Functional Characterisation of the Muscle Giant Protein Obscurin

Dissertation

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass ich diese Arbeit selbständig und nur mit den angegebenen Hilfsmitteln angefertigt habe.

Dortmund, Iuni 2004

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1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Movement is a feature all living being share throughout the evolutionary scale from the simplest unicellular organisms to the highly evolved mammals equipped with many types of muscle tissues. Intracellular protein motion processes can lead to movement at the level of the entire organism during muscle contraction. At the basis of this process is a universal mechanism that employs chemical energy for the generation of mechanical force. In muscle, the cellular pool of adenosine tri-phosphate (ATP) constitutes the energy reservoir while the proteins able to use it and produce mechanical force are myosin in interaction with actin. They are organized in thick and thin filaments, respectively, arranged in a highly regular manner inside the muscle cell that allows their sliding past each other during contraction. The description of the mechanism of contraction or its regulation pathways will not be referred to here, as they might have no direct and strong connection with this work, as suggested by our data at this time. Instead, the organization of muscle and sarcomeres, myofibrillogenesis, as well as GTP-binding protein signalling pathways and cytoskeletal aspects of the muscle cells will be discussed.

1.1.1 Muscle organization: different types of muscle

Mammals have 3 types of muscle tissues developed to suit their needs: skeletal, cardiac and smooth muscle (Cormack, 1987; Alberts et al., 2002). In all types of muscle cells, the contractile apparatus is based on actin and myosin but its organisation as well as the arrangement of the muscle cells themselves differs, reflecting divergent functions.

Skeletal muscle. All skeletal muscles of the body are of mesodermal origin and with the exception of some head muscles, are derived from somites (Bellairs et al., 1986). In the mouse embryo the somites appear at about day 8 and further differentiate to generate the dermomyotome and the sclerotome, which later contribute mainly to skin and muscle, and skeletal structures, respectively (Buckingham, 1992). Quiescent mononucleated precursor cells become determined as myoblasts and migrate from dermomyotome, giving rise to 1) the pre-muscle masses in the limb buds - from, which the limb muscles will derive - and to 2) the myotome in the central region of the somite - from, which the body muscles develop - (Kaehn et al., 1988; Buckingham, 1992). After a period of proliferation these myoblasts fuse together to form multinucleated young muscle cells called multinucleated syncytia or

myotubes (reviewed by Abmayr et al., 2003). These myotubes then undergo further differentiation and (when innervated) mature to form fully functional muscle fibres (ca. 50μm in diameter and several centimetres in length) (**Figure 1-1 A, B**). Muscle cells, like neurons and cells of the eye lenses, are arrested in G0 phase of the cell cycle, and are not dividing anymore in adult organisms. They are called postmitotic cells. Muscle tissues can be though renewed (e.g. in case of injuries) by the recruitment of a subpopulation of myogenic stem cells, which can divide and differentiate into normal muscle cells in adult organisms as response to muscle damage (Buckingham et al., 2003).

The cytoplasm of a muscle fibre is comprised of myofibrils, which represent linear arrays of adjacent sarcomeres linked by rigid structures called Z-disks. The sarcomeres represent the smallest contractile units of both skeletal and cardiac muscle and are able to contract simultaneously under electrical nerve impulses that lead to Ca^{2+} release from the sarcoplasmic reticulum. This regular arrangement of sarcomeres gives these muscles their striated appearance under the microscope (**Figure 1-1 C**). The neighbouring Z-bands of the sarcomeres are connected through desmin/vimentin intermediate filaments to form aligned myofibrils across the width of the cells (Granger and Lazarides, 1979). The structure of the sarcomere is presented in **Figure 1-2** and discussed below.

Each myofibril (typically 1-2 μm in diameter) is surrounded by sarcoplasmic reticulum (SR) and by invaginations of sarcolemma, called T-tubules that transport the nerve impulses to the myofibrils and SR (**Figure 1-1 D**). Like most cell types, muscle cells possess an intracellular network of Golgi and ER membranes, but they in addition developed a specialised compartment, the sarcoplasmic reticulum (SR), which is responsible for Ca^{2+} releasing and up taking during contraction and relaxation, respectively. The SR derives from ER but differs from it, as specific markers are occupying their lumen (Fleischer and Inui, 1989; Sitia and Meldolesi, 1992). Therefore an interesting functional difference from other cell types is that the striated muscle fibres contain both ER and SR as linked but individual compartments (Volpe et al., 1992; Sitia and Meldolesi, 1992).

Cardiac muscle. Formation of cardiac muscle begins soon after gastrulation with the commitment of precursor cells from the anterior lateral plate mesoderm to the cardiac fate (De Haan, 1965; Olson and Srivastava, 1996; reviewed by Mohun and Sparrow, 1999). These precursor cells form bilaterally symmetrical structures that develop into the parallel cardiac primordia. Fusion of the cardiac primordia at the midline gives rise to the

primitive cardiac tube by embryonic day 7.5 in mice. This straight tubular heart soon initiates rhythmic contractions and undergoes rightward looping, which is followed by chamber formation (De Haan, 1965; Olson and Srivastava, 1996). Cardiac muscle is also a striated muscle, containing sarcomeres arranged in myofibrils that are very similar to those in skeletal muscle. However, cardiac myoblasts do not fuse to form multinucleated cells and instead are connected by intercalated disks, which join the sarcomeres in one cell to those in a neighbouring cell (Fawcett, 1986) and connect the cells mechanically and electrically.

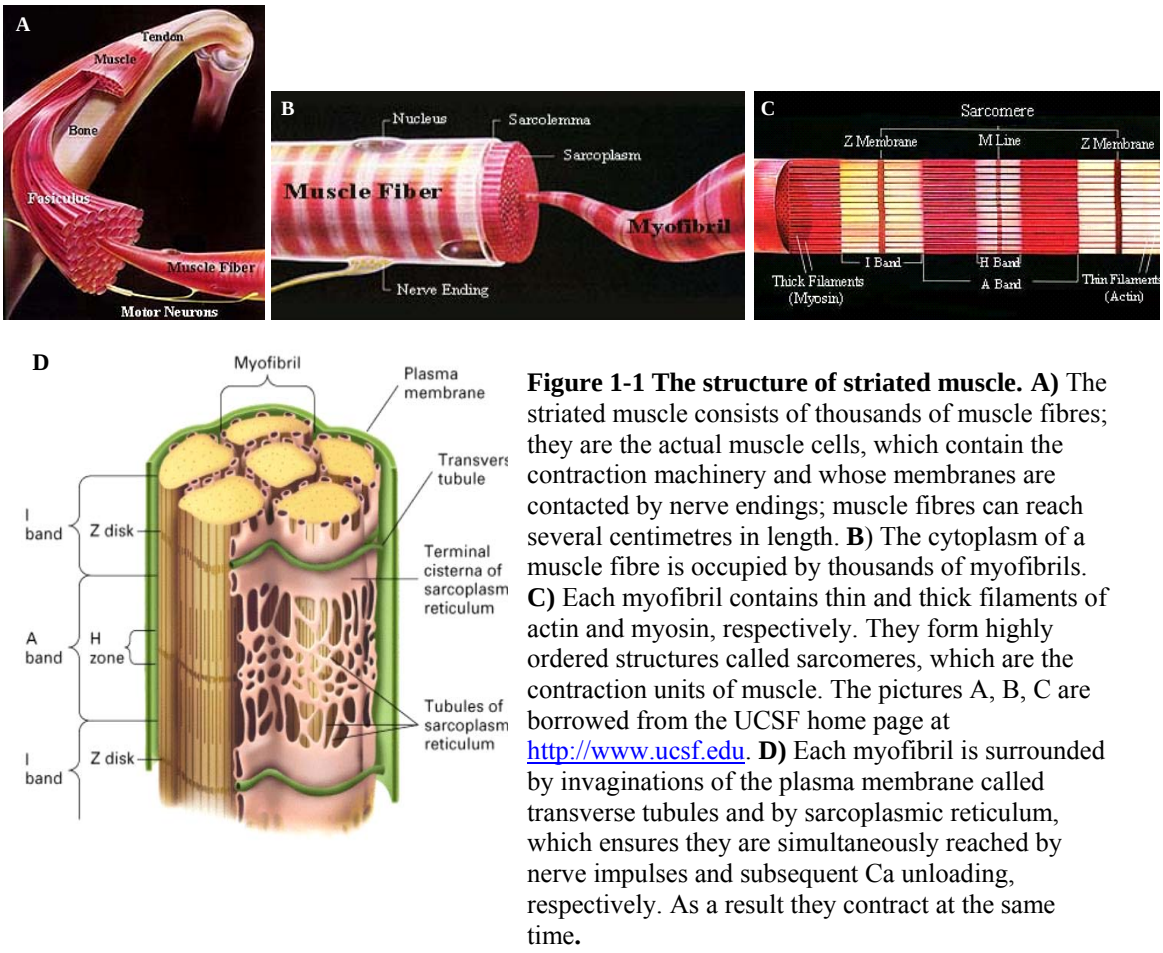

Figure 1-1 The structure of striated muscle. A) The striated muscle consists of thousands of muscle fibres; they are the actual muscle cells, which contain the contraction machinery and whose membranes are contacted by nerve endings; muscle fibres can reach several centimetres in length. **B**) The cytoplasm of a muscle fibre is occupied by thousands of myofibrils. **C)** Each myofibril contains thin and thick filaments of actin and myosin, respectively. They form highly ordered structures called sarcomeres, which are the contraction units of muscle. The pictures A, B, C are borrowed from the UCSF home page at http://www.ucsf.edu. **D)** Each myofibril is surrounded by invaginations of the plasma membrane called transverse tubules and by sarcoplasmic reticulum, which ensures they are simultaneously reached by nerve impulses and subsequent Ca unloading, respectively. As a result they contract at the same time**.**

Smooth muscle. Smooth muscle is derived from the lateral mesoderm and consists of elongated and spindle shaped mono-nucleated cells (Cormack, 1987). In these cells actin and myosin filaments are not found in ordered sarcomeric structures bur rather are loosely arranged along the long axis of the cell. Smooth muscle is so called because it lacks the striations of cardiac and skeletal muscle. This arrangement of actin filaments does not allow for rapid contraction but permits a greater degree of shortening than in striated or

cardiac muscle. Smooth muscle forms, for example, the contractile portion of the stomach, intestine and uterus, the walls of arteries and other tissues characterised by slow sustained contractions (Alberts et al., 2002).

1.1.2 The structure of the sarcomere

The striated aspect of skeletal and cardiac muscle arises from alternating ordered arrays of actin and myosin filaments (Amos, 1985). In polarised light (electron micrographs), actin thin filaments appear as light bands called I-bands (from their isotropic appearance) while myosin thick filaments appear as dark bands called A-bands (from their anisotropic appearance). Actin filaments of opposite orientation meet in the middle of the I-band in a structure called Z-disk or Z-line (from the German "zwischenlinie"). The centre of the A-band is called M-band or M-line (mittellinie). The sarcomere is defined as the contractile region found between two immobile Z-disks in skeletal and cardiac muscle (Squire, 1981). The length of a mature sarcomere can be between under 2 to 3 μm depending on its passive extension (Amos, 1985).

Myosin filaments of opposite orientation are anchored in the M-line by proteins like M-protein, myomesin and titin (Obermann et al., 1997) (**Figure 1-2**). The region in the middle of the A-band in which the myosin filaments have no heads is called H zone or bare zone. At the extremities of the A-band, the myosin filaments overlap with and contact the actin filaments. Along this distance the thick filaments are decorated by regularly spaced myosin binding protein C (MyBP-C).

The thin filaments are associated over their entire length with the regulatory protein tropomyosin and troponin complex, which are able to regulate in a Ca^{2+}-dependent manner the contact between actin and myosin filaments during contraction. Tropomyosin is an α-helical protein that spans 7 actin monomers and has one troponin complex attached (TnI, TnT, and TnC).

A key molecule in maintaining the highly ordered structure of the sarcomere is titin, the largest protein discovered so far (Maruyama et al., 1977a; Maruyama et al., 1977b; Wang et al., 1979; Labeit and Kolmerer, 1995). Titin has a molecular weight of about 3 MDa and stretches over 1μm from the Z-disk to the M-band being often refered to as the third filament system of muscle (**Figure 1-2**). Titin is in a unique position to relay spatial information during sarcomere assembly because of its size and position in the sarcomere (Gautel et al., 1999).

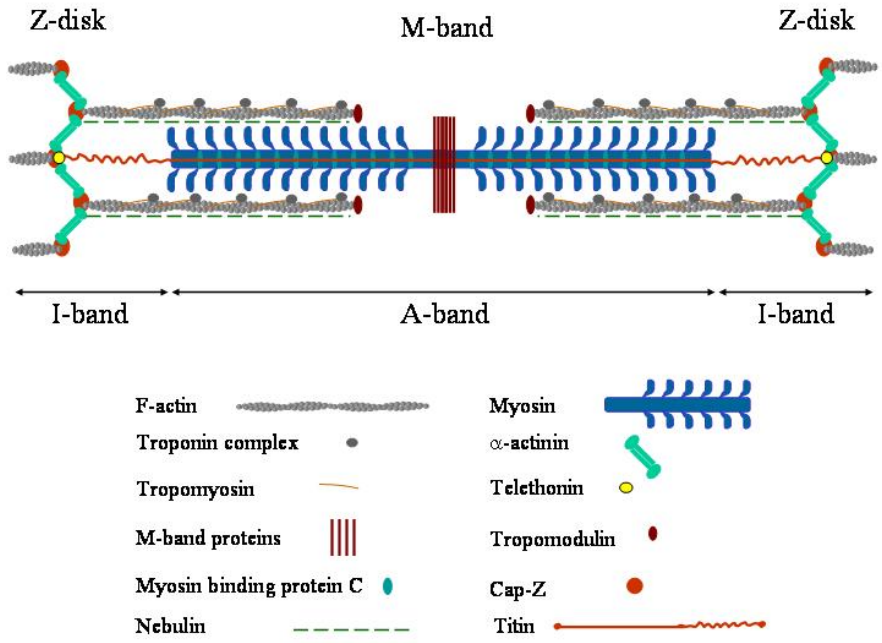

Figure 1-2 The structure of the sarcomere. The myosin region is called the A-band and the actin filament region is called the I-band. Myosin filaments of opposite orientation meet in the M-band while anti-parallel actin filaments arranged with their pointed ends towards the middle of the sarcomere are cross-linked in the Z-disk, where their barbed ends are inserted. The region between two Z-disks is defined as a sarcomere. The localisation of some sarcomeric proteins is schematically represented.

1.2 MYOFIBRILLOGENESIS

The functional achievement of muscle is due to the highly regular spatial organization of the thin and thick filaments into sarcomeres able to contract by the sliding filament mechanism. Myofibrillogenesis is the process of spatial organization of the contractile and structural muscle proteins into functional sarcomeres and myofibrils. Although much has been learned about this process, its initiation, intermediate steps and control mechanisms are to date still incompletely understood.

During embryogenesis, myofibrillogenesis takes approximately one week in skeletal muscle (Fischman, 1986) while a beating heart needs only a few hours to mature (Tokuyasu and Maher, 1987). Therefore different mechanisms of myofibrillogenesis might be employed during cardiac muscle development. This is also why, sometimes, intermediate stages can be seen more clearly in the not so fast skeletal myogenesis. Furthermore, there appear to be distinct differences between the *in vivo* situation in developing muscles and cultured myocytes (discussed in the end of this Section). For

instance, non-muscle myosin-II is found in premyofibrils in cultured cardiomyocytes but this intermediate is not observed *in vivo* (Ehler et al., 1999).

In skeletal muscle, the first sign of myogenesis is the expression of desmin in myoblasts, which are still proliferating (Gard and Lazarides, 1980; Furst et al., 1988). Most other muscle-specific proteins only start to appear in postmitotic myoblasts (Grove et al., 1985; Hill et al., 1986). The biochemical appearance of a protein does not necessarily correspond to its assembly into or its attachment to myofibrils. Desmin, for example, as one of the first muscle protein expressed is also one of the last proteins to attach to myofibrils (Hill et al., 1986). Myosin shows a similar fate (van der Ven et al., 1999). Myofibrillogenesis has been studied in cultured cells derived from embryonic tissue as well as in developing embryos. As a result, several models have been proposed to describe the expression sequence of muscle proteins and the order of incorporation into striated muscle myofibrils.

1.2.1 Stress fibre-like structures act as a scaffold in myofibrillogenesis

This model proposes that myofibrils are assembled in close association with stress fibre-like structures (SFLS). In cultured chick cardiomyocytes, Dlugosz et al (1984) observed for the first time thick non striated actin bundles, which were called SFLS. They appear early in myofibrillogenesis and disappear completely from mature myofibrils, a phenomenon that suggests them to act as a scaffold during sarcomere assembly. Later, the SFLS were also detected in other cultured cells types, e.g. rat skeletal (Handel et al., 1989) or human skeletal (Handel et al., 1989; van der Ven et al., 1993) myogenic cells. It is not clear whether similar non-striated actin filament structures serve as template for myofibril assembly *in vivo*.

Titin is the first sarcomeric protein expressed in avian (Tokuyasu and Maher, 1987; Colley et al., 1990) as well as in mammalian myofibrillogenesis (Furst et al., 1989; Schaart et al., 1989; Handel et al., 1989; van der Ven et al., 1992). In human skeletal myoblasts, titin is first detected as dots (sometimes named aggregates) initially associated with actin filaments in a specific manner (Sanger et al., 2000). At this early stage, microtubules are already found to closely follow the tracks of actin and the intermediate filament protein desmin (van der Ven et al., 1993), and later will appear parallel to the nascent myofibrils.

Aggregated titin molecules, initially located in close proximity to the nucleus (van der Ven et al., 1992), are targeted to α-actinin/α-actin decorated SFLS that are localized

closely to the sarcolemma (these new complexes are also named I-Z-I complexes). Titin gradually unwinds after association with the SFLS, thus forming a scaffold for the integration or remodelling of other sarcomeric proteins. The SFLS association is thought to be crucial for titin unwinding**.** In mouse skeletal (Furst et al., 1989) as well as both cardiac (Schultheiss et al., 1990) and skeletal (Soeno et al., 1999) chicken myogenesis first the Z-disk region (N terminus) of titin becomes organized and then the M-line end (C-terminus).

Concomitantly with titin unwinding the sarcomeric proteins myomesin and then C-protein (MyBP-C) form a cytoskeletal scaffold on SFLS (van der Loop et al., 1996; van der Ven et al., 1999) in human skeletal muscle. Immediately, and only after this regularly arranged scaffold gets completed, the sarcomeric myosin heavy chain (MHC) pattern becomes periodic, characteristic for mature myofibrils. It seems therefore that the correct assembly of a cytoskeletal scaffold is a prerequisite for correct thick filament formation and for the integration of the contractile apparatus into the myofibril (van der Ven et al., 1999). Thick filaments were observed to assemble separately in myofibrillogenesis and only integrate at the right moment into myofibrils in cultured cardiomyocytes (Schultheiss et al., 1990)**,** and in situ (Hiruma and Hirakow, 1985). Evidence for this phenomenon is also provided by experiments with isolated myosin molecules that proved to form bipolar filaments even *in vitro* (Goldfine et al., 1991) but failed to assembly proper A-bands in the absence of further organising proteins.

The sarcomeric actin filaments seem to assemble independently from the myosin filaments and chronologically before them in chick skeletal myogenic cells (Komiyama et al., 1992; Nwe et al., 1999). Again, a scaffold protein seems to be necessary. The giant actin binding protein nebulin displays striated pattern sooner than actin filaments. This indicates that nebulin plays no role in the initiation of I-Z-I complexes on SFLS or in the subsequent steps of myofibril maturation.

Desmin is among the last proteins to become organized in striated pattern and laterally links mature myofibrils mainly at the Z-disk level.

1.2.2 Premyofibril Model

More recently, another model for myofibrillogenesis was proposed by Rhee et al. (1994) reviewed by Sanger et al. (2000). In cultured cardiomyocytes, three types of myofibrils were detected occupying specific regions in the cell: 1) premyofibrils that are closest to the cell periphery, 2) fully formed mature myofibrils that are centrally

positioned, and 3) nascent myofibrils, containing most sarcomeric proteins but lacking their precise arrangement in striations. These are spatially arranged between the premyofibrils and the mature myofibrils (**Figure 1-3**). A similar myofibrillogenesis model is believed to be true for skeletal muscle cells as well (Sanger et al., 2002).

The premyofibril is characterized by mini-sarcomeres composed of alpha-actinin (Z-bodies) and actin filaments held together by short non-muscle myosin IIB filaments. Premyofibrils lack titin. They are believed to be transformed into nascent myofibrils by their capture of titin and muscle-specific myosin II filaments aligned in non-periodic arrays. Further, nascent myofibrils are thought to transform into mature myofibrils by the loss of non-muscle myosin IIB, the fusion of the Z-bodies into Z-bands, and the periodic alignment of muscle myosin II filaments into A-bands (LoRusso et al., 1997). The distance between the Z-bodies increases to reach the distance normally observed in the sarcomere. The role of non-muscle myosin IIB seems to be important, at least in cultured cells, as inhibitors preventing its assembly into filaments led to lack of mature myofibrils in Xenopus skeletal myocytes (Ferrari et al., 1998). However, the inhibitors used in these studies are somewhat unspecific, and the exact importance of these observations remains to be evaluated. The premyofibril theory postulates a positional information role for non-muscle myosin II isoforms in myofibrillogenesis (Rhee et al., 1994). The association of premyofibrils with the plasma membrane provides a surface on which the initial components can be anchored and organised.

The *in vitro* models of myofibrillogenesis serve as a basis for understanding the *in vivo* process and were developed as a result of difficulties encountered during direct work on embryos. Despite the difficulties, *in vivo* muscle development was characterized in mouse (Furst et al., 1989), chicken (Holtzer et al., 1990) and, more recently, in the frog Xenopus (Martin and Harland, 2001). Myofibrillogenesis *in vitro* and *in situ* are in general similar but differences are observed, suggesting that the process is not always faithfully reflected in detail in culture. For example, in a comparative study culture vs. *in situ* myofibrillogenesis, Ehler at al. (1999) observed that no mini-sarcomeres (premyofibrils) form in the developing chicken heart, i.e. the spacing between neighbouring α-actinin dots resembles mature sarcomeric distances even before beating. Moreover, the authors suggested that non-muscle myosin IIB is not essential as "space-holder" for muscle myosin isoforms, also observed by (Tullio et al., 1997) in mouse heart.

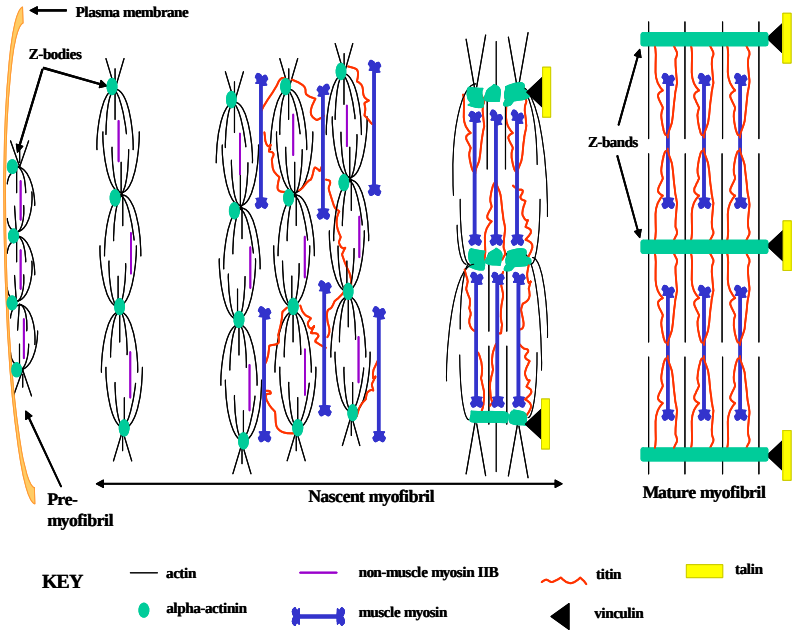

Figure 1-3 Model for the assembly of a mature myofibril. The position of alpha-actinin (green circles) relative to actin, non-muscle myosin IIB, muscle myosin II and titin is shown for each stage of myofibril development. Near the spreading edge of the cell (far left), alpha-actinin is found in mini-sarcomeric arrays, the premyofibrils. In the nascent myofibril, the alpha-actinin-rich Z-bodies have begun to laterally associate, possibly through interaction with titin. Overlapping muscle myosin II filament bound to titin are present at this stage. In the mature myofibril, the alpha-actinin containing Z-bodies fuse to form the wide lateral arrays of mature Z-bands; vinculin and talin are detected at costameric Z-bands of the mature myofibrils; non-muscle myosin IIB is lost, whereas muscle myosin filaments are now aligned into A-bands. The Z-bodies grow in size in the progression from premyofibrils to mature myofibril. The shape of the Z-band changes after the Z-bodies fuse to form a linear structure. The figure was reproduced from Sanger et al (2000).

1.2.3 Signalling in myofibrillogenesis

The myofibrils are not assembled simply as a result of correct protein expression, or the intrinsic self-assembly property of their constituent polymeric filaments composed of actin or myosin subunits (Reviewed in Trinick J. (1994) ; Trinick, J. (1996) ; Trinick, J. and Tskhovrebova, L. (1999). Some of the sarcomeric proteins are expressed early in myofibrillogenesis but are incorporated only at later stages (see above), which suggest that tight regulation pathways control their sequential integration into sarcomeres. Moreover, titin, nebulin, myomesin, M-protein and telethonin are phosphorylated during myofibrillogenesis, but still relatively little is known about the upstream signalling pathways or the significance of this phosphorylation.

Myomesin and M-protein anchor the titin C-terminus in the region of the M-band, but their binding to titin is regulated. In the case of myomesin, the phosphorylation at a specific site is known to block its interaction with titin (Obermann et al., 1997). The M-protein is phosphorylated by PKA at a specific serine residue, which further inhibits its

constitutive binding to myosin (Obermann et al., 1998). These events along with the phosphorylation of the C-terminal region of titin (see below) could provide a mechanism that controls the events during sarcomeric M band assembly.

Sommerville and Wang observed that titin and nebulin are phosphorylated in Xenopus laevis (1987) or mouse (1988) skeletal muscle during myofibrillogenesis. The C-terminal (i.e. M-line) end of titin is phosphorylated by a cdc2-like kinase and possibly other SP-directed kinases at up to four KSP repeats (Gautel et al., 1993). The Z-disk region of titin is also phosphorylated *in vitro* at a site or four x-S-P-x-R sequences by extracellular signal regulated kinases (ERKs) (Gautel et al., 1993; Sebestyen et al., 1995). Phosphorylation of these sites may regulate the binding capacity of the neighbouring domains to other proteins in or near the Z-disk.

Apart from phosphorylation sites, titin contains at its C-terminal end a protein kinase domain. The dual activation mechanism of titin kinase has been described (Mayans et al., 1998). Two events are necessary to unlock the double autoinhibited conformation: 1) the phosphorylation of an autoinhibitory tyrosine that blocks the substrate binding site and 2) the sterical rearrangement of the C-terminal regulatory tail as a result of Ca^{2+}/calmodulin binding. The key tyrosine of titin kinase is phosphorylated by extracts from differentiating myocytes but not by extracts from adult muscle, which suggests that titin kinase is activated specifically during myofibrillogenesis. The *in vivo* upstream activator is still unidentified.

Titin kinase was shown to phosphorylate telethonin during myofibrillogenesis in mouse (Mayans et al., 1998). Telethonin is a sarcomeric protein found at the level of Z-disks in mature myofibrils (Valle et al., 1997; Mues et al., 1998) The C-terminal, kinase-containing end of titin is anchored in the M-band, but telethonin is phosphorylated in the early stages of the sarcomeric assembling while it still coexists with titin in dot like aggregates on SFLS. The significance of this phosphorylation is not yet understood. In mature sarcomeres, telethonin specifically binds to a Z-disk titin fragment of two Ig domains, Z1-Z2 (Mues et al., 1998) and anchors these fragments in the rigid structure of the Z-disk in an anti-parallel fashion (Zou et al., 2003).

