, they become longer at a time between 2 and 5 DIV, which might represent a homeostatic mechanism trying to compensate for inefficient energy supply at specific sites. Likewise, ultrastructural examination of axonal mitochondria revealed similar differences in mitochondrial length in the absence of Cap23 (Fig. 27).

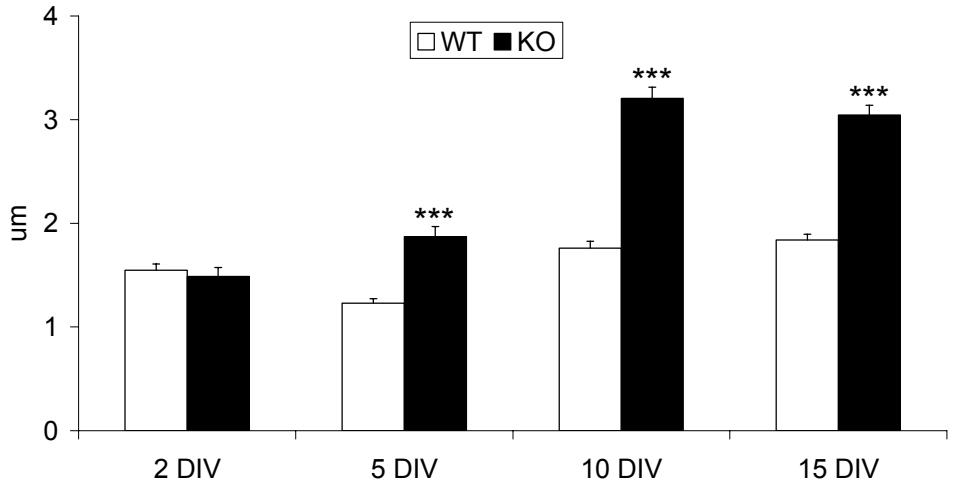

Figure 26. **Mitochondrial length.**
Hippocampal neurons were cultured for 2 to 15 DIV. Mitochondria were labeled using Mitotracker and axonal mitochondrial length was measured. ***=p<0.001.

It has been suggested at least for dendrites, that structural changes of mitochondria regarding their length could result in a more efficient distribution of energy to e.g. multiple, closely apposed sites of high metabolic demand (Skulachev, 2001). Since improper docking of mitochondria at sites of ATP demand would lead to a suboptimal energy support of these sites, it is likely that such cells would aim for compensation for the loss of

this essential mechanism, one possibility being the formation of longer mitochondria. Mitochondrial length is mainly regulated by fusion or fission. These mechanisms are known to occur rapidly and can cause a change in volume and functional capacity by loss or gain of mitochondrial proteins. This idea was further investigated by comparing the proteome of WT and Cap23 KO brains and is discussed in section 2.4.

To rule out the possibility that increased mitochondrial biogenesis contributes to enhanced fusion and/or length of mitochondria, we decided to examine total mitochondrial DNA contents. To this end, we isolated and purified genomic DNA from brains of WT, Cap23 heterozygous and Cap23 KO mice and performed quantitative PCR using two different sets of primers, each directed against a unique mitochondrial DNA region, as well as against nuclear 28S-DNA, which we used as an internal control for normalization. Two individual sets of experiments revealed similar results, showing no difference between any of the genotypes (Fig. 28).

This result thus indicates that total mitochondrial contents are unaltered by the absence of Cap23 and suggests that structural changes of mitochondria rather are a consequence of increased fusion and/or reduced fission of mitochondria.

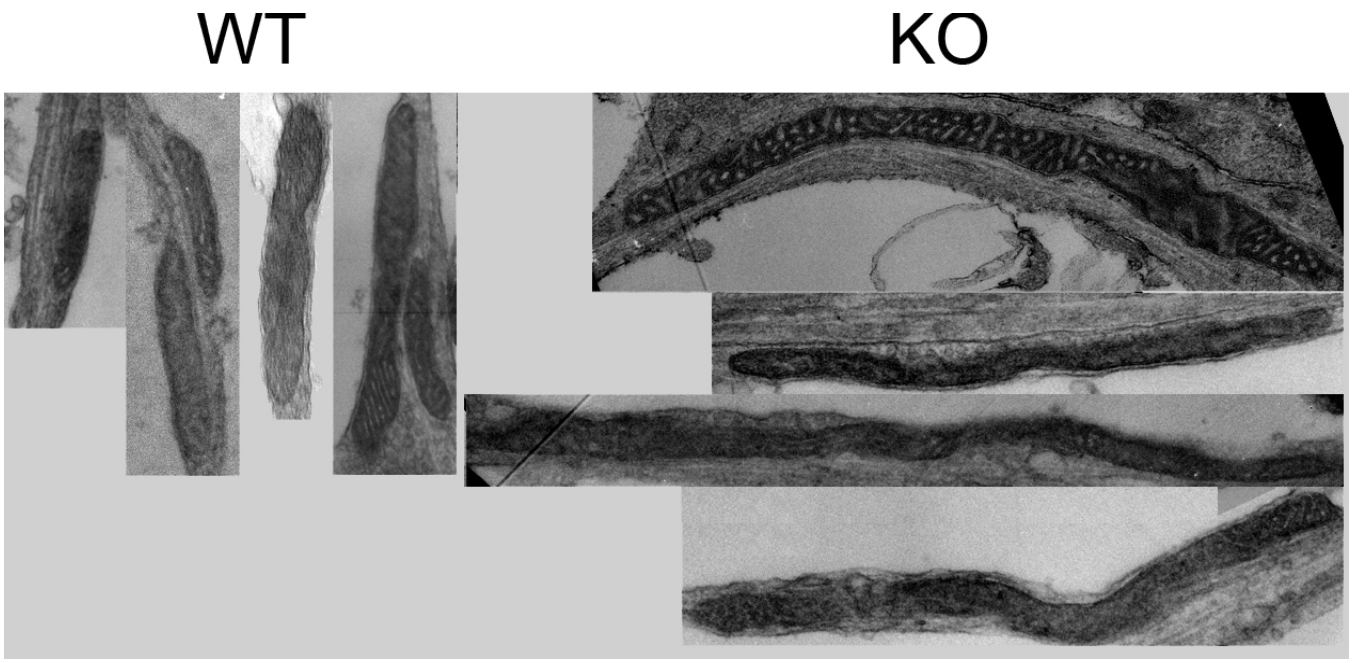

Figure 27. **Mitochondrial length as observed by Electronmicroscopy.**
Hippocampal neurons were cultured for 36 DIV and then processed for EM sectioning (see. 4.10). Scale bar=1μm

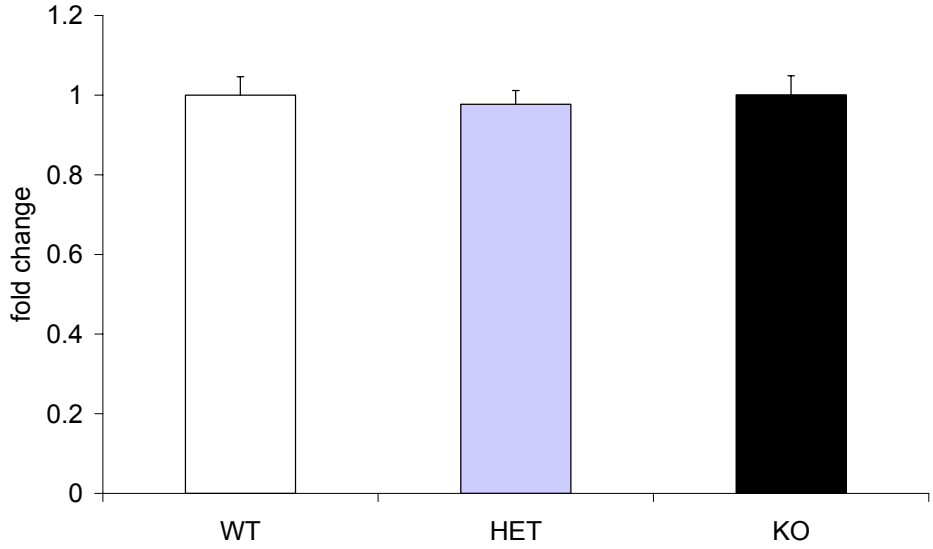

Figure 28. **Mitochondrial DNA Content is Independent of Cap23 Levels.**
Quantitative Real-Time PCR was performed with two independent primer sets directed against mitochondrial DNA and 28S DNA used as internal control. Depicted is the fold change of the ratio mitochondrial DNA / 28S DNA.

Taken together, these results provide compelling evidence that the absence of Cap23 severely affects mitochondrial docking. This is especially relevant in a physiological scenario like neurite outgrowth, where polarized distribution of mitochondria inside actively elongating neurites is necessary for proper neurite outgrowth and the continuous support of energy by mitochondria for growth-associated events like axonal elongation.

What then is the causative origin of this docking phenotype? A good candidate for regulating mitochondrial docking is the actin cytoskeleton, since pharmacological intervention affecting the actin network has been demonstrated to result in a similar docking phenotype (Chada et al., 2004). This model is in perfect agreement with our observations, which demonstrate a defect in mitochondrial docking as well as a disorganized f-actin cytoskeleton, making it likely that the disruption of the actin cytoskeleton in the absence of Cap23 directly and/or indirectly affects docking of mitochondria. The impairment of actin-based structures, which probably together with other proteins serve as a docking site for mitochondria, might then be translated into a defect of mitochondrial docking and retention at growth cones, branching points or synapses.

2.4 Cap23 Involvement in the Regulation of Lipid Rafts

2.4.1 Cap23 Modulates Cholesterol-rich Clusters

In order to assess the subcellular localization of Cap23, we performed immunocytochemistry on dispersed hippocampal neurons, using an antibody directed against Cap23 and filipin complex III, which is a fluorescent compound specifically binding to cholesterol and thereby labeling lipid rafts enriched by this lipid.

Cholesterol was found to localize all along the membrane of axons and dendrites, and appeared in discrete clusters in what seemed to be membrane exvaginations (Fig. 29). In addition, cholesterol was frequently found at the tips or bases of membrane protrusions.

Cap23 accumulated in the same plasma membrane clusters and protrusions, thus showing nearly 100% of colocalization (Fig. 29).

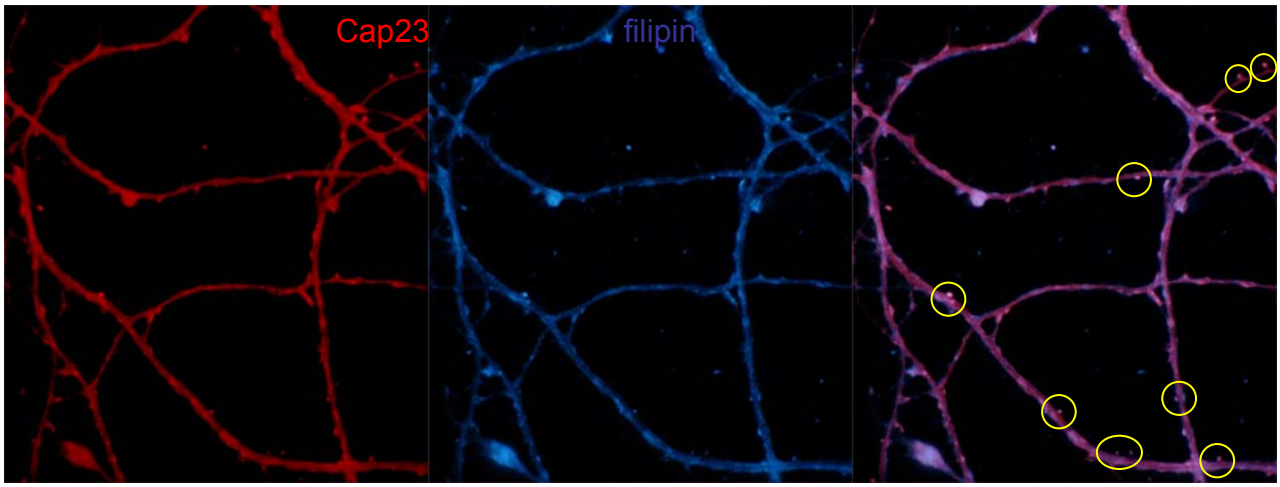

Figure 29. **Cholesterol localizes to discrete clusters along axonal and dendritic plasma membranes and codistributes with Cap23.**
Hippocampal WT neurons were cultured for 14 DIV and then stained with a polyclonal Cap23 antibody and filipin. Colocalization at discrete is indicated by yellow circles.

When we examined the distribution of cholesterol in hippocampal neurons, we found that in the absence of Cap23, dendritic shafts (Fig. 30) as well as axons (Fig. 31) displayed a marked reduction of filipin labeling intensity. In addition, cholesterol-labeled clusters on

Cap23 KO dendrites which seemed unaltered in total number were smaller than WT clusters (Fig. 30). WT Growth cones frequently contained defined cholesterol clusters at the most distal part of lamellipodial GC structures, whereas KO GCs contained irregular, amorphous cholesterol structures (Fig. 30, blue insets).