With its immense number of specific protein-protein interactions and complex dynamics, myofibrillogenesis remains a challenging task of understanding it in detail along the way that leads to mature muscle.

1.3 CYTOSKELETAL ASPECTS OF MUSCLE CELLS

Apart from the sarcomeric proteins that perform contractile functions and regulation of contraction, muscle cells possess, like all other types of cells, a multitude of structural proteins implicated in cell shape maintenance, intracellular trafficking of organelle or cargo vesicles, and there is growing evidence that they are implicated in cell signalling as well. These are called cytoskeletal proteins and form three main types of networks: actin, microtubule and intermediate filament networks, which in adult skeletal muscle account together for nearly 30% of the protein mass. This subchapter will address structural and functional aspects of cytoskeletal proteins relevant for this work without intending to exhaust the subject.

1.3.1 Microtubule network and vesicular traffic –General principles

1.3.1.1 The microtubules

In all types of cells the microtubule network is a major component of the cytoplasmic compartment. In muscle cells, microtubules have been assigned many functional roles, for example, in protein synthesis, intracellular trafficking, and intracellular signalling (Rogers and Gelfand, 2000), roles also true in other types of cells. Microtubules are hollow protein cylinders of α and β tubulin heterodimers about 25nm in diameter (Goldstein and Entman, 1979; Rappaport and Samuel, 1988) aligned predominantly along the longitudinal axis of the adult myocyte and adjacent to the perinuclear space (Cartwright, Jr. and Goldstein, 1985). In most cells, microtubules exist as a large, dynamic population and a small subset of drug- and cold-stable microtubules (Schulze and Kirschner, 1987). With the exception of this small stable population, microtubules are in a state of dynamic instability with addition of subunits to the plus (A) end and loss at the minus (D) end (Alberts et al., 2002). Addition of subunits is powered by GTP hydrolysis, each α and β tubulin monomer binds GTP, but it is the GTP on β tubulin, which is hydrolysed. Microtubules are found in association with GTP-binding proteins such as Gi and Gs (Rasenick et al., 1981; Rasenick et al., 1990; Wang et al., 1990), microtubule associated proteins (MAPs) that promote microtubule stability, predominantly MAP4 in cardiac muscle (Olmsted, 1986; Sato et al., 1997), Tau protein (Yang et al., 2003; Delacourte and Buee, 1997), kinases (Pitcher et al., 1998). Microtubules also associate with other components of the cytoskeleton such as actin (Cunningham et al., 1997) and

intermediate filaments (Gurland and Gundersen, 1995). Microtubule associated protein has been shown to recognize both actin microfilaments and microtubules.

1.3.1.2 Molecular motors and direction of movement

During muscle formation, membranous organelles such as the Golgi and ER vesicles, lysosomes and mitochondria are moved around the cell along cytoskeletal filaments with the help of molecular motors, which hydrolyse ATP to power their movement. Kinesin and dynein family of proteins move their cargo along microtubules while myosin motors (e.g. myosin V - reviewed in Bridgman, 2004) are known to accomplish the same function along actin microfilaments. Myosin V has also the capacity to interact with either kinesin or dynein suggesting the existence of a transitioning mechanism between the actin and microtubule system (Fan et al., 2001). The structural organization of kinesins (e. g. Kull, 2000) and dyneins (e. g. Harrison and King, 2000) was intensively studied. Dynein is a massive molecular complex assembled in mammals out of usually 2 heavy chains (DHC) that bind microtubules and are responsible for ATPase activity and force production; 2 intermediate chains (DIC) important in binding the cargo; four light intermediate chains (DLIC); and a number of light chains (DLC) believed to participate in cargo selection and binding (reviewd by Kamal and Goldstein, 2002). Kinesin is comprised of one HC with ATPase function and several LC.

Kinesins are, with some few exceptions, plus-end directed (anterograde) motors, while all dyneins tested move in the opposite direction (retrograde) towards the minus-end at the cell periphery. However, the same organelles (e.g. pigment granules from melanocytes) can bidirectionally move on microtubules, and surprisingly have both kinesin and dynein attached (Hirokawa et al., 1990). This raises the question how the direction of movement is regulated. Various studies proposed that vesicles bearing both kinesin and dynein move towards the plus end of microtubules, while vesicles with dynein but not kinesin move towards the minus end, the direction being dictated by the presence of kinesin (Hirokawa et al., 1990; Hirokawa et al., 1991; Dahlstrom et al., 1991; Muresan et al., 1996). Other studies however, show evidence that kinesin and dynein remain attached to vesicles during both anterograde and retrograde transport and their activity might be regulated instead (Reese and Haimo, 2000; Ligon et al., 2004). There is growing evidence for the existence of a remarkable physical link between kinesin and dynein motors that possibly regulate the direction of movement: dynactin. Dynactin was initially reported to be essential for dynein anchorage to cargo vesicles (Gill et al., 1991) and represents a large

multimeric complex of at least 10 polypeptides with the best characterised subunits $p150^{Glued}$ and p50 (dynamitin). Dynactin is an activator of dynein-mediated transport (King and Schroer, 2000). Through its $p150^{Glued}$ subunit, dynactin associates with microtubules, the intermediate chain of dynein (King et al., 2003) and the membrane of cargo vesicles. Recently, the research on pigment granule transport showed that, surprisingly, dynactin also interacts with kinesin II (a smaller member of the kinesin family also named KIF3) and that dynactin presence is necessary for plus end-directed motion (Deacon et al., 2003). Although kinesin II and dynein can be present on the same melanosomes (Gross et al., 2002) they cannot, however, bind dynactin simultaneously (Deacon et al., 2003). This suggests that dynactin is not needed for recruiting kinesin II and dynein to melanosomes but is rather involved in regulating the activation or organisation of motors already bound to membrane (reviewed by Gross, 2003). Therefore, proteins other than dynactin might bind kinesin II and dynein to membranes (see below).

1.3.1.3 Motor cargo attachment

The nature of the motor-cargo interaction and the identity of the membranous cargo each motor carries are still poorly understood aspects of microtubule-dependent trafficking. The attachment of vesicular cargo to a motor protein is generally an interaction between the motor and a cargo-bound receptor, usually through an adaptor protein or complex. The receptor is usually a transmembrane or scaffold protein and the adaptor is any protein that links the motor to the receptor. (**Figure 1-4** summarises the general attachment mechanisms existing). The first reported potential receptor for a motor protein was kinectin, an integral membrane protein localised to the endoplasmic reticulum (Toyoshima et al., 1992) that binds kinesin I. The transmembrane amyloid precursor protein (APP) (Kamal et al., 2000; Kamal et al., 2001), sunday driver (syd) (Bowman et al., 2000) and Jun amino-terminal kinase (JNK)-interacting protein (JIP) (Verhey et al., 2001) are other scaffolding proteins directly interacting with the light chain of kinesin I thus mediating the anchorage of the cargo.

Dynactin is considered to be a membrane vesicle receptor for dynein (see above) and was recently shown to interact directly with Golgi-associated spectrin bIII (Muresan et al., 2001) present on vesicles indirectly anchored through ankyrin. Large isoforms of ankyrins are Golgi attached membrane proteins that mediate vesicle binding to the dynein motor, however, there exist small ankyrin isoforms specific to the sarcoplasmic reticulum membrane of striated muscle (Gallagher et al., 1997; Zhou et al., 1997). Other molecules

can bind directly to DIC or DLC, suggesting a cargo-binding mechanism independent of dynactin. Rhodopsin (Tai et al., 1999) and neurotrophin receptor TrkA (Yano et al., 2001), bind to Tctex-1, one of the light chains of the mouse dynein complex. Also viral molecules may bind directly to the dynein IC (Ye et al., 2000), dynein LC8 (Raux et al., 2000; Jacob et al., 2000), or Tctex-1 (Mueller et al., 2002). In A7r5 smooth muscle cells stimulated to elevate the cytosolic Ca^{2+} level, the inositol 1, 4, 5-triphosphate receptor 1 (IP3R1) localised in the membrane of the sarcoplasmic reticulum undergoes massive re-localisation in a microtubule-dependent manner (Vermassen et al., 2003). The authors propose that mechanisms of vesicle trafficking be involved in the re-localisation of IP3R1 in prolonged Ca^{2+} stimulated muscle cells. This might be a general mechanism of re-organisation of sub-cellular components since the redistribution of Ca^{2+} stores in newt eggs also require the microtubular network (Mitsuyama and Sawai, 2001). In addition, protein kinase C (PKC) (involved in the IP3R1 redistribution) positively regulates the microtubule polymerisation (Vermassen et al., 2003; Kabir et al., 2001) and is involved in ER reorganisation after excessive Ca^{2+} release (Putney, Jr. and Ribeiro, 2000).

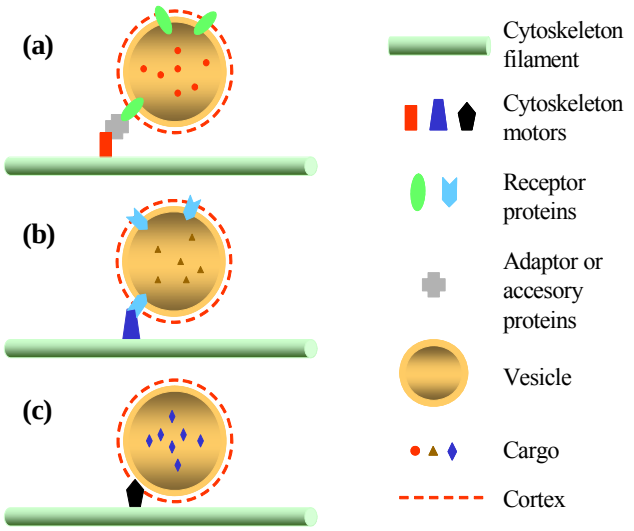

Figure 1-4 Models of vesicle anchoring to motors. The vesicles can attach to molecular motor complexes directly **(c)**, through a membrane receptor **(b)** or with the help of an adaptor molecule able to recognise both receptor and motor **(a)**.

1.3.2 Actin binding proteins

The actin cytoskeleton generally plays a central role in many cell functions such as the maintenance of cell shape, cell division, cell adhesion, motility and signal transduction. In muscle cells however, a distinction must be made between sarcomeric and non-sarcomeric actin as 6 isoforms are known to exist: four so called α-actins are found in sarcomeric contractile structures (namely skeletal, cardiac, vascular, and enteric actin)

while β and γ actins are thought to be predominantly cytoplasmic, i.e. form non-muscle actin filaments (Stromer, 1998). The non-sarcomeric actin structures are necessary for cell spreading and generation of new myofibrils as well as for cell signalling (see below). In order to accomplish these functions, the actin cytoskeleton needs to permanently assembly and disassembly in conformity with the cell necessities. Its dynamic re-organisation is regulated by a variety of actin severing and sequestering molecules that influence the length of polymerised actin (also called filamentous actin, F-actin) (Ayscough, 1998). At a further level of organisation, the localisation and cross-linking of F-actin into bundles and networks is mediated by multiple families of cytoskeletal proteins (reviewed by Otto, 1994).

Alpha-actinin is the main actin binding protein in muscle; however, in this study an obscurin-filamin interaction is presented and therefore the description of actin binding proteins will focus on filamin. Filamins represent a family of cytoskeletal proteins able to homodimerize and in this form to cross link actin filaments (**Figure 1-5**). Obscurin, the muscle protein studied in this work was found to be an interaction partner of muscle filamin C isoform in yeast two-hybrid screening of both cardiac and skeletal cDNA libraries (Section 3.7). The implications of this interaction are discussed in Section 4.2 while an introduction to filamin structure and function is given below.

Filamins posses an N-terminal actin binding domain (ABD) per monomer and a C-terminal rod domain implicated in filamin tail-to-tail dimerization (Gorlin et al., 1990; Tyler et al., 1980), orientation, flexibility and spacing of the cross-linked actin filaments. The rod domain, although composed almost exclusively of Ig-like repetitive domains, has one or two so called hinge regions (H1 and H2) depending on the filamin isoform. Filamin ABD is evolutionarily related to those of the α-actinins, spectrins, and dystrophin (Noegel et al., 1989) being composed of two calponin homology (CH) domains also present in signal transduction proteins such as Vav and IQGAP (Banuelos et al., 1998).

The filamin family has three members: filamin A, B and C encoded in humans by three genes FLNA, FLNB and FLNC. Over the years, no unitary system of nomenclature has been used in publications, different names being assigned to the same filamin. Synonyms of the three filamins in different species have been reviewed by van der Flier (2001). In humans, filamin A, B, and C show 70% homology over their entire sequence with the exception of hinge-1 (H1) and hinge-2 (H2) (only 45% homology). The diversity of the filamin family is increased by alternative splicing of filamin mRNA. For example, in

humans the H2 region is present in all filamin isoforms while H1 is absent in some splice variants of filamin B and filamin C (called ΔH1 variants) (Xie et al., 1998; Xu et al., 1998). In addition, filamin C contains an 80 amino-acid insertion in its Ig-like domain 20 of the rod domain (Chakarova et al., 2000)

At tissue level, the human filamin A and B are the most ubiquitously expressed while filamin C expression is largely restricted to skeletal and cardiac muscle (Thompson et al., 2000), in which the mRNA encoding the filamin-C (ΔH1) splice variant dominates (Xie et al., 1998). In skeletal muscle, filamin C is enriched at the Z-lines, I bands immediately adjacent to the Z-lines (Thompson et al., 2000; van der Ven et al., 2000a) and myotendinous junctions, being also detected at low level at the plasma membrane in association with the cortical actin (Thompson et al., 2000; van der Ven et al., 2000a; Gomer and Lazarides, 1983; Gomer and Lazarides, 1981). In cardiac muscle, filamin is present at Z-lines and intercalated disks (Price et al., 1994; van der Ven et al., 2000a) and in avian smooth muscle filamin-C-like proteins are found in the dense plaques and dense bodies (Tachikawa et al., 1997).

Filamins accomplish many important functions in the cell. They are implicated not only in F-actin cross linking but also in receptor anchoring and cell signalling. Their functions are summarised in **Figure 1-5**.

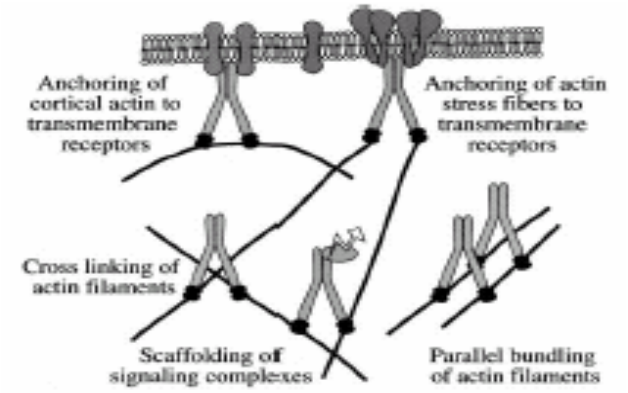

Figure 1-5 Schematic model of filamin functions. Filamin dimers link actin filaments in orthogonal networks and parallel bundles depending on actin-filamin ratio and the flexibility of the filamin isoform. In addition, filamin links transmembrane receptors to the F-actin cytoskeleton and serves as a scaffold for several signalling molecules (indicated by thunderbolt) (reproduction from van der Flier, 2001).

First, filamin cross-links actin filaments, thus participating in actin cytoskeleton organization. The overall ratio of filamin to actin in the cell determines which type of actin filament organisation will be adopted. The ratio is estimated to be 1:80-140 in melanoma cells (Cunningham et al., 1992) and interestingly a 1:10-50 ratio leads to formation of tight parallel bundles, while a stoichiometry of 1:150-740 promotes formation of more loosely spaced orthogonal actin networks (Dabrowska et al., 1985). The type of actin network is

also determined by the type of filamin. Muscle filamins lacking H1 region have a limited flexibility compared with other filamins and can therefore lead to rigid actin fibres instead of orthogonal actin networks.

Second, filamins can stabilize cellular membranes by anchoring through their C-terminal tails the actin cytoskeleton to a) integrins (Pfaff et al., 1998; Loo et al., 1998) (**Figure 1-6**) or to b) transmembrane receptors like glycoprotein Ibα, a subunit of the von Willebrand factor receptor (Ezzell et al., 1988; Xu et al., 1998). In muscle, filamin C specifically binds to γ and δ sarcoglycans (**Figure 1-6**). These are glycosylated transmembrane proteins that together with α and β sarcoglycans are part of a multimeric complex called dystrophin-glycoprotein complex (DGC) located in the sarcolemma of cardiac and skeletal muscle cells. DGC has three main components: a) dystroglycan b) dystobrevin-syntrophin and c) sarcoglycan complex. In smooth muscle, the sarcoglycan complex of DGC is composed of β, δ, ε and ξ sarcoglycans (Straub et al., 1999). Through its γ and δ sarcoglycan interaction, filamin C could strengthen the dystrophin–mediated linkage of the cytoskeleton to the membrane. Filamin C also interacts with β-integrins at the membrane (**Figure 1-6**). Cycling between membrane and cytoskeletal locations is signalled through integrins or DGC. In myopathies caused by mutations in γ or δ sarcoglycans, signalling through DGC is impaired and filamin C fails to translocate back to bind F-actin (Thompson et al., 2000). Its accumulation at the membrane can also represent a mechanoprotection mechanism taken over by filamin in the DGC altered muscle fibres (Glogauer et al., 1998). Thompson et al (2000) found filamin C both subsarcolemmal, associated with sarcoglycans, and at the Z-disk. It seems therefore that filamin C is capable of shuttling, suggesting a highly dynamic exchange between the submembraneous cytoskeleton and the contractile apparatus. Indeed, filamin C accumulates also inside muscle fibres in so called core lesions in central core disease (CCD) (Bonnemann et al., 2003).

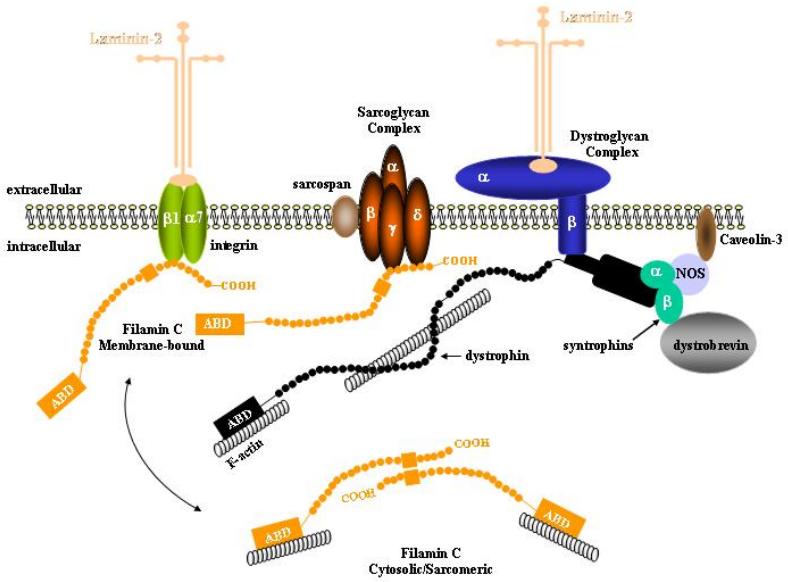

Figure 1-6 The dystrophin-glycoprotein complex (DGC). The mechanic linkages from the muscle cell cytoskeleton to the extracellular matrix include dystroglycan bound to laminin 2 (a component of the extracellular matrix) and binding of muscle-specific integrin α7/β1 to laminin 2. The sarcoglycan complex interacts on the extracellular face with dystroglycan (a heavily glycosylated, bipartite transmembrane protein) and with filamin C and dystrophin on its intracellular face. Dystrophin and filamin C have a number of additional binding partners.

At the Z-disk, filamin C interacts with myotilin (van der Ven et al., 2000b) as well as FATZ (also named myozenin, calsarcin or Z) (Faulkner et al., 2000; Takada et al., 2001). Myotilin is able to cross-link F-actin and moreover, apart from filamin C can interact with α-actinin leading to the formation of a unique complex of three actin cross-linkers in the Z-disk (Salmikangas et al., 2003). It is suggested that this complex forms late in myofibrillogenesis and links thin filaments in the Z-disks conferring rigidity to this structure in order to resist mechanical stress upon muscle contraction. Filamin C interaction with FATZ/myozenin is believed to regulate dimerisation of filamin C and perhaps modulate the spacing between actin thin filaments (Takada et al., 2001). Moreover, like myotilin, FATZ/myozenin interacts with both filamin C and α-actinin but also with telethonin probably serving as an adaptor for binding these proteins to other Z-disk proteins (Valle et al., 1997; Faulkner et al., 2000). Mutations in either myotilin (Hauser et al., 2000; Salmikangas et al., 1999) or telethonin (Moreira et al., 2000; Valle et al., 1997) lead to limb-girdle muscular dystrophies, suggesting also a potential participation of filamin-C in these diseases.

Third, filamin is able to interact through its C-terminal repeats with several Rho proteins of the Ras superfamily (Ueda et al., 1992) thus scaffolding signalling complexes (**Figure 1-5**) (the GTP-binding proteins are discussed in Section 1.4). The GTPase RalA (Ohta et al., 1999) binds filamin only in a GTP-dependent manner while binding of Rho-like GTPases Cdc42 and Rac1 is GTP-independent. In addition, Trio, a tandemly organised

guanidine exchange factor for RhoG, Rac and RhoA, binds by its pleckstrin-homology (PH) domain (Bellanger et al., 2000). These data suggest that the carboxy-terminal end of filamin serves as a GTPase docking site, which ensures correct localisation of GTPases and other regulatory factors for actin nucleation, actin dynamics and vesicle transport.

1.4 SMALL GTP-BINDING PROTEINS IN MUSCLE SIGNALLING

Small GTP-binding proteins represent a large family of molecular switches present in all types of cells and involved in numerous signal transduction pathways. Their regulatory function is accomplished by cycling between active (GTP-bound) and inactive (GDP-bound) states. In the GTP-bound form the proteins can interact with and transmit the signal to various downstream effectors to initiate a cellular response after which they return to the inactive state by hydrolysing the bound GTP to GDP. In this activity they are assisted by GTPase activating proteins (GAP proteins), which only accelerate the process. The activation of the G-proteins occurs by release of the bound GDP and exchange for the cytosolic GTP with the help of GDP/GTP exchange factors (GEFs).

The superfamily of small monomeric G-proteins comprises over 150 members distributed in six subfamilies: Ras, Rho, Ran, Rab, Arf and Rad. All these proteins share similar sequence elements and have similar overall three-dimensional folding as demonstrated by the structures of Ras (Pai et al., 1989; Milburn et al., 1990), Rap (Nassar et al., 1995), Arf (Amor et al., 1994; Greasley et al., 1995), Ran (Scheffzek et al., 1995), Rac1 (Hirshberg et al., 1997) and Rab (Brachvogel et al., 1997; Neu et al., 1997). Each of this subfamily of proteins is implicated in specific signalling pathways with particular effect at cellular level also different from one cell type to another. Briefly, members of the Ras subfamily regulate complex signalling pathways implicated in cell proliferation and differentiation, Rho subfamily of proteins mediate cytoskeletal rearrangements, Rab proteins are involved in vesicular traffic, Ran proteins regulate the transport of molecules through the nuclear membrane. The signalling pathways regulated by GTP-binding proteins are though not simple, linear flows of information but form rather complex networks with cross talk nodes, these proteins cooperating with each other in promoting a coordinated biological response.

Obscurin contains a DH domain, which, although not yet confirmed in this protein, is generally known in many other proteins to function as Rho GDP/GTP exchange factor. The mammalian Rho family of proteins currently comprises ten distinct members: Rho (A,

B, C isoforms), Rac (1, 2, and 3 isoforms), and Cdc42 (G25K, Cdc42Hs isoforms), Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, RhoD, RhoG, TC10 and TTF (Ridley A.J., 2000). The most extensively characterized members are Rho, Rac and Cdc42. Many cellular processes (e.g. cell division, cell migration, cell contraction, secretion or phagocytosis) need cytoskeletal rearrangements and these are achieved through coordinated Rho GTPases signalling. In mammalian cells, these proteins undergo different cellular responses: Rho regulates the assembly of actin stress fibres, Rac regulates the polymerization of actin at the cell periphery to produce lamellipodia and membrane ruffles, while Cdc42 triggers filopodia formation (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Kozma et al., 1995; Puls et al., 1999). In addition, all three GTPases regulate the formation of cell-matrix adhesion sites called focal adhesions, which are intimately associated with the actin structures (Nobes and Hall, 1995; Hotchin and Hall, 1995).

In addition to their effects on the cytoskeleton, Rho GTPases contribute to the regulation of cell cycle progression. In cultured fibroblasts and epithelial cells, Rho, Rac and Cdc42 contribute to G1 cell cycle progression. All three can promote entry into G1 and progression to S phase when expressed in quiescent fibroblasts, whereas inhibition of any of the three blocks serum-induced G1 progression (Olson et al., 1995). In antigen stimulated T-cells, Rac is activated by Vav and stimulates the JNK MAP kinase cascade. This leads to activation of the transcription factor NF-AT and expression of the cytokine interleukin-2 (IL-2), which in turn stimulates G1 progression of the quiescent T cell and promotes clonal expansion of the appropriate antigen-specific lymphocytes (Cantrell, 1998). Rac and its exchange factor Vav are also essential for proliferation of B cells; in this case, Rac regulates expression of the cyclin D2 gene through an unknown pathway (Glassford et al., 2001). The roles of Rho proteins in these dividing cell types differ partly from the ones they play in muscle cells, which, in contrast, are cells that have terminally withdrawn from the cell cycle.

During muscle formation, Rho proteins play a crucial role in regulating the commitment toward myogenesis. RhoA, Rac1 and Cdc42 have different but coordinated roles during this process. In the developing embryo, the precursor cells migrate from the dermomyotome to the limb buds and during this migration process their differentiation is inhibited. Myogenesis involves subsequent withdrawal of myoblasts from the cell cycle, a prerequisite for differentiation and myoblast fusion. Rac1 and Cdc42, as described above,

regulate migration in various cell systems (Nobes and Hall, 1995). Likewise, during muscle formation, they are potent regulators of migration but in addition, they are activated by TGFβ, an inhibitor of skeletal myogenesis (Meriane et al., 2002b), which supports earlier observations that constitutively active Rac1 and Cdc42 inhibit skeletal myogenesis (Gallo et al., 1999; Meriane et al., 2000). Moreover, active Rac1 and Cdc42 expression impairs cell cycle exit of myoblasts (Meriane et al., 2002a). Therefore, their activation might be important for both migration and inhibition of myogenesis during development. RhoA, in contrast, positively regulates the skeletal muscle cell differentiation by promoting the expression of MyoD (Carnac et al., 1998; Takano et al., 1998; Wei et al., 1998). MyoD and Myf5 are muscle-specific transcription factors that play crucial roles during muscle differentiation process. The myogenic cells start expressing MyoD and Myf5 only when they reach the limb sites (Tajbakhsh and Buckingham, 1994). RhoA activation is achieved through signalling from adhesion molecules of the cadherin family of proteins (Charrasse et al., 2002), which are glycoproteins essential in cell-cell adhesion, cell growth, migration, and differentiation during embryonic development. N-cadherin-dependent cellular adhesion was shown to play a major role in cell cycle exit and in the induction of the skeletal muscle differentiation program (Knudsen, 1990; Mege et al., 1992; George-Weinstein et al., 1997; Goichberg and Geiger, 1998). Therefore, an attractive function for RhoA in early myogenesis could be to inhibit cell migration and to favour differentiation. Later, during cardiac myofibrillogenesis, RhoA was proposed to play an important role in myofibril formation by providing the necessary non-striated actin stress fibre like structures on which the sarcomeric proteins are assembled (Wang et al., 1997). (Section 1.2)

Depending on the type of cell, GTP binding proteins elicit different effects. For example, Ras plays a major role in many signal transduction pathways, which lead to cell growth and differentiation in neuronal, adipocytic or myeloid cells, in some cases accompanied by growth arrest, while particularly in muscle Ras inhibits differentiation when active before induction of differentiation. The differentiation-defective phenotype is a consequence of the down-regulation of the MyoD1 determination gene (Konieczny et al., 1989) by MEK1, which forms an inhibitory complex with MyoD transcriptional complex in the nucleus (Perry et al., 2001). If Ras becomes pathologically activated after the muscle differentiation started, i.e. after the cells withdrawn from the cell cycle, instead of aberrant division and cancer (like in other cell types), Ras elicits muscle hypertrophy. However, the

role of Ras subfamily of proteins in myogenesis seems more complex since another member of this group, R-Ras, was reported to positively regulate myogenesis when active before muscle differentiation started (Suzuki et al., 2000).