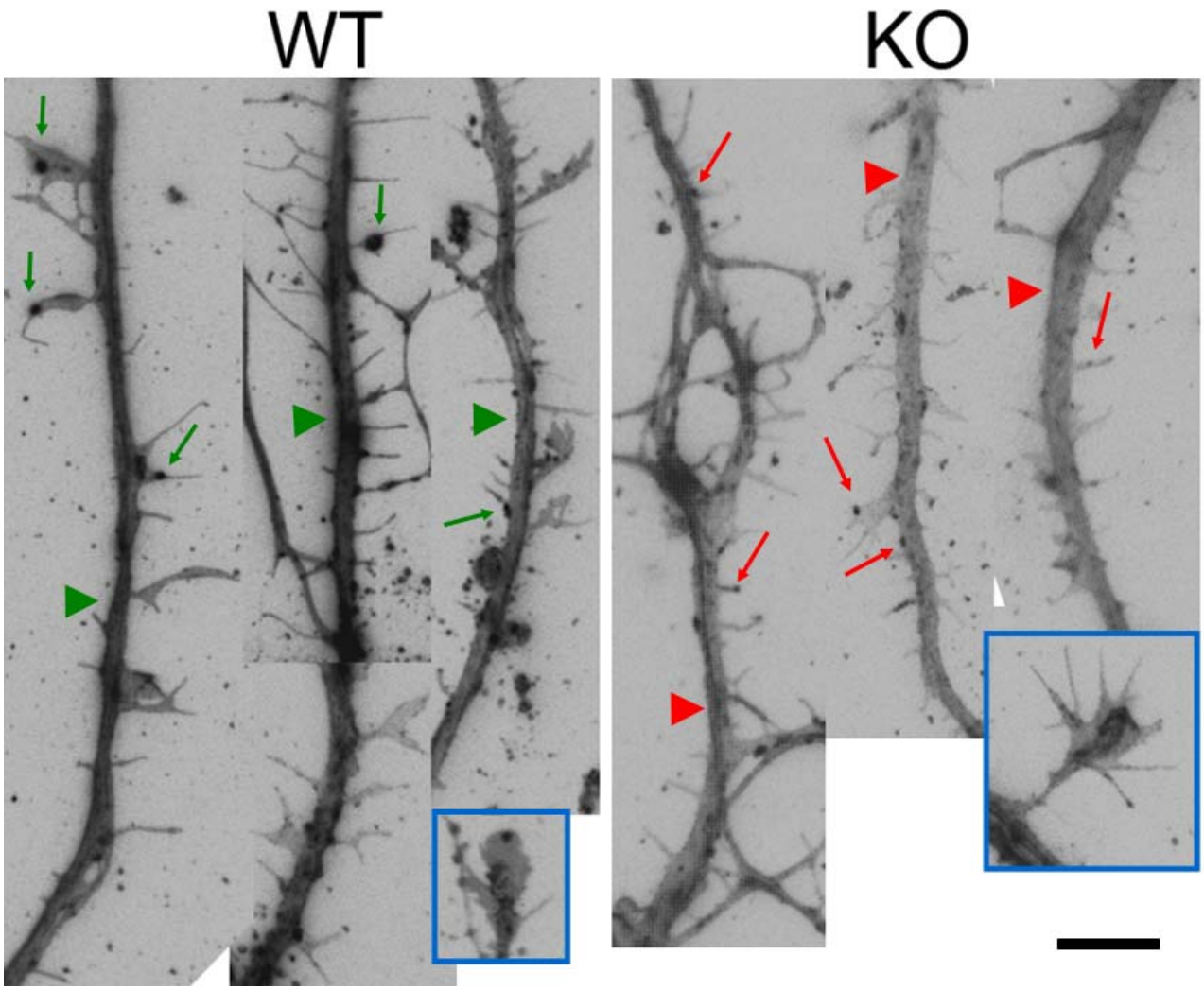

Figure 30. **Reduction of cholesterol clustering in the absence of Cap23 in Dendrites and Growth Cones.**
Hippocampal neurons were cultured for 4 DIV and then stained with filipin. Large arrowheads indicate dendritic shafts (high concentrations green, low concentrations red). Arrows indicate discrete cholesterol clusters. Scale bar =10μm

Clusters along WT axons were regularly spaced and seemed to consist of multiple smaller domains. These assemblies were less frequent in KO axons and seemed less well confined, as they occupied larger areas of decreased staining intensity, suggesting

that Cap23 serves to confine lipid raft platforms by promoting the local accumulation of cholesterol.

In various studies using artificial lipid membranes (Khan et al., 2003), Cap23 was shown to attract cholesterol into defined clusters. Furthermore, examining the cluster formation of different lipid on lipid bilayers, it was shown that the domain formation of $PI(4,5)P_2$, an important process involved in regulation of the actin cytoskeleton, is facilitated by cholesterol (Epand et al., 2004). The combination of these results thus strongly suggests that Cap23 plays an important role in the organization of lipid rafts by sequestering cholesterol into defined plasma membrane domains, which then can attract and accumulate $PI(4,5)P_2$. Through their ability to attract various actin-binding proteins, lipid domains with such particular composition are able to modulate actin polymerization and thus regulate actin-based cellular morphology in a pronounced manner.

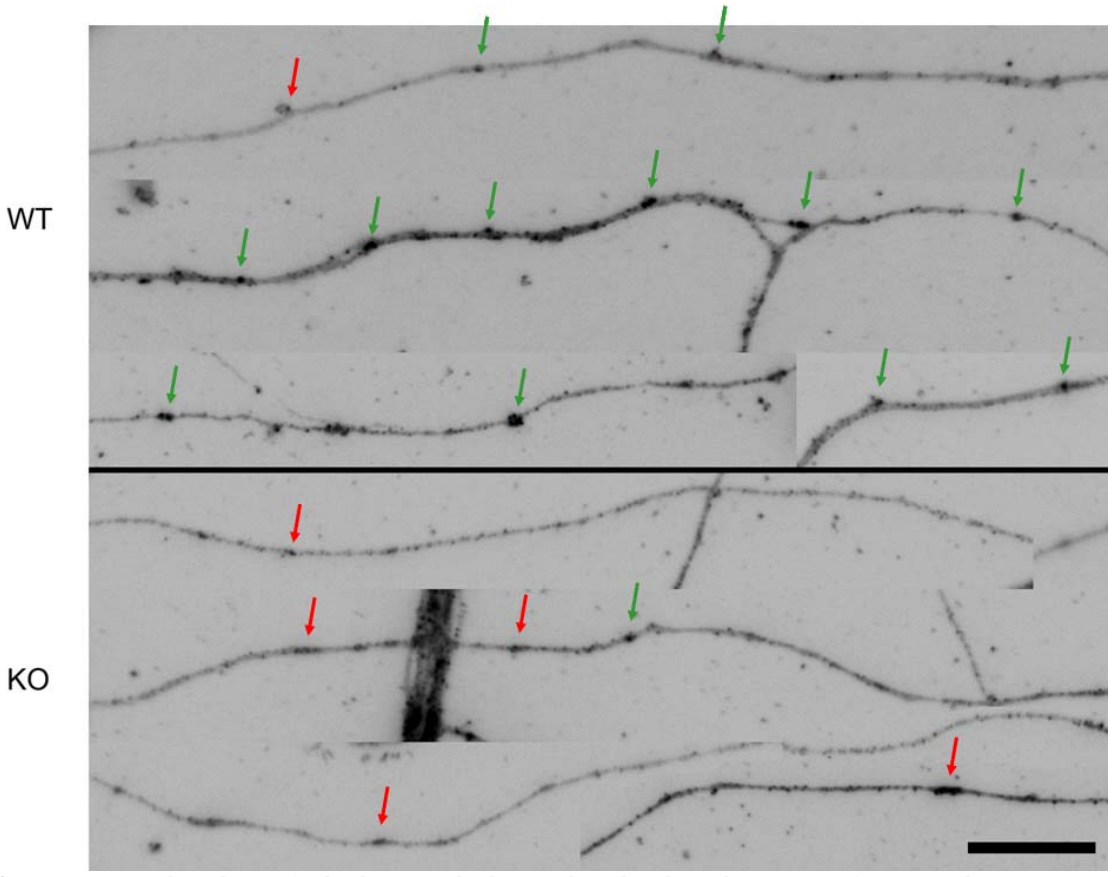

Figure 31. **Reduction of cholesterol clustering in the absence of Cap23 in axons.** Hippocampal neurons were cultured for 4 DIV and then stained with filipin. Arrows indicate cholesterol clusters (large/confined accumulations green, small/dispersed accumulations red). Scale bar=10μm

As it is well established that lipid raft-clustering affects cytoskeletal behavior, and since cholesterol-rich raft organization is disrupted in the absence of Cap23, we wondered whether the subcellular localization of Cap23 and f-actin in hippocampal neurons might help our understanding of how these mechanisms are correlated. Hence, we performed immunocytochemistry on hippocampal neurons, co-labeling Cap23 and actin in the same cells. We observed that Cap23 clusters, besides colocalizing with cholesterol (Fig. 30), were almost exclusively in close apposition to accumulations of f-actin structures (Fig. 32). Usually, Cap23 accumulations were found at the tip of f-actin rich filopodia or at the surface of lamellipodial structures, where the presence of either Cap23 or actin seemed to be mutually exclusive, but directly adjacent to each others. As can be appreciated in Fig. 32, also non-neuronal cells were labeled, displaying intensely labeled actin stress fibers and some filopodia extending from its surface, which at their tips contained Cap23-positive clusters as well, indicating that a) Cap23 is also expressed in non-neuronal cells, and b) the localization pattern of Cap23 relative to actin is conserved also in non-neuronal cells.

Does direct modulation of raft clusters downstream of Cap23 affect the actin cytoskeleton? In order to investigate this possibility, we analyzed the morphology of cholesterol-rich lipid rafts, actin and Cap23 clusters in hippocampal neurons of *Thy1*-Cap23 mice. Cap23-clusters were increased in size and density (Fig. 33), which is likely to be a consequence of the more than twofold increase of the Cap23 protein levels in these transgenic mice (Fig.9). These clusters were usually close to f-actin structures (Fig. 33), indicating that overexpression of Cap23 does not alter its subcellular distribution.

In some dramatic examples the size of cholesterol- and Cap23 clusters displayed major size increases, while their density was reduced along dendrites (Fig. 34), probably representing potent aggregation of Cap23 along with cholesterol structures at the extent of their density.

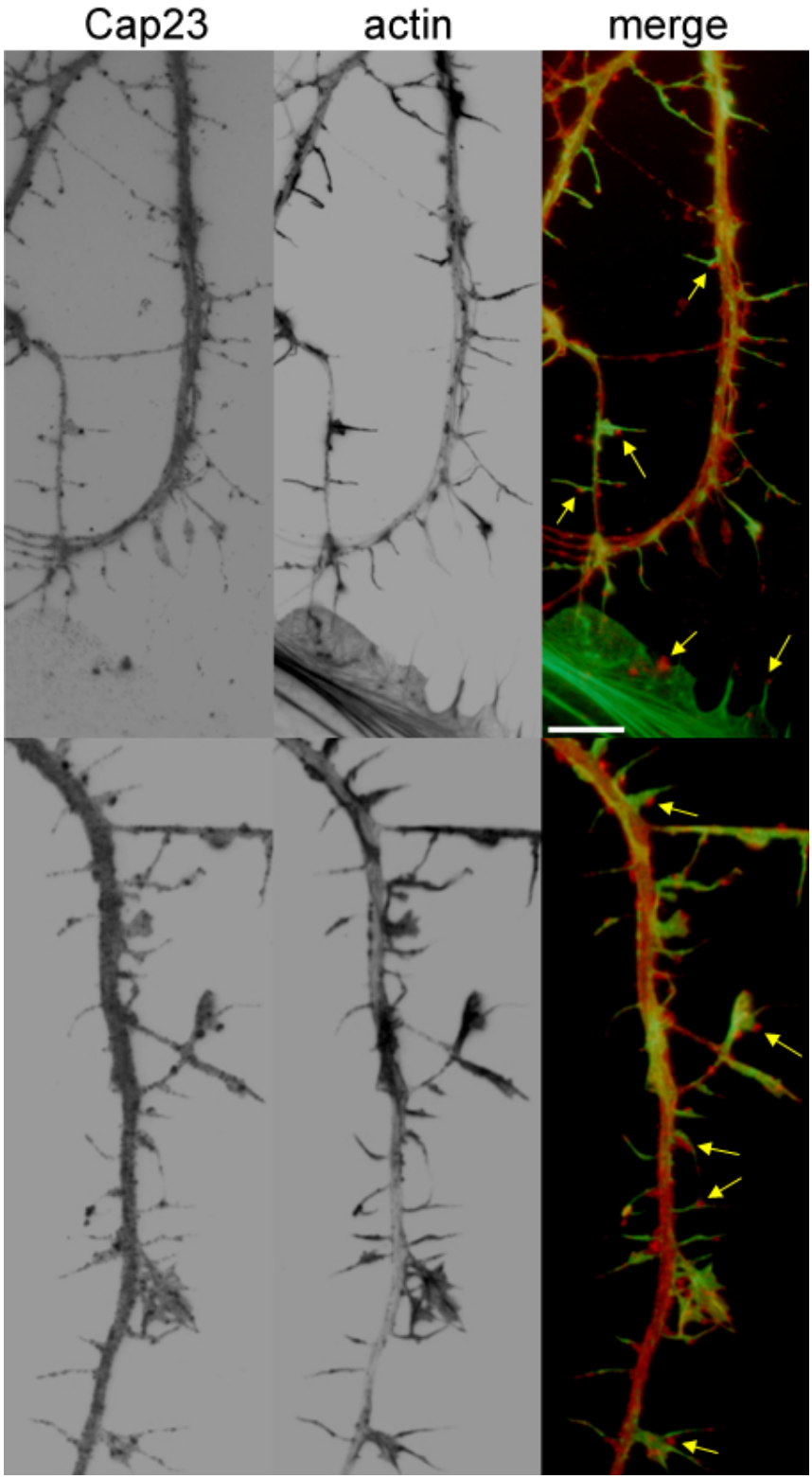

Figure 32. **Cap23 clusters correlate with regions of reduced actin polymerization.** Hippocampal neurons were cultured for 6 DIV and then stained with a Cap23 antibody and Alexa488-phalloidin. Arrows indicate Cap23 clusters, which are located at terminal f-actin structures. Scale bar=10μm

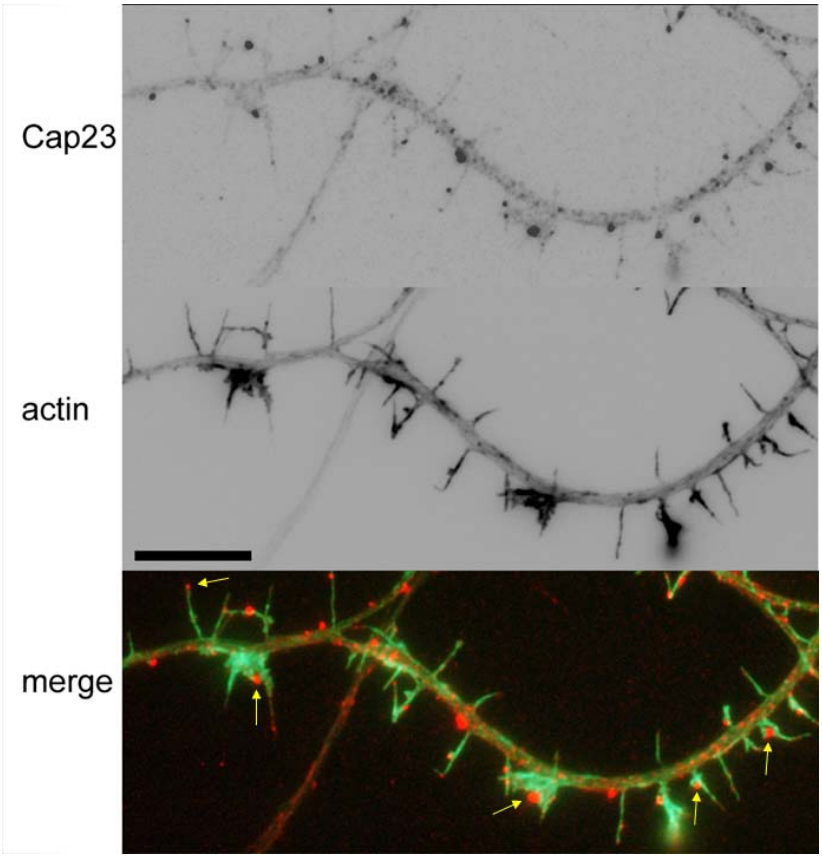

Figure 33. **Overexpression of Thy1-Cap23 slightly increases density and size of Cap23 clusters in dendrites.**
Hippocampal neurons from *Thy1*-Cap23 mice were cultured for 9 DIV and then stained with an antibody recognizing chCap23 and Alexa488-phalloidin. Arrows indicate Cap23 clusters, which are located at terminal f-actin structures.

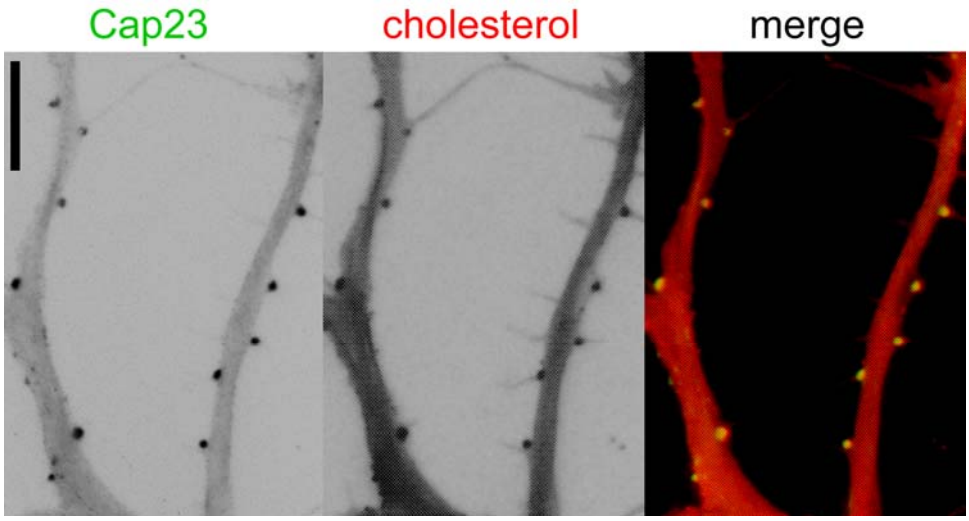

Figure 34. **Overexpression of *Thy1*-Cap23 can increase size at the extent of density of Cap23- and cholesterol clusters in dendrites.**
Hippocampal neurons from *Thy1*-Cap23 mice were cultured for 5 DIV and then stained with an antibody recognizing chCap23 and filipin. Scale bar=10μm

Also in axons, similar increases in density and size of cholesterol were observed upon overexpression of *Thy1*-Cap23 (Fig. 35a). Interestingly, this phenotype extended to growth cones as well, where cholesterol-rich clusters were increased in density and size, and were located at sites which presumably represent domains rich in f-actin-dynamics (Fig.35b).

As already discussed in chapter 2.1, *Thy1*-Cap23 mice displayed dramatic morphological changes on the level of the actin cytoskeleton, which is a further implication that Cap23 levels, through modulation of cholesterol-rich lipid rafts affect cytoskeletal dynamics and morphology.

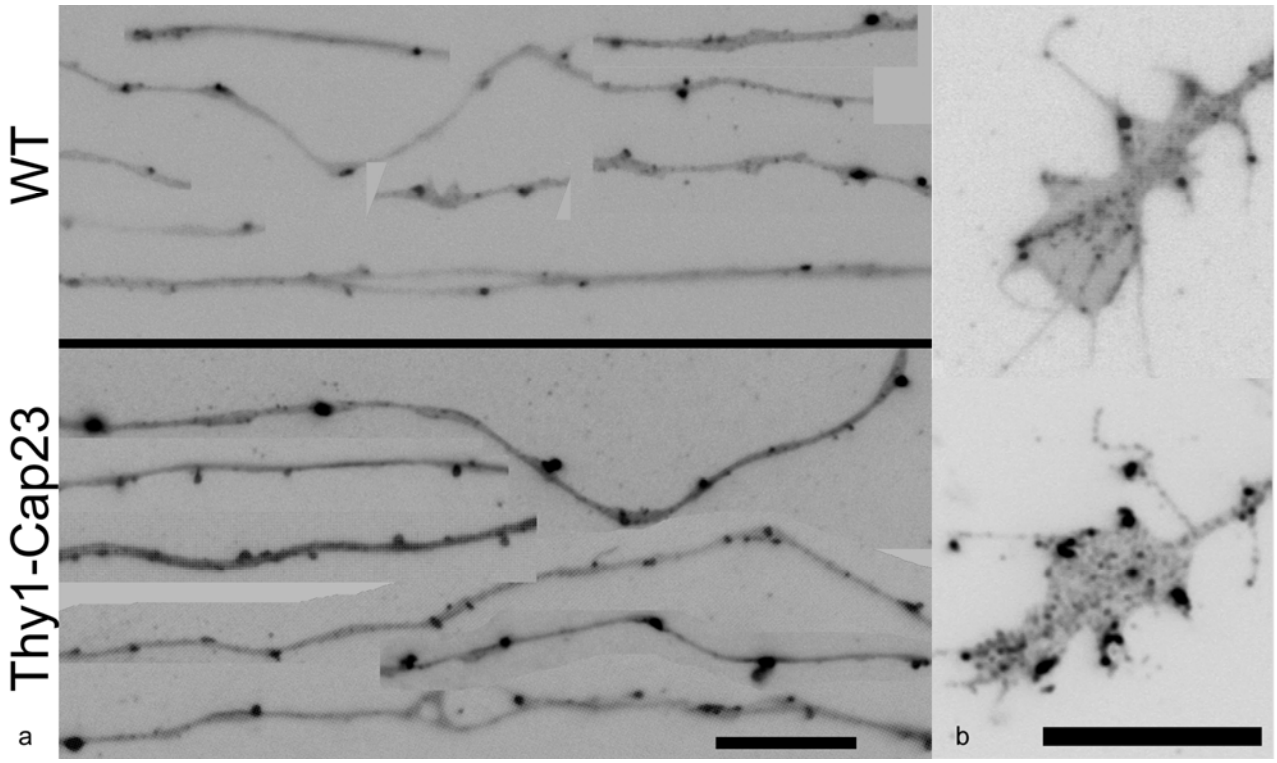

Figure 35. **Overexpression of *Thy1*-Cap23 increases size and density of cholesterol clusters in axons and growth cones.**
Hippocampal neurons from *Thy1*-Cap23 mice were cultured for 5 DIV and then stained with filipin. a) axons and b) growth cones are shown. Scale bars=10μm.

2.4.2 Protein composition of Lipid Raft and Non-Rafts is Altered by Cap23 Protein Levels

2.4.2.1 Alteration of Cytoskeletal Protein Levels

We wondered whether the effect of different levels of Cap23 protein on lipid levels we had observed on the level of cholesterol clusters would affect the protein composition of raft platforms, since it is well known that clustering of individual raft units can induce various proteins to co-distribute with raft lipids. We therefore analyzed raft fractions, obtained by fractionation of brains lysates from WT and Cap23 mutant mice. Analyses were performed either by 2D-gelelectrophoresis followed by protein staining and sequencing or by western blot.

Beside IF proteins, which were already discussed in section 2.2, we found a small set of cytoskeletal proteins altered in lipid rafts. It might seem unusual to find cytoskeletal proteins in raft fractions, since they are usually detected in non-raft fractions. Nevertheless, association with lipid rafts has been reported for a variety of cytoskeletal proteins. Among other cytoskeletal proteins, spectrin association with $PI(4,5)P_2$ and cholesterol have been investigated (Grzybek et al., 2006), the regulation of cofilin by $PI(4,5)P_2$-binding has been established (Yonezawa et al., 1991) and tropomyosin3, among other proteins, has been found to rapidly distribute into raft domains upon stimulation with PDGF (MacLellan et al., 2005).

In our analysis, spectrin $\alpha 2$ protein levels were increased to about 230% of WT levels in both, Cap23 heterozygous and Cap23 KO rafts (Fig. 36). Since spectrins form part of the membrane cytoskeleton and are important for neurite stability, it is conceivable that an increase in spectrin protein levels could result in a stabilization of the neuritic membrane. We interpreted this result as a homeostatic upregulation of spectrins in response to a less stable cortical actin network, as we have demonstrated in section 2.1.

Cofilin is a ubiquitous actin-binding factor required for the reorganization of actin filaments, which upon activation by dephosphorylation, severs and depolymerizes actin filaments, therefore driving directional cell motility. We found cofilin to be downregulated in lipid raft fractions of Cap23 heterozygous and KO mice to 52 and 39% of WT protein

levels, respectively. The graded reduction in heterozygous and KO mice implies that a stepwise reduction of Cap23 seems to have a proportional effect on cofilin levels, while the similarly enhanced levels of spectrin in either background may point to a more essential function being disturbed by deleting only one allele of Cap23.

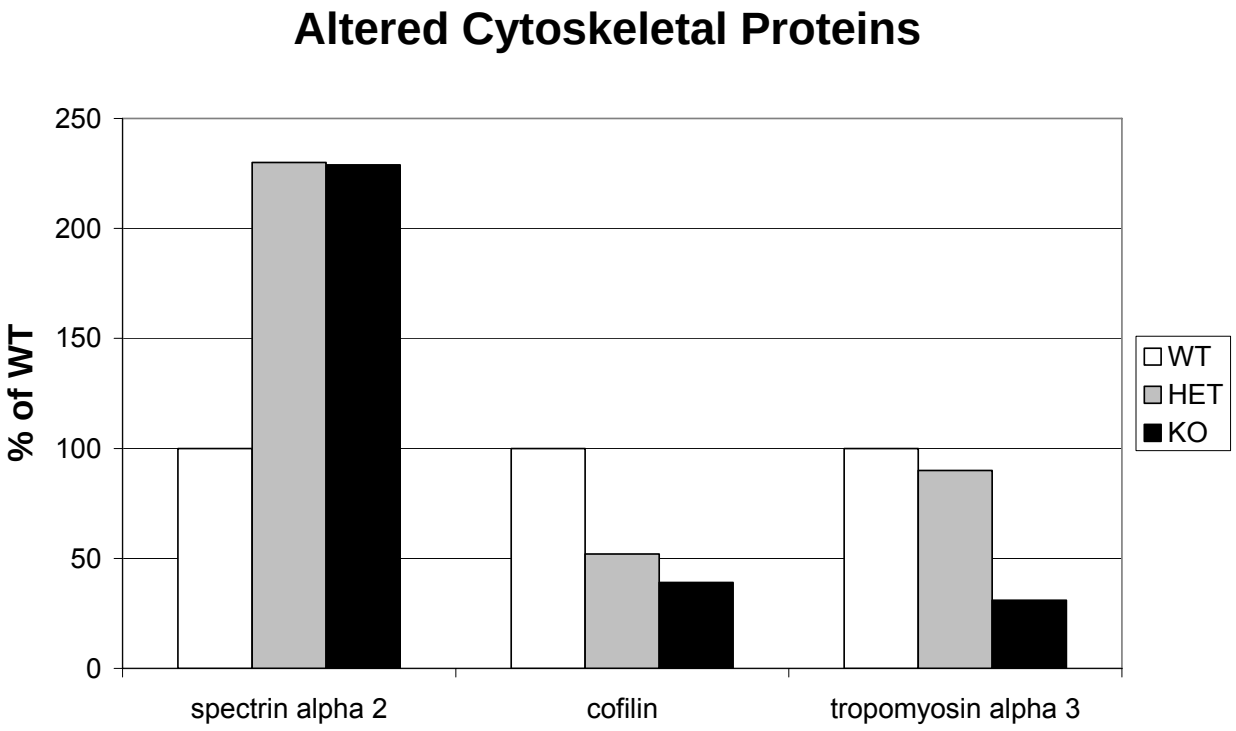

Figure 36. **Cytoskeletal proteins are differentially distributed in protein fractions of mice with altered Cap23 protein levels.**
Protein spots from 2D-gels were quantified and sequenced. Spectrin α2 and cofilin were found in lipid raft fractions, tropomyosin α3 was found in non-raft fractions of P16 mice.

The third cytoskeletal protein revealed in our screen was tropomyosin α3 (synonym Tm5NM), which was downregulated to 90 and 31% in Cap23 heterozygous and KO mice, respectively. Tropomyosins are rod-like helical proteins which stabilize actin filaments by binding laterally to their sides and probably protecting them from severing. There are about 20 tropomyosin isoforms, 10 of which are expressed in neurons, where besides stabilizing actin filaments, they compete with drebrin for actin binding (Shirao 1995). This raises at least two possibilities of interpretation: reduced levels of tropomyosin could lead to actin filaments being less protected by tropomyosin, thus having a destabilizing effect

on the actin network. This would be in line with our data showing unstable actin structures in neurons of Cap23 KO mice. A decrease of tropomyosin could on the other hand also result in an imbalance of drebrin binding to actin as a consequence of improper competition. However, since the fourth protein we find to be downregulated in Cap23 KO mice is drebrin itself, competition might not be altered to such a big extent. Tm5 has also been reported to be enriched in filopodia and growth cones, and its overexpression in neurons results in enlarged growth cones accompanied by an increase in the number of dendrites and axonal branching (Schevzov et al., 2005). The filopodial localization of Tm5 is consistent with our finding, that f-actin structures display instability in the absence of Cap23, a process which is likely to be aggravated by a reduction of tropomyosin in these structures.

Drebrin, which has been reported to compete with tropomyosin for actin binding, is downregulated to 84 and 64% of WT levels in Cap23 heterozygous and KO non-raft fractions, respectively (Fig. 37). In fact, drebrin is a developmentally regulated actin binding protein and is enriched in dendritic spines, promoting their maturation and stability. Its reduced protein levels in Cap23 mutant mice could therefore indicate a delayed or unsuccessful maturation of dendritic spines, which might be secondary to the instability of f-actin structures as discussed in 2.1.