1.5 OBSCURIN

1.5.1 Discovery

Titin and nebulin are now generally accepted as being major scaffolding proteins of the sarcomere, which through their size and complex structure are able to coordinate the complicated process of sarcomeric assembly (discussed in Section 1.2)

Recently, a novel protein joined the family of titin and nebulin. Obscurin is the third giant sarcomeric protein, discovered in a search for titin interaction partners in the Z-disk region (Young et al., 2001). In a yeast two-hybrid screen, the peripheral Z-disk fragment of titin identified a novel titin-interacting protein. The new protein was termed obscurin, after the adjective obscure, defined in the New Oxford Dictionary as meaning: a) difficult to see or make out, b) not well known, or c) not easily understood. All three meanings seem to be appropriate to obscurin, which has proven difficult to characterise because of its large size, complexity and relatively low abundance.

1.5.2 Obscurin at cDNA and molecular level

Obscurin is a complex, highly modular protein built from adhesion modules and signalling domains (**Figure 1-7**).

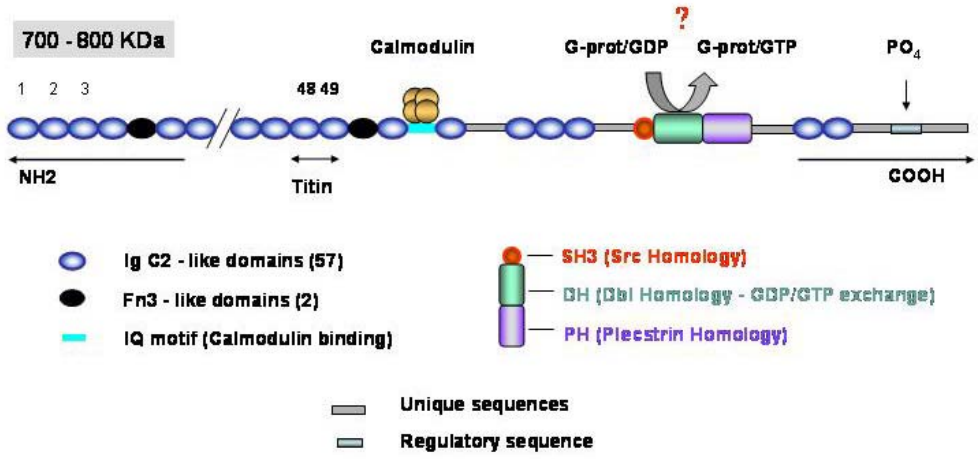

Figure 1-7 A schematic representation of obscurin domains. The N-terminus of the cardiac isoform of obscurin is exclusively formed out of Ig-like and Fn3 –like domains, while a series of signalling domains is concentrated at its C-terminus. No upstream regulator or downstream activator of obscurin GEF was identified to date. All domains were defined as described by Young et al (2001); the binding site for the ligand partner titin is shown.

The N-terminal region is a highly repetitive region consisting of 49 Ig and 2 Fn3 domains, arranged mostly in tandem. Concentrated in the C-terminal part, there are a number of signalling domains. First, an IQ motif is present, so called for the conserved sequence Ile-Gln. This motif is a recognized binding motif for calmodulin or calmodulin-like proteins such as myosin light chains (Rhoads and Friedberg, 1997). In the case of obscurin, this binding takes place in a Ca^{2+} independent manner. Second, obscurin has a three-domain region composed of an SH3 domain next to tandem dbl homology (DH) and pleckstrin homology (PH) domains. The SH3 domain is 43% identical to the SH3 domain of *C.elegans* protein UNC-89. The DH domain, generally known as activator of the small RhoGTP-binding protein family (RhoGEF), is followed in obscurin by a PH domain, as it is invariably in all DH-domain containing proteins. These two domains are most homologous (~25% identity) to the similarly arranged domains in dbl, Vav, trio, kalirin and UNC-89. Obscurin is the first human sarcomeric protein reported to possess a RhoGEF domain. This makes it a possible regulator implicated e.g. in the cytoskeletal rearrangements that accompany the formation of myofibrils. Apart from this, there are many other known functions of the Rho proteins, and obscurin could be potentially implicated in many of them (Section 1.4). No upstream activators or downstream effectors of obscurin DH domain were reported to date. Third, the very C-terminal end of the protein consists of two tandem Ig domains followed by a non-modular region of 417 amino-acids, containing several copies of a consensus phosphorylation motif (SPXR) for ERK kinases

similar to that found in the N-terminal region of titin (Gautel et al., 1996). The non-modular interdomain sequences have no clear homology to known protein domains.

This is the first isoform of obscurin described in the literature (Young et al., 2001). Two further splice variants were sequenced at that time and showed variation in the middle Ig repetitive region of the molecule. Recently, Russell et al. (2002) reported the identification of an obscurin isoform that contains two further serine-threonine kinase domains (SK) at the C-terminal end: SK1, similar to kinase domains of the Dbl family of GEFs and SK2, similar to twitchin kinase domain of the titin family of kinases. Because of the high amino-acid similarity of these SK domains with myosin light chain kinases (MLCK) from titin or dbl families, the authors named this longer isoform, obscurin-MLCK. It is expressed in both skeletal and cardiac muscle, like the originally described isoform but with a different pattern. The potential roles of the MLCK-like domains are to date entirely enigmatic. It seems, therefore, that obscurin is not a unique protein, but rather a member of a growing family of kinases with potential roles in sarcomere function. The nematode unc-89, which bears the closest similarity to obscurin was very recently shown to also have kinase domains (Sutter et al., 2004). Unc-89 is implicated in *C. elegance* in the assembly of the thick filaments into A-bands and the assembly of M-band proteins (Benian et al., 1996).

1.5.3 Obscurin at the sarcomeric level

Obscurin is expressed in cardiac and skeletal muscle. The expression level is very low: for example, the ratio between expressed nebulin and obscurin is estimated to be of at least 10:1 in skeletal muscle. Detection of obscurin sarcomeric localisation in myogenesis or in mature muscle helps understanding its function. Transfections of the Ob48-51 fragment in neonatal rat cardiomyocytes show that the protein co-localises with the titin Z-disk epitope T12, which further supports the notion that Ob48-49 constitutes a functional Z-disk targeting signal in obscurin. In agreement with the transfection data, an anti-Ob48-49 antibody detected obscurin at the Z-disc in early stages of chicken and mouse embryonic heart development (up to about 10-somite stage, or E10.5 in mouse).

Although obscurin was discovered as a ligand of titin in the Z-disk region, corroborated by transfection experiments and epitope detection of hearts at early stages of development, in neonatal rat cardiomyocytes the endogenous Ob48-49 epitope is surprisingly detected at the M-band. Furthermore, further four antibodies raised against

different epitopes along the entire obscurin molecule stain M-band, suggesting that the Z-disk association of obscurin in early embryonic stages is only transient. This localisation also suggests that the molecule must be largely transversal to the myofibril axis. Indeed, during muscle differentiation, the staining of the M-band with anti-Ob48-49 increases concomitantly with a decrease of Z-disk staining up to a complete loss in mature myofibrils.

Why such an unusual behaviour for a giant protein? The answer might reside in its different and largely unknown functions during muscle development and in a peripheral rather than central localisation at the myofibril.

Recently, Bagnato et al. (2003) and Kontrogianni-Konstantopoulos et al. (2003) reported an interaction between obscurin and small ankyrin-1 (sAnk-1), a membrane protein of the sarcoplasmic reticulum (SR). This is the first indication of a direct link between the SR and myofibrils. The family of ankyrins is composed of giant or smaller isoforms encoded by three genes (*ANK1*, *ANK2* and *ANK3*) and have many functions among which the rearrangement of Ca^{2+} receptors on SR membranes (Mohler et al., 2002). Kontrogianni-Konstantopoulos et al. propose a model in which obscurin, with its N-terminus bound to the M-band, anchors through its C-terminus the network SR in a firm position with respect to the myofibrils by interacting with sAnk1. Along this line, it is tempting to speculate that the existence of obscurin IQ domain in the proximity of the SR might indicate a calcium sensor role in a so far not identified process that requires regulation of Ca^{2+} levels.

1.6 AIM OF THE THESIS

Relatively little is known to date about the roles that the giant protein obscurin may play during muscle development. The main aim of this study is to better characterise obscurin from the functional point of view. This may be accomplished by searching for new ligands and investigating the role of such new interactions inside an appropriated cell system. Ideally, muscle cells are used for this purpose, but due to ease of transfection and maintenance in culture, other mammalian cell lines can be used, as they can also provide insightful information about particular aspects of the functionality of a mammalian protein in a ubiquitous process.

Titin is the first binding partner of obscurin reported by Young et al (Young et al., 2001). Its Z-disk region interacts with two of the obscurin Ig-like domains. A further

interaction of obscurin was recently described in the literature with the small, M-line associated protein ankyrin 1.5 (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003). Despite its giant size (700-800 kDa), obscurin seems to change its localisation during myofibrillogenesis, chronologically, from the Z-disk to the M-band, this being allowed by the perisarcomeric rather than integrated localisation of the molecule. Neither the significance nor the mechanisms of this process are clearly understood to date. However, the two mentioned ligands of obscurin, residents of sarcomeric regions 1µm away from each other are indicating that, during development, obscurin may play various roles at different points in time and space. The complex domain structure of obscurin suggests that other interactions are very likely to be still unidentified, and may help understanding the reasons of this unusual behaviour.

In this study, two new ligand partners of obscurin will be described. First, the attention will focus on the interaction with Tctel, a human dynein light chain. Obscurin has a so far unknown mechanism of adopting its striated pattern. The giant protein titin becomes organised in striations with the help of the stress fibre-like structures (van der Ven et al., 1992), but obscurin may use another intracellular network for this purpose, the microtubule network. The question will be addressed whether obscurin may play specific signalling roles during this transportation process.

Second, as part of a collaborative project with the group of Dr. Peter van der Ven from the University of Potsdam, the interaction with the muscle-specific filamin C isoform will be described. Filamins are actin binding proteins that can switch between subsarcolemmal and Z-disk localisation. Membrane association is brought about through interaction with the dystrophin-glycoprotein complex. Interactions with sarcomeric proteins were to date unknown. During myofibril formation, intense actin cytoskeleton remodelling is required, and the question arises whether obscurin may play a role in this process. The interesting question of ligand binding regulation will be briefly addressed, as both filamin C and ankyrin 1.5 bind in the same region of obscurin.

While a complete characterisation of obscurin function is beyond the scope of this thesis, the new interactions presented and their initial characterisation will serve as a platform for further studies.

2 MATERIALS AND METHODS

2.1 CLONING OF CDNA CONSTRUCTS

The cDNA constructs for yeast two-hybrid analysis, protein expression and cell immunofluorescence were amplified by polymerase chain reaction (PCR; (Saiki et al., 1985)) using the Expand Polymerase from Boehringer Mannheim. For all constructs (Tctel, RagA and obscurin), total human cardiac cDNA (Matchmaker cDNA library no HL 4042AH from Clontech) was used as template. Primer design was based on the obscurin sequence (EMBL database HSAJ2535), Tctel (EMBL database D50663), RagA (EMBL database X90529). All primers were synthesised by MWG Biothech and are listed in the Table 2.2. PCR products were purified in agarose gels, digested with the appropriate restriction enzymes and ligated (using T4 DNA Ligase, Fermentas) directly into yeast two-hybrid or expression vectors.

For expression, obscurin Ig55 domain, Tctel and RagA cDNAs were cloned in a modified version of the pET 8c vector (Novagen), pET8c-6HTev, which introduced the MHHHHHHSTENLYFQGSS amino acid sequence upon expression. This sequence was useful for subsequent affinity purification of the proteins (Section 2.4.2). For cell fluorescence, Ig55 was cloned in pEGFPN2 (Clontech) in fusion with the green fluorescent protein containing a double mutation that enhances its fluorescence (Clontech). The expression of this hybrid protein upon transfection allowed the direct visualisation of obscurin Ig55 at cellular level. The obscurin 56-C construct was cloned in a pCMVT7 vector (Promega) in fusion with the 11 amino acid T7 tag which was subsequently detected with the help of an anti-T7 antibody. For yeast two-hybrid analysis, the bait proteins were cloned in a modified version of the pLexA vector (Clontech), in fusion with a DNA binding domain, while the prey proteins were cloned in a pGAD10 or pACT2 vector (both from Clontech) in fusion with the Gal4 transcription activation domain (Table 2.1).

Amplification and isolation of plasmids followed standard procedures for Mini- and Midi preps using the appropriated Quiagen kit and the electrocompetent *Escherichia coli* strain XL-1 Blue (for preparation of competent cells see Section 2.3). Upon ligation and amplification, the identity of the derived constructs was checked by restriction digestion and by sequencing.

Table 2- List of cDNA constructs used in this study.

Vector name and origin	Insert name	Comments
pLexA (Clontech)	Ig55	
	Ig55-linker	
	Ig55-SH3	
	Linker	
	Linker-SH3	
	SH3	
	Ob 56-57	Paul Young
	Ob 56-C	Paul Young
pACT2 (Clontech)	Filamin A Ig19-21	Peter van der Ven
	Filamin B Ig19-21	Peter van der Ven
	Filamin C Ig19-21	Peter van der Ven
pGAD10 (Clontech)	Tctel	Mathias Gautel
pET8c-6HTev (Novagen)	Ig55	
	Tctel	Nathalie Bleimling
	RagA	Nathalie Bleimling
pEGFPN2 (Clontech)	Ig55	
pCMV T7	Ob 56-C	Mathias Gautel

Table 2- List of primers used for cloning of the above mentioned constructs.

Primer name	Primer sequence
Ig55-5	5'-TTTCTCGAGCCAGCTGGTGCCGCCCCGAATGCTG-3'
Ig55-5b	5'-TTTCTCGAGCGTGCTGGTGCCGCCCCGAATGCTG-3'
Ig55-3	5'-TTTGGATCCACGCGTCAGGCCCGAGACGTGCAGGCT-3'
Ins5	5'-TTTCTCGAGTCCTAAGGTGGAGGAGCAGGA-3'
Ig55ins-3 (Ins-3)	5'-TTTGGATCCACGCGTCTCGCCTCGGGCATCGCCGTC-3'
Obsh3-5	5'-TTTCTCGAGTATCTTTGACATCTACGTGGTC-3'
Obsh3-3	5'-TTTACGCGTGGATCCTTACTTGAGCCTCCTGTCCAGG-3'
Tctel 5	5'-TTCTCGAGCGAAGACTACCAGGCTGCGGAG-3'
Tctel-3	5'-TTTGGATCCACGCGTCAATAGACAGTCCGAAGGCA-3'

Primer name	Primer sequence
RagA-5	5'-TTTCGCGAGCCCAAATACAGCCATGAAGAAAAAGG-3'
RagA-3	5'-TTTGGATCCACGCGTCAACGCATAAGGAGACTGTG-3'

Table 2- The pair of primers used for each construct.

Construct	Primers used	Construct	Primers used
pETev6H Ig55	Ig55-5b	pLexA "Ig55insert" *	Ig55-5
	Ig55-3		Ig55ins-3
pETev6H Tctel	Tctel 5	pLexA Ig55sh3	Ig55-5
	Tctel-3		Obsh3-3
pETev6H RagA	RagA-5	pLexA "insert"	Ins5
	RagA-3		Ig55ins-3
pEGFPN2 Ig55	Ig55-5	pLexA "insert-sh3"	Ins5
	Ig55-3		Obsh3-3
pLexA Ig55	Ig55-5	pLexA SH3	Obsh3-5
	Ig55-3		Obsh3-3

***** "Insert" = obscurin linker region between the Ig55 and SH3 domains.

2.2 GENETIC ANALYSIS IN YEAST CELLS

For mapping the interaction sites of both Tctel and filamin on obscurin (Sections 3.1 and 3.7.1), L40 yeast cells were employed. The genetic system permits the activation of the nutritional HIS3 reporter gene and the subsequent growth of yeast cells on medium lacking histidine only when the proteins encoded by the prey and bait vectors are interacting with each other (Fields and Song, 1989).

From the glycerol stock, cells were plated on YPD medium plates and incubated at 30^{o}C. Colonies appear usually overnight. A single colony was picked and grown in 50 ml YPAD medium 30^{o}C under shaking till maximum OD^{600}=1. For each transformation, 1.5ml L40 cell suspension was used. Each aliquot was spun down at 14000g for 30 seconds and the pellet was mixed with 10µl transforming DNA (2µg plasmid DNA + 8µl herring sperm DNA 10mg/ml, previously boiled for 10 min and cooled to room temperature). 500µl of PEG/Lithium acetate (PEG/LiAc) solution (given below) were

added to the cell suspension and the tubes were shaken 15 min at 30°C. Before transformation, 50µl DMSO solution were added and briefly vortexed, then the cells were heat shocked for 15 minutes at 42°C and pelleted 30 seconds at 14000g. Each pellet was washed once with sterile H_2O, recentrifuged and resuspended in 50 µl SD (synthetic dropout) medium (given below). For each transformed bait plasmid, cells were plated on two different sets of plates: 1) on selection medium lacking Trp, the auxotrophic marker present on the pLexA plasmid – in order to check the efficiency of transformation and 2) on SD medium lacking both Trp and His – to check for auto-activation capacity of the insert in the absence of His. The plates were incubated at 30°C for 3 to 5 days until colonies appeared.

For double transformation experiments, the prey and the bait vectors were either simultaneously co-transformed in L40 competent cells as mentioned above or simple transformants of the bait vectors (plated to check for auto-activation) were re-suspended as single colonies in YPD medium and subsequently transformed with the prey vector in a similar way. The double transformants were checked both for co-transformation efficiency (SD/-Trp -Leu), and protein-protein interactions (SD/-Trp –Leu -His). For the bait proteins that were able to autonomously activate the genetic system, the minimum quantity of 3-aminotriazole (3-AT) (Sigma Aldrich) was determined and was always added to the selection medium on which the double transformants were plated.

YPD: 20g/l Difco peptone
10g/l Yeast extract
20g/l Agar (for plates only)
In bidest. H_2O
Adjust pH to 5.8

SD: 6.7g/l Difco yeast nitrogen base without amino acids
20g/l Agar (for plates only)
In Bidest. H_2O
Adjust pH to 5.8
100ml 10x dropout solution
40% dextrose

10x LiAc: 1M LiAc (Sigma Aldrich)
In bidest. H_2O

PEG/LiAc: 40% PEG 4000 (w/v)
0.1M LiOAc
In TE

1xTE: 10mM TRIS/HCl pH7.5
1mM EDTA

10x dropout solution:	Met	0.2g/l	Leu	1g/l
	Phe	0.5g/l	Val	1.5g/l
	Thr	2g/l	Lys HCl	0.3g/l
	Trp	0.2g/l	Arg HCl	0.2g/l
	Tyr	0.3g/l	Ade* hemisulfate salt	0.2g/l
	Ura	0.2g/l	His HCl monohydrate	0.2g/l
	Ile	0.3g/l		

Filter assays for β galactosidase activity were performed as follows. Bait transformed, and double transformed L40 yeast cells were restreaked on nylon membranes (NEN Life Sciences) and grown for 1-2 days on SD/-W-H or SD/-W-L-H, respectively. The membranes were then frozen twice in liquid nitrogen to lyse cells, placed on a filter paper soaked in Z buffer in a Petri dish containing the appropriate selection medium and incubated for 30min to 6h at 37°C. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used as colour substrate.

Z buffer:	130mM Na_2HPO_4/NaH_2PO_4 pH7	X-gal stock:	20mg/ml X-gal In DMF (dimethylformamide)
	10mM KCl		Stored at -20°C
	1mM $MgSO_4 \cdot 7H_2O$		

Z buffer/X-gal:	100ml Z buffer
(freshly prepared)	0.001% β-Mercaptoethanol
	1.67ml X-gal solution

2.3 ELECTROCOMPETENT CELLS AND TRANSFORMATION

All solutions and media were sterile, media were chilled prior to usage where indicated. For protein expression or amplification of plasmids, competent cells were prepared as follows. 100ml Luria Broth (LB) or SOC medium was inoculated with a single colony *Escherichia coli* BL21[DE3] or XL-1 Blue and grown overnight at 37°C under continuous shaking. The next day, 5 ml preculture were diluted with 500ml SOC medium and further grown to optical density OD^{600nm} = 0.8. The cells were harvested by

centrifugation at 3000xg 15 minutes. The pellet was resuspended in 500ml sterile H_2O at 4°C and centrifuged again as above. The procedure was repeated with 250ml H_2O and the pellet was resuspended in 10ml 10% glycerol at 4°C and recentrifuged. The resulting cell pellet was finally resuspended in approximately 2.5ml 10% glycerol 4°C and 60 µl aliquots were shock frozen in liquid nitrogen and stored at -80°C for months.

LB medium: 20g/l LB broth (Sigma Aldrich)
In deionised H_2O

SOC medium: 1.55g Luria Broth
250mM KCl
2M $MgCl_2$
1M Glucose
In deionised H_2O

The competent cell aliquots were mixed with the DNA, transformed through electroporation using a BioRad Gene Pulser apparatus, then resuspeded in 1ml antibiotic free LB and shaken for 30-60min at 37°C. Usually 100ml of this suspension was then plated on a selection medium containing the appropriate antibiotic for selection of the plasmid. For transformation of ligation mixtures, also the rest of 900ml cell suspension was pelleted 3 min at 2000xg and plated out.

2.4 PROTEIN EXPRESSION AND PURIFICATION

2.4.1 Protein Expression

All proteins were expressed in *Escherichia coli* strain BL21[DE3]. Usually, an overnight preculture from a single colony was always made and diluted the next morning with fresh antibiotic medium - final ratio 0.01 volumes preculture in new medium. The cells were further grown at 37°C under continuous shaking until they reached maximum OD^{600} = 0.7. Induction of expression was generally carried out at 20°C overnight (14h), using 0.2mM isopropyl β D thiogalactoside (IPTG). The cells were harvested by centrifugation 15 minutes at 4500xg. When not immediately lysed for protein purification, the pellets were shock frozen as it is or resuspended in cell lysis buffer and stored at -80°C.

2.4.2 Obscurin Ig55, Tctel and RagA Purification

The cell pellet was resuspended in the corresponding buffer A (given below) containing 1mM PMSF to protect the overexpressed protein from imminent proteolysis upon cell lysis. The cells were opened by passing the suspension 3 times through a fluidizer. 1h ultracentrifugation at 100000xg (Beckman ultracentrifuge model Optima L-70K, rotor Ti60) removed the cell debries and the resulting supernatant was filtered through 0.2μm filter. Typically 5ml of Ni-NTA agarose matrix (Qiagen) was used to purify protein from 3 litter expression culture. The column was pre-equilibrated with buffer A on a GP-250 Plus FPLC system (Pharmacia) then the filtered supernatant was applied at 2ml/min.

Purification of Tctel protein raised no complications, while Ig55 and RagA proved to need additional helping steps. Under native conditions, no binding of Ig55 to the column occurred, most probably because the histidine tag (6H) was not properly exposed in the folded protein. Thus, the purification of this protein required denaturing reagents to ensure full exposure of the tag and to facilitate the specific interaction with the column trapped Ni^{2+}. The binding of both Ig55 and RagA occurred in a 6M urea buffer. The column was washed with 8-10 column volumes (CV) of buffer A containing 1% buffer B (5mM imidazole final concentration) and the bound proteins were eluted in gradient of imidazole from 1 to 100% buffer B over a volume of 100ml.

Ig55/RagA Buffer A:	100mM Na_2HPO_4/NaH_2PO_4 pH8
	10mM TRIS/HCl pH8
	6M urea
Tctel Buffer A:	The above Buffer A without urea
Buffer B:	Buffer A + 500mM imidazole

Removal of the N-terminal tag was achieved after the initial Ni-column purification using the Tobacco Etch Virus (Tev) protease (Gibco BRL, (Parks et al., 1994). The digestion removes the 6xHis tag and leaves only 3 additional residues (Gly-Ser-Ser) at the N-terminus of the expressed protein (Section 2.1). For this cleavage, the proteins had to be dialysed against an optimal Tev cleavage buffer (given below). Obscurin Ig55 carrying N-terminal His tag proved to be insoluble in the Tev cleavage buffer in the absence of urea, while the tag-free obscurin Ig55 was soluble. For this reason the Tev protease was added

directly to the purified Ig55-6His in 6M urea solution and dialysed stepwise against minimal volumes of Tev cleavage buffer for short periods of 4 x 1h at 4°C. This treatment ensured that the dialysis took place slowly and the Tev protease had enough time to cut the tag before the buffer without urea replaced the initial one. The Tev protease was already mildly active in 6M urea and gradually recovered its full activity as the dialysis took place. The last dialysis step took place overnight, against a big volume of Tev buffer. The protein was ultracentrifuged next day for 1h at 50000xg to remove possible precipitation and the supernatant was repurified on Ni-NTA column. Only the flow through was collected, which contained the cleaved Ig55 protein, dialysed against the storage buffer, concentrated in an Amicon chamber using a 3kDa cut off membrane and stored at -80°C.

For solubility reasons, RagA always required denaturant reagents in the buffer both during the cleavage of the His tag (urea) and after (guanidinium hydrochloride). Many attempts of purifying this protein yielded no results when no denaturant reagents were added. Therefore, for rabbit immunisation, this antigen solution contained 6M guanidinium hydrochloride.

Tev cleavage buffer:	25mM NaH_2PO_4/Na_2HPO_4 pH 8
	Tris/HCl pH 8
	10% Glycerol
	1mM EDTA
	10mM β-mercaptoethanol
	100mM NaCl
Ig55 storage buffer:	25mM HEPES
	50mM NaCl
	1mM EDTA
	1mM DTT
RagA storage buffer:	PBS pH7
	6M Guanidinium hydrochloride
	5mM β-mercaptoethanol
	1mM EDTA
Tctel storage buffer:	20mM TRIS/HCl pH8
	100mM NaCl
	1mM DTT

2.5 GEL FILTRATION

This method was used for the isolation of Ig55-Tctel complex obtained *in vitro* (Section 3.2.1). Recombinant tag cleaved Tctel and Ig55 in their storage buffers (Section 2.4.2) were mixed 1:3, respectively, in a total amount of 1mg protein and diluted with gel filtration buffer (given below) to decrease the salt amount present in the Tctel buffer. The ratio 1:3 for the Tctel-Ig55 complex was determined after the first gel filtration attempt revealed that Tctel forms trimers. Denaturing reagent urea (4M) was added during the complex formation to mildly disrupt the Tctel oligomers. The volume of the complex was 800µl and needed to be concentrated before gel filtration. This was achieved with a Pall FILTRON Nanosept Microcentricon cut off 3 kDa until the volume was reduced to 200µl, which represented less than 1% of the gel filtration column volume.

The gel filtration took place on a 24ml SUPEROSE 12 HR 10/30 column (Amersham Pharmacia Biotech) pre-equilibrated overnight with 90ml gel filtration buffer at 0.1 ml/min on a Waters 600S FPLC system. The complex was loaded and ran on the column at 0.4 ml/min, while 0.4ml fractions were collected. The gel filtration buffer didn't contain denaturants in order to ensure the separation of the urea molecules from the applied protein mixture and to allow the formation of the Ig55-Tctel complex on the column. Control runs of each Ig55 and Tctel alone were performed and the calibration of the column was achieved with a commercial gel filtration standard from BioRad (Tyroglobulin (bovine) – 670kDa, γ-Globulin (bovine) – 158kDa, Ovalbumin (chicken) – 44kDa, Myoglobin (horse) – 17kDa, Vitamin B12 -1.35kDa.) The protein fractions were analysed through SDS PAGE (Section 2.6.3).