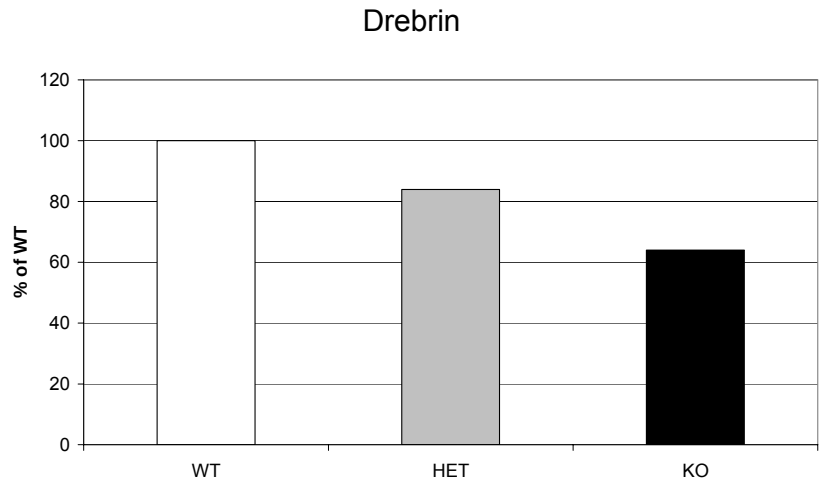

Figure 37. **Drebrin Protein Levels are Downregulated in Mice with Reduced Cap23 Levels.** Non-raft fractions of P28 mice were analyzed by immunoblotting using a monoclonal drebrin antibody. Resulting bands were quantified by densitometric scanning.

The last actin-binding protein downregulated in the absence of Cap23 is calcium/calmodulin-dependent protein kinase II α (CaMKII α). Interestingly, this protein was present in rafts as well as non-rafts, and was downregulated in both fractions. Levels were decreased more dramatically in raft fractions (11% of WT levels) than in non-rafts (58% of WT levels; Fig. 38), indicating that whereas there seemed to be a global downregulation of this kinase, its localization to lipid rafts might be especially labile in the absence of Cap23.

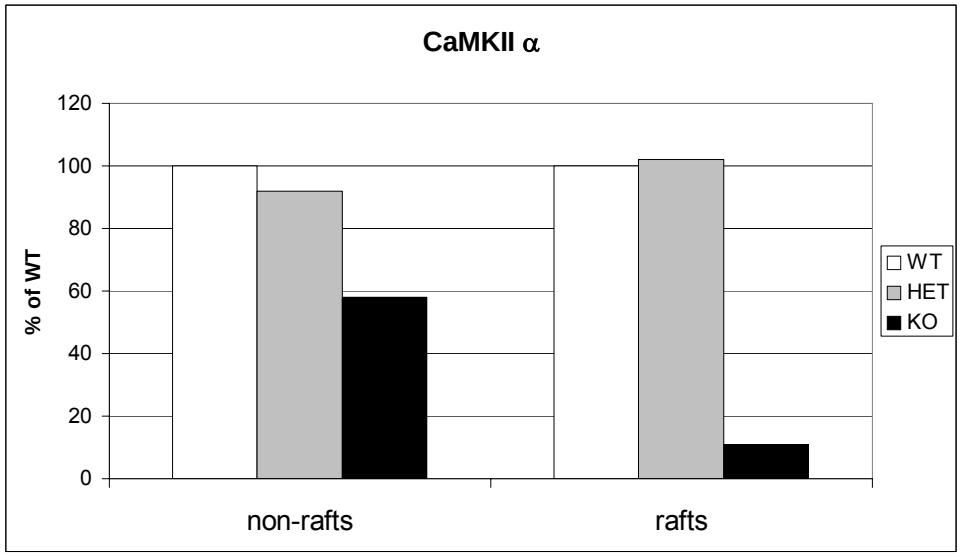

Figure 38. **CamKII α Protein Levels are Downregulated in Mice with Reduced Cap23 Levels.** Raft- and non-raft fractions of P16 mice were analyzed by immunoblotting using a monoclonal CamKII α antibody. Resulting bands were quantified by densitometric scanning.

CaMKII is an important component for synapse stabilization, being especially enriched in those synapses which have undergone potentiation due to previous activation. CaMKII consists of one alpha and one beta subunit with the alpha subunit containing an actin-binding site, by which it attaches to the actin scaffold at the postsynaptic density (PSD), to which it translocates upon activation. Once recruited to activated synapses, it further promotes synaptic potentiation by phosphorylating AMPA-type glutamate receptors, which in consequence are inserted at the PSD and contribute to synaptic strength. Therefore, CaMKII is regarded as a critical modulator of learning and memory formation. Its reduced protein levels we find in different protein fractions of Cap23 mutant mice could therefore have an impact on the stability of activated synapses.

2.4.2.2 Alteration of Mitochondrial Protein Levels

Besides cytoskeletal proteins, we also found a large group of proteins which are involved in energy homeostasis and/or are localized to mitochondria. Analyzing 2D-gels of raft- and non-raft fractions obtained from P16 and P28 mice, several proteins differentially regulated in WT and Cap23 KO mice were found (summarized in Fig. 39). Strikingly, 11 of 12 proteins were mitochondrial proteins and 9 of these 12 proteins are involved in various pathways related to energy production including ATP synthesis, glycolysis, electron transport and the citric acid cycle.

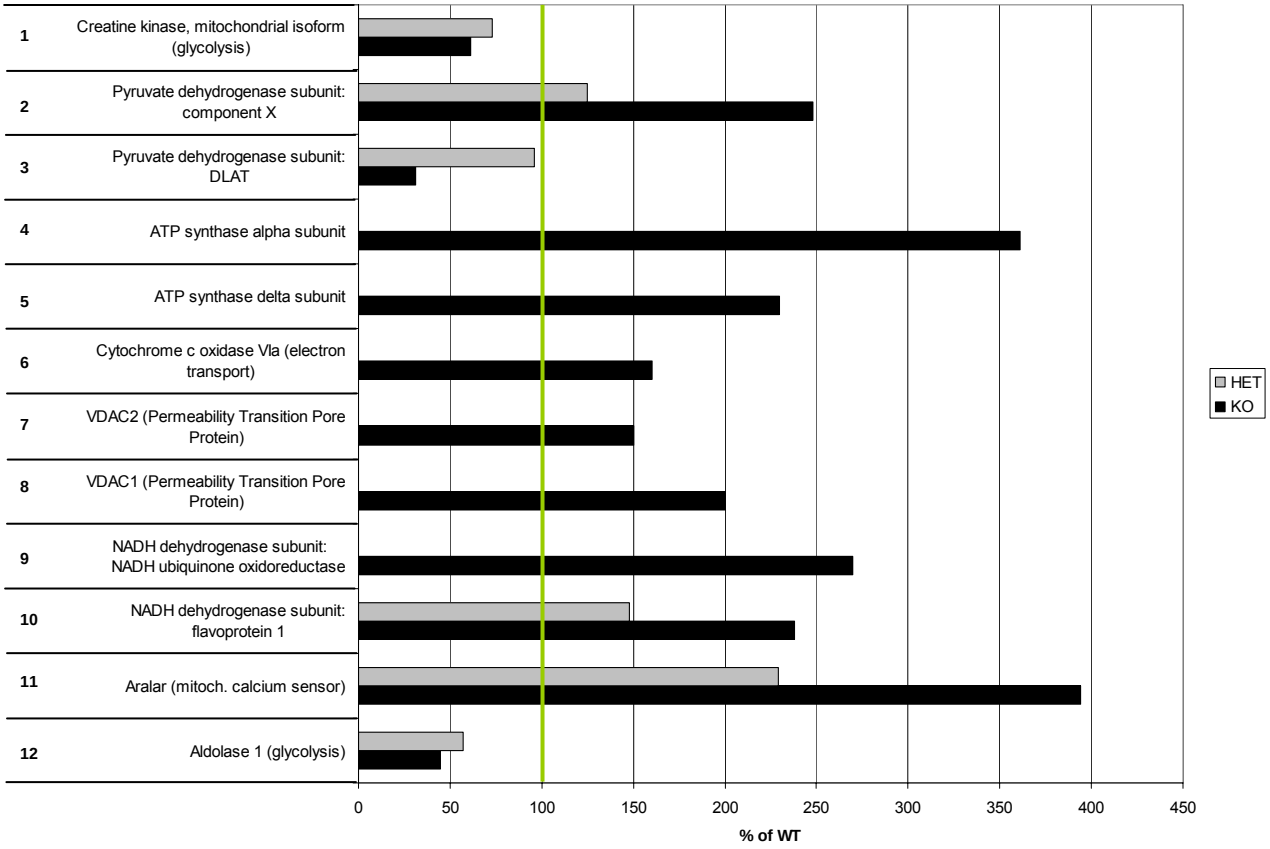

Figure 39. **Altered Mitochondrial Proteins Levels in the Absence of Cap23.**
Individual protein spots differentially regulated on 2D-gels were quantified and sequenced. Proteins 4-12 were found in rafts and proteins 1-3 in non-rafts. Proteins 1-3 and 10-12 were from P16 mice and proteins 4-9 from P28 mice. Values are normalized to WT (100%, indicated by green line). Proteins 2-12 are known to be mitochondrial, whereas only aldolase 1 is situated in the cytoplasm.

As demonstrated in 2.3, the absence of Cap23 has multiple effects on mitochondrial structure, motility and docking inside axons. Apart from phenotypes regarding their motile versus stable behavior, mitochondria showed prominent length increase in Cap23 KO neurons. This, along with an increase in mitochondrial protein levels might be an effort of regulatory mechanisms in order to restore proper mitochondrial functioning. Since most regulated proteins we found in this screen are involved in energy production rather than structural mitochondrial proteins, this gives rise to the hypothesis that Cap23 neurons suffer from suboptimal energy supply as a consequence of failure of proper mitochondrial targeting and retention.

This statement is supported by the fact that unfractionated whole brain protein lysates similarly display altered levels of proteins involved in energy homeostasis, which again are mainly localized to mitochondria (Fig. 40).

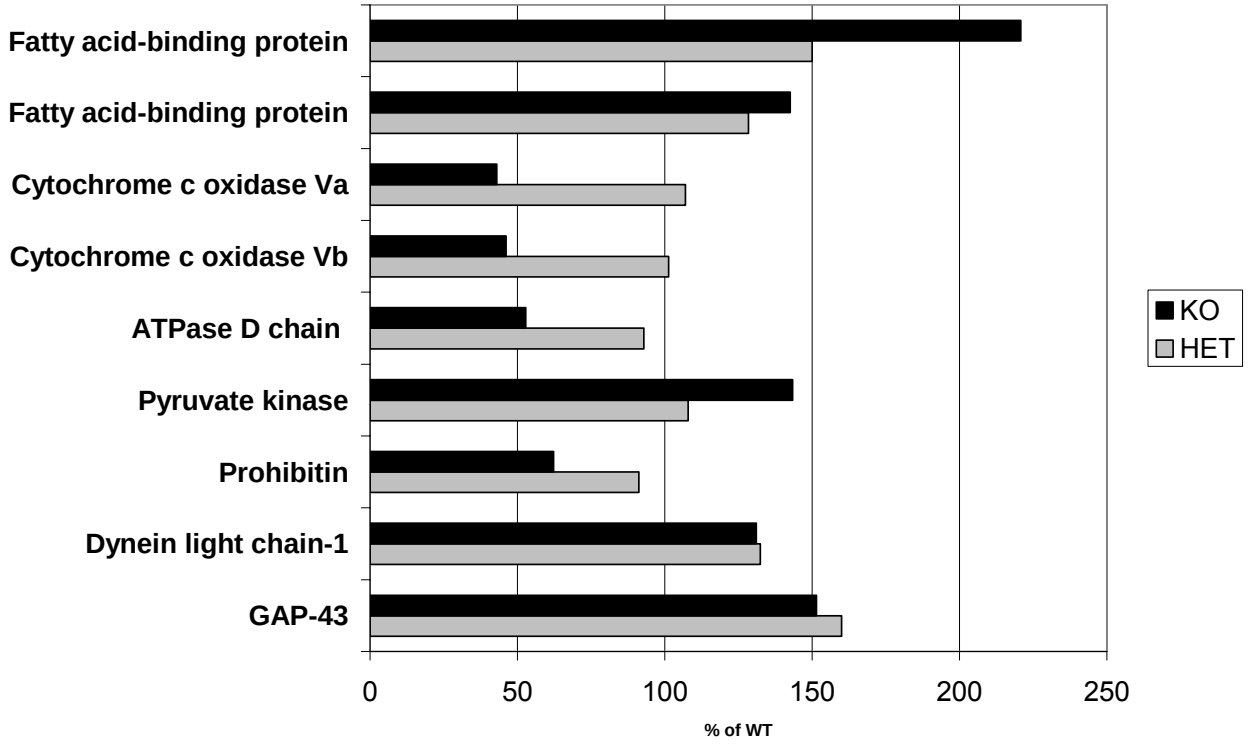

Figure 40. **Altered Proteins Levels of Brain Homogenates in the Absence of Cap23.**
Whole brain homogenates of P34 mice were prepared and analyzed by 2D-gelelectrophoresis, followed by DIGE-staining, quantification and sequencing by MALDI-TOF/MS. Fatty acid-binding protein was isolated at two different spots on the same gel, displaying slightly different pI.

Prohibitins can target to lipid rafts and may have a role modulating and maintaining mitochondrial function (Mishra et al., 2006). The fact that we find it downregulated to 62% of WT levels (Fig. 40) might indicate partial loss of this regulatory function, giving rise to inefficient energy production by mitochondria. This would be in line with our observations on mitochondrial structure and motile behavior (section 2.3). Similar arguments apply for cytochrome c oxidase Va, Vb, the ATP synthase D chain and pyruvate kinase, which all have well established functions in either oxidative phosphorylation or glycolysis and need to be tightly regulated in order to maintain proper ATP production. Failure of this regulation is likely to result in energy deficits at certain sites of the cell.

An interesting feature was observed for fatty acid-binding protein (FABP). This protein was isolated from two different spots on 2D-gels, which slightly differed in their isoelectric point (pI), and will therefore be referred to as FABP-A (for more acidic) and FABP-B (for more basic). First, both isoforms seemed to be developmentally regulated, as FABP-A and FABP-B were downregulated at P34 to 58 and 28% of values at P20, respectively (Fig. 41). Strikingly, mice with reduced levels of Cap23 showed a very consistent upregulation in both spots at both timepoints analyzed. This upregulation was inversely proportional to Cap23 levels, as protein levels from heterozygous mice showed always intermediate values between WT and KO, indicating that Cap23 seems to regulate FABP protein levels. FABP facilitates uptake of fatty acids across the plasma membrane and to regulate their intracellular trafficking. Furthermore it has been shown, that transfer of fatty acids from fixed liposomes to mitochondria is promoted by FABP (Veerkamp et al., 2001). Since fatty acids are the main structural and energy sources of the human body, and their cellular uptake is the first step in energy production in the form of ATP, FABP is involved at a critical process of energy supply to the cell, and maybe specifically to mitochondria.