Gel filtration buffer: 25mM Hepes/KOH pH 7
50mM NaCl
1mM DTT
1mM EDTA

2.6 PULLDOWN AND COLUMN BINDING ASSAYS

2.6.1 Pulldown assays with HeLa lysates

Due to difficulties in observing the binding, pulldown assay was performed in two variants: 1) when recombinant Tctel was used as "antigen" bound to the column and a

cytosolic lysate of transfected HeLa cells containing the Ig55-EGFP hybrid protein was passed through; and 2) when recombinant Ig55 was bound to the column and a lysate of HeLa cells was passed through to pulldown endogenous Tctel. For these experiments, both Ig55 and Tctel were previously cleaved of tags. Column preparation: 200µg recombinantly expressed Tctel was coupled to 200µl NHS-Sepharose Fast Flow 4B columns (Pharmacia) following the manufacturer's instructions (see also Section 2.8.1 - Antibody Purification). The column was equilibrated with 5 column volumes lysis buffer. Cytosolic lysates of HeLa or pEGFPN_2-Ig55 transfected HeLa cells were prepared as following: 3 x 10 cm dishes of transfected cells were washed with 37^{o}C DMEM; the cells were carefully scraped, resuspended in DMEM and centrifuged for 2 minutes at 800xg. The cell pellet was lysed on ice in 3ml lysis buffer for 30 minutes and passed once through a 0.2µm syringe needle.

Lysis buffer:	20mM Hepes/KOH pH 7	20µM Nocodazole
	150mM NaCl	2mM AMP-PNP
	1mM EGTA	10µg/ml Leupeptin
	3mM $MgCl_2$	0.5mM PMSF
	0.1% NP40	1mM Na vanadate
Wash buffer:	lysis buffer without NP40	

Cell debries were removed by 10 minutes centrifugation at 16000xg in a table top centrifuge at 4^{o}C. The supernatant obtained was passed on the equilibrated antigen-coupled column and the flow-through was collected. Washing of the non-specifically bound material was carried out with 5 CV of lysis buffer and 2 CV of wash buffer. The specifically bound material was then stepwise eluted with 4 X 1 CV of 100mM glycine/HCl pH3. For both variants of the assay, a control run was performed on columns without the bound antigen.

For Western Blot analysis, the eluted fractions needed to be concentrated by adding 80% TCA final concentration, followed by incubation on ice for 30 minutes and centrifugation at 16000xg for 20 minutes. The protein pellet was washed twice with 1ml acetone, air dried for 5 minutes, and re-dissolved in minimal volume Laemmli Buffer. The samples were analysed by SDS PAGE/Western Blot (Section 3.2.2, 3.3.1).

Laemmli buffer: 62mM TRIS/HCl pH6.8
2% SDS
10% glycerol
5% β mercaptoethanol
0.001% Bromophenol Blue

2.6.2 Column binding assay with Ig55 and Tctel recombinant proteins

Recombinantly expressed Ig55 cleaved of His6 tag was coupled to NHS-Sepharose (Pharmacia) as described above (Section 2.6.1), typically, 100µl matrix for 100µg protein. In this case, the experiment was performed only with Ig55 bound to the column due to the lack of an antibody able to recognise Ig55 without a tag. A solution of 100µg Tctel in pulldown buffer was passed through the column. The flow through was collected. Similarly, a control column having no antigen bound was treated in an identical way to check whether, under the same conditions, Tctel can bind directly to the matrix. Extensive column washing was carried out with 20 CV of pulldown buffer to ensure that no material remained unspecifically bound to the matrix. The bound protein was eluted with 2 x 1 CV of Laemmli buffer at 96^{o}C and analysed by SDS PAGE/Western Blot.

Pulldown buffer: 25mM Hepes/KOH pH7
100mM NaCl
1mM EDTA
1mM DTT
0.05% Tween20

2.6.3 Preparation of rabbit heart lysates

The heart powder was obtained from adult rabbit hearts by crushing the tissue under liquid nitrogen until homogeneity. For obtaining cytosolic extracts containing soluble Tctel dynein light chain, 0.3g heart powder was incubated with 5ml hypotonic lysis buffer for 10 minutes on ice. Centrifugation in a Beckman Optima L-70K ultracentrifuge (Ti50 rotor) for 30 minutes at 90000xg pelleted the organelles and the cell debries. The supernatant was kept separately while the pellet was further extracted with 5ml high salt buffer and 5ml high salt/detergent buffer. The two extracts were pooled together, filtered and shock frozen for storage at -80^{o}C.

Lysis buffer:	20mM HEPES pH7
	2mM $MgCl_2$
	1mM EGTA
	2mM DTT
	1mM Na vanadate
	1mM PMSF
	5μM Pestatin
	20μM Leupeptin
High Salt buffer:	Lysis buffer + 500mM NaCl
High Salt/detergent buffer:	Lysis buffer + 500mM NaCl + 0.05% Triton X-100

2.6.4 SDS PAGE and Western Blotting

The protein samples from the rabbit heart cytosolic lysate and from the pulldown/column binding assays (Sections 3.3.1 and 3.2.2) were loaded onto 15% SDS PAGE gels and electrophoresis was carried out according to Laemmli (1970). The running gel was composed of 15% acrylamide, 0.4% bisacrylamide, 0.1% (w/v) SDS and 375mM Tris, pH 8.8. The polymerisation of the gel material was initiated by adding 14μM /μl TEMED and 0.5 mg/ml ammonium persulphate (APS). The gel was casted by pouring it into the casting chamber and covering it with isopropanol/water 1:1. After polymerisation, the isopropanol/water mixture was replaced by the stacking gel: 5% acrylamide, 0.13% bisacrylamide, 0.1% (w/v) SDS, 125mM Tris, pH 6.8, 14μM/μl TEMED and 0.5% mg/ml APS. Before applying on the gel, the protein samples were denatured 5 minutes at 95^{o}C in sample buffer (given below). The protein molecular weight marker (LMW-Standard, Amersham Biosciences) was composed of protein phosphorlylase b (97 kDa), albumin (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa). The protein electrophoresis took place at 50 mA in SDS Running buffer. The gels that were not further blotted were coloured with a Coomassie Blue solution and then destained with destaining solution to display the protein bands.

SDS Sample buffer:	50mM Tris, pH = 8.9
	100mM DTE
	2% (w/v) SDS
	0.25% (w/v) Bromophenol blue
	10% Glycerine
SDS Running buffer:	25mM Tris, pH = 8.3
	192mM Glycine
	0.1% (w/v) SDS
Coomassie staining solution:	0.25 % (w/v) Coomassie Brilliant Blue R 250
	45 % Ethanol
	10 % Acetic Acid
Destaining solution:	5% Ethanol
	10% Acetic Acid

The proteins from the gels destined to Western blotting, were directly after SDS-PAGE transfered onto nitrocellulose membranes for 2 to 3h, at 0.8 mA/cm^2, in a Semi Phor blotting apparatus (Hoefer Scientific Instruments).

The blot transfer buffer was:	24mM TRIS base
	192mM glycine
	20% methanol

The major bands on the blots were detected by Ponceau Red (Sigma) and labelled with the pencil. Prior to antibody probing, the membrane was blocked for 30 minutes in 2% BSA, PBS/Tween20 (0.05%). Anti-Tctel antibody was used at a dilution 1:1000- 1:500 in 1% BSA/PBSTween20 (0.05%) and incubated with the membrane at 4^oC overnight. The membrane was then washed 3 times for 15 minutes with PBST and a secondary pig-anti-rabbit antibody coupled with horse radish peroxidase was applied for 30 minutes. An enhanced chemiluminescence (ECL) kit from Amersham Life Sciences was used for detection.

Stripping of blots for subsequent reprobing was achieved by boiling the membrane for 3 minutes in the above SDS PAGE running buffer + 50mM β- mercaptoethanol, in a microwave oven.

2.7 CELL CULTURE

2.7.1 Maintenance of cell lines and preparation for transfection

All media were sterile; media were warmed up previous to usage where indicated.

HeLa human epithelial cells and **COS-1** monkey kidney cells (both from ATCC, Manassas, USA; www.ltcpromochem.com/atcc) were cultured in complete medium: DMEM (Sigma Aldrich) 1gGlc/l, 10% fetal calf serum (FCS; GIBCO BRL), 1X Penicillin/Streptomycin/Neomycin (i.e. 500/0.5mg/1mg per ml penicillin, streptomycin, neomycin, respectively), in 10cm culture dishes and incubated at 37^{o}C in 5% CO_2, until they reached a cell density of approximately 70%. The cells were passaged (for procedure see below) on glass cover slips (11mm in diameter) in 6 well dishes (Falcon) one day prior to transfection at a ratio of 1:2, which yielded the next day a cell density of 50-70%, proved to be optimal for transfection.

H9C2 rat cardiac cells (ATCC) were maintained in culture in DMEM 4.5g Glc/l, 10%FCS, 1XP/S/N at a cell density of less then 60% in order to prevent spontaneous differentiation. For transfection experiments, H9C2 cells were plated the previous day on glass cover slips as described above for HeLa/COS-1 cells. These cells yielded only 40-50% confluence in 24h but this proved to be a good cell density for transfection.

Cardiomyocytes from neonatal rats were prepared according to the manufacturer's protocol (Worthington).

For propagation (except for primary cells), the cells were passaged as following. The cells from each 10cm dish were washed with 37^{o}C plain DMEM and detached by trypsinisation at room temperature with 2 ml Trypsin/EDTA solution (Sigma Aldrich) per dish for about 2 minutes. As this treatment is harmful for the cells if prolonged, visual control under a light contrast microscope ensured that the incubation period didn't exceed the time necessary for detaching the cells. The reaction was stopped by adding the desired volume of 37^{o}C complete medium per dish, according to the desired dilution factor to be achieved. The homogeneous cells suspension was then plated at 10ml/dish.

For storage (except for the primary cells), DMSO stocks were prepared as following: 5x10cm dishes of cultured cells at a density of 60% were trypsinised with only

1.5ml Trypsin/EDTA per dish, as above. The reaction was stopped with 10ml complete medium per dish. The cell suspensions were centrifuged in separate tubes for 2 minutes at 100xg and the medium removed. The cell pellet from each 10cm dish was resuspended in 1ml FCS + 7% DMSO and moved into sterile cryotubes. The tubes were chilled for 15 minutes on ice and then wrapped in paper, placed inside a thick-wall box and moved at -80^{o}C. This ensured that the freezing process took place gradually, in days.

The cells were not maintained in culture for more than 20 passages. To restart a cell culture from the DMSO cell stock, one vial of frozen cells was rapidly thawed in a 37^{o}C water bath, diluted with 10ml 37^{o}C complete medium and centrifuged 1 minute at 100xg. The DMSO containing medium was then removed, and the cell pellet was resuspended in 37^{o}C complete medium and plated in a 10cm Petri dish.

2.7.2 Differentiation of myoblasts in the absence or presence of nocodazole

H9C2 cardiac myoblasts are able to form mature sarcomeres under appropriate conditions in culture. This is a property that was explored for visualising the localisation of obscurin during myofibrillogenesis (Section 3.5). These cells were able to form mature sarcomeres after 7 days and the cardiac phenotype was confirmed by immunostaining several of the sarcomeric markers. For differentiation analysis, H9C2 cells were plated on 35mm dishes previously covered with a solution of 0.1% rat tail collagen in 0.5% acetic acid and dried/sterilized in the laminar flow hood under ventilation and UV illumination for 1h. The cells were grown at 37^{o}C in 5% CO_2 until 100% confluence (approximately 3 days). They were then briefly washed in plain DMEM and differentiation was induced by switching them to low serum medium DMEM 4.5g Glc/l, 1% horse serum, 1% Nutridoma (Roche Applied Science), 1X Penicillin/Streptomycin/Neomycin (Sigma Aldrich). Differentiation took place over a period of 7 days, the medium being changed daily.

In parallel with the normal conditions, H9C2 cells were also submitted to differentiation in the presence of nocodazole. For this, 10μM nocodazole was added 6h after the differentiation was induced by low serum medium. This lag period was introduced to allow the cells to exit the cell cycle. The medium was changed daily and the concentration of nocodazole was maintained throughout the entire differentiation period (7 days).

2.7.3 Transfection of adherent cells and subsequent nocodazole treatment

Adherent cells in culture are able to take up foreign DNA when this is properly delivered across the cell membrane. Different methods were tested in this study like $Ca_3(PO_4)_2$ and transfection with the help of liposomal reagents, the later method yielding the best results for HeLa, COS-1 and H9C2 cells. However, the transfection of cardiomyocytes was not successful during this thesis work regardless of the method applied.

The liposomal reagents are able to form micelles enclosing the desired plasmid DNA and in this form to fuse with the cell membrane, thus delivering the DNA into the cell. Further on in the cell, the plasmid is transcribed from the gene promoter present in the vector and translated, resulting in the appearance in the cell of the encoded desired protein. Using the manufacturers' protocols, different transfection reagents were tested in culture for best transfection yields with the desired plasmids. Gene Shuttle 40 (Qbiogene) (equivalent to Escort IV – Sigma Aldrich) and SuperFect (Qiagen) provided in the case of HeLa and COS-1 cells best transfection yields of 30-40% or higher. H9C2 cells were best transfected using Escort III (Sigma Aldrich) at a ratio of 1:4 (μg plasmid DNA: μl transfection reagent) according to the manufacturer's protocol. The transfection efficiency in this case was always around 15-20%.

Transfected HeLa cells were allowed for 24-48h to express the desired protein. At the end of this period, nocodazole was added directly to the culture medium at a final concentration of 10μM. Further on, the optimal incubation period necessary to destabilize the microtubule network and simultaneously preserve the cells alive was determined to be 1h.

2.7.4 Cell fixation

In order to analyse the cellular localisation of constitutive or transfected proteins, the cell content must be fixed as it is at a desired moment (e.g. after a given number of days in culture during differentiation or at the moment of optimal protein expression level after transfection). Two fixing methods were applied.

First, the COS-1 cells were washed twice with the Protection buffer specified below and then fixed 10 minutes in 4% para-formaldehyde (PFA) prepared in Protection buffer. This method preserved both the cytoskeletal structures and the cytosolic content intact. For fixation, the solutions were 37^{o}C warm to prevent microtubules

depolymerisation. After fixing, the solution was replaced with Protection buffer and the dishes were stored in this way at 4°C for several days; however, for storage up to 1 month, 1mM azide (NaN_3) was added to maintain sterility.

Protection buffer:	65mM PIPES
	25mM HEPES
	10mM EGTA
	3mM $MgCl_2$
Fixing solution:	4% PFA
	in Protection buffer

Second, transfected HeLa/H9C2 cells, and differentiated H9C2 cells were fixed in 37°C BRB80 buffer (given below). Due to the presence of the NP40 detergent in the fixing buffer, this method extracted the cytosolic content while fixing the cytoskeletal proteins. The cells were washed twice with BRB80 buffer and fixed in the fixing/permeabilisation solution for 10 minutes. The reaction was subsequently quenched for 5 minutes with the specified Quenching solution. When necessary, cells were stored till further use in PBS/NaN_3 at 4°C for up to 1 month.

BRB80 buffer:	8mM Pipes/KOH pH6.8
	1mM EGTA
	1mM $MgCl_2$
Fixing/permeabilization solution:	0.1% NP40
	3.7% PFA
	in BRB80
Quenching solution:	50mM NH4Cl
	in BRB80

2.8 ANTIBODIES AND IMMUNOFLUORESCENCE

2.8.1 Affinity purification of antibodies

Polyclonal sera against Tctel and RagA recombinant proteins were raised in rabbits using a standard immunisation protocol (Harlow E. and Lane D., 1988) (BioScience). For affinity purification of antibodies from a single bleeding serum (ca 10ml), 2ml Sepharose NHS 4 Fast column (matrix purchased from Amersham Pharmacia) was activated with 2 column volumes (CV) 1mM HCl and the antigen was bound to the column in an appropriate buffer (specified below) with the pH in the basic range. In the case of RagA, the binding was performed in the presence of 6M guanidinium hydrochloride required for protein solubility (as described in Section 2.4.2). Subsequently, the unoccupied matrix groups were blocked with 1M TRIS/HCl pH8 for 1h on ice and the antigen fraction that didn't bind properly was eluted with 3 CV elution buffer 100mM Glycine/HCl pH3, the pH on the column being immediately restored with PBS. After the serum was applied, the column was washed - it was observed that in contrast with simple PBS, a more stringent washing in 2 steps (buffers given below) dramatically improved the purity of the antibody prep and was always performed for this purpose during this thesis work. The elution was done stepwise, with 1CV (2ml) at a time, 100mM Glycine/HCl pH3, the fractions being collected in tubes already containing 1ml TRIS/HCl pH8 to quickly restore the pH. The fractions having the highest amount of antibody were immediately dialysed against PBS with 5mM NaN3 overnight, and then concentrated to minimum 2mg/ml in a Centriprep device (Millipore) of 30 kDa cut off. The antibody was stabilized with 1-5mg/ml BSA, 5mM NaN_3. For storage at -20^{o}C, 50% glycerol was added.

Coupling buffers:	A) Tctel: 50mM Na_2PO_4/$NaHPO_4$ pH9 100mM NaCl
	B) RagA: 6M Guanidinium hydrochloride 5mM β Mercapto-ethanol 1mM EDTA in PBS pH7.4
Washing buffer step 1:	PBS/Tween20 (0.05%), 20 CV
Washing buffer step 2:	PBS with 500mM NaCl,10 CV

2.8.2 Immunofluorescence methods

Transfected and differentiated cells were investigated with the help of fluorescently labelled antibodies. The fixing solution was removed, the cells were washed with PBS and blocked for 20 minutes in 0.5% Triton X-100, 1% BSA in PBS. The primary antibodies were diluted in the below given buffer and incubated with the cells for 1h at room temperature. For the secondary antibodies, incubations of 30-40 minutes were sufficient. The cells were imbedded in the Imbedding solution containing 1X anti-bleach solution specified below. Washing steps of 3 times for 5 minutes with PBS/Tween20 (0.05%) (PBST) were performed after blocking and all antibody incubation steps. When the actin cytoskeleton was stained, phalloidin Alexa 633 was added with the secondary antibodies at a dilution of 1:50. The affinity purified anti-Tctel and anti-RagA antibodies as well as all commercial antibodies were tested for optimal dilution factors and these are specified in Table 2.4 and Table 2.5. Control stainings were always performed as described above but omitting the primary antibodies – these stainings were consistently negative.

Antibody dilution solution:	0.1% BSA 0.02% Tween20 in PBS
Cell Imbedding solution:	50% glycerol 1X anti-bleach solution in PBS
Anti-bleach solution 10X:	10mg/ml N-propyl-gallate 60% glycerol 20mM TRIS/HCl pH8 in PBS

Table 2- Primary antibodies and their dilution factors.

Primary Antibody Name and Organism		Source	Dilution
Tctel	Rabbit, affin. purified	BioScience	1:1000 – 1:500
RagA	Rabbit, affin. purified	BioScience	1:50 – 1:20
Alpha Tubulin	Mouse monoclonal	Sigma Aldrich	1:500
Ob27 (ObIQ)	Rabbit, affin. purified	BioScience	1:1000
Calreticulin	Goat	AbCam	1:200
Dynein IC	Mouse monoclonal	Sigma Aldrich	1:100
T7 tag	Mouse monoclonal	Novagen	1:10000

Phalloidin Alexa 633 (purchased from Molecular Probes) was used at a dilution of 1:50.

Table 2- Secondary antibodies and their dilution factors.

Secondary Antibody Name and Origin		Dilution
Goat anti-Mouse Alexa 546	Molecular Probes	1:500
Goat anti-Mouse Alexa 633	Molecular Probes	1:500
Goat anti-Rabbit Alexa 546	Molecular Probes	1:500
Goat anti-Rabbit Alexa 488	Molecular Probes	1:500
Donkey anti-Rabbit Cy3	Jackson ImmunoResearch	1:200
Donkey anti-Goat Alexa 488	Molecular Probes	1:500
Donkey anti-Mouse Cy5	Jackson ImmunoResearch	1:500
Rabbit anti-Mouse Alexa 488	Molecular Probes	1:500
Rabbit anti-Mouse Alexa 546	Molecular Probes	1:500

2.8.3 Confocal microscopy and image processing

The cells were analysed and pictures were taken with the help of a Leica confocal inverted microscope model DM IRB with a TCS SP 2 Confocal Scanner (Leica, Mannheim Germany) and with the help of a Leica confocal inverted microscope model DM IRB equipped with a CARV Confocal cell imaging system (Atto Bioscience, Rockville, MD,

USA) and a Hamamatsu ORCA ER CCD camera using a 63x/1.32 oil immersion or a 63x/0.7 long distance lens. The recording software was AQM from Kinetic Imaging, Nottingham, UK.

The images were further processed using the Adobe Photoshop software (Adobe Systems, USA).

3 RESULTS

3.1 MAPPING THE TCTEL BINDING SITE ON OBSCURIN

In a classical Yeast Two Hybrid screening for obscurin interaction partners, Tctel was discovered as ligand (Young P. and Gautel M., unpublished). Tctel is the putative human homologue of the mouse dynein light chain Tcte 1 (Watanabe et al., 1996). The obscurin fragment used as bait in the Yeast Two Hybrid Screen – spanning from Ig55 to the SH3 domain. In order to determine whether the entire bait or only one of its three sub domains (Ig55 domain, SH3 domain or the linker region) are Tctel specific binding site, yeast two hybrid analysis was performed in L40 host cells.

The obscurin fragments Ig55, Ig55-linker and Ig55-SH3 cloned in a modified pLexA bait vector (Young, 2000) were checked for unspecific HIS3 reporter gene activation by plating simple L40 transformants on double selection medium lacking Trp and His. None of them was able to induce false positive growth of the host cells at 30^{o}C, ensuring that the co-transformation experiments with Tctel prey vector will produce reliable results. Tctel cloned into pGAD10 prey vector was co-transformed with each of the obscurin fragments in pLexA and plated on triple selection medium (-W-L-H) at 30^{o}C. Under these conditions all co-transformants grew, suggesting that the minimal obscurin fragment to which Tctel can bind is the domain Ig55 (**Figure 3-1**).

In order to verify these results, three further fragments of obscurin lacking the Ig55 domain were cloned into pLexA to be subsequently used in a forced yeast two-hybrid assay with the Tctel prey vector. First, the new bait vectors were checked whether their encoded proteins are able to autonomously activate the HIS3 reporter gene. Of all, only the SH3 domain, due to its acidic pH, was able to promote L40 cells growth on His deficient agar. This ability could be inhibited by plating the simple transformants on selection medium supplemented with 5mM 3-AT. All new bait plasmids were each co-transformed with the Tctel prey vector and checked for growth in the absence of Trp, Leu and His (the agar contained 5mM 3AT in the case of pLexA SH3 vector) (**Figure 3-1**). In agreement with the first observations, none of these obscurin bait vectors was able to induce host cell growth under appropriate selection, suggesting that the absence of obscurin Ig55 domain abolished the interaction with Tctel.

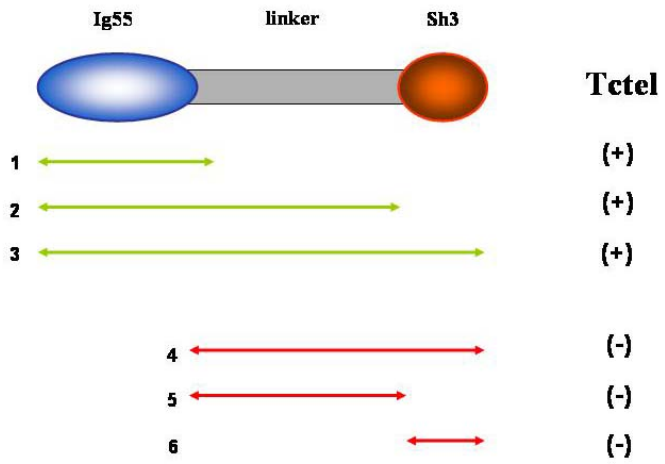

Figure 3-1 Obscurin Ig55 contains the Tctel binding site. The pGAD10 Tctel prey vector was co-transformed with each of the pLexA bait vectors containing obscurin DNA fragments Ig55 (1), Ig55-linker (2) and Ig55-SH3 (3). The co-transformants were plated on triple selection medium lacking Trp, Leu and His. All co-transformants grew, suggesting that the smallest obscurin fragment necessary for Tctel binding is the domain Ig55. In parallel, the bait vectors were checked for auto-activation capacity of their encoded proteins. None of them was able to grow suggesting that the co-transformations reflect a true protein-protein interaction. Bait vectors of obscurin fragments 4, 5 and 6 lacking the domain Ig55 were checked for growth in co-transformants with Tctel prey vector. This control experiment confirmed that Tctel doesn't interact with the linker fragment or with the SH3 domain of obscurin. SH3 domain autonomous activation of the HIS3 gene was inhibited by 5mM 3AT.

Taken together, these results indicate that the possible docking site of Tctel on obscurin is the domain Ig55, since the yeast host cells always grew in the presence of the Ig55 domain but the linker and the SH3 domain failed to activate HIS3 reporter gene when co-transformed with Tctel.

3.2 OBSCURIN IG55 AND TCTEL INTERACT *IN VITRO*

3.2.1 Gel filtration assay

The interaction between obscurin Ig55 and Tctel was verified *in vitro.* However, the first attempts of obtaining evidence for the existence of the complex *in vitro* failed. The two recombinant proteins mixed at 1:1 molar ratio were applied on a Superose 12 HR gel filtration column. The equimolar complex was expected at a molecular weight of approximately 24kDa corresponding to the added calculated molecular weights of Ig55 (11kDa) and Tctel (12.5kDa), but under native conditions no complex was visible (**Figure 3-2**). One explanation may be that Tctel formed trimers and eluted at approximately 40.17kDa instead of expected 12.5kDa, as shown in **Figure 3-2**. In control runs, each of the proteins was applied separately on the column, which confirmed the existence of Tctel exclusively as oligomer under the buffer conditions used, while Ig55 eluted at a lower

molecular weight than expected for its monomeric form, probably due to compact folding of the molecule under the buffer conditions used. Oligomerisation of dynein light chains was previously reported for Tctex1/Tctex2 dynein light chains (DiBella et al., 2001) although the authors have reported a dimerization of the protein.

In addition, another explanation for the lack of visible complex may be that under the buffer conditions used, the affinity of Ig55-Tctel complex is much lower than that of the Tctel homotrimer and the amount of complex might be under the detection limit. Moreover, the formation of the Ig55-Tctel complex might be regulated *in vivo* by factors that could not be present in the gel filtration assay.

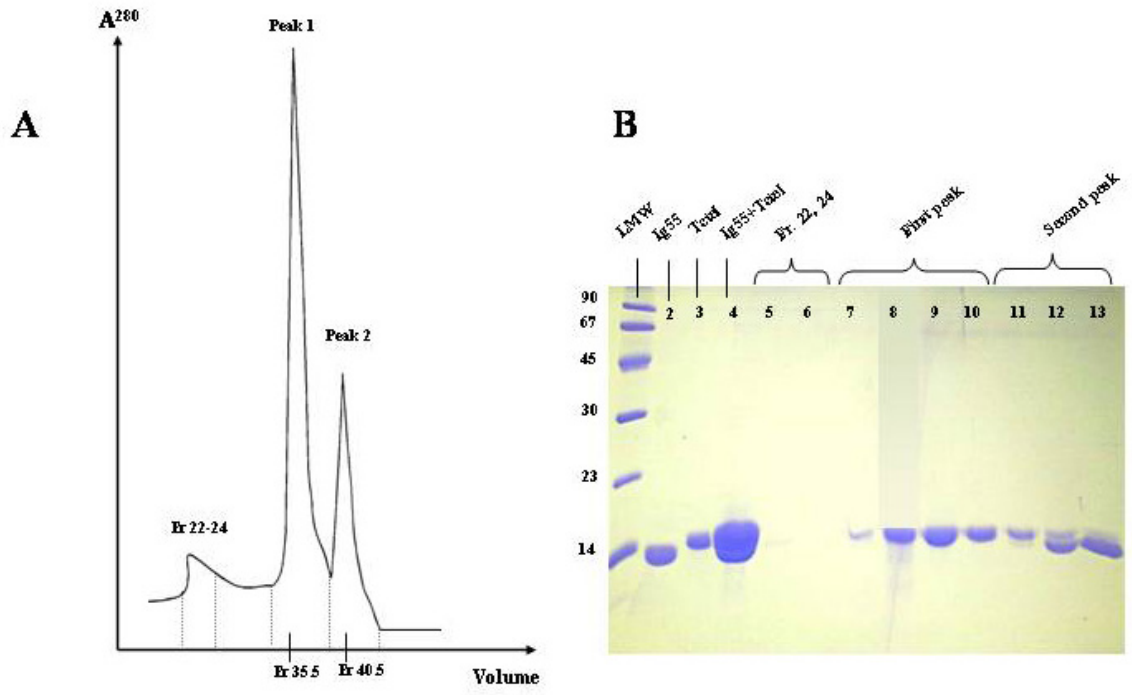

Figure 3-2 Gel filtration of the Ig55-Tctel complex. A) An equimolar mixture of Tctel and obscurin Ig55 was loaded on a Superose 12 HR column. Surprisingly, the two similarly sized proteins were eluted in separate peaks – Tctel at 40.17 kDa (Peak 1) and Ig55 at 8 kDa (Peak 2), which corresponded to Tctel trimeric and Ig55 monomeric forms, respectively. No peak was visible at 24kDa as expected for the complex. The maximum of the peaks was marked (Fraction 35.5 and 40.5, respectively). Dash lines indicate the limits of each peak loaded on SDS PAGE gel; **B)** SDS-PAGE of the gel filtration fractions shows that Tctel oligomers (Peak 1) were denatured under electrophoresis buffer conditions and migrated at ca 14 kDa, while Ig55, eluted as monomer, migrated slightly faster, at about 12kDa. In the last three lanes (11, 12 and 13), a double band is visible corresponding to Tctel and Ig55, but because they are fractions of the second peak (eluted at about 8 kDa) they do not represent the complex of the two proteins, which was expected at about 24 kDa. The double bands are rather due to partial overlapping of the two peaks also suggested by the decreasing amount of Tctel present in the Ig55 peak (lane 11 through 13). Independent control runs with each protein alone resulted in similar chromatograms; Tctel was found exclusively in oligomeric form and eluted at 40.17kDa, while Ig55 was monomeric and eluted at 8 kDa.

Further gel filtration attempts to visualise the complex were made with modifications of the protocol: 1) adjustment of the Ig55:Tctel molecular ratio to 3:1, and 2) the addition of 4M urea to the Ig55-Tctel complex, which would mildly disrupt the Tctel aggregates and favour the formation of the complex on the column as urea elutes. The complex was further on not visible – not shown. A possible explanation might be, in addition to a low affinity of the complex, the unspecific binding of both proteins to the column (in the second case). As urea separated from the protein mixture on the column, none of the proteins was eluted anymore, most probably due to unspecific attachment to the column matrix during refolding.

3.2.2 Pulldown assays with HeLa cell extracts

In pulldown experiments, Ig55 or Tctel proteins were covalently bound to mini Sepharose NHS activated columns and attempts were made to isolate from HeLa cell lysates the endogenous Tctel or the transfected EGFP-Ig55, respectively. The cell lysates would also provide the possible regulation factors necessary for the interaction between obscurin and Tctel. Both methods however failed to fish out the soluble interaction partner. In the case of cytosolic Tctel, an explanation might be that, even though nocodazole depolymerised the microtubules and solubilised dynein (see Materials and Methods), it might be very difficult to dissociate Tctel from the dynein complex. The use of a non-hydrolysable ATP analogue, AMP-PNP, did not lead to positive results. In the case of expressed soluble Ig55 fused with EGFP, no binding was observed probably due to the low affinity of the complex under the buffer conditions used. In the cellular context however, the affinity of this complex might be higher when Tctel is complexed with other dynein chains, as suggested by transfection experiments.

3.2.3 Pulldown assays with recombinant proteins

In an alternative approach, both recombinant Ig55 and Tctel proteins were used as fixed and mobile phases, respectively. Ig55 was covalently-bound to a Sepharose NHS activated column and a Tctel solution was passed through. An identical control column with no Ig55 coupled was used to check whether Tctel is able to unspecifically bind to the matrix. The eluted Tctel protein was visualised through immunoblotting (**Figure 3-3**). Fractions eluted from the identically treated control column show that under the buffer conditions used, Tctel can only weakly bind to the matrix alone (lane 1) while the amount

of Tctel eluted from the Ig55-coupled column is significantly higher (lane 2). This indicates that column-coupled Ig55 is able to retain soluble Tctel even though the gel filtration assay showed that Tctel forms homo-oligomers in solution. Along this line, the use of Tctel as fixed phase was not possible due to the lack of an antibody able to detect the eluted Ig55 domain not fused with any tag. Nevertheless, the pulldown method with recombinant proteins shows that obscurin Ig55 and Tctel are able to interact with each other with low affinity, although i*n vivo,* the affinity of this complex might be higher when Tctel is complexed with dynein.

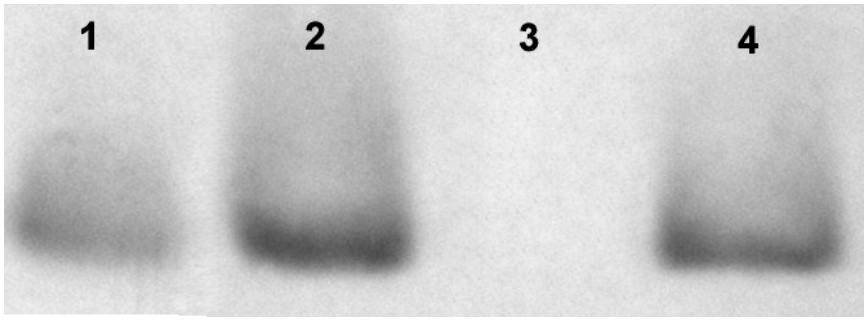

Figure 3-3 Obscurin Ig55-Tctel interaction visualised through immunoblotting. 1) Control column – uncoupled matrix, **2)** Ig55-bound column, **3)** recombinant Ig55 as negative control for the antibody – the anti-Tctel antibody didn't recognise Ig55, **4)** recombinant Tctel as positive control. Although in lane 1 Tctel binds weakly to the column matrix, the amount of eluted protein is significantly higher in the case of the experimental column (lane 2). It seems therefore possible that Ig55 column retains Tctel, suggesting the formation of a complex between these two proteins.

3.3 OBSCURIN IG55 AND TCTEL CO-LOCALISE IN MAMMALIAN COS-1 AND HELA CELLS

The obscurin Ig55 domain was shown to interact with Tctel in indirect yeast two hybrid experiments or *in vitro*, as recombinant proteins. The significance of their interaction, though, is relevant exclusively in the context of the cell. For this reason, investigations were made to further establish their relative localisation at the cellular level with the help of transfection experiments and immunofluorescent labelling. For this purpose an anti-Tctel antibody was raised in rabbit (BioScience) and affinity purified as described in Material and Methods.

3.3.1 Rabbit anti-Tctel antibody tests

3.3.1.1 Immunostainings in HeLa cells

Before directly comparing the localisation of transfected Ig55 and endogenous Tctel, obtaining a correctly reactive anti-Tctel antibody was a prerequisite. First, the localisation of the antigen stained by the antibody was analysed in cells and in a second approach, the antibody was also tested on rabbit heart cell lysates (Section 3.3.1.2).

Tctel is one of the light chains of the human dynein motor complex and thus a constitutive component of all types of human cells, including HeLa cells. Antibodies against Tctel were raised in rabbit, immunopurified on recombinant Tctel-Sepharose columns and tested directly on fixed HeLa or H9C2 cells relative to dynein complexes and microtubules. Tctel was reported to bind directly to the dynein intermediate chain (~ 74kDa) (Mok et al., 2001). This interaction was used to verify the correct reactivity of the anti-Tctel antibody.

Commercial monoclonal antibodies against intermediate chain of dynein (DIC) stained a dotty pattern (Fig 3-4 A, B), in agreement with the role of dynein as motor protein complex implicated in the transport of vesicles on microtubules. The dotty pattern stained by the anti-Tctel antibody (most likely, also corresponding to transported vesicles) overlapped partly with DIC (Fig 3-4 A, see for example the arrow). Complete co-localisation is unlikely to be observed as the dynein subunit composition is highly variable. Depending on the identity of the cargo molecule that is be transported, the heavy chains are different isoforms of DIC, which in turn are able to form complexes with different types of light chains (King et al., 1998; King et al., 2002; Tai et al., 2001). Because both primary anti-dynein IC and anti-alpha tubulin antibodies were raised in mouse, it was only possible to independently compare their relative localisation with respect to Tctel (**Figure 3-4 A, B** and **Figure 3-5**).

In HeLa cells, the anti-Tctel antibody stained the endogenous human protein, while in H9C2, COS-1 cells, or in rabbit cytosolic extracts, it stained the mouse, monkey or rabbit homologues of the human Tctel. These Tctel homologues were recognised by the antibody due to cross-reactivity, as these proteins are highly homologous (e.g. human Tctel and mouse Tctex-1 are 94% identical, 100% similar at protein level (Watanabe et al., 1996).

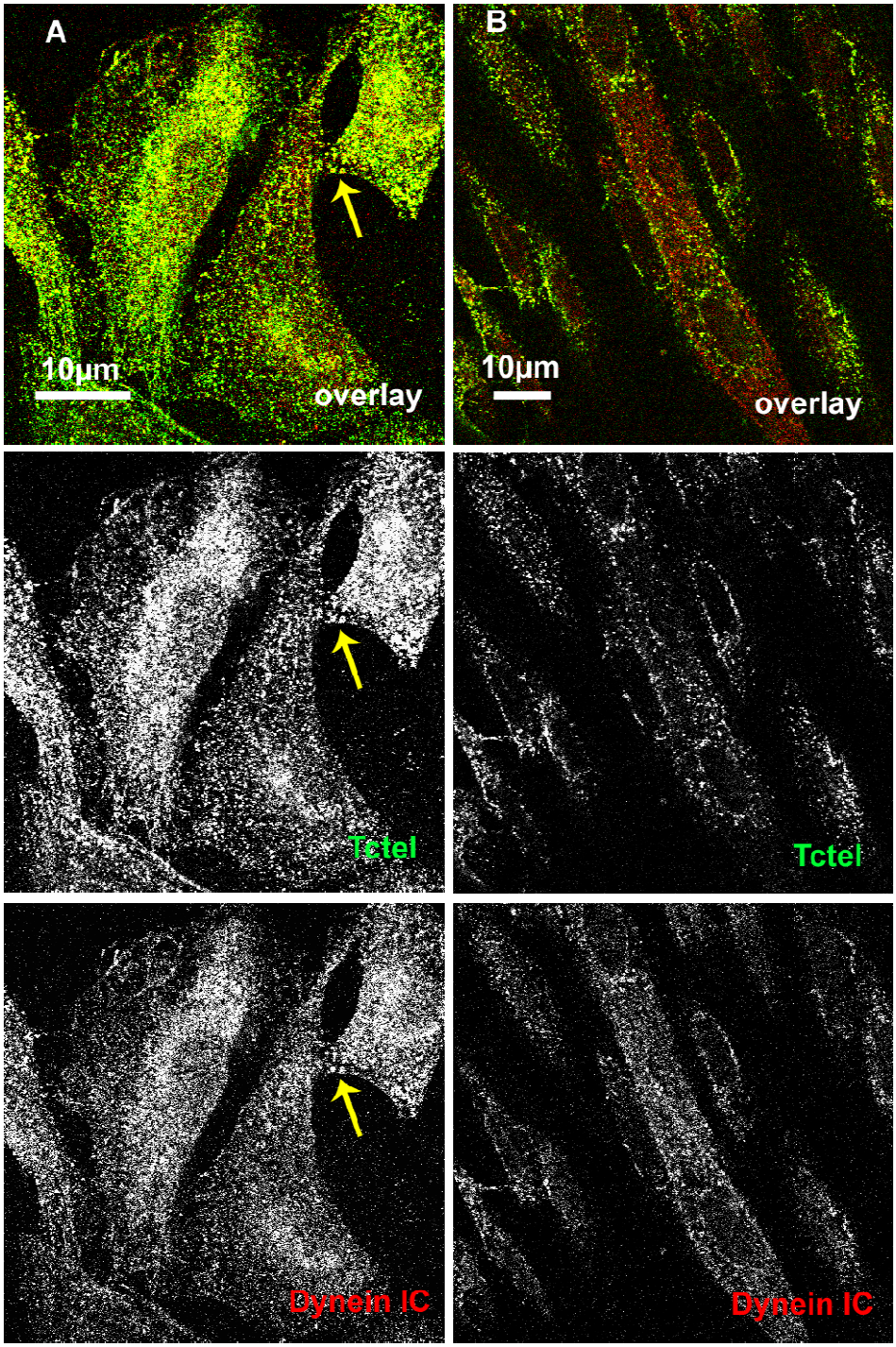

Figure 3-4 Rabbit anti-Tctel antibody test on HeLa cells and H9C2 cells reveals a dotty pattern that partly co-localises with dynein IC. A) and **B)** In HeLa and H9C2 cells respectively, anti-Tctel antibody reveals Tctel decorated vesicles (visible as dots) largely co-localised throughout the cytoplasm with dynein IC as indicated by the yellow colour resulted from the superposition of green (Tctel) and red (dynein IC) (e.g. the arrows). Bars 10 µm.

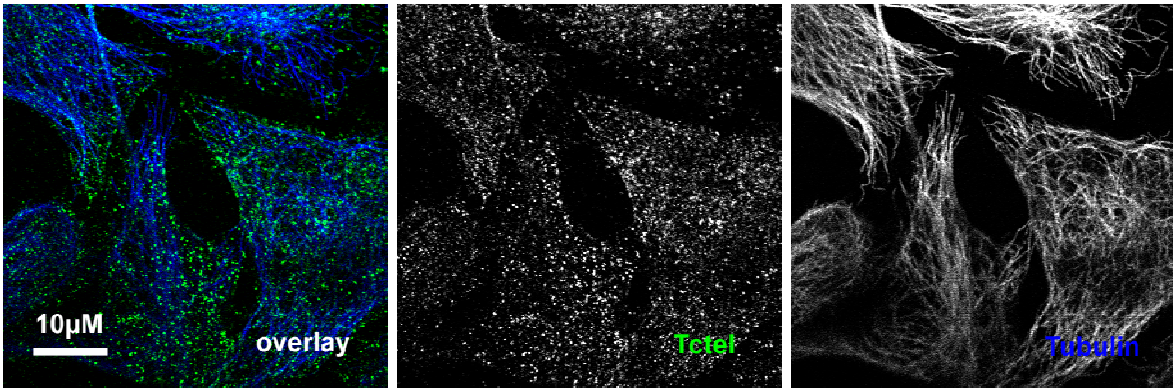

Figure 3-5 Rabbit anti-Tctel antibody stains vesicles on microtubules in HeLa cells. The Tctel dots are only present along anti-tubulin stained microtubules (HeLa cells). These results taken together are confirming that Tctel antibody raised in rabbit stains vesicles transported by dynein complexes on microtubules. Bar 10µm

3.3.1.2 Western blot test on rabbit heart lysates

The anti-Tctel antibody was further tested by immunoblotting for its ability to recognise the endogenous Tctel antigen in cytosolic extracts obtained from adult rabbit heart tissue (Materials and Methods). The antibody successfully detected a band slightly below 14kDa (**Figure3-6**), the calculated molecular weight of Tctel being 12.5 kDa. An additional strong band was also visible at a molecular weight of about 40kDa, possibly corresponding to an oligomeric form of Tctel also observed in the gel filtration assay (Section 3.2.1). This homotrimer was not disrupted by SDS/β-mercaptoethanol denaturing reagents present in the electrophoresis buffer, indicating that the affinity between monomers might be very high, maybe increased by posttranslational modifications (e.g. disulfide bridges, which in some cases may resist denaturation). In contrast, the recombinant form of Tctel is easily denatured by the SDS PAGE buffer conditions (**Figure 3-6**), although the same protein prep, loaded on gel filtration column, produced exclusively oligomers under native buffer conditions. Therefore, oligomerisation might be an intrinsic property of Tctel, as the recombinantly obtained protein can form trimers, but these complexes might be stabilised in vivo by posttranslational modifications.

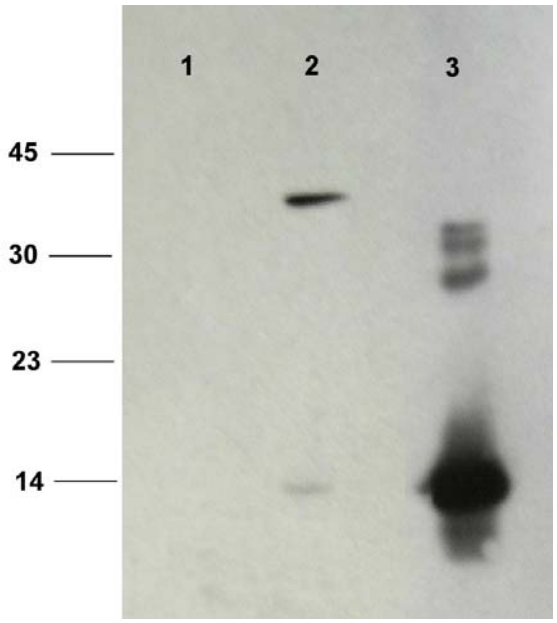

Figure 3-6 Rabbit anti-Tctel antibody test on rabbit heart cytosolic lysates. 1) LMW marker (also negative control for the anti-Tctel antibody which didn't unspecifically recognise any of the proteins), **2)** Cytosolic extract of rabbit heart tissue, **3)** Recombinant Tctel. The heart lysates were prepared as described in Materials and Methods (Section 2.6.3). The anti-Tctel antibody recognised in the cell lysate (lane 2) a band at about 14kDa corresponding to recombinant Tctel, loaded as positive control (lane 3). In the same lane, another band is visible at a molecular weight of about 40kDa, most probably representing an oligomeric form of Tctel (also detected by gel filtration – Section 3.2.1), which was not disrupted by the denaturing conditions of the SDS PAGE. Both monomeric and oligomeric Tctel were solubilised from microtubules by the lysis buffer.

3.4 TRANSFECTED IG55 CO-LOCALISES WITH TCTEL IN MAMMALIAN CELLS

3.4.1 Ig55-Tctel co-localisation in monkey COS-1 cells

After establishing the correct reactivity of the anti-Tctel antibody, it was possible to proceed to transfection experiments of mammalian cells with EGFP-coupled Ig55 domain. Initially, COS-1 cells were used for this experiment, being a cell line very easy to handle in culture and to transfect. The transfected green fluorescent cells were fixed with a protocol that protected thde cytoskeleton structures as well as the soluble cytosolic proteins (Materials and Methods) and were subsequently counter-stained with anti-Tctel and anti-tubulin antibodies in order to determine whether the Ig55 domain is targeted to microtubules through the interaction with Tctel (**Figure 3-7**). The obscurin Ig55 domain displayed a cytosolic as well as nuclear localisation. The later, however, doesn't represent in this case a real targeting to this cellular compartment, but an artefact often encountered

in the case of over expressed GFP (**Figure 3-8**). The cytosolic pattern of Ig55 was relatively diffuse, but the regions enriched in Ig55-EGFP were largely overlapping with Tctel, and both together were only present on microtubules (for example, the 3 “spike”-like structures indicated by arrows). This suggests that in cellular context, Ig55 and Tctel may be interaction partners, as they co-localise in the same cytosolic structures.

Generally, the dynein light chains can simultaneously exist in a motor-bound or soluble, possibly inactive state, depending on the cellular request of the moment. In the cytosolic, soluble fraction of the transfected cells, a co-localisation of the two proteins was not visible both proteins being largely diffuse (**Figure 3-7**). Obscurin Ig55, therefore, might interact with the active, motor-bound form of Tctel.

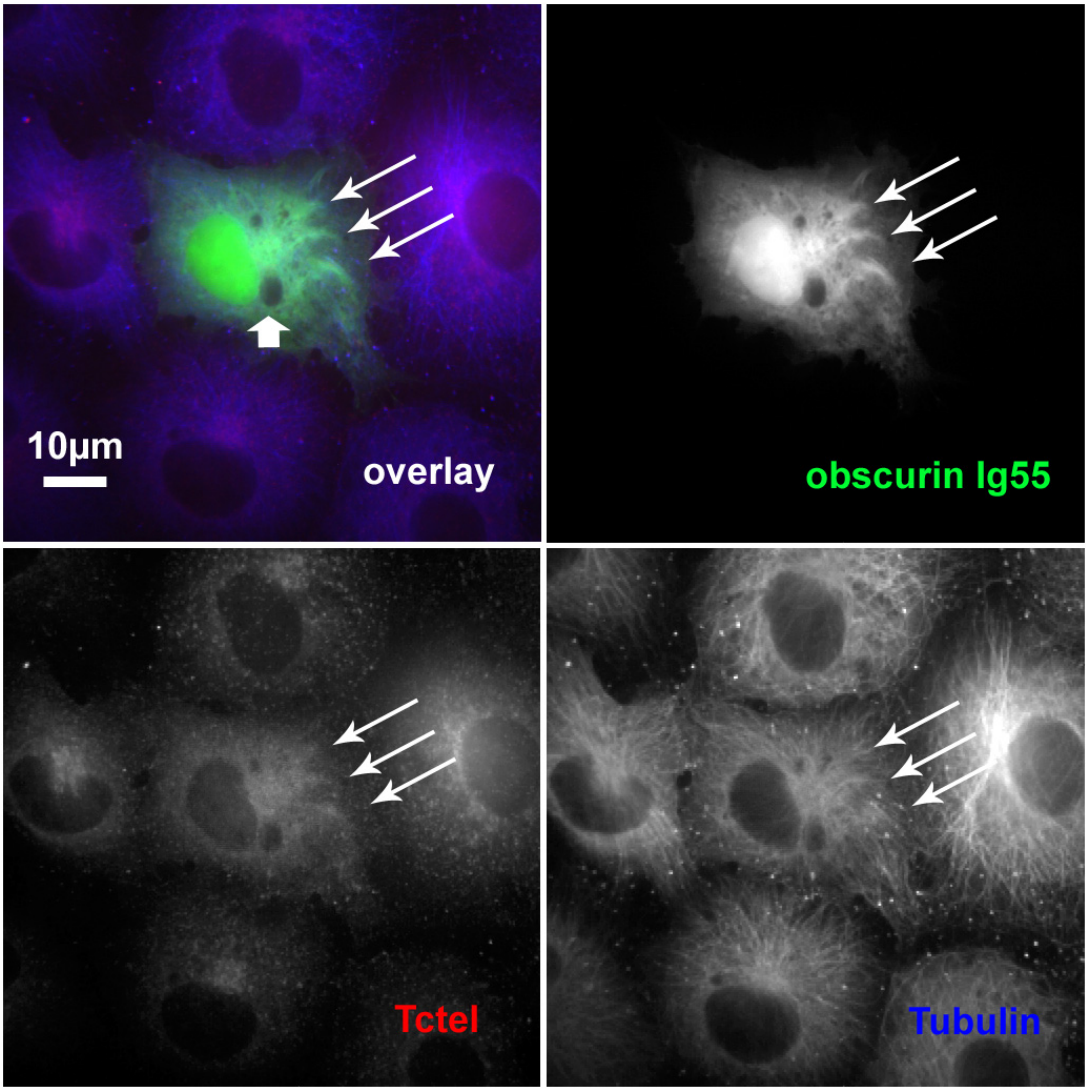

Figure 3-7 Transfected obscurin Ig55 co-localises with Tctel in monkey COS-1 cells. Upon expression of the hybrid protein Ig55-EGPF, the transfected mammalian cells were fixed using a protocol that preserved both the cytoskeletal structures and the cytosolic content (Materials and Methods) and were stained for Tctel/alpha-Tubulin. The transfected construct was largely diffuse, but some co-localisation was observed with Tctel and tubulin as they are all present in structures indicated by thin arrows. Moreover, it seems that simultaneously all avoided "empty" regions visible in all channels as black round areas in the vicinity of the nucleus (e.g. thick arrow in the overlay picture), this suggesting that were no microtubules are present, neither Tctel nor Ig55 are visible. Bar 10µm.

This targeting effect was not caused by the GFP half of the chimera construct, as transfection of EGFP alone did not lead to co-localisation with microtubules (**Figure 3-8A**), The construct was distributed in the cytosol and was almost with no exceptions diffuse, but also produced a localisation artefact by accumulating inside the nucleus (already observed in **Figure 3-7**). Sporadically (under 1%), the EGFP protein was also observed attached to actin stress fibres (**Figure 3-8B**) and these cells were not considered in the analysis of the transfected Ig55-EGFP construct.

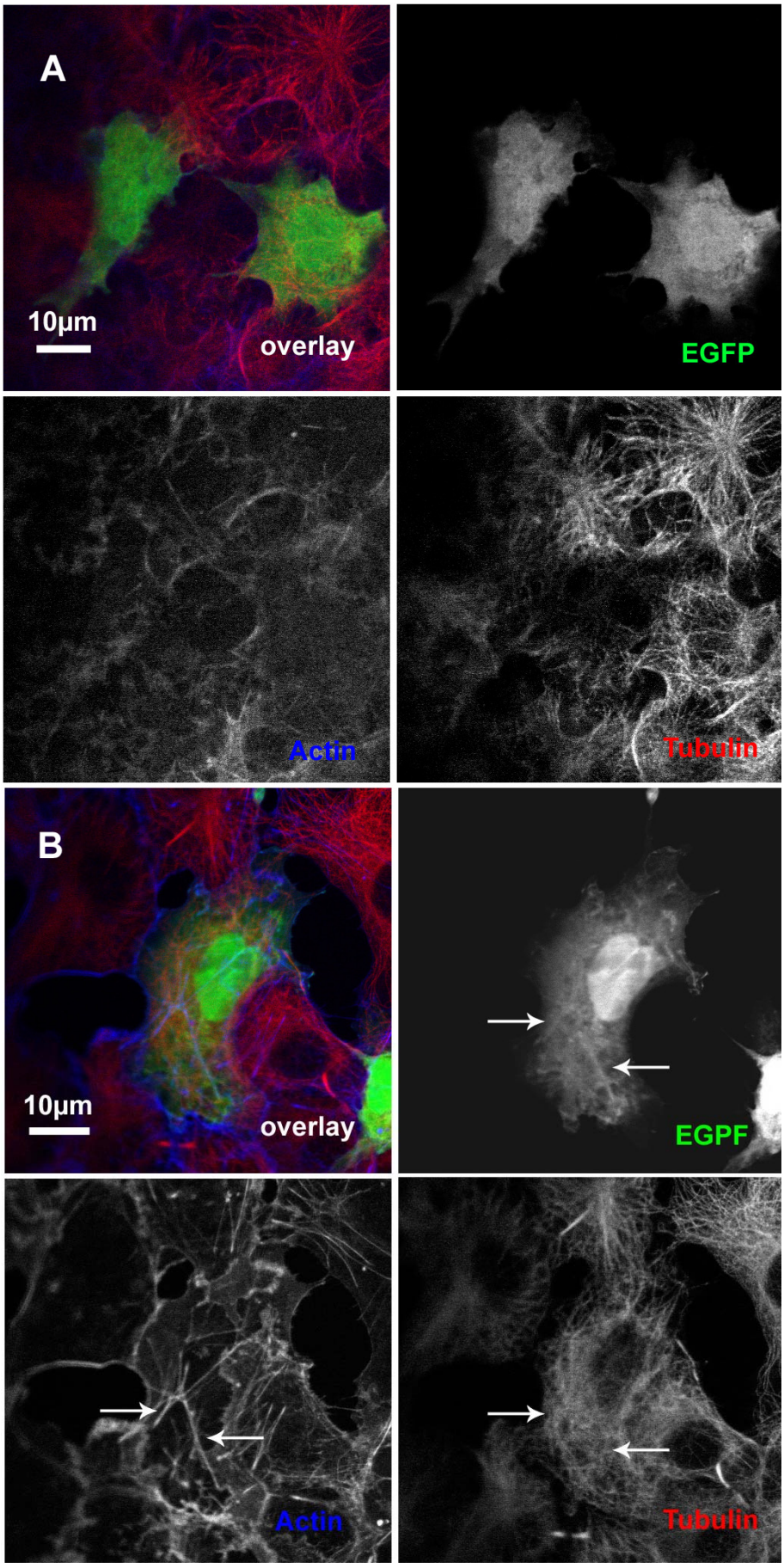

Figure 3-8 Control transfection of COS-1 cells with EGFP. (A) and **(B)** COS-1 cells transfected with the pEGFPN2 vector encoding the EGFP protein alone. In (A) it is shown that, in the majority of cases, EGFP cytosolic distribution was diffuse, the protein not being associated with any cellular structures. In (B) one of the few exceptions is shown when EGFP was targeted to actin stress fibres (indicated by arrows). When encountered in transfections of the fused Ig55-EGFP construct, the cells displaying this artefact were not considered.