The observed misregulations of the proteins described here and in 2.4.2.1 are likely to be an effect of altered protein stability due to interference with their microenvironment including composition, dynamics and stability of lipid rafts as well as of the actin and intermediate filament cytoskeleton. However, an alternative scenario would be regulation at the level of mRNA transcripts, which theoretically could be induced by the inactivation of the Cap23 allele and has been observed in various gene KO backgrounds. To distinguish between these possibilities, we performed microarray analysis (2.4.3).

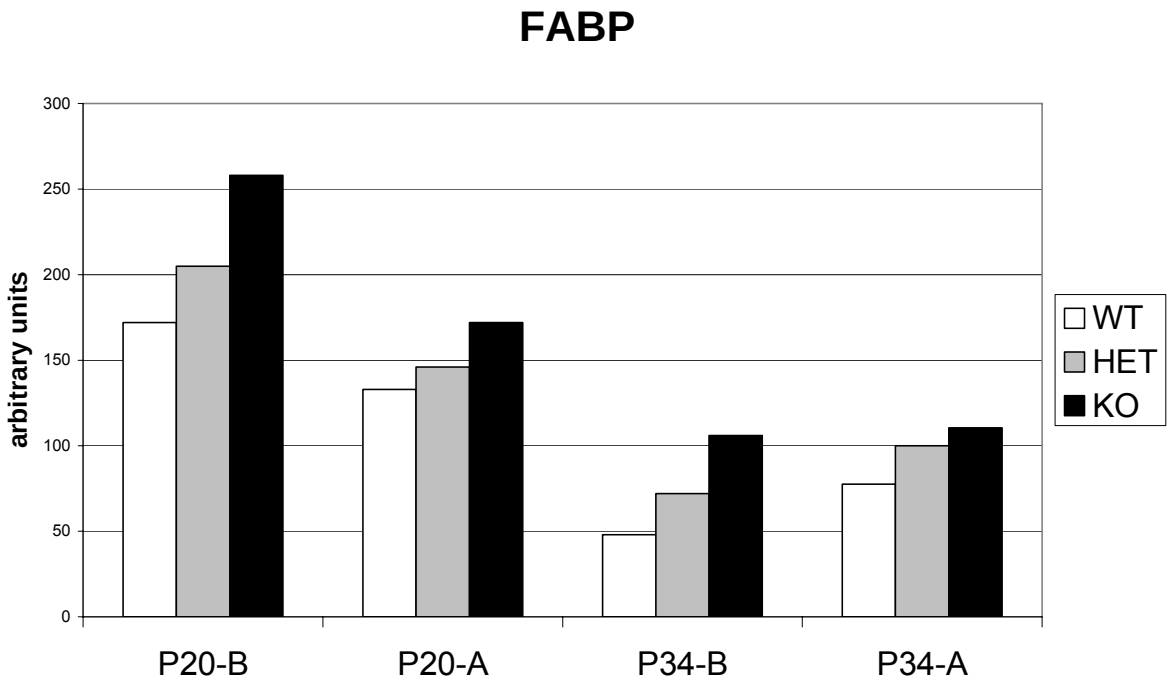

Figure 41. **Fatty Acid Binding Protein (FABP) Level Regulation in the Absence of Cap23.** Individual protein spots differentially regulated on 2D-gels were quantified and sequenced. FABP appeared at 2 distinct spots of different pI, one spot being more acidic (-A), the other more basic (-B). Both spots are downregulated with age, but the B-spot is reduced to a larger extent than the A-spot, so that at P34, there is more FABP-A than FABP-B, resulting in an inverted ratio compared to P20.

The suggestion, that mitochondria contain lipid raft-like domains has been controversial for some time, but more recent data seem to confirm this hypothesis (see introduction). The mitochondrial proteins we found in the lipid raft fraction (8 out of 9) could therefore be either fractionation artefacts or represent real distribution of these proteins into detergent-resistant membrane fractions.

In the light of our results demonstrating diverse defects regarding the size and distribution of lipid rafts and downstream pathways affected, it is appealing to speculate that even though there is no evidence of Cap23 regulating mitochondrial membrane domains, modulation of the composition of raft lipids and proteins therein at the plasma membrane might have some effect on the lipid and protein composition in mitochondria.

2.4.3 Microarray analysis of Cap23 mutant mice

We performed Affimetrix-based microarray analysis in order to understand whether transcript regulation was altered by the deletion of Cap23. To this end, we isolated RNA from brains of WT, Cap23 heterozygous and KO mice at the age of P20 and P34, which was close to the age at which we had observed misregulations at protein level.

Hybridization of purified RNA to DNA chips revealed that a large fraction of mRNA transcripts were preserved during RNA isolation (present calls ~60%). When we performed statistical analysis of genes being significantly ($p<0.01$) regulated more than 1.5 fold between genotypes, only 54 genes passed this criterion. 6 of these genes were upregulated and 48 genes were downregulated in Cap23 KO mice. Filtering for a factor of 2-fold regulation left only 1 gene upregulated and 8 genes downregulated, which with the exception of Cap23 itself and Mlc1 were mostly unknown or little characterized genes (Table 1). Myosin light chain 1 (Mlc1) is an essential component of the myosin motor complex and thus might affect actin-based transport processes. Negatively affecting the myosin machinery would be expected to slow down actin-based transport. On the contrary, we observed an increase in actin-based motility in the absence of Cap23 and did not find Mlc1 protein levels to be altered in our screens, thus making it unlikely that Mlc1 is directly involved in the phenotypes described here.

fold change	gene
-7.82	deaminase domain containing 1 (Receptor for TNFSF6/FASLG)
-4.59	spermatogenesis associated glutamate (E)-rich protein 7, pseudogene 1
-2.58	myosin, light polypeptide 4 (=Mlc1)
-2.51	brain abundant, membrane attached signal protein 1 **(=Cap23)**
-2.30	Transcribed locus
-2.07	DnaJ (Hsp40) homolog, subfamily B, member 4
-2.05	FYVE, RhoGEF and PH domain containing 2
-2.02	RIKEN cDNA 4833409A17 gene
2.32	melanoma inhibitory activity 1

Table 1. **Regulation of mRNA transcripts >2-fold with p<0.01 in Cap23 KO mice.**
Microarray results were analyzed using Expressionist software. Values are relative changes compared to WT levels.

Given that more than 29.000 genes were included in our analysis (as characterized by having a detection value of p<0.04), these few alterations proved to us, that Cap23 is not involved in regulating mRNA transcripts and that deletion of its gene does not cause gross changes in transcript levels. Two conclusions can be drawn from this result: First, this strongly indicates that any changes observed on protein levels are not the consequence of altered gene expression on the mRNA level; and second, the absence of Cap23 therefore does affect the stability of several proteins in a pronounced manner.

However, we were interested to see whether among the genes failing to pass our stringent filter settings, we might find some of the genes which we had demonstrated to be affected by the absence of Cap23 in our proteomic screens. We could not find any alteration for mitochondrial proteins, but transcripts for spectrin β2 and CamKII α were slightly but consistently downregulated, while vimentin transcripts were upregulated (Fig. 42). These results indicate that there are some small alterations in gene expression in response to reduced cytoskeletal instability, since vimentin, spectrin and CamKII stabilize the IF cytoskeleton, the axonal cortical membrane and synaptic stability, respectively.

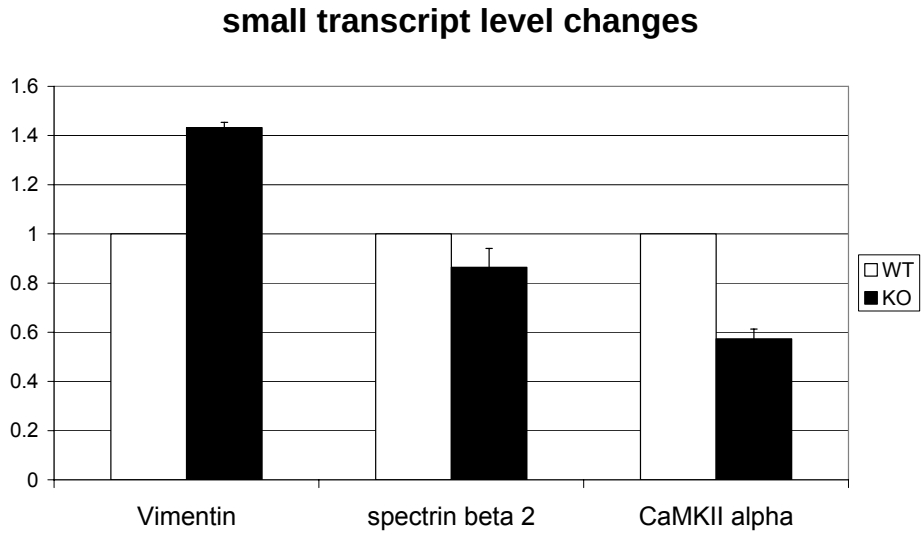

Figure 42. **Small but non-significant changes in transcript levels of some candidate genes.** Manual inspection of microarray data beyond hits that passed stringent filters (2-fold change and p<0.01) reveals small changes in transcripts for vimentin (+43%), spectrin β2 (-14%) and CaMKII α (-43%).

2.4.4 Colocalization of Cap23 and the Exocyst Complex

Since we had realized that Cap23 is localized along axons and dendrites in quite regularly spaced, distinct clusters and demonstrated that by either reducing or increasing Cap23 protein levels, the frequency and size of cholesterol-based lipid rafts at these very sites can be similarly altered, we decided to make an effort to identify the nature of these clusters. We therefore performed immunolabeling of Cap23 together with several candidate proteins. We were not able to find codistribution of Cap23 together with adhesion markers, but finally could successfully identify the exocyst complex member Sec6 to colocalize to about 100% with Cap23 clusters.

The exocyst is an octameric complex involved in targeting proteins and vesicles to specified plasma membrane domains, thereby acting as a polarization cue. The exocyst complex has been suggested to selectively regulate the docking of insulin-containing vesicles at sites of release close to the plasma membrane in adipocytes (Tsuboi et al., 2005). In a more recent study, Sec6 was demonstrated to be targeted to adipocyte lipid rafts and to be required for glucose uptake and Glut4 docking at the plasma membrane (Inoue et al., 2006). It was suggested, that exocyst assembly at lipid rafts sets up targeting sites for Glut4 vesicles important for proper insulin signaling. In neurons, the small GTPase RalA has been shown to promote neurite branching through the exocyst complex, involving protein kinase C-dependent phosphorylation of Gap43 (Lalli & Hall, 2005).

As illustrated in Fig. 43, Sec6 and Cap23 both localize to the very same defined clusters along axons in WT. Whereas the cluster shape is defined and regular in WT, KO axons show enlarged accumulations of Sec6 or amorphous shape. In fact, KO exocyst clusters are more than 7-fold larger in average, and using a mathematical shape factor we could demonstrate that the irregularity of the clusters are significant. These data show that Cap23 is necessary for the confinement of exocyst shape.

The colocalization of Cap23 and Sec6 is remarkable, and probably highly significant since no other proteins have to date been shown to be located at exactly the exocyst site. That a cytoskeleton-associated protein, involved in axon elongation and an exocyst member, involved in neurite branching and polarization are found to cluster together is

unanticipated and could have important implications in further understanding various processes involved in the formation of specialized compartments, polarization at the plasma membrane, and consequently polarized growth processes.

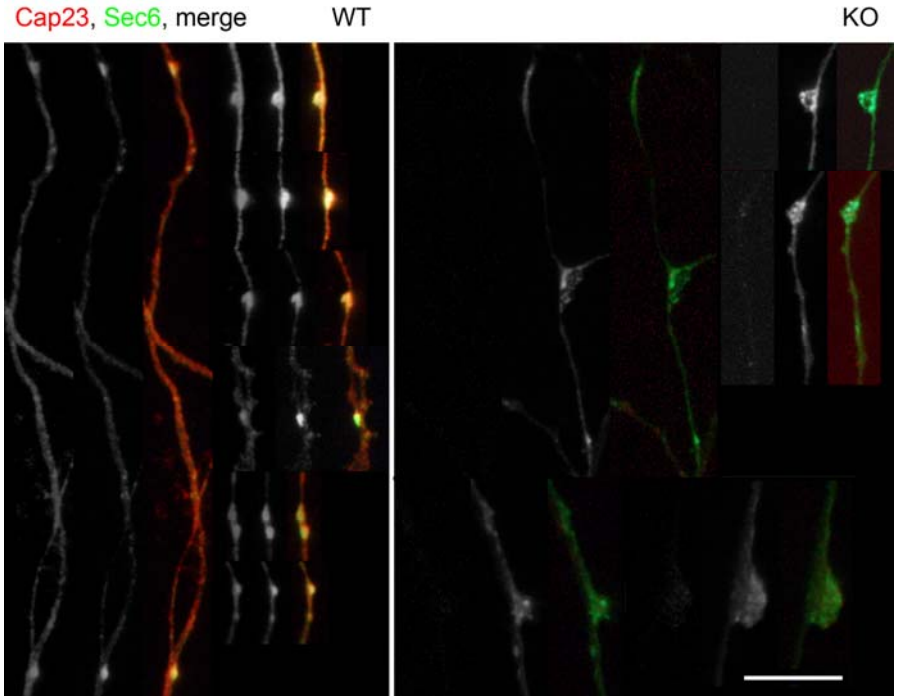

Figure 43. **The exocyst complex member Sec6 colocalizes with Cap23. Absence of Cap23 results in size increase and irregularity of clusters.**
Hippocampal neurons were cultured for 5 DIV and stained with antibodies against Cap23 and Sec6. Scale bar=10μm.

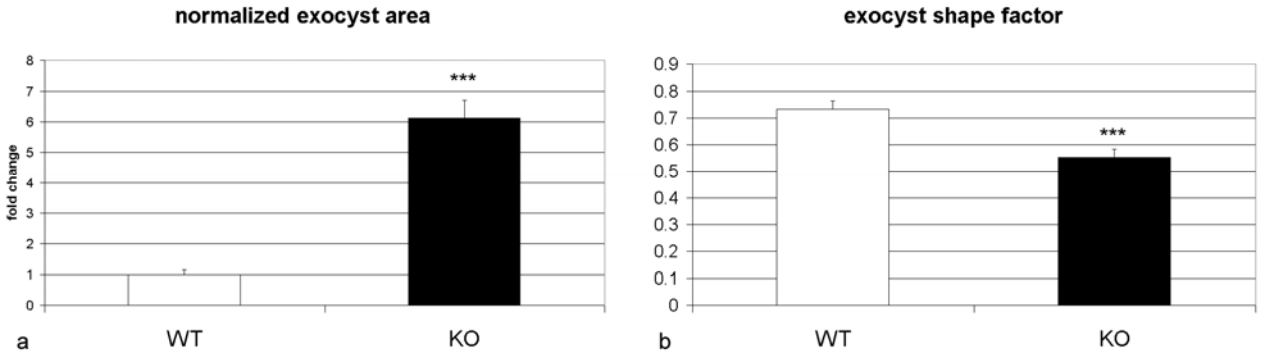

Figure 44. **Absence of Cap23 increases exocyst size and leads to loss of its confinement.**
Exocyst area was measured using Metamorph, which was also used to calculate the shape factor (shape factor=$4\pi A/(P^2)$, where A is area and P is perimeter; 0=flattened object, 1=perfect circle) ***=p<0.001

2.4.4 Unique Properties of Cap23

Using bioinformatics tools, we analyzed the secondary structure of Cap23, and found that it is made up of a very uncommon amino acid (AA)-composition. This is characterized by having an unusual high fraction of lysines, alanines, prolines and glutamic acids (Fig. 45). All other AAs are underrepresented, compared to an average of all known proteins (zero-line in Fig. 45).

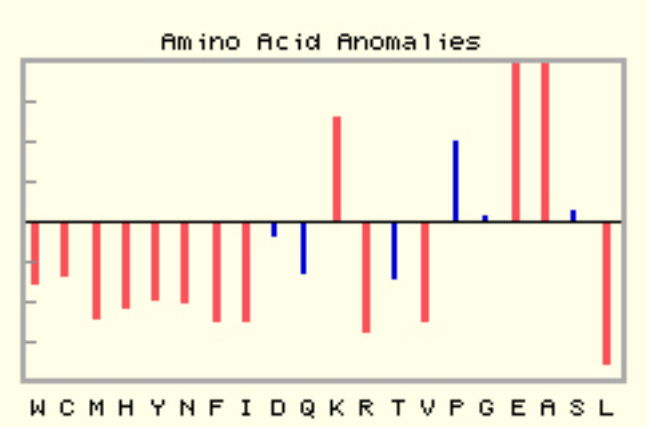

Figure 45. **Amino Acid Anomalies of Cap23 Protein Sequence.**
The zero-line of the X-axis represents the mean frequency of all proteins known in the SWISS-PROT database, the Y axis shows how far the frequency of a given amino acid deviates from the average.

Performing a database search (SWISS-PROT entries) in order to identify proteins with an AA composition which is similar to Cap23 in terms of AA anomaly revealed MARCKS, Gap43, NFH and tropomyosin. MARCKS and Gap43 are similar to Cap23 in that they are raft-associated proteins and involved in growth-associated processes (see introduction). However, these three proteins share very little protein homology, raising the possibility that their close similarity concerning AA anomaly serves as a structural basis for their involvement in partially homolog functions. The similarity of Cap23 to NFH and tropomyosin concerning their AA anomaly was unanticipated. NFH and tropomyosin protein levels are altered in Cap23 KO mice, but the significance of this *in silico* result is unclear. It is though tempting to speculate that like for the similarity shown for Cap23, Gap43 and MARCKS, the similar AA anomaly might reflect some so far unidentified structural commonalities between these proteins, which could result in certain protein-

protein interactions or alternatively in similar functions by adopting similar 3D structures, which is known to be a feature of proteins with intrinsic disorder.

In the protein sequence of Cap23, no motifs like alpha-helices or beta-sheets were identified, thus no 3D-structure could be predicted. Even more remarkable, a special algorithm (DisProt Predictor, VL3H-neural network based) predicting intrinsic disorder revealed that the entire structure of Cap23 was predicted to exhibit intrinsic disorder (Fig. 46). Intrinsic disorder is a measure of lack of 3D structure and has been shown to serve as the structural basis for hub protein promiscuity. Hub proteins are defined as proteins interacting with more than 10 partners, and are significantly more disordered than end proteins, defined as those that interact with just one partner (Haynes et al., 2006). They thus play important roles in networking via hubs by interacting with multiple proteins.

As illustrated in Figure 45, the three proteins Cap23, Gap43 and MARCKS exhibit striking similarity in their degree and distribution of intrinsic disorder with values close to 1 (=total disorder) over almost the entire stretch of the protein. In contrast, actin is a protein which has regions of very high order, which is expected for a protein with essential roles in preserving the structure of the cytoskeleton. Depicted is also the predicted disorder analysis for spectrin αII, to illustrate that the majority of proteins contain mixed regions of both, order and disorder, further strengthening the importance and uniqueness of the unprecedented disorder being exhibited especially for Cap23, but also for Gap43 and MARCKS. NFH and tropomyosin contain large regions of disorder as well, and especially the C-terminal part of NFH is extremely disorder. This is not surprising, since the C-terminus of NFH is known to take part in multiple protein-protein interactions with other cytoskeletal proteins, and disorder has been reported to be a feature of protein regions or entire proteins which undergo multiple protein-protein interactions.

These results indicate a high probability for Cap23 to take part in multiple (probably weak and/or transient) protein-protein interactions, as it has been suggested for hub proteins with a high degree of intrinsic disorder. This might also explain why we were unsuccessful in identifying direct interaction partners in immunoprecipitation experiments (not shown).

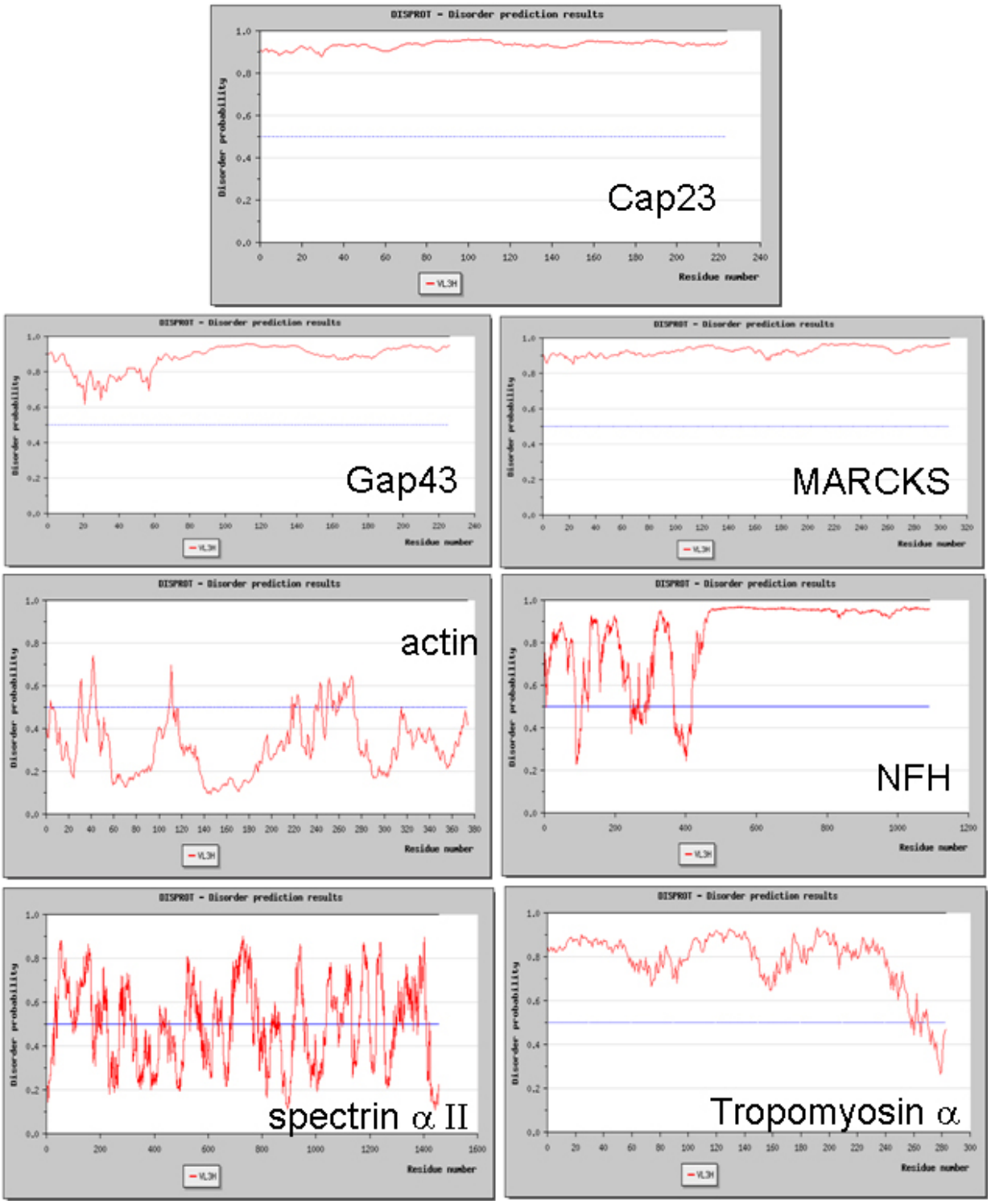

Figure 46. **Absence Amino Acid Anomalies of Cap23 Protein Sequence.**
The X-axis represents the N- to C-terminal protein length, on the Y-axis the degree of predicted intrinsic protein disorder is plotted. Actin is shown as an example of a protein containing extended regions of highly ordered motifs, whereas spectrin αII represents an example of short fragments of unordered versus ordered regions. Values near 0 indicate high order, values near 1.0 indicate high disorder.

3 DISCUSSION

3.1 Overview

The cytoskeleton in general has many diverse functions like establishing polarity of the cell, acting as transport tracks for various organelles, stabilizing the cellular morphology but also promoting regulated remodeling at certain sites including outgrowth and stabilization of fine processes emanating from its surface. Remodeling of the neuronal cytoskeleton is of special importance during major morphological changes, i.e. during initial process outgrowth at early developmental stages and during axonal regeneration following lesions in adult neurons. The latter critically depends on the expression of growth associated proteins like Cap23. The mechanisms by which Cap23 contributes to cytoskeletal remodeling and stability have so far been obscure. We were able to demonstrate in this work, that Cap23 regulates cytoskeletal plasticity in axons and dendrites by affecting the intermediate filament branch of the cytoskeleton as well as f-actin structures, respectively. Upon interference with Cap23 protein levels, cytoskeletal elements display reduced stability, which in consequence results in the reduced ability of mitochondrial docking, reduction of anterograde mitochondrial transport and reduced retention of mitochondria in distal axons.

3.2 The Actin Cytoskeleton Adopts Distinct Morphologies Dependent on Levels of Cap23

Studying dispersed hippocampal neurons cultured for several weeks *in vitro*, we could demonstrate that the actin cytoskeletal morphology is severely affected if Cap23 is reduced, absent or overabundant. A very obvious phenotype was dendritic spine morphogenesis. The shape of dendritic spines needs to be tightly regulated, as it is directly related to synaptic function in respect to Ca^{2+} compartmentalization and therefore the localized specificity of a synaptic signal. In the absence of Cap23, dendritic filopodia, which are the precursors of dendritic spines at their initial outgrowth phase, are not altered yet in their gross appearance. Around the time of intense synaptogenesis though,

when synaptic contacts and thus spines need to gain stability in order to become consolidated, Cap23 KO spines fail in this process and either produce amorphous, complex shapes, which exhibit increased actin-based motility, as we could demonstrate by live imaging, or they collapse onto the dendritic shaft, giving rise to abnormally large f-actin rich accumulations. Either way, these dendritic spines have obviously lost some form of regulatory function, which should promote their stabilization and influence their growth or shrinkage in a regulated manner.

With the use of transgenic mice overexpressing *Thy1*-Cap23, we could demonstrate that this spine morphogenesis phenotype is directly related to Cap23 levels, as this phenotype was not only rescued but even showed opposite morphogenic effects. These were obvious in the length of dendritic filopodia and spines, as well as in an increase of stability, as demonstrated by live imaging, as the motility of these structures was decreased as compared to Cap23 KO and even WT neurons.

We could further demonstrate that the effects of Cap23 on the actin cytoskeleton were broader than being restricted to f-actin-based outgrowth in the form of filopodia and spines. Specifically, the cortical actin cytoskeleton showed a very similar phenotype in the absence of Cap23, which was characterized by amorphous outgrowth and collapse of both short filopodial and lamellipodial structures.

We thus conclude that Cap23 stabilizes f-actin structures, when their maturation becomes critical.

3.3 Cap23 Organizes Lipid Rafts by Accumulation of Cholesterol

Cap23 is situated at the inner leaflet of the plasma membrane via a myristoylation anchor, which, besides electrostatic interactions with acidic phospholipids, renders Cap23 associated with lipid rafts. Various earlier studies have demonstrated that Cap23 exhibits cholesterol-binding ability, by which it concentrates cholesterol molecules into distinct domains on the plasma membrane, termed lipid rafts. These membrane domains exhibit unique functions including sustained signal transduction, which e.g. can affect regulation of the actin cytoskeleton to name just one among many other downstream effects.

The phosphoinositide $PI(4,5)P_2$ has a well established role in lipid raft-associated processes, like the regulation of actin polymerization. The phosphoinositides $PI(4,5)P_2$ and $PI(3,5)P_2$ are quite similar in structure, yet only $PI(4,5)P_2$ is sequestered by Cap23 (Epand et al., 2004). Thus, attracting $PI(4,5)P_2$ to cholesterol-rich rafts will likely affect local regulation of actin polymerization.

Since we have demonstrated, that cholesterol clusters are changed in size and frequency when Cap23 is absent, whereas their size and frequency increases upon Cap23 overexpression, we conclude that Cap23 directly affects lipid rafts by promoting the sequestration of cholesterol and $PI(4,5)P_2$ into aggregates on plasma membrane surface lipid rafts.

It is therefore also conceivable that actin regulation is affected by this phenomenon. This is supported by studies showing that interfering with lipid rafts by cholesterol depletion inhibits actin-based processes like T-cell migration (Gomez-Mouton et al., 2001). Since in the present work we see profound alterations on the level of actin dynamics and stability when we either increase or decrease Cap23 levels, we conclude that Cap23-modulated raft assembly is directly related with downstream regulation of the actin cytoskeleton.