3.4.2 Ig55-Tctel co-localisation in human HeLa cells

For improving the visualisation of a possible co-localisation between Ig55 and Tctel, the cell fixation method was modified and the soluble content of the cytosol was extracted with detergent, while all cytoskeleton structures were protected and fixed (Material and Methods). Simultaneously, the cell line was changed to HeLa, a human cell line which would provide more accurate information about a possible co-localisation of obscurin and Tctel, as these cells display a better microtubule cytoskeleton than the COS-1 cells. Except the fixation method which differed, HeLa cells were transfected and stained in a similar way with the COS-1 cells. In HeLa cells, all three proteins stained strikingly similar patterns as observed in all three colour channels and in the overlay pictures (**Figure 3-9**). The expressed EGFP-coupled Ig55 domain displayed a structured cytosolic, network-like distribution, suggesting its targeting to a pre-organised cytosolic network – the microtubule network as shown by this experiment. Tctel is also present together with Ig55 on microtubules, potentially mediating its attachment to this cytoskeleton network (**Figure 3-9 A)** and **B)**).

Transfection of H9C2 myoblasts with EGPF-Ig55, show similar obscurin targeting to microtubules and co-localisation with Tctel (**Figure 3-9 B**).

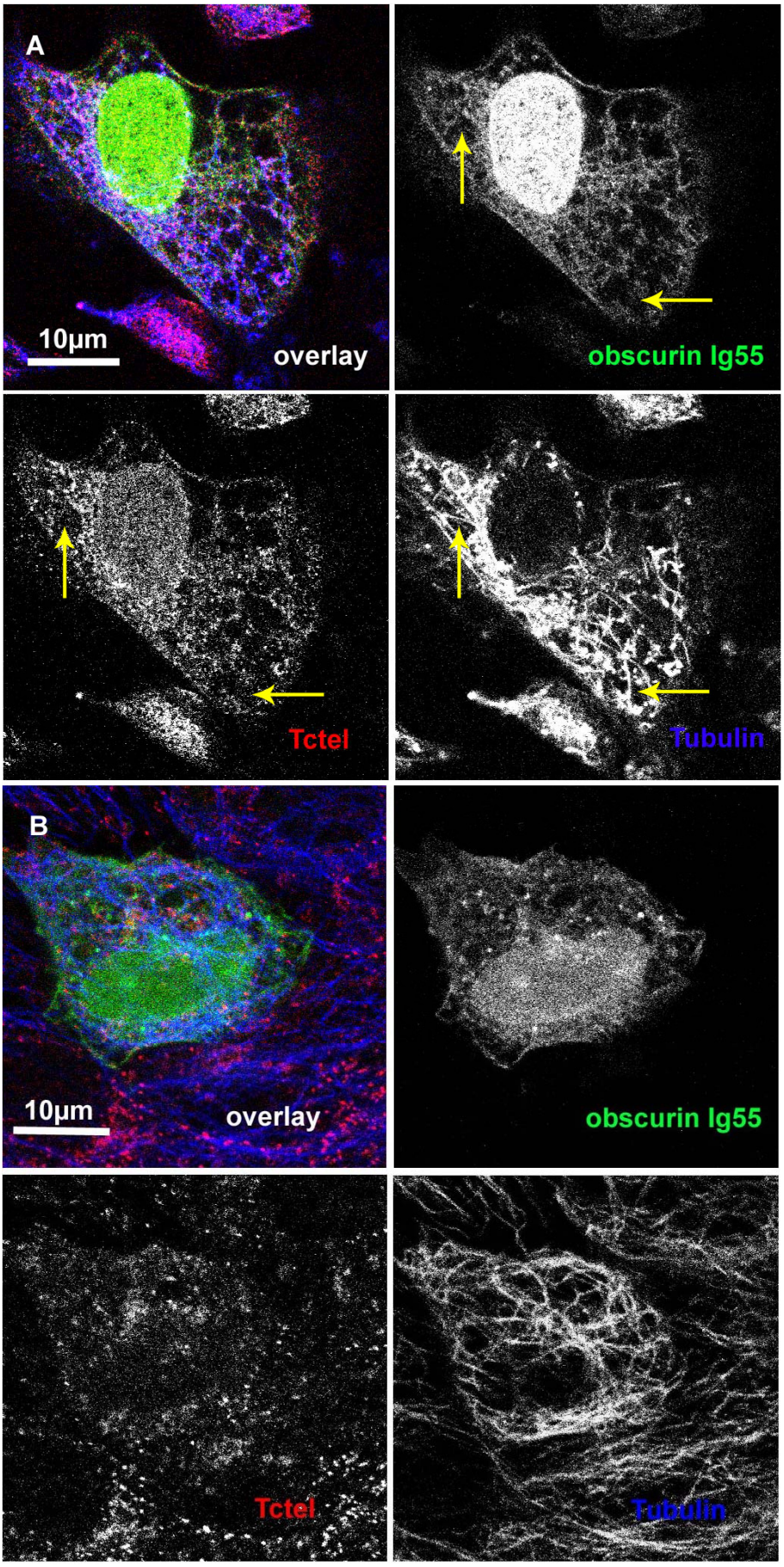

Figure 3-9 Tctel specifi-cally targets obscurin Ig55 to microtubules. After changing the fixation method and extracting the cytosolic proteins, the co-localisation of Ig55 and Tctel became visible. In both HeLa cells **(A)** and H9C2 myoblasts **(B)**, transfected EGFP-coupled Ig55 domain (green) stains similar cytosolic structures as Tctel and both together follow the microtubule tracks, suggesting that obscurin Ig55 is targeted to dynein motor complexes on microtubules through its specific interaction with Tctel. The presence of Ig55 on microtubules is conditioned by the presence of Tctel as indicated by the arrows – on microtubules that are not decorated by Tctel, Ig55 is also missing. (Note: the observed nuclear localisation of Ig55 represents, as shown in COS-1 cells and control experiments with HeLa cells, an artefact due to overexpression of EGFP). Bars 10µm

A control of the EGFP protein alone was also made for HeLa cells using the new fixation/extraction method and similar results were obtained like with COS-1 cells. The construct localised in the nucleus or it was diffuse at the cytosolic level, while some actin stress fibre association was observed in even less cases than with COS-1 cells (not shown).

The specificity of Ig55 targeting to microtubules was verified by destabilisation of the tubulin network with the help of nocodazole. The drug is able to specifically inhibit the formation of new microtubules and, at a concentration higher than 1μM, can also depolymerise the existing tubulin network if maintened long enough in contact with the cells (Webster and Patrick, 2000). This would result as well in the detachment of the dynein complexes from microtubules. Nocodazole was added at the end of the optimal expression time of transfected EGFP-Ig55 protein and incubated with the cells for one additional hour. **Figure 3-10** shows the effect of microtubule depolymerisation on the distribution of Ig55. Two confocal planes of the same cell show aspects of a complete and incomplete nocodazole effect at different cellular depths. The disruption of the tubulin network affected the distribution of Ig55 which became diffuse where no microtubules were anymore present, while being still partly co-localised with Tctel and fragments of yet unfragmented microtubules. Moreover, it seems that under the nocodazole effect, the interaction between Ig55 and Tctel was not disrupted, but only their complex was detached from tubulin polymers. Ig55 and Tctel still co-localised in structures not visible in the tubulin channel.

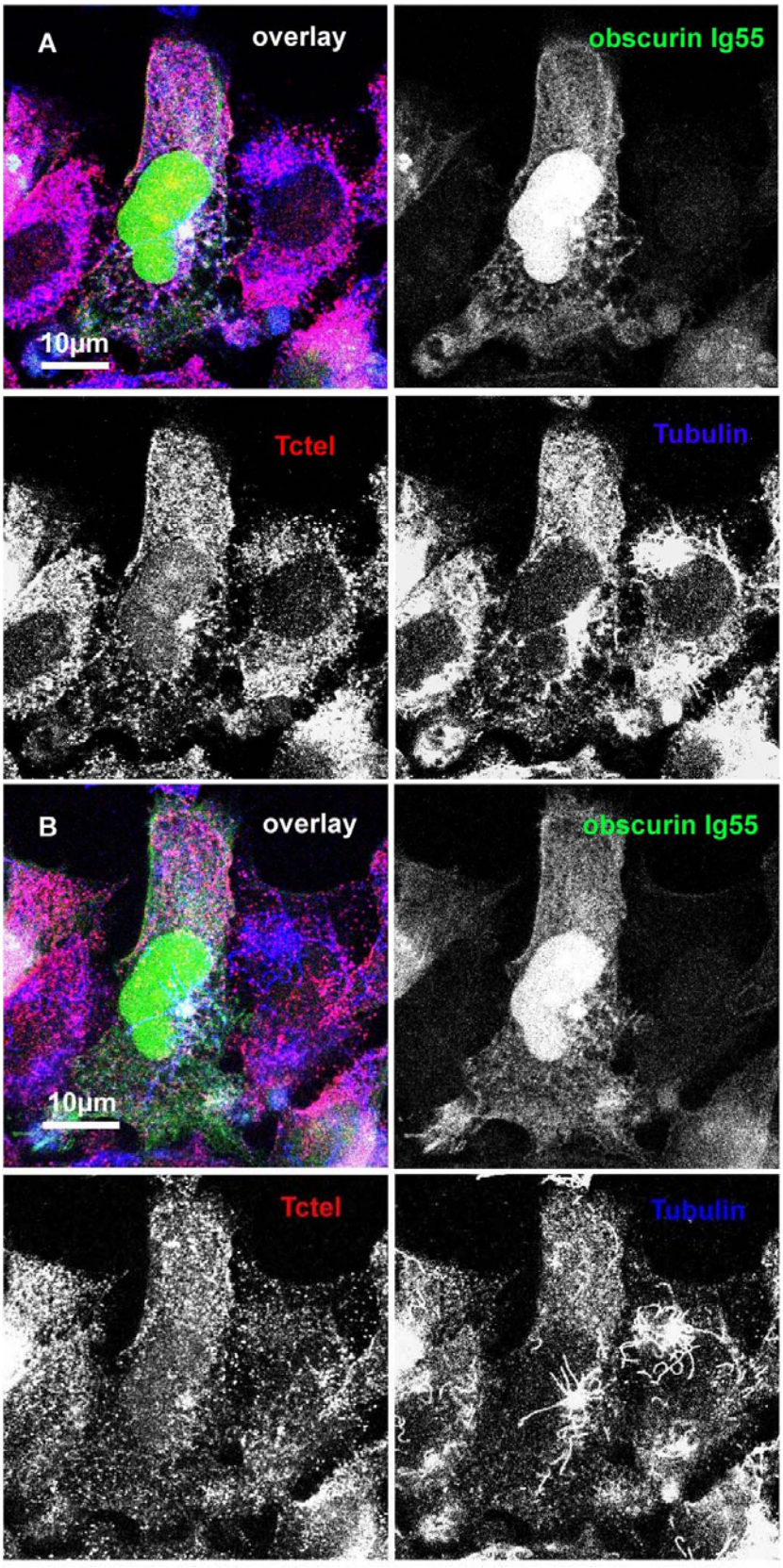

Figure 3-10 Nocodazole specifically breaks down the attachment of obscurin to microtubules. A) and **B)** Two confocal plans at different cellular depths show that nocodazole disrupts the interaction Ig55/Tctel-tubulin. In **A)**, the upper half of the cell displays total depolymerisation of the microtubules visible as diffuse tubulin, while the lower half still has small fragments of microtubules as well as completely diffuse tubulin. Obscurin and Tctel seem both diffuse where no microtubules exist anymore but are still organised in a network-like pattern co-localising with tubulin where this one was not depolymerised. **B)** In a different confocal plane the same cell displays only diffuse tubulin (with the exception of the centrosome) and at this level both Tctel and Ig55 are mostly diffuse as well; the only poorly structured regions of Ig55 and Tctel are still largely co-localised in the lower half of the cell and seem to have detached from tubulin as there are no microtubules visible to support them in that area. Therefore, the nocodazole effect confirms the specificity of Tctel mediated Ig55 targeting to microtubules. Bars 10µm

The co-localisation of obscurin Ig55 domain and Tctel in the cytosol suggests that a direct specific interaction is possible between them in the cellular context confirming that the yeast two hybrid analyses and the biochemical assay indicate the existence of a biologically significant complex. The targeting of obscurin Ig55 to microtubules therefore seems to occur through Tctel. Moreover it seems possible that the Ig55 domain would be necessary and sufficient to target the entire obscurin molecule to vesicles transported by dynein motor complexes on microtubules during myofibrillogenesis. Obscurin was recently reported to interact through its C-terminal end with the small ankyrin 1.5, a membrane protein of the sarcoplasmic reticulum located, in muscle, at the periphery of myofibrils, predominantly at the M-band (Bagnato et al., 2003). It therefore seems possible that this interaction might play a role in the vesicular transport during myofibrillogenesis, as the ankyrin molecules are known to mediate attachment of the sarcoplasmic reticulum vesicles. (Section 1.3.1).

3.5 OBSCURIN IS FOUND ON MICROTUBULES IN MYOBLASTS

3.5.1 Myoblasts differentiation under native conditions

To further investigate whether Ig55 domain is able to target the entire obscurin molecule to microtubules, differentiating myoblast cells were used. For this purpose, cultured H9C2 cardiac myoblasts were induced by low serum to initiate differentiation and then stained for endogenous obscurin using an antibody against the obscurin Ig-IQ-Ig epitope, distinct from the Ig55 domain, to determine the localisation of other epitopes than Ig55 (for obscurin domain pattern, see **Figure 1-7**). As shown in **Figure 3-11**, in the cells that switched on the differentiation program and started to express obscurin, this epitope is found as dots that closely follow microtubule tracks along the muscle cell. The figure displays a series of intermediate steps of myofibrillogenesis in which the distance between obscurin dots increases but has not yet reached the usual distance displayed by mature myofibrils while, simultaneously, the lateral alignment improves from A) to C).

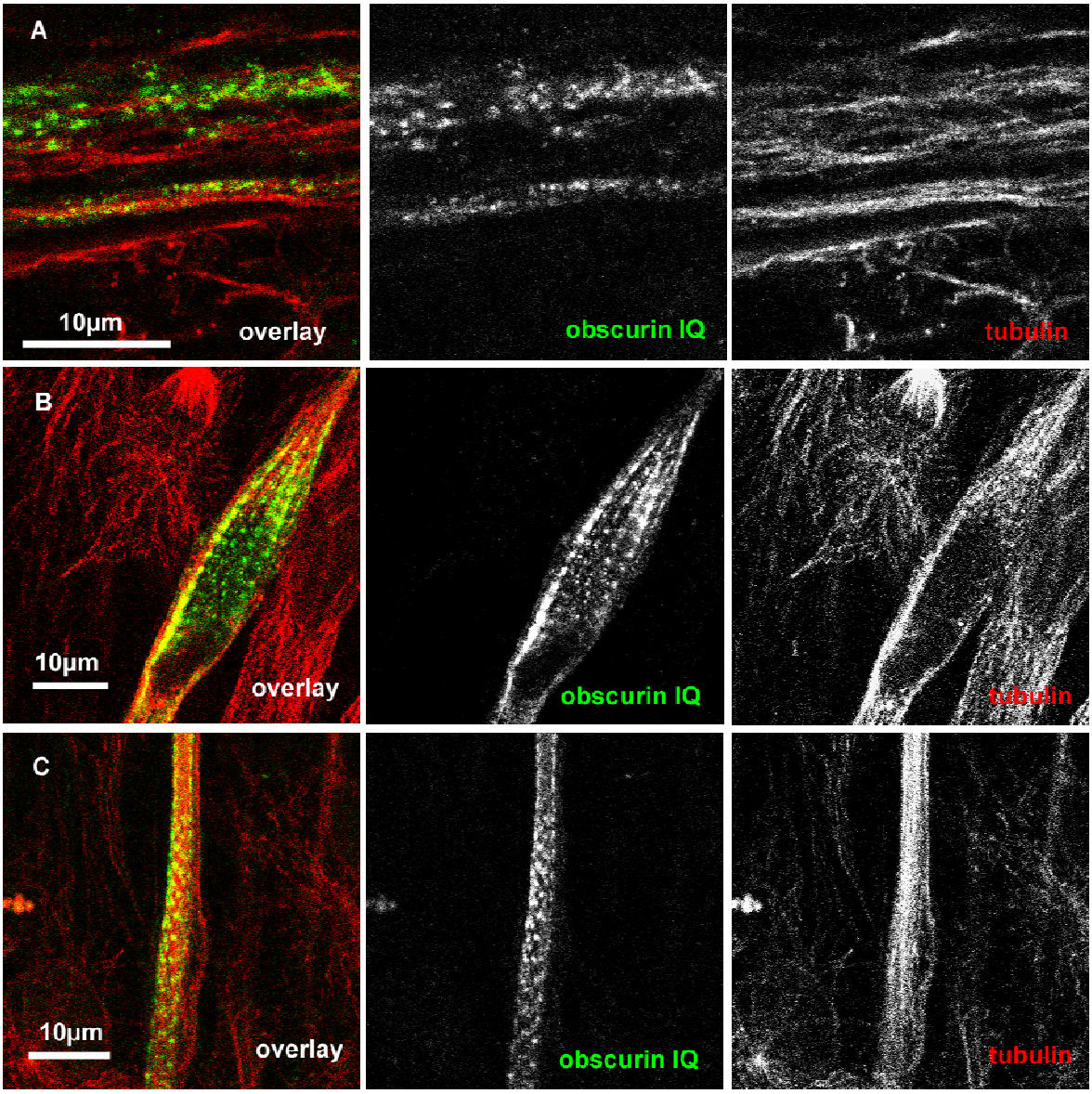

Figure 3-11 Obscurin is located along the microtubules in differentiating myoblast cells. A), B), C) During differentiation of H9C2 cardiac myoblasts another epitope than Ig55, the IQ domain, is found distributed along microtubule tacks in a dotty pattern at distances that do not span yet the full width of a mature sarcomere. In this series of pictures the maturation stage evolves from A) to C) as suggested by the increased organisation of the sarcomeres and suggests that during myofibrillogenesis obscurin may travel along microtubules from the cytosolic sites of synthesis to the sarcomeric sites of its final localization in mature myofibrils

3.5.2 Myoblasts differentiation in nocodazole medium

The specificity of obscurin targeting to the microtubules was verified in differentiating myoblasts by nocodazole depolymerisation of the microtubule network. For this purpose the H9C2 cells were differentiated in low serum medium containing 10μM nocodazole from the first day throughout the entire differentiation period. In most of the cases, the presence of nocodazole in the differentiation medium produced rounded up H9C2 cells that didn't display any striations although the differentiation program was initiated and the obscurin epitope Ig-IQ-Ig was expressed (**Figure 3-12**). Moreover, unlike in the absence of nocodazole (**Figure 3-11**), during differentiation with nocodazole obscurin is found completely segregated from tubulin as shown in **Figure 3-12**. This suggests that the association between obscurin and tubulin is specific and that during myofibrillogenesis in cardiac muscle cells the presence of microtubules is required for transportation of obscurin and proper integration at its perisarcomeric sites.

In isolated cases, some H9C2 cells (less then 1% per dish) submitted to differentiation in the presence of nocodazole formed striations (not shown). These striations, however, could be due to spontaneous differentiation observed in some culture dishes before switching to low serum conditions. The explanation might reside in the fact that these cells, apart from low serum, are easily activating their differentiation program when the cell density is high. Confluence is not likely to be reached simultaneously in the entire culture dish and thus, the slightly more dense areas could allow the initiation of differentiation even in rich serum conditions. Those cells will be then able to preserve their striations in the presence of nocodazole as microtubules are not required for the structural maintenance of the sarcomeres (Rothen-Rutishauser et al., 1998). This is why the few cells displaying proper myofibrils in nocodazole were not considered.

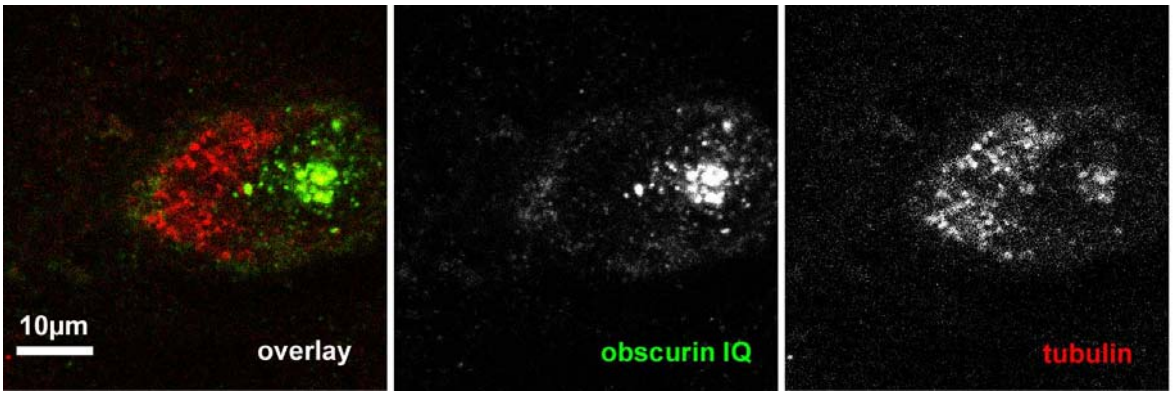

Figure 3-12 Obscurin co-localisation with tubulin is disrupted by nocodazole in myoblast cells. H9C2 cells were submitted to differentiation in the presence of 10µM nocodazole throughout the entire culture time in low serum. With almost no exceptions, the cells rounded up (some even detached and were washed away with the fresh medium). The cells that initiated the differentiation programme and expressed obscurin displayed virtually no myofibrils and the IQ epitope was completely segregated from tubulin in contrast with the native conditions presented in Figure 3-10.

In myofibrillogenesis, obscurin is expressed at an early time shortly after titin and seems to become organised in a striated pattern only after this one (Young et al., 2001). Titin moves along actin stress fibre-like structures to the sites of the future Z-disks to stretch from there its C-terminal end across the sarcomere up to the M-band (discussed in Section 1.2). Obscurin on the other hand has an unknown way of adopting its striated pattern but the interaction between its Ig55 domain and Tctel observed might indicate the implication of microtubules in this process.

3.6 OBSCURIN MAY BE INVOLVED IN THE VESICULAR TRANSPORT

3.6.1 Tctel co-localises with calreticulin-containing vesicles

During differentiation, the organization of the cellular organelles and the plasma membrane of the myoblasts changes dramatically, with the consequent formation of a single functional unit. This process involves extensive reorganization of the constituents of the cytoskeleton, the microtubule organizing sites being re-localised at the surface of the nuclei in myotubes, in marked contrast with the classical pericentriolar localization(Lu et al., 2001). Also new muscle specific organelles such as the sarcolemma, the sarcoplasmic reticulum (SR) and the transverse (T-) tubules are generated. (Tassin et al., 1985). Apparently, also novel membrane trafficking pathways are needed to communicate with

the new organelles and membrane domains. SR forms gradually from the ER compartment (Sitia and Meldolesi, 1992; Volpe et al., 1992) starting in early stages of myofibrillogenesis and being completed approximately 14 days after birth, time that in small mammals largely corresponds with the onset of motor activity (Villa et al., 1993). This process involves changes in the ER/SR endomembrane system at both morphological and molecular level (Damiani et al., 1992; Flucher, 1992; Franzini-Armstrong et al., 1987; Jorgensen et al., 1977; Schiaffino and Margreth, 1969) and evidence exists these may be accomplished by vesicle generation form ER that will fuse and align along myofibrils (Nori et al., 2004). The observation that obscurin travels along the microtubules, corroborated with the newly reported interaction with ankyrin (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003, Section 1.5) suggests that this interaction may not only play a role in SR anchoring to the myofibrils in mature muscle, as proposed by the authors, but it may possibly take place also at earlier stages of muscle differentiation and be involved in the transport of vesicles from ER to SR. In this process, the role of obscurin might be that of an adaptor protein between the motor and the vesicle, and may be important for the correct targeting of the cargo to a specific cellular docking site.

To investigate whether obscurin is implicated in vesicular transport, both the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR) lumen were stained for calreticulin. Calreticulin and calsequesterin are, apart form their chaperon functions, the two major Ca^{2+} binding proteins responsible for Ca^{2+} sequestering in the ER and SR during muscle relaxation. While calsequesterin resides only in the ER and is a muscle specific protein, calreticulin is ubiquitously found in most of the cell types but, particularly in muscle, is found in both the ER and SR lumen (Milner et al., 1991; Allen and Katz, 2000). Therefore, differentiating H9C2 cardiomyocytes, if stained with calreticulin, display the localisation of both those compartments, while HeLa cells as a non-muscle cell line, will only reveal calreticulin-containing ER.

Due to the fact that our obscurin Ig55 construct was fused with the EGFP and because the lack of an appropriated secondary antibody other than green fluorescently labelled, the direct observation of Ig55 and calreticulin relative localisation was not possible. Therefore, the use of a reference molecule with already established localisation was necessary. Tctel, which co-localises with Ig55 (**Figure 3-9**), was subsequently considered as reference and visualised in relation with vesicles of the ER in HeLa cells or ER/SR in myoblast H9C2 cells (**Figure 3-13 A** und **B**). Commercial antibodies

recognising calreticulin were used for visualisation of these cellular compartments. In both HeLa and myoblast cells they displayed the reticular nature of ER/SR throughout the cytoplasm (**Figure 3-13 A** and **B**). Tctel decorated vesicles matched tightly the position of calreticulin-containing-vesicles, indirectly suggesting that obscurin Ig55 domain – which co-localises with Tctel – participates in the transport of ER/SR vesicles.

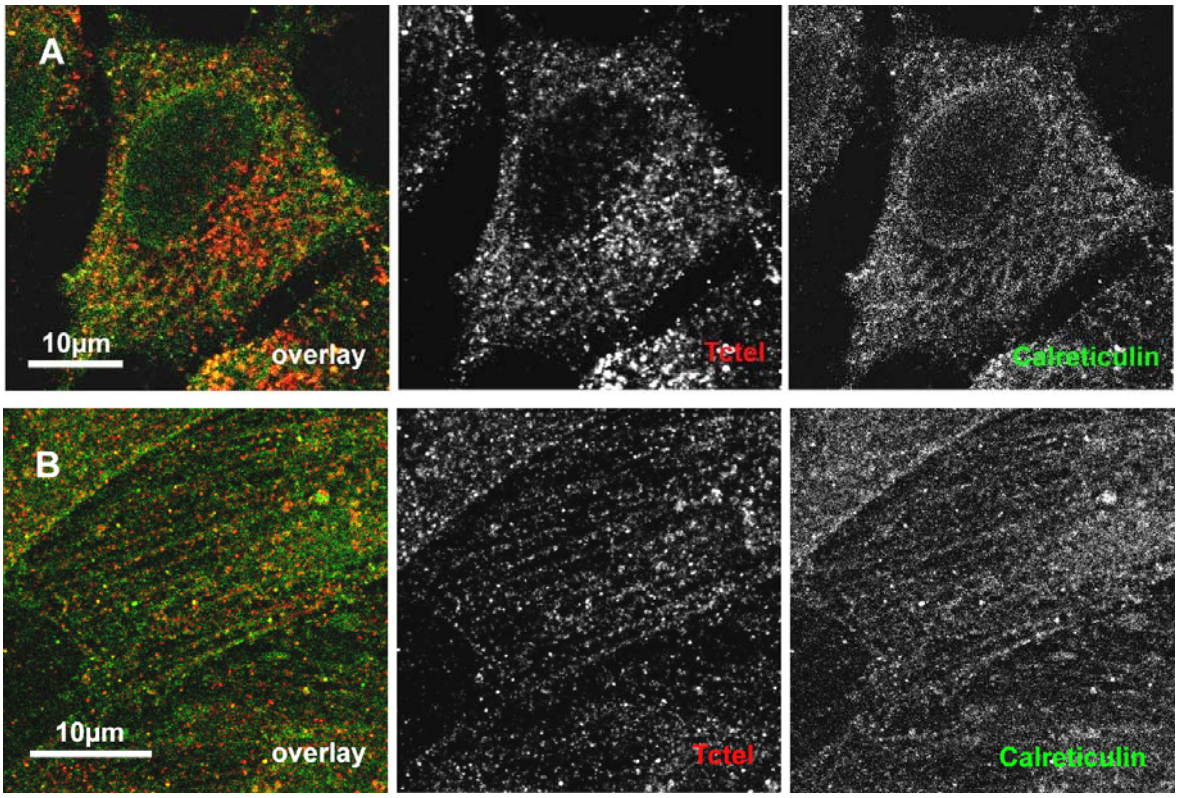

Figure 3-13 Tctel co-localises with calreticulin containing vesicles. In both HeLa **(A)** and myoblast H9C2 cells **(B),** endogenous calreticulin displays a network like pattern corresponding to ER and ER/SR compartments, respectively. The vesicles stained by Tctel, which were previously shown to co-localise with transfected Ig55 on microtubules (**Figure** 3-9), follow closely the network-like pattern of calreticulin throughout the cytoplasm. This suggests that Tctel is involved in the transport of ER/SR vesicles and represents an indirect indication of a possible involvement of obscurin in this process.

3.6.2 RagA might play a role in vesicle attachment regulation

Since the cargo must be loaded onto and released from the dynein motor complexes, some regulation will be required. How the cargo attaches to the motors is generally a still poorly understood issue, but adaptor molecules and regulatory proteins may be involved (discussed in Section 1.3.1). Tctel was reported to interact *in vivo* with the GTP-binding protein RagA (Lukashok et al., 2000) during adenovirus infection of human

host cells. The significance of this interaction is not clear but the authors propose that Tctel/RagA complexes participate in transporting the viral protein Ad E3-14.5 to its site of action upon viral invasion. However, very little is known about RagA or the closely related proteins RagB, C and D. The presence of the obscurin GEF domain (SH3DHPH triad) in the vicinity of the Ig55 domain (**Figure 1-7**) suggests that a GTP binding protein recruited by Tctel would coexist largely in the same place.

To investigate whether RagA might play a role in vesicle transport, anti-RagA antibodies were raised in rabbit. From immune sera, the antibody was affinity purified as described in Material and Methods. Western blot test with rabbit heart cell extracts was performed in a similar way as for the Tctel antibody (Section 3.3.1). This antibody recognised a single band at 29 kDa, the calculated molecular weight of RagA, which confirmed a correctly reacting antibody (not shown).

After establishing the correct reactivity of the anti-RagA antibody, HeLa cells were immunostained to display endogenous RagA in relationship with the ER calreticulin-containing vesicle in order to determine whether RagA might play a role in vesicular transport. RagA GTP-binding protein was found in a dotty pattern (**Figure 3-13**) similar with the one observed for Tctel (see for comparison **Figure 3-9, 3-13**) and co-localised with calreticulin, suggesting that it is as well, like Tctel, attached at least partly to ER vesicles.

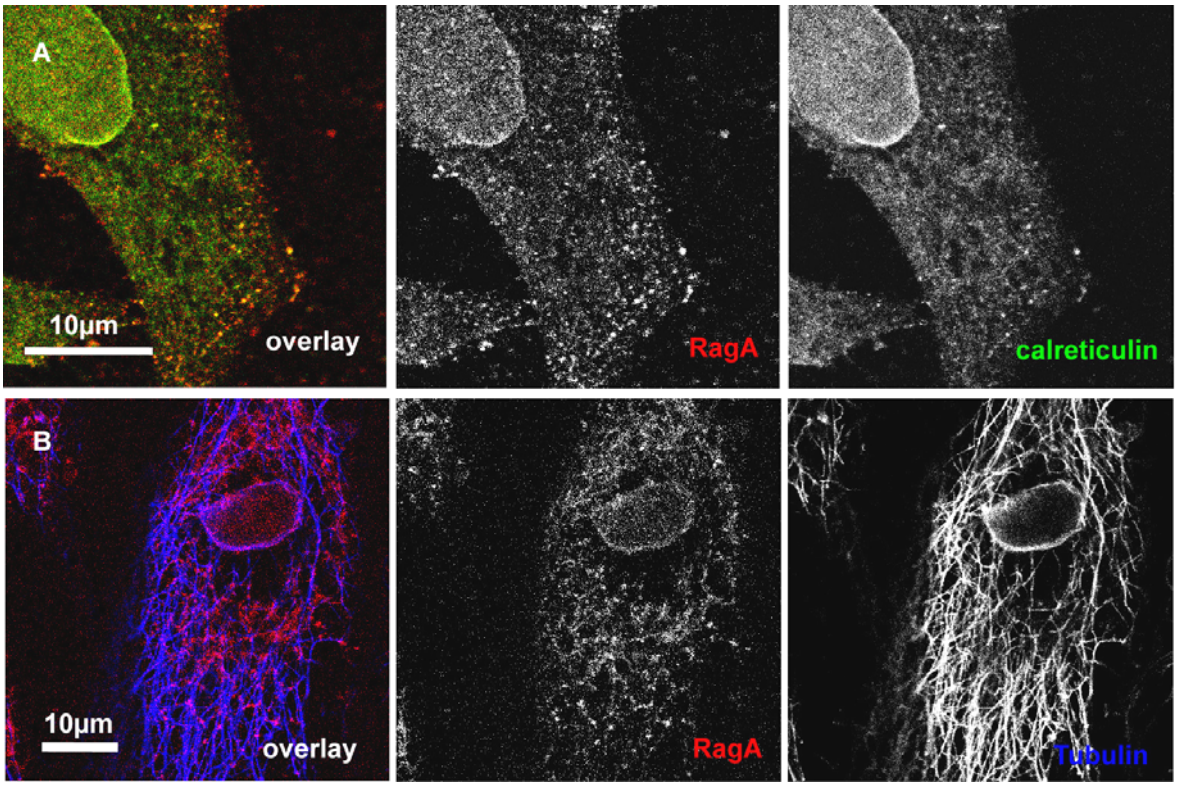

Figure 3-14 Endogenous GTP-binding protein RagA associated with ER vesicles. In HeLa cells **(A)** the anti-RagA antibody stains a dotty pattern very similar to the one observed in the case of anti-Tctel antibody. Calreticulin displays a network-like pattern corresponding to the ER compartment. The largely similar cytosolic pattern stained by the two antibodies suggests, like in the case of Tctel-calmodulin (**Figure 3-13**), that the dotty pattern of RagA reveals most probably RagA decorated calreticulin containing vesicles. This might also be an indirect indication of a co-localisation between Tctel and RagA; **B)** H9C2 cells were stained with the anti-RagA antibody and display a dotty pattern of the endogenous RagA GTP-binding protein present exclusively on microtubules. This suggests that RagA is associated at least partly with ER vesicles.

3.6.3 Transfected Ig55 co-localises with RagA on microtubules

Even though Tctel and RagA were previously shown to interact with each other (Lukashok et al., 2000), it was not possible to directly compare their localisation as both anti-Tctel and anti-RagA antibodies were raised in rabbit. In future studies, labelling the antibodies with the help of commercially available fluorescent kits will help directly observe their cellular localisation. However, a good indication of a possible co-localisation of the two proteins is the reference to a component with which each of them co-localises. Therefore, to confirm that Rag A co-localises with Tctel, obscurin Ig55 (which is targeted to Tctel), was transfected into HeLa cells and myoblast H9C2 cells. In both cell lines the expressed EGFP-coupled Ig55 domain co-localises with the RagA-vesicle complexes on microtubules (**Figure 3-15 A** and **B**). This also indirectly confirms that Tctel and RagA are co-localising and suggests the formation of a specific complex.

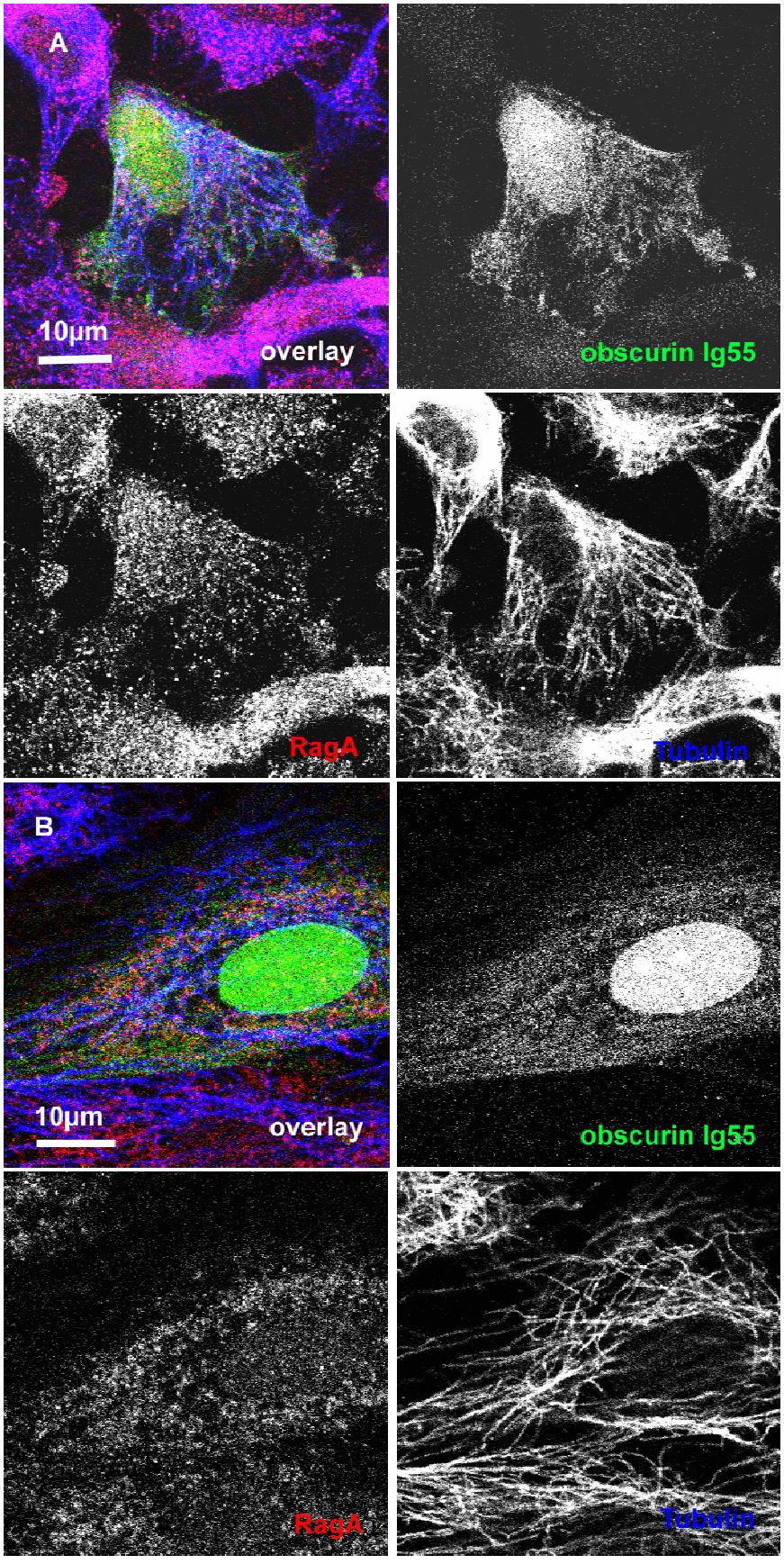

Figure 3-15 Endogenous RagA is involved in vesicular transport and co-localises with obscurin Ig55 on microtubules. In both HeLa **(A)** and H9C2 **(B)** cells, transfected Ig55 domain is targeted to the microtubules through the specific interaction with Tctel, as previously established (**Figure 3-9**). Endogenous GTP-binding protein RagA stained a dotty pattern very similar to the one displayed by Tctel staining (**Figure 3-9**). Its visible partial co-localisation with the transfected Ig55, and its co-localisattion with calreticulin (**Figure 3-14**), strongly suggests that these dots represent RagA-decorated vesicles transported by the dynein motor on microtubules. Bars 10µm.

3.7 THE OBSCURIN C-TERMINUS INTERACTS WITH FILAMIN C

3.7.1 Mapping of filamin C binding site on obscurin

In a collaborative action with the group of Dr. Peter van der Ven from the University of Potsdam, attempts were made to find new muscle-specific interaction partners for filamin C. For this purpose, filamin Ig-like domain 20 was employed as part of the bait in a classical yeast two-hybrid screen. This domain contains a muscle-specific insertion of 80 amino acids (Chakarova et al., 2000) and attempts were made to identify specific ligands for this region. The bait was however constructed with the flanking Ig19 and Ig21 domains for stability reasons, also eliminating a bait unspecific HIS3 activation of the Ig20 alone. The filamin C 19-21 bait identified as interaction partner in both cardiac and skeletal cDNA libraries the C-terminal end of obscurin. However, these identified obscurin target fragments differed in length: one of them started at nucleotide 16341 (Acc. No. XM047536) while the other, at nucleotide 19444, in cardiac and skeletal cDNA libraries, respectively. A more exact mapping of the filamin C binding site on obscurin was therefore necessary. For this purpose, the fragments ob56-57 and ob56-C (**Figure 1-7**) cloned into pLexA (see Material and Methods) were submitted to a forced two-hybrid interaction with the filamin C construct 19-21 cloned into pACT2. The ob56-C bait protein was able to autonomously activate the His3 reporter gene, but this capacity was abolished by 10mM 3-AT, while ob56-57 didn't require this treatment. The filamin C 19-21 construct interacted with ob56-C only, as this fragment combination activated the HIS3 reporter gene, while ob56-57 did not (**Figure 16A**), suggesting that the filamin binding site is placed inside the non modular C-terminal tail of obscurin. These observations are also supported by biochemical data from our group in which the recombinant C-terminal fragment ob 56-C interacts with recombinant filamin C Ig 19-21, 20-21, but not with filamin C Ig 19-20 (unpublished data).

To test whether the muscle-specific amino acid insert is required for binding of obscurin, another two filamin 19-21 constructs were tested for interaction with ob56-C, both being derived from non-muscle filamin isoforms A and B which lack the insert inside Ig domain 20. Similar co-transformations were performed with the new baits and ob56-57, ob56-C obscurin fragments (**Figure 16B**). Both non-muscle filamin isoforms were able to activate the HIS3 reporter gene when co-transformed with ob56-C but not with ob56-57, in

a similar way with filamin C. It seems therefore plausible that the insert present in the Ig-like domain 20 of muscle filamin C is not required for obscurin binding.

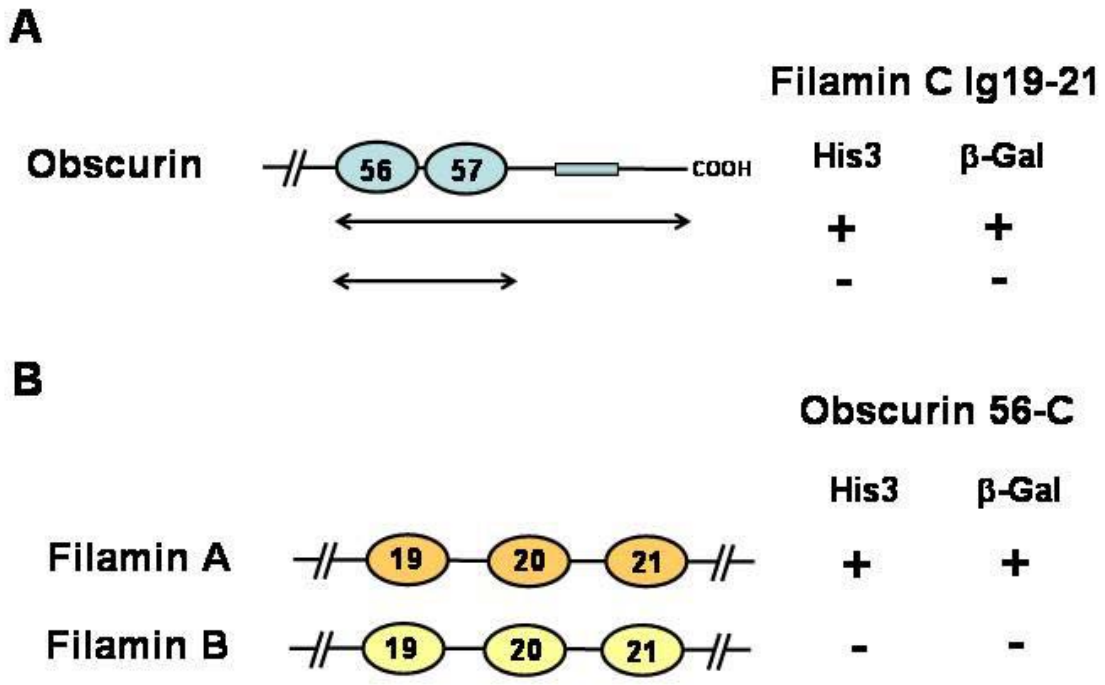

Figure 3-16 Obscurin interaction with filamin. A) Mapping of filamin C binding site on obscurin. Two C-terminal bait constructs were co-transformed in L40 cells with the prey filamin bait containing the Ig-like domains 19 to 21. Only the bait construct including the non-modular C-terminal sequence was able to interact with filamin C, suggesting the binding site of filamin might reside in this region of obscurin. This observation is also supported by biochemical data (see the text). **B) Filamin C insert of the Ig-like domain 20 is not essential for obscurin binding**. Two constructs of non-muscle filamin A and B lacking the 80 amino acid filamin C-specific insert in the Ig-like domain 20 were tested for binding with the obscurin fragment ob56-C. Both of them were able to bind obscurin, suggesting that the insert of the filamin C isoform is not essential for this interaction.

Conversely, the minimal binding site of obscurin on filamin C was also mapped with the help of forced two-hybrid assay aiming to establish whether all three Ig-like domains of filamin C are essential for obscurin binding. Fragments of filamin C spanning from Ig-like 19 to 21 and shorter constructs of Ig19-20 and 21 alone, were tested for binding with obscurin 56-C. The results suggest that a larger context than simply Ig20 domain is necessary for binding (**Figure 3-17**).

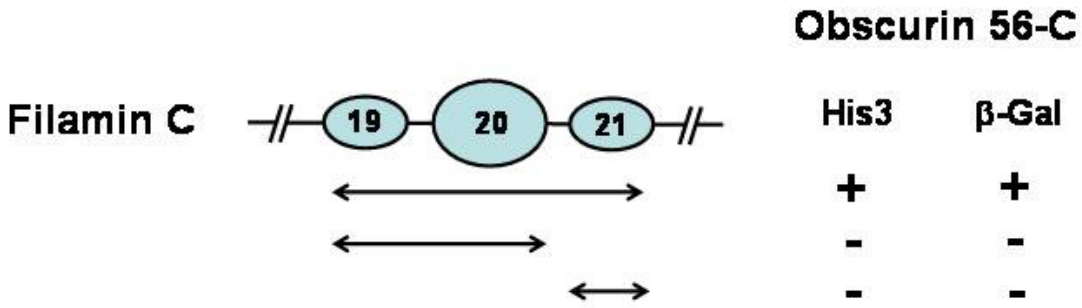

Figure 3-17 Mapping of obscurin binding site on filamin C. In a forced two-hybrid assay, filamin C constructs of different lengths were checked for binding with ob56-C. Consistent with the biochemical data (see text above), the fragment Ig19-20 didn't bind obscurin. The Ig21 domain alone was also not able to interact. Only the presence of all three domains allowed HIS3 reporter gene activation, suggesting that a larger context is needed than the Ig20 domain in order to allow obscurin 56-C binding. The domain Ig20 was depicted larger than the others, to suggest the presence of the muscle-specific insertion of 80 amino acids.

3.7.2 Obscurin C-terminus co-localises with subcortical actin

The functional interaction between obscurin C-terminus and filamin C analysed by yeast two-hybrid assays or by biochemical *in vitro* assays (not shown) gains meaning only in a cellular context. Therefore, attempts were made to preliminarily establish the localisation of the two interaction partners in COS-1 cells. As already showed by yeast two-hybrid assay, not only the muscle-specific filamin C isoform can bind obscurin but also oubiquitous filamin A and B (**Figure 3-16 B**). Therefore, this non-muscle cell line was used in preliminary cellular localisation experiments for reasons of ease in transfection and maintenance in culture in comparison with the muscle cell lines.

Ob56-C construct in fusion with a T7 tag was transfected into COS-1 cells and attempts were made to determine the localisation of the expressed protein in relation with the filamin cytoskeleton. However, the filamin-positive cytoskeleton of these cells was very poor and a possible co-localisation of the two proteins was difficult to observe. For this reason, the actin cytoskeleton was stained instead (**Figure 3-18**). Transfected obscurin 56-C fragment was distributed in a diffuse way throughout the entire cytosol, but enrichment of the protein was clearly visible at the cell periphery in membrane ruffle structures in which it co-localised with actin (**Figure 3-18**).

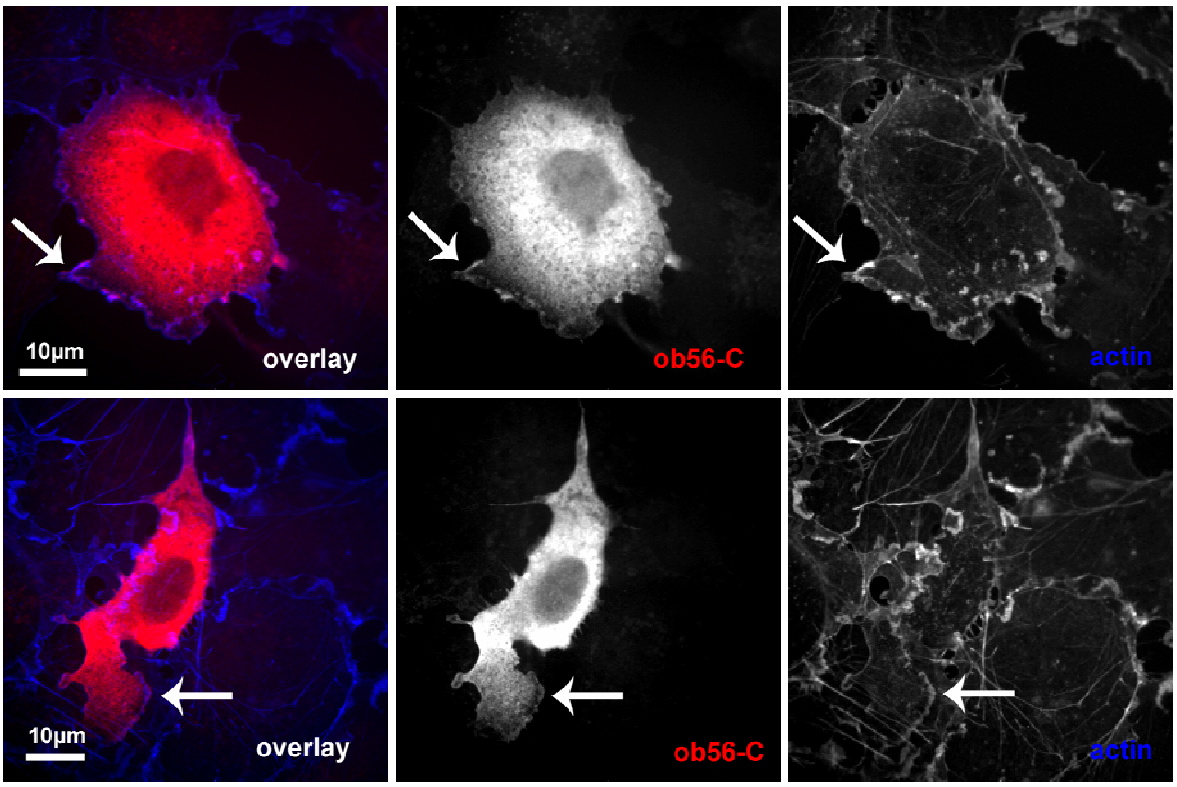

Figure 3-18 Obscurin C-terminus is targeted to the subcortical actin cytoskeleton in COS-1 cells. Both pictures show the generally diffuse localisation of transfected obscurin 56-C throughout the cytosol of the COS-1 cells, but in both pictures it is visible that obscurin becomes enriched at the cell periphery where also actin-rich membrane ruffles are present (arrows). This indirectly suggests that obscurin may co-localise with filamin in these subcortical actin structures. Bars 10µm.

As the formation of membrane ruffles is mediated by filamin (Vadlamudi et al., 2002), the co-localisation of obscurin 56-C with subcortical actin is a strong indication that this protein might be targeted to the actin bundling protein filamin in COS-1 cells. This data confirm the interaction between the two proteins observed in *in vitro* tests and suggest that obscurin C-terminus and filamin might form a biologically significant complex.

4 DISCUSSION

4.1 THE INTERACTION OF OBSCURIN WITH TCTEL

4.1.1 A model for obscurin participation in the ER/SR vesicular transport

The present study investigated a previously discovered interaction between obscurin and the human dynein light chain Tctel, the homologue of the better known mouse Tctex-1. This interaction was identified in our group in a yeast two-hybrid screening of a human cardiac cDNA library aiming to find further obscurin partners apart from titin (unpublished to date). The obscurin bait used in the screening was composed of three distinct regions – an immunoglobulin-like domain (Ig55), an SH3 domain, and the linker region between them – therefore a more precise mapping of the binding site was performed in this work and concluded that obscurin Ig55 domain alone suffices for Tctel binding (**Figure 3-1**). No combination of two or all three regions seems to be required for the binding site formation. Further on, the interaction between Ig55 and Tctel was as well confirmed by *in vitro* binding assay with the two proteins in recombinant form (**Figure 3-1**) and was also visualised in the cellular context, in transfection experiments (**Figure 3-8**). This result is in agreement with the observation that also other obscurin Ig-domains are implicated in ligand interaction: titin binds to two obscurin Ig-domains Ob48 and Ob49 both of which are necessary for binding. Similarly, two titin Ig-domains in the Z-disk region of the molecule, Z9 and Z10, are required for this homotypic interaction (Young et al., 2001). In muscle, other examples of doublet Ig-domains interactions are known, in the case of titin, with the Z-disk protein telethonin (Mues et al., 1998), the A-band protein myosin binding protein-C (Freiburg and Gautel, 1996) and the M-band protein myomesin (Obermann et al., 1997). This requirement of two Ig-domains for ligand interaction seems to be as well the case of telethonin in its interaction with titin (Mues et al., 1998). All these interactions are related with the functioning of titin or telethonin as integrated sarcomeric components. In contrast, the binding of Tctel is mediated by a single obscurin Ig-domain suggesting a different role in muscle and possibly relating to the stability of this complex. Like all dynein interactions it is most probably only transient during microtubule-assisted assembly. Such transient microtubule associations are known for myosin, which associates with microtubules via MURF2 (Pizon et al., 2002). The low abundance of obscurin

suggests furthermore that its signalling roles may be brief during myofibrillogenesis (Young et al., 2001).

What could be the significance of obscurin interaction with a dynein light chain? In the past years, it became clear that the existence of different DLCs is important for the specificity of cargo binding, each selecting a particular cargo that will be transported by the dynein motor. For example, Tctel and RP3, another dynein light chain, compete for binding to dynein intermediate chain in such way that over expressed RP3 displaces Tctel. This prevents the translocation of Tctel-interacting rhodopsin from the inner segment to the outer segment of MDCK cells, suggesting that dynein cargo specificity can be altered by dynein subunit composition (Tai et al., 2001). Therefore, a complex formed between a particular protein and a dynein light chain would suggest that, in most cases, the respective protein might represent the cargo or the dynein receptor of a membranous cargo in a vesicle transport process (see also Section 1.3.1); however this is not necessarily always true.