3.4 Cap23 is Involved in Proper Organization of Intermediate Filament Assembly

Intermediate filaments support the actin cytoskeleton and are probably especially important in axonal stability, since axons, in contrast to dendrites are rich in IFs but comparably poor in f-actin structures. IF instability is observed in a plethora of neurodegenerative diseases, but in most cases, unambiguous causal relations to disease origins have not been determined conclusively.

In the present work we demonstrate that IF instability is one consequence of reduced lipid raft organization mediated by Cap23. Since a large part of mitochondria become immobilized near Cap23 accumulations and interactions between mitochondria and IFs have been observed as well, it is likely that Cap23 promotes the formation of a scaffolding complex, at which mitochondria and IFs can interact. As we have never observed that IFs fuse during motile states but fusion and in consequence formation of elongated IF-networks occurs during resting periods, it is very likely that immobilization of IFs at certain sites is a necessary prerequisite for the production of a stable IF-based axonal cytoskeleton. Even though f-actin levels in axons are relatively low compared to dendrites, the actin cytoskeleton is an important structural component of axons, since we could demonstrate that by weakening the actin network pharmacologically, axonal structures, especially neurofilaments become unstable and do not assemble properly, appearing in ring-like structures all along the axon. Even though they become upregulated upon such treatments, the remaining axonal cytoskeleton without a properly functioning f-actin network seems to fail to stabilize axonal elements.

It therefore follows that intermediate filaments critically depend on f-actin for assembling into the typical elongated filamentous network, which as such provides additional support of the axonal cytoskeleton.

Since we also observed that WT neurons with an experimentally induced weakened actin cytoskeleton partially resemble untreated Cap23 KO neurons, but treating KO neurons the same way results in an even more pronounced phenotype, it is likely that besides a proper actin network, an additional component is involved in IF assembly.

3.5 A Potential Role for the Exocyst in Cytoskeletal Organization

Due to its ability to promote raft assembly, Cap23 promotes the polarized concentration of certain components necessary for polarization of growth and remodeling. As one example, clustered Cap23 assists to confine domains of exocyst assembly, a lipid raft-associated complex (Sans et al., 2003; Riefler et al., 2003). The exocyst acts as an important landmark at the plasma membrane for the targeting of secretory vesicles, whose fusion at the surface directly results in membrane expansion and therefore cellular growth. Additional roles for the exocyst are being continuously revealed. So it has been shown in neurons, that the exocyst attracts vesicles carrying specialized cargoes like neurotransmitter receptors (Sans et al., 2003). Small GTPases of the Ral family have been reported to use the exocyst as an effector for signaling pathways leading to exocytosis (Wang et al., 2004), neurite branching (Lalli & Hall, 2005), membrane fusion events (Shipitsin et al., 2004) and even cell migration (Rosse et al., 2006). Furthermore, interactions between RalA and the exocyst complex have been shown to mediate Cdc42-dependent filopodia formation in fibroblasts (Sugihara et al., 2002), which renders Ral an attractive candidate molecule likely to coordinate cytoskeletal reorganization using the exocyst as an effector. RalB colocalization in Gap43-patches along neurites has also been reported (Lalli & Hall, 2005). These reports indicate the exocyst complex as a central effector of Ral-GTPases with potential regulatory function of the cytoskeleton.

In the present work we identified Cap23 and the exocyst complex to codistribute at distinct axonal patches. We could further demonstrate that this colocalization was not mere coincidence of two components being present at these sites independently from each others by finding that the confinement of exocyst structure critically depends on Cap23. It is thus to be expected that disruption of exocyst structure induced by the absence of Cap23-mediated confinement affects downstream signaling pathways of the exocyst, which might precipitate in improper organization of cytoskeletal structures, like the actin- or the intermediate filament network.

3.6 Cap23 plays an Important Role in Determining Docking Sites for Axonal Mitochondria

Mitochondrial localization is vital for various cellular functions involving cellular growth and thus is especially critical in regions of the cell which are remote from the site of mitochondrial biogenesis, which in neurons can be as distant as 1mm from the cell body. Nevertheless, neurons seem to be capable of perfectly orchestrating mitochondrial targeting to those sites. Energized mitochondria are attracted to and retained at sites of high energy demand, de-energized and damaged mitochondria are targeted towards the cell body for degradation and especially during neurite outgrowth, constant mitochondrial density is achieved by intercalated mitochondrial positioning by a yet unknown mechanism (Miller & Sheetz, 2006). All of these mechanisms face the challenge of how to get hold of a mitochondrion that is passing by at high speed and retaining it for as long as necessary. Thus, a docking mechanism has been postulated, but little is known about its nature. A recent study of Chada and Hollenbeck (2004) is so far the only one suggesting a potential mechanism. They demonstrated that stimulation by NGF caused axonal mitochondria to become immobilized at sites where NGF signaling was elicited and showed that the f-actin cytoskeleton is involved at least partially in mediating mitochondrial docking. Though several other studies have suggested that a cytoskeleton-associated component might be involved in mitochondrial docking, the identification of such a factor has not been reported so far.

In the present work, we identify Cap23 as an intrinsic determinant of mitochondrial docking in axons. We demonstrated that stably docked mitochondria are preferentially located at sites of Cap23 accumulation and that motile mitochondria have a high probability to stop near Cap23 clusters. In addition, we observed that Cap23 clusters are sites of transient mitochondrial pauses during extended excursions.

One would postulate that removal of a mitochondrial docking factor should decrease the frequency of observed mitochondrial pauses. This in consequence should result in longer excursion distances, concomitant with instability of mitochondria at sites of critical importance as e.g. the growth cone. We could demonstrate that all of these postulated

phenotypes can be observed in neurons lacking Cap23, thus unambiguously identifying Cap23 as a critical factor involved in regulating mitochondrial docking.

Since mitochondrial docking is vital for the proper functioning of neurons, it is very likely that evolution will have provided backup mechanisms for mitochondrial targeting, and since there still exist stable mitochondria in the absence of Cap23, these other mechanisms remain to be determined. It is clear though that the lack of Cap23 affects a very large fraction of mitochondria, since even though the majority of mitochondria do not travel for long distances in the absence of Cap23, many show oscillating behavior on a scale of a few μm.

We find the exocyst to adopt a similar shape as Cap23 clusters, and this confined shape is lost in the absence of Cap23, being much enlarged and irregular. In trying to explain this oscillation behavior, an appealing possibility is that the increased and irregular size of the exocyst is reflected in the increase of mitochondrial oscillation, if the exocyst as a downstream effector of Cap23 would be involved in mitochondrial docking. If mitochondria were connected to a scaffold involving the exocyst complex, one would expect that a less densely packed exocyst cluster could cause mitochondria to be less stably docked at a certain site and therefore oscillate around this region. Since our observations fit to these postulations, a role for the exocyst in mediating mitochondrial docking at Cap23-induced cholesterol clusters is an attractive model.

Mitochondrial bidirectional motility is well described, but little is know about its regulation. Sheetz and coworkers have proposed that $PI(4,5)P_2$-clustering could play a role in deciding, in which direction the mitochondrion will be transported (De Vos et al., 2003). They reported that by expressing PH-domains in N2A cells, which is thought to induce $PI(4,5)P_2$-clustering, anterograde mitochondrial transport is increased (De Vos et al., 2003). This effect was achieved by two independent $PI(4,5)P_2$-specific PH domains, implying that $PI(4,5)P_2$-clustering by various means can enhance anterograde mitochondrial translocation. Unfortunately, this study did not identify the location of $PI(4,5)P_2$ clustering, but suggested the mitochondrial membrane to be involved. In an alternative scenario, PH domains could concentrate $PI(4,5)P_2$ at the plasma membrane, where actually most of this phosphoinositide is found. Speculating that induction of lipid raft clusters at the surface could influence the affinity of mitochondria to docking

complexes near Cap23 clusters, stable docking of mitochondria would be promoted along the axon by this means. Since the largest fraction of mitochondria being transported retrogradely are of low transmembrane potential (Miller & Sheetz, 2004) and therefore incompetent in ATP-generation, an increased fraction of anterogradely transported mitochondria, as is the case upon expression of PH-domains would therefore represent the more competent fraction of mitochondria for energy supply.

In fact, we could observe a trend of mitochondria in the absence of Cap23 to have lower transmembrane potentials, using the transmembrane voltage-sensitive dye JC1. As also reported by Miller & Sheetz (2004), despite being able to obtain interesting observations about the energy level of mitochondria, this labelling technique bares inherent difficulties due to a strong tendency of this dye to bleaching, followed by rapid degradation of labeled neurons. We could thus not conclusively show that Cap23 KO mitochondria have lower energy levels, which might be a consequence of less stable docking, though this is still an interesting possibility and would be in agreement with above-mentioned arguments.

Taken together, PH-domain-induced raft clustering might increase stable docking of mitochondria, which could positively influence their energization level, and therefore have an impact on their bidirectional motile behavior.

3.7 Synopsis

In the present work, we identified the lipid raft-associated protein Cap23 to be involved in several important neuronal functions like the organization of lipid raft platforms, the maturation and stabilization of the actin- and intermediate filament cytoskeleton, and the formation of a scaffolding complex, which promotes mitochondrial docking.

Cap23 was so far considered to play an important role in promoting axonal elongation during initial neurite outgrowth and especially during axonal regeneration. This protein was studied in mouse models of different genetic backgrounds or dorsal root ganglions in lesion experiments, which mainly focussed on anatomical alterations at the synapse or on the axonal regenerative response and concomitant changes in gene expression patterns. The findings in these studies demonstrated that Cap23 acts as an important intrinsic determinant of cytoskeletal regulation and growth-related processes in axons. Yet, the manner by which Cap23 could modulate these processes remained largely obscure.

In the present work we took a closer look at diverse cell biological processes in cultured neurons, which bare the advantage that the development of single neurons and small networks can be easily studied at very high subcellular resolution using a combination of immunocytochemistry and live imaging. This approach thus enabled us to define cytoskeletal alterations caused by the absence of Cap23, besides providing a detailed study of mitochondrial trafficking in axons at high spatiotemporal resolution.

3.7.1 Proposed Model of Cap23 Involvement in Axonal Regeneration

Axonal growth depends on the concerted formation and remodelling of the cytoskeleton in order to form a neurite which is continuously stabilized behind the leading growth cone, which is responsible for pathfinding and thus the formation of proper connectivity to other neurons in the target region. This cytoskeletal remodelling has to permit f-actin-based outgrowth at a few defined locations, while restricting outgrowth along the major portion of the neuritic cortex. Cap23 is involved in this regulatory mechanism by restricting ectopic actin-outgrowth by stabilizing cortical actin structures. This might be achieved by the formation of lipid raft clusters of special composition, including and/or excluding certain actin-binding proteins. Cap23 promotes clustering of cholesterol and $PI(4,5)P_2$ into defined domains at the plasma membrane surface, which as we have shown, also includes the exocyst complex. Its shape and size is defined by Cap23, either directly by concentrating raft components, to which the exocyst is attached, or as a secondary effect by facilitating the formation of a raft-associated scaffolding complex.

Either way, a complex of such composition close to the plasma membrane surface acts as a docking signal for mitochondria, which are stably anchored at this site or become immobilized as they are passing by. The significance of this process for a growing neurite are obvious: Locations of high energy demand like developing synapses, growth cones and branch points critically depend on energy supply in the form of ATP by mitochondria. The presence of a mitochondrial docking signal at these sites would therefore prevent energy supply to become a limiting factor of growth processes.

Regenerating nerves need a lot of energy in order to grow over long distances, especially in less permissive substrates like lesioned regions, where glial scars and inhibitory molecules like Nogo-A impose big challenges on growing neurites. Speeding up axonal growth by providing growing regions with sufficient energy is therefore beneficial, though probably not essential.

However, increased mitochondrial retention in growing axons is a valid postulation and has been described in regenerating DRG neurites, where a redistribution of the mitochondrial pool into regenerating axons was observed (Dedov et al., 2000). Increased expression of Cap23 during axonal regeneration is therefore likely to facilitate

redistribution of mitochondria into regenerating axons as well as their retention, though this has not been addressed directly.

Certainly also of beneficial effect to growing and regenerating axons is the cytoskeletal stabilization via the intermediate filament system. Besides microtubules, the IFs form the largest fraction of cytoskeletal components, thus axonal integrity critically depends on a proper IF network. Cap23 ensures proper assembly of IFs, since in its absence, misassembled IF structures appear in the form of numerous ring-like structures in axons, which have been reported to appear in a wide range of neurodegenerative diseases, lesioned and aged neurons (Lee et al., 1996; Migheli et al., 1993; Vickers et al., 1996, respectively). Spheroid IF structures are thus characteristic for axonal instability of multiple origins.

IF stability is achieved by lateral and longitudinal alignment and subsequent fusion of individual protofilaments into a longer network (Herrmann & Aebi, 1998). We observed fusion events taking place during immobilization of at least one fusion partner, but never of motile filaments, and therefore suggest that IF fusion requires a certain degree of immobility or docking. Docking of NFs is reduced in the absence of Cap23, and we propose that this results in inefficient maturation of the NF network, as we also observe shorter stable filaments in Cap23 KO neurons. It follows that such an immature-like IF cytoskeleton would be disadvantageous to neurons with an already affected actin cytoskeleton, as is the case in the absence of Cap23, whereas overexpression of Cap23, which leads to upregulation of neurofilaments, would probably enhance axonal cytoskeletal stability.

Taken together, these newly identified functions of Cap23 might promote axonal regeneration by stabilizing both the actin and the intermediate filament cytoskeleton, as well as facilitating mitochondrial redistribution into regenerating axons, retention at various axonal sites with high energy demand and thus fueling the regeneration process.

3.8 Outlook

Having elucidated several processes in which Cap23 is involved, morel open questions remain. One interesting question would be to study mitochondrial trafficking in DRG neurons upon challenging them with a preconditioning lesion of the peripheral branch, since this has been shown to trigger a regenerative response of a subsequent central branch lesion. One might expect a higher density of cholesterol-rich and Sec6 positive clusters, and a redistribution of mitochondria towards the regenerating branch, since Cap23 upregulation in these settings has been shown (Bomze et al., 2001). Likewise, DRG neurons of mice double transgenic for Cap23 and Gap43, which have been demonstrated to regenerate the central axon without the need of a peripheral preconditioning lesion and the concomitant change in gene expression (Bomze et al., 2001), might display elevated mitochondrial retention and higher levels of Sec6-containing exocyst as compared to WT DRGs.

Following a similar logic, it might be worth analyzing crossbreedings between *Thy1*-Cap23 mice and various mouse models for neurodegenerative diseases like ALS (amyotrophic lateral sclerosis), which show similar IF spheroid appearance like Cap23 KO mice, since overexpression of Cap23 could have a positive effect on docking of IFs in these cases, and therefore might ameliorate defects in IF assembly, which are known hallmarks of disease.

Improving the assessment of mitochondrial transmembrane potential in Cap23KO neurons might confirm our preliminary observations that less stably docked mitochondria tend to be less effective generators of ATP and might likewise confirm our hypothesis that the causal origin of mitochondrial proteins we find upregulated in Cap23 KO mice is the consequence of improper mitochondrial function. In such a case it should be examined if diseases known to involve mitochondrial dysfunction such as Alzheimer’s disease, Parkinson’s disease or ALS could be alleviated by attempting to increase mitochondrial stability by targeted expression of Cap23 in affected neurons.

Mitochondria are also needed to fuel synapses and have been observed both pre- and postsynaptically (Chang et al., 2006). It might be of interest, whether synapses in neurons lacking Cap23 contain fewer mitochondria, or whether dendritic subsynaptic mitochondria

display similar increases in length as the axonal pool we had focussed on, which would probably indicate a homeostatic response, resulting in wider distribution of energy supply in presence of fewer (stable) mitochondria.

Since in the present work we found the exocyst member Sec6 to be almost exclusively localized to axons, but dendritic exocyst localization has been reported (Sans et al., 2003), it would be interesting to identify postsynaptic markers of exocyst and thus to examine, whether the axonal and dendritic composition of the multimeric exocyst complex differ from each others.

Since we observed that most mitochondria are stably docked, but sometimes in ca. 1μm distance from Cap23 clusters, it remains to be identified which direct interaction mediates physical docking. It is possible, that a component of the exocyst complex itself mediates binding to mitochondria, so one could probe for other known members of the exocyst and assess by RNAi, if downregulation of single components leads to a reduction of mitochondrial docking, though interference with other exocyst functions might complicate this analysis.

An unambiguous identification of the mitochondrial docking machinery on a molecular basis would definitely be of much help in understanding the complex behavior of regulated bidirectional mitochondrial motility and may perhaps even be of interest as a candidate for pharmaceutical industries in developing new targets for neurodegenerative disease in which mitochondrial instability is involved.

4 EXPERIMENTAL PROCEDURES

4.1 Astroglial cultures

Usually, brains from 10-12 newborn Wistar rat pups were dissected in cold HBSS and the meninges and white matter removed. Hemispheres were cut into small pieces before trypsinizing them in 13ml trypsinizing solution (warm HBSS containing final conc: 0.07% DNAseI, 0.19% Trypsin) for 10 mins at 37°C, continuously shaking the Falcon tube. After pipetting the trypsinized tissue 10 times, incubation at 37°C was continued for another 5 mins. Then, the cells were poured through a Nitex screen in order to get rid of large tissue debris. After centrifugation at 800 rpm for 7 min, cells were resuspended in Glia-MEM and 7.5x106 cells were plated into one T75 Primaria tissue culture flask. Flasks were shaken vigorously every 2nd day in order to separate strongly adherent glia from nonglial cells, which would lift off easily, and new medium was added. After 8-10 days in culture, a confluent monolayer of pure astrocytes was obtained, which was trypsinized, washed and aliquoted (2 aliquots per confluent flask) in freezing medium (10% DMSO, 20% horse serum in MEM). After freezing the aliquots overnight at -70°C, they were transferred to liquid nitrogen and 1 vial was thawed 8 days before hippocampal neurons were prepared. To this end, 12-well plates were coated with Poly-L-Lysine (100ug/ml) overnight and washed extensively with ddH2O before thawed glia were set out (1 aliquot/4-8 x 12-well plates). After the first and at every 2nd day, glia were fed by removing all medium and replenishing with fresh Glia-MEM. On the day before adding hippocampal neurons (optimal: 16h earlier), when cells had grown into a confluent monolayer, all medium was removed and 1.5ml HC-MEM was added per well in order to equilibrate the medium.

4.2 Preparation of Coverslips

Coverslips were placed in a custom-built Teflon rack accommodating 80 coverslips. They were immersed in concentrated nitric acid for 48 h and then washed with ddH2O 2 x 1h and with 70% EtOH for 30-45 mins. Then, they were air-dried in a laminar hood and

baked in an oven at 180°C for 5 h. After cooling down, ca. 0.5 mm thick paraffin-dots were applied to coverslips, which served as spacers between neurons and glia, which were sitting at the bottom of the 12-well plate. After application of ca. 75ul Poly-L-Lysine (1mg/ml) per coverslip, coating was allowed to take place overnight in the incubator. The next morning, coverslips were washed extensively with ddH2O in a bacterial dish and then 10ml of CS-MEM were added, to prepare for neuronal plating.

4.3 Dissociated Hippocampal Neurons Cultures

Hippocampal neuron cultures were prepared using a protocol developed by Gary Banker, with modifications.

Briefly, mice ranging from E16.5 to P0 were decapitated and the hippocampi were dissected in cold HBSS. Hippocampi from individual mice were treated separately and trypsinized (0.125% final conc.) at 37°C for 15 mins. After washing twice with cold HBSS, hippocampi were triturated 10 times using a fire-polished glass Pasteur pipette. 400.000 cells were plated into a bacterial dish containing 12 13mm glass coverslips in 10ml CS-MEM. After attachment of neurons for 2-4h, coverslips with adherent neurons were flipped into wells of a 12-well plate, containing a confluent glia feeder layer and 1.5ml MEM-N2.1. Two to three days later, 500ul MEM-N2.1 containing 20uM AraC were added to each well to further suppress glial growth. Feeding was performed every 3-4 days by exchanging a third of the medium.

4.4 Transfection Of Hippocampal Neurons

500 ul of medium were transferred to a new well of a 12-well plate, and the coverslip placed inside, cell-side facing up. 4ug DNA/coverslip were mixed with 6.1ul 2M CaCl2 and water in a total volume of 25ul, then 25ul of 2xBBS were added dropwise to the DNA solution during slow vortexing. The transfection solution was thereafter dropwise added to the well containing the coverslip to be transfected and put back to the incubator for 15-30 mins. Formation of the calciumphosphate-precipitate was followed under the microscope,

until a precipitate of medium size had formed. At this point, the coverslip was flipped cell-side down into a new well containing prewarmed MEM-N2.1 medium for 2x10min in order to wash away excess precipitate. After that, the coverslip was returned to its home well and incubated further until used for imaging.

4.5 Live Imaging

The coverslip to be imaged was put into a “Ludin Chamber Type 1” (LIFE IMAGING SERVICES), and 1ml of prewarmed MEM-N2.1 containing glutamate but no phenol red, was added immediately. The imaging chamber was inserted into the according chamber holding inset at the Zeiss “LongRUN” microscope (Till5, Axiovert200M), equipped with a temperature- and CO2-control chamber. Temperature was kept at 37°C, and CO2 flow was set to 5% at 8 liters/min. In order to be able to re-identify imaged cells after fixation and staining, X,Y and Z coordinates were recorded after calibrating the Zero-point to a fixed reference point.

The light intensity was minimized in order to prevent phototoxicity and bleaching, which was usually a value of 20% of maximum intensity. Exposure times were chosen as short as possible, which varied according to the fluorophore used between 100-700ms.

For timelapse recordings, images were usually acquired every 5 s, for a total duration of 5 mins. A 63x magnification lense of 1.4 numerical aperture was used.

Images were aquired with Metamorph 6.2r5, and resulting stack files were deconvolved using Huygens. Typical deconvolution settings were: signal/noise ratio = 5, removal of background = 5%, 10 iterations. “8 bit tiff” was chosen as output file format.

4.6 Immunocytochemistry

Neurons were fixed with either cold MeOH for 5 mins at -20°C (for IF/NFs and most synaptic proteins) or with “Cap-PFA” (see 4.11) for 10-30 mins at RT. After washing with PBS, primary antibodies were diluted in Antibody Solution and incubation was done

overnight. Secondary antibodies were added in Antibody Solution and added for 45 min – 1h at RT after washing again in PBS. Coverslips were embedded in Airvol on glass slides. To label cholesterol, filipin (Fluka) was prepared freshly at 10mg/ml in cold MeOH and added to secondary antibodies at 1:500. To label actin, Alexa488-phalloidin was added to secondary antibodies at 2 units/ml.

4.7 Proteomics

Mouse brains were rapidly dissected and homogenized in cold lysis buffer using a glass homogenizer. Postnuclear supernatants were obtained by 10 min centrifugation at 14.000 rpm at 4°C. Samples were analyzed by the FMI Proteomics Facility using 2D-Gelelectrophoresis, Coomassie- or DIGE staining of gels and sequencing of excised spots by mass spectrometry or MaldiTOF.

4.8 Microarray analysis

Mouse brains were rapidly dissected and cut into small pieces, before immersing them in RNAlater (Qiagen) for RNA stabilization and storage until all samples could be processed in parallel. RNAlater was then disposed of, and RNA was purified according to the RNeasy Midi Kit (Qiagen). RNA concentration was measured using the Nanodrop spectrometer, and precipitated in order to increase the concentration. 2ug RNA were used for hybridization of an Affimetrix gene chip (GeneChip® Mouse Genome 430 2.0 Array), and results were analyzed using Expressionist Pro 3.1.2 software.

4.9 Quantitative real-time PCR

Mouse brains were rapidly dissected and digested overnight in tail lysis buffer at 55°C. DNA was purified according to standard procedures and cleaned up with chloroform in a

second step. DNA concentration was quantified using the Nanodrop spectrometer and dilutions prepared at a concentration of 10ng/ul. In order to quantify mitochondrial DNA, the following primer sets were used:

mito antisense1: AGTCCTCCTCATGCCCCTAT
mito sense1: AAGGGATCCCACTGCACATA
mito antisense2: CCTATGTGGGCAATTGATGA
mito sense2: AGGGATCCCACTGCACATAG

In order to normalize mitochondrial DNA to 28S DNA, the following primer sets were used:

28S antisense1: CTTGGGCGGATTCTGACTTA
28S sense1: GGGTTTAGACCGTCGTGAGA
28S antisense2: AACCTGTCTCACGACCGTCT
28S sense2: TGGGTTTTAAGCAGGAGGTG

Q-RT-PCR was performed using an ABI Prism 7000 system, using saved default settings.

4.10 Embedding for Electron microscopy

Coverslips with hippocampal neurons were fixed for 10 min at 37°C 3.5% glutaraldehyde, diluted in medium. Postfixation was performed for 30 min in 3.5% glutaraldehyde containing 1% osmium tetraoxide at RT in the dark. Coverslips were then rinsed in phosphate buffer for 5 min and further incubated for 30 min in 1% osmium tetraoxide and 1.5% potassium hexacyanoferrat (III) in the dark at RT. After rinsing with 3.6% NaCl twice for 5 min and with ddH2O twice for 5 min, coverslips were stained with 5% uranyl acetate (in ddH2O) for 1h. Then, they were rinsed with ddH2O three times for 5 min and then dehydrated in acetone (50, 70, 95 and 100% 5 min each). Finally, dehydration was repeated with 100% acetone 3 times for 10 min. Coverslips were then transferred to a 1:1 mixture of acetone and resin I at RT for 15 min followed by incubation in a 3:1 mixture of propylene oxide and resin I for 15 min, followed by incubation in a 1:1 mixture of propylene oxide and resin I for 15 min, followed by incubation in a 1:3 mixture of propylene oxide and resin I for 15 min. After that, coverslips were submerged in resin I for 30 min at 37°C, then in resin II for 15 min at 60°C, and then mounted onto resin II-filled

pads and put at 60°C for 48 h in order to let the resin polymerize. Material was sectioned with a diamond knife. 75-85 nm thick sections were deposited on slot grid coat with formvar. The sections were stained with 9% Iso-butanal (v/v) for 10 min and lead citrate for 12 min. Images were aquired with a Zizze EM900 at 100kv on Kodak electron image plates.

4.11 Media

HBSS (Ca^{2+}-Mg^{2+}-free)

20.0 ml	10x Hanks's BSS (Ca^{2+}-Mg^{2+}-free)
2.0 ml	1M HEPES pH 7.3
178.0 ml	sterile H2O

GLIAL MEM

500 ml	MEM with GlutaMAX I
5 ml	Penicillin/Streptomycin
6.7ml	45% (w/v) glucose
50 ml	horse serum (heat inactivated)

CS-MEM (for attachment of neurons to coverslips)

500 ml	MEM
6.7ml	45% (w/v) glucose
50 ml	horse serum

HC-MEM

45 ml	1x MEM with GlutaMAX I
5 ml	N2.1

N2.1

13.5 ml	glucose 45%	Sigma G-8769
5 ml	Albumax 20%	Invitrogen 11020-021
10 ml	ITS-A Supplement	Invitrogen 51300-044
1 ml	putrescine (16.1mg/ml)	Sigma P-5780
1 ml	progesterone (6.3ug/ml)	Sigma P-8783
69.5 ml	MEM with GlutaMAX I	Invitrogen

Cap-PFA (for staining of Cap23, actin etc.)

PFA 4%: freshly dissolved in H_2O, containing:

Sucrose	50 mM,
$NaPO_4$	10 mM
$CaCl_2$	0.4 mM

Lysis buffer

Tris pH 7.4	25mM
NaCl	0.15M
EDTA	5mM
TX-114	1%
Protease Inhibitor cocktail	
PMSF	40ug/ml

Tail lysis buffer

Tris pH 8.0	50mM
NaCl	36mM
EDTA	100mM
SDS	1%
ProteinaseK	30ug/ml

Antibody Solution

Glycine	50mM
FCS	1%

BSA	0.1%		
Saponin	0.2%		
dissolved in PIP_2-PBS:		NaCl	137mM
		KCl	2.7mM
		Na_2HPO_4	8mM
		KH_2PO_4	1.5mM

4.12 Labeling of mitochondria in living neurons

Mitochondria were labeled by adding Mitotracker (CM-H2TMRos; M7511, Invitrogen) to coverslip-containing wells (1 □M final conc.) for 5-10 mins and then washed with warm imaging solution before mounting the coverslip into the imaging chamber. Time-lapse recordings of mitochondrial motility were performed as described in Live Imaging (see 4.5).

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7 # CURRICULUM VITAE

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