A relatively recent study (Tang et al., 2002) reports that the interaction between the transforming growth factor-beta receptor (TGFβR) and LC7 dynein light is rather a mechanism of phosphorylation-activation of LC7 necessary for its subsequent recruitment to a specific DIC for the transport of a so far unidentified cargo - possibly a downstream signalling molecule in the TGFβ pathway. The activated receptor itself is not transported by this particular motor complex, the interaction with LC7 being only transient and necessary for the phosphorylation and subsequent recruitment of LC7 to the dynein motor. In the case of obscurin, however, the transfected Ig55 domain is targeted to Tctel on the microtubular network (**Figure 3-8**) and co-localises as well with calreticulin containing vesicles of the ER/SR compartment along microtubule tracks as indicated by the Tctel co-localisation with calreticulin (**Figure 3-12**). Therefore, in contrast to the TGFβR activation of LC7 where the TGFβR is not the cargo (Tang et al., 2002), the localization data of Ig55 strongly suggest an interaction with the pre-localized Tctel on vesicular membranes. It seems therefore possible that obscurin might be transported by the dynein complex to its sarcomeric location sites. Furthermore, during this process, obscurin may be involved in the dynein-mediated transport of a so far unidentified cargo along microtubules during myofibrillogenesis. The Ig55 domain, being a Tctel targeting signal, would therefore be sufficient to target the entire obscurin molecule to dynein complexes on microtubules. This

idea was verified in differentiating myoblasts where another obscurin epitope than Ig55 was observed along microtubules (**Figure 3-10**) and where nocodazole leads to complete segregation of tubulin and obscurin (**Figure 3-11**).

The Ig55 domain represents only the docking site of obscurin on dynein so far identified, but it cannot be ruled out that during muscle differentiation obscurin might activate Tctel by phosphorylation thus promoting dynein-mediated transport of cargo, given that recently reported isoforms of obscurin bear Ser/Thr kinase domains at their C-terminus (Russell et al., 2002). Tctex-1, the mouse homologue of Tctel, was reported to be phosphorylated *in vivo* and *in vitro* by both a tyrosine kinase named Fyn (Mou et al., 1998) and by a Ser/Thr kinase, the bone morphogenetic protein receptor II (BMPR-II) (Machado et al., 2003). This phosphorylation processes are thought by the authors to represent an activation mechanism for Tctex-1. Alternatively, other early Tctel activation mechanisms might be employed in the striated muscle cells before the interaction with obscurin occurs. These hypotheses were however not explored in this work and will need further investigation.

Other regulation points of the cargo loading and releasing onto and from motor complexes may include as well phosphorylation of dynein intermediate chains (Vaughan et al., 2001), dynein light intermediate chains (Dell et al., 2000; Addinall et al., 2001) or may, alternatively, be mediated by GTP-binding proteins. The recently reported interaction between Tctel and the small GTP-binding protein RagA (Lukashok et al., 2000) is essential for the indirect attachment of the adenoviral molecule Ad E3-14.7 to Tctel on the dynein complex and for its transport to the host cell nucleus even if it is puzzling that this molecule is not a capside component. RagA is the human homologue of the yeast Gtr1 protein known to be implicated in the Ran GTPase cycle (Nakashima et al., 1996) and was proposed to shuttle between the nucleus and the cytoplasm, depending on the bound nucleotide state (Hirose et al., 1998). RagA is the new member of the GTP-binding protein family but it is characterised by the lack of C-terminal cysteine residue, which precludes its posttranslational prenylation or geranylgeranylation (Schurmann et al., 1995) and may allow it to function exclusively in the cytosol. RagA can form homodimers as well as heterodimers with other members of the Rag family like RagC and RagD (Sekiguchi et al., 2001). In the present study, antibodies against RagA were generated and it was observed that the ER/SR calreticulin vesicles were decorated not only with Tctel but also with RagA

molecules (**Figure 3-13**), suggesting a so far not reported implication of RagA in vesicular transport, a fuction somewhat related to the transport of the viral protein E3-14.7 to the nucleus on microtubules (Lukashok et al., 2000). Moreover, the transfected obscurin Ig55 domain colocalised with RagA in a very similar way like with Tctel (**Figure 3-8** and **3-14**). RagA is not necessary for the binding of obscurin to Tctel, but it might play a regulatory role in the vesicular transport itself or it may represent a signalling molecule that functions upstream or downstream of obscurin DH domain found in the proximity of the Tctel binding site (**Figure 1-7**). Because the anti-Tctel and anti-RagA antibodies were both raised in rabbit, the colocalisation of the two proteins was observed only indirectly. For future studies, it might be very helpful to fluorescently label the two primary antibodies, separately, with the help of commercially available labelling kits. Also biochemical studies are still necessary to confirm the formation of a ternary complex, which could not be carried out in this study due to the insoluble properties especially of RagA.

The transport mechanism in which obscurin seems to be involved is most probably a very general one, ubiquitously existent in most types of cells, which would explain why it is possible to visualise it as well in non muscle cells like HeLa or COS-1 cells, but also why cDNA of obscurin is as well found in brain and testis. This general mechanism might have been only particularized in striated muscle according to the specific signalling requirements of this type of cells. There is growing evidence that motor proteins generally utilise signalling molecules for cargo binding and transport (Bowman et al., 2000; Goldstein, 2001). For example, p53 was found to be localized to the microtubules and its transport to be dynein dependent, suggesting that the interaction of p53 with dynein facilitates its accumulation in the nucleus after DNA damage (Giannakakou et al., 2000). Jun N-terminal kinase (JNK)-interacting proteins (JIPs) are thought to serve as scaffolding proteins for the JNK signalling pathway (Davis, 2000). The JIP proteins also bind with high affinity and specificity to the motor protein kinesin (Verhey et al., 2001). It was proposed that kinesin carries the JIP scaffolding proteins preloaded with cytoplasmic and transmembrane signalling molecules. In a similar way, obscurin might be necessary as a scaffold for the transport of other signalling molecules that are newly synthesised or rearranged during muscle formation/signalling. Examples of such processes in muscle, for which an implication of obscurin is presently not known, are the rearrangement of IP3R1 inside the smooth muscle SR membrane upon Ca^{2+} stimulation (Vermassen et al., 2003) or

the routing of skeletal muscle calsequesterin (a Ca^{2+} sequestering protein of the SR lumen) from its synthesis sites in the ER compartment to the SR terminal cisternae (Nori et al., 2004) (discussed below). Both relocation processes employ vesicle transport mechanisms and are taking place in a microtubule-dependent manner.

The interaction of obscurin with Tctel and titin, and the absence of a signal peptide at the N-terminus excludes the possibility that obscurin is transported inside the ER/SR vesicles together with the chaperone calreticulin during vesicular transport. At the level of the PH domain, in both obscurin and UNC-89, the conserved lysine residue crucial for PiP_2-binding is exchanged for arginine, which in other PH containing proteins abolishes PiP_2-binding (Hyvonen and Saraste, 1997). However, interaction of obscurin PH with other phospholipids is a possibility, as well as a function purely as a protein interaction domain.

The C-terminal end of obscurin was shown to interact with the cytosolic domain of small ankyrin 1.5 (also named sAnk1.5), a short isoform of ankyrin 1, specific for the SR membrane (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003). These authors suggest an implication of this interaction in the alignment of the SR to the sarcomeres at the M-band in mature muscle. The obscurin N-terminus might simultaneously stabilize the thick filaments. Ankyrin 1.5 seems to be a key molecule in the alignment of SR membrane to the contractile apparatus as another giant protein of the sarcomere was discovered to be a ligand for it in the Z-disk, titin (Kontrogianni-Konstantopoulos and Bloch, 2003**)**. The implication of obscurin in the transport of ER/SR vesicles presented in this study and the given interaction with the sAnk1.5, suggest that the later might represent the receptor molecule in the SR membrane that helps anchoring the vesicles to the dynein motor. More experimentation is necessary to establish the relationship between obscurin and ankyrin 1.5 during muscle development.

In summary, all localisation data suggest that obscurin acts as an adaptor molecule implicated in the transport of molecules from the ER to the SR compartment, through its molecular architecture simultaneously scaffolding various signalling pathways as depicted in **Figure 4-1**.

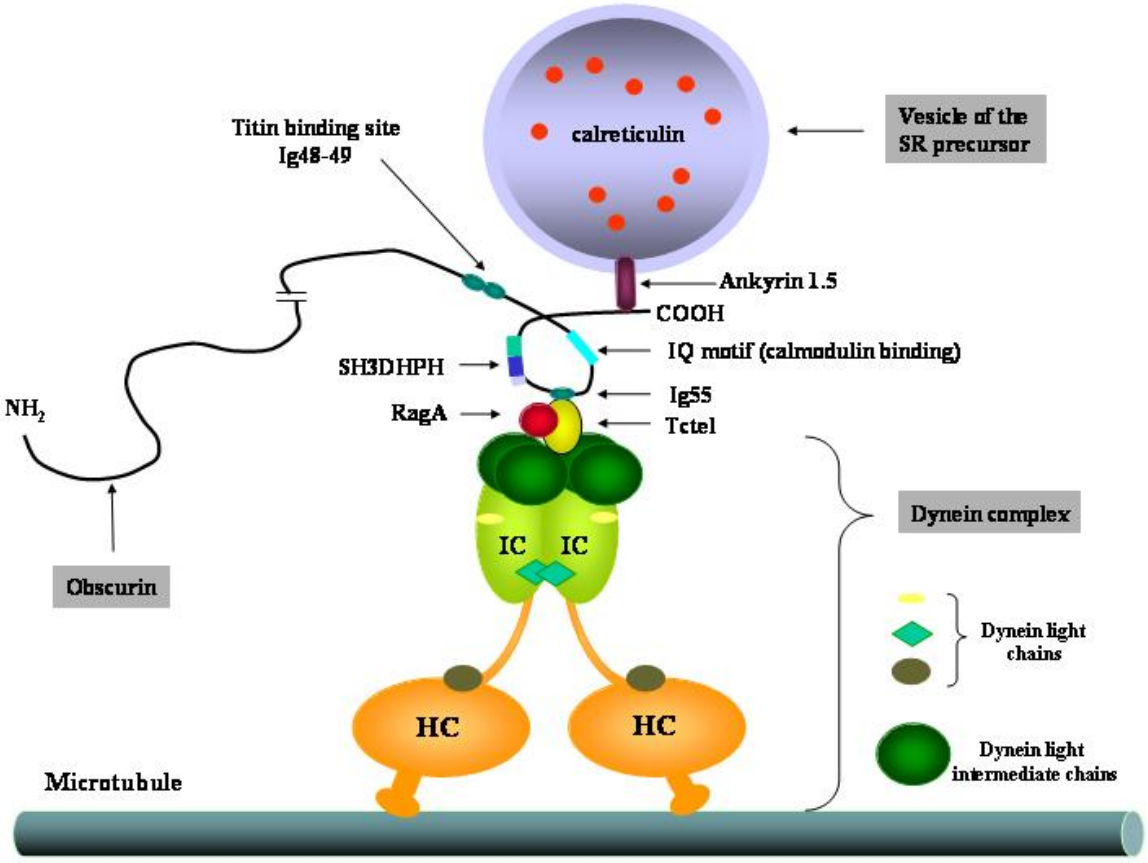

Figure 4-1 Model of obscurin scaffolding role in the transport of ER/SR vesicles during myofibrillogenesis. As shown experimentally, obscurin directly binds through its Ig55 domain the dynein light chain Tctel, which is found as part of the macromolecular motor complex. The interaction between Tctel and RagA (Lukashok et al., 2000) brings a GTP-binding protein in the vicinity of the obscurin GEF domain (the DHPH tandem), and raises the question of a possible regulatory mechanism in the loading/unloading of the cargo onto/from the dynein motor. Further experiments are necessary to answer this question and eventually establish which of the two proteins functions upstream the other. Ankyrin 1.5 might mediate the attachment of the vesicle to the motor by functioning as a specific receptor molecule integrated in the membrane of the transported ER/SR vesicle and recognized by the C-terminal end of the adaptor protein obscurin in the way described by Bagnato et al (2003) and Kontrogianni-Konstantopoulos et al (2003). Except for possibly calreticulin, the exact nature of the cargo carried with the help of obscurin is not yet clear, as other proteins are likely to be simultaneously transported inside the vesicles. The presence of the IQ domain in the proximity of the SR vesicle might be necessary for bringing the calcium sensor calmodulin in place for a mechanism that regulates the Ca^{2+} concentration at perisarcomeric sites. For simplicity, RagA and Tctel are shown as monomers and the represented obscurin way of folding is only hypothetical. The protein size was not always faithfully depicted.

4.1.2 Which events or signalling pathways?

The identity of the cargo molecule(s) transported by dynein with the help of obscurin remains to be identified, which would help understanding the process for which obscurin is transiently required during muscle formation, e.g. possibly the organisation of SR membranes or the assembly of other sarcomeric structures.

Striated muscle contraction is not only sustained by the high degree of order inside each sarcomere and by the precise lateral alignment of the myofibrils (Sommer and Jenings, 1992) at the level of the Z disks and M bands. The perisarcomeric structures, like the T-tubules and the terminal cisternae of the SR are also precisely aligned with the myofibrils in this way ensuring the simultaneous contraction of the sarcomeres when the mature muscle starts functioning under electrical nerve impulses (see also **Figure 1-1**). A key question in the biology of the striated muscle is how the SR membranes become perfectly aligned with the contractile apparatus. There must be tissue specific differences given the fact that in cardiac muscle the triads are aligned with the Z-disks but in skeletal muscle are placed at the junction of the A- and I-bands (Sommer and Jenings, 1992).

The striated muscle cells have both endo- and sarcoplasmic reticulum compartments. Mesaeli and coworkers (2001) hypothesized that SR Ca^{2+} stores could be responsible for control of excitation-contraction coupling whereas the ER compartment may provide Ca^{2+} for housekeeping and transcriptional functions. The SR is the key compartment for excitation-contraction coupling, especially the junctional SR at the level of triads, which contains the sites mainly responsible for regulating the Ca^{2+} concentration during muscle excitation. The ryanodine-sensitive calcium channels in the membrane of its terminal cisternae release the Ca^{2+} necessary for initiation of contraction, while the Ca-ATPase (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase, SERCA) is pumping it back into the SR in the relaxation phase (Berridge et al., 2000). During muscle development, the SR compartment gradually develops from the ER, the continuity of the ER-SR membrane being evident during development (PORTER and PALADE, 1957) and being further maintained after maturation (Kaprielian and Fambrough, 1987; Flucher, 1992). Two models for the biogenesis of the SR have been proposed (reviewed by Flucher, 1992). First, SR could be formed by the outgrowth of distinct membrane structures from ER. A second hypothesis is that the incorporation of proteins involved in Ca^{2+} regulation leads to a transformation of the membrane system into a specialized Ca^{2+}–regulating organelle

(Flucher, 1992). During skeletal muscle differentiation, it seems that the sites at which calsequesterin is delivered are transformed in SR (Koyabu et al., 1994). The two major Ca^{2+} binding proteins of the muscle, calsequesterin and calmodulin, are differentially expressed and localized in the cardiac tissue (Allen and Katz, 2000) or in the smooth muscle (Michalak et al., 1980). While calsequesterin is primarily detected in the terminal cisternae of the SR at the level of Z-disks in triads (Allen and Katz, 2000), calreticulin is present in both the ER and SR, in the later being distributed exclusively in the longitudinal SR that surrounds the myofibrils like a network (**Figure 1-1**).

Recently, it was observed that calreticulin is transported from the ER synthesis sites directly to the SR through a novel pathway that skips the classical routing through the Golgi complex, this mechanism being dependent on the integrity of the microtubule network (Nori et al., 2004). The present study describes an involvement of obscurin in the transport of calreticulin-containing vesicles in a microtubule dependent way that points to a role in the organization of the SR compartment during myofibrillogenesis. In the same context, it must be mentioned that Bagnato et al (2003) proposed a mechanism in which obscurin participates through its interactions with ankyrin 1.5 and ankyrin 2.2 in the localization of Ca^{2+} release channels (e.g. ryanodine and IP3 receptors) and eventually other proteins at specific sites of the SR.

At the very beginning of muscle differentiation, the cytoplasm of the muscle fibre is not yet occupied with myofibrils and the positioning of nuclei, organelles as well as Golgi and ER compartments generally resembles the one in other cell types. As the myofibrils start forming, the SR differentiates around nascent myofibrils and the nuclei and the organelles are moved towards the cell periphery to make place for the contraction machinery. In the very early phases of myofibrillogenesis, the polarity of microtubules is oriented, as in most of other cell types, with the plus end at cell periphery and the minus end towards nucleus (Lu et al., 2001). In later stages of differentiation or in mature muscle, the orientation of microtubules is not known, difficulties being encountered due to myofibrils abundance. In the present study, a possible implication of obscurin in the transport of SR precursor vesicles to myofibrils is presented. This process would be expected to be carried out with the help of a plus end directed motor protein but the present data indicate an interaction with dynein, a minus end directed motor (Section 1.3.1). All dynein motors carry the cargo towards the cell nucleus but can also participate in

bidirectional processes in which the presence of kinesin might regulate the direction of movement (Section 1.3.1). Although it cannot be ruled out that calreticulin-containing vesicles transported with the help of obscurin have also kinesin attached, more experimentation is necessary to answer the question whether both motors are simultaneously present on the membrane of these vesicles and to further dissect the complex role dynein might play in membrane morphogenesis, when ER vesicles fuse and align along the myofibrils.

Very recently, the non-modular C-terminal end of obscurin was reported to be essential in the assembly of thick filaments into regular A-bands in rat skeletal myofibrillogenesis (Kontrogianni-Konstantopoulos et al., 2004) in a similar way with the nematode UNC-89, the protein which bears the closest homology to human obscurin, (Benian et al., 1996). The C-terminal end of obscurin can weakly bind myosin although it is not clear whether the binding is direct or indirect (Kontrogianni-Konstantopoulos et al., 2004) as pulldown experiments with cytoskeletal preparations are not unambiguous. Therefore, although at this moment only a speculation, obscurin might also be implicated in the transport of signalling molecules essential for the assembly of the A-band.

Further studies would have to define more precisely the moment in myofibrillogenesis when this signalling function of obscurin is required. It is however very likely that the implication of obscurin in vesicular transport takes place very early in muscle differentiation for two reasons. First, obscurin is abundantly expressed early in embryogenesis and this declines with the developmental stage and second, this low abundance corresponds to late moments in myofibrillogenesis when obscurin becomes regularly organised around M-lines and only weakly around Z-disks. The obscurin mediated vesicle transport might therefore take place only during differentiation, later in mature muscle obscurin playing more of a structural role.

4.2 OBSCURIN INTERACTION WITH FILAMIN C

4.2.1 Filamin C – a new sarcomeric ligand for obscurin

In the present study a second interaction of obscurin with a new ligand, filamin C, is described. This interaction was recently discovered with the help of yeast two-hybrid techniques (van der Ven, unpublished) and the investigations of this binding are currently at their very beginning. With filamin C, the list of known binding partners of obscurin has raised up to five, along with titin, calmodulin (Young et al., 2001), small ankyrin 1.5 (Kontrogianni-Konstantopoulos et al., 2003), and Tctel (unpublished).

Filamin C is the striated muscle-specific isoform of filamin family of proteins, a multifunctional dimeric protein distributed at the level of the Z-disks and I bands (Thompson et al., 2000; van der Ven et al., 2000a) and in the subsarcolemmal space (Bonnemann et al., 2003). Interestingly, filamin C can shift between locations at the plasma membrane and contractile apparatus, this property being necessary for its different functions at these cellular sites: at the sarcolemma, filamin C has a stabilizing role for the cell membrane by interacting with the DGC (Thompson et al., 2000), while at the sarcomeric sites it stabilizes the Z-disks through interactions with myotilin (van der Ven et al., 2000b) and FATZ (Faulkner et al., 2000; Takada et al., 2001) (discussed in Section 1.3.2).

Obscurin binds filamin C in the non modular C terminal end, in a region (our unpublished data) that is partly overlapping with the previously described ankyrin 1.5 binding site (Kontrogianni-Konstantopoulos et al., 2003) (**Figure 3-15A**). This interaction seems not to be directed by the muscle-specific insert of filamin C, as forced two-hybrid assays show as well binding of the Ig20 domain from filamin A and B (**Figure 3-15B**) that lack the insertion. Transfections of COS-1 cells with the obscurin C-terminal tail show enrichment of this fragment in membrane ruffles at the subcortical actin cytoskeleton, this indirectly suggesting an interaction between obscurin C-terminus and filamin (**Figure 3-17**) as this type of actin cytoskeleton reorganization is always mediated by filamin (Vadlamudi et al., 2002). The COS-1 cells had a poor filamin-positive cytoskeleton, which hindered the observation of the co-localisation with obscurin. More transfection experiments, using other cell lines, will be necessary for clearly confirming the co-localisation of the two proteins.

While ankyrin 1.5 is mainly situated at the M-band, filamin C is a Z-disk associated protein (Bagnato et al., 2003; van der Ven et al., 2000a). The question arises whether these two proteins could bind simultaneously to obscurin C-terminus. Unfortunately, the answer is not provided in this study but biochemical assays from our laboratory indicate that they can only interact separately with obscurin (unpublished data), suggesting therefore that their binding sites function independently and that precise temporal and spatial regulation mechanisms must exist for coordinating their binding to obscurin during muscle development. How is this accomplished is still an open question but the presence of multiple KSP/RSP phosphorylation consensus sites similar to those found in M-line titin (Gautel et al., 1993) suggests that ligand binding in this region might be phosphorylation-controlled.

4.2.2 A panel of interactions

Filamin C plays an important role as a scaffolding protein: various Rho and Rho GEF proteins bind to its C-terminus like the GTPase RalA (Ohta et al., 1999) or Trio, a GEF for RhoG, Rac1 and RhoA (Bellanger et al., 2000) (discussed in Section 1.3.2). These features place filamin in a key position to spatially coordinate different Rho signalling pathways related to actin cytoskeleton reorganization. In muscle, this is a particularly important aspect as during myofibrillogenesis extensive cytoskeleton remodelling accompany and support sarcomeres formation (Sanger et al., 2000; Sanger et al., 2002) and is, therefore, a process that requires precise regulation likely to be achieved with the help of scaffolding proteins. Due to its DH domain, obscurin is a predicted Rho GEF in muscle and, therefore, the interaction with filamin C seems to support once more the idea that filamin may function as scaffold for the spatial organization of Rho GEF mediated signalling pathways. However, the Rho signalling function for which obscurin is required at the level of the Z-disk is still unknown, as no downstream effectors or upstream activators of the DH domain were identified to date. Obscurin GEF shows little similarities with other small G-proteins GEF domains and is therefore likely to be regulated in a novel way and may interact with non-classical Rho proteins (Young et al., 2001).

The data presented in this study, combined with findings by other groups available in the literature lead to a very complex picture in which obscurin seems to play important roles in both microtubule mediated transport and actin cytoskeletal rearrangements. All

ligands known to date as well as the emerging functions of obscurin as a scaffolding protein in muscle signalling are represented in **Figure 4-2**.

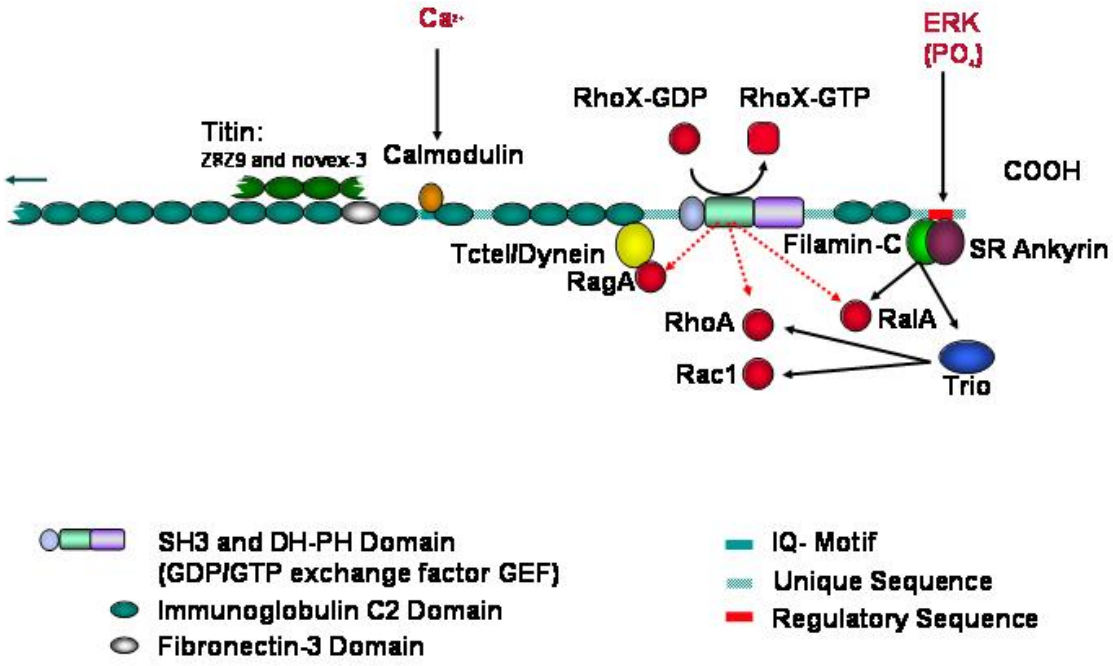

Figure 4-2 Obscurin interactions. The novel obscurin ligands described in this study and the interactions reported by other groups are synthesised in this panel. Dashed lines indicate pathways under question mark; the already established interactions are represented by solid lines. Obscurin interaction with calmodulin is not dependent on the Ca^{2+} concentration (Young et al., 2001), indicating this binding might be rather necessary for bringing calmodulin in a place where a Ca^{2+} sensor is needed. Which Ca^{2+} signalling pathway requires this function and at which moment in muscle development/functioning remains to be established but the proximity of obscurin with the SR (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003) might suggest its implication in some signalling pathways that regulate the Ca^{2+} concentration around myofibrils. Moreover, the participation in the dynein-mediated transport of ER/SR vesicles described in this study points as well towards a possible function of obscurin in Ca^{2+} signalling related to the SR. The presence of RagA in the proximity of obscurin GEF raises the question whether this GTP-binding protein is an upstream activator or downstream effector of obscurin. This issue is currently under investigation. The mutually exclusive filamin C and ankyrin 1.5 bind to obscurin at the level of the Z-disk and M-band, respectively, possibly in this chronological order during muscle development. Their binding is likely to be phosphorylation-regulated as the C-terminal segment of obscurin contains multiple KSP/RSP sequences recognised by ERK kinases. The functioning of filamin as a scaffolding protein might bring further GTP-binding proteins in the proximity of the obscurin GEF. Both RalA (Ohta et al., 1999) and the RhoGEF Trio (Bellanger et al., 2000) act upstream of filamin in signalling pathways that target the actin cytoskeleton remodelling. In muscle, these signalling events might be transiently required during myofibrillogenesis**.**

4.2.3 A possible sequence of events

From this complex picture emerges the question of the timing of all identified obscurin interactions during the tightly regulated process of muscle formation. Despite its giant size, obscurin appears to relocate during myofibrillogenesis from the early Z-disks where it interacts with titin, to the M-band (Young et al., 2001), although it is not clear to date whether this shift regards the entire molecule or only a specific splice variant (Section 1.5). This very unusual behaviour is only possible due to the localization of the giant molecule around myofibrils rather than inside the sarcomeric space (Young et al., 2001; Kontrogianni-Konstantopoulos et al., 2003). At the two cellular locations, obscurin has different ligands: filamin C/titin and ankyrin 1.5, respectively. It is therefore likely that the interactions with filamin C and titin at the Z-disk are transient (Young et al., 2001) and take place chronologically before the organisation of obscurin around M-bands. This idea is also supported by the notion that the intensive cytoskeletal rearrangements during muscle formation (for which obscurin might be necessary at the Z-disk) are largely finished after birth when obscurin localizes almost exclusively at the M-band. Some exceptions exist however, when obscurin is occasionally observed at the Z-disk also in mature muscle (our group, unpublished observations); this leaves the question open if there is an exact moment or time window when the M-line or the titin interaction can occur.

In contrast with the filamin C/ankyrin 1.5 binding sites which partly overlap, the binding of Z-disk titin and filamin C on obscurin are not mutually exclusive, the Ig48-49 and the non modular tail of obscurin (**Figure 1-7**) being distinct and theoretically allowing the simultaneous binding of the two proteins in the Z-disk space. Further studies will establish if this is the case *in vivo* and will help to further characterise the spatial and temporal relationship between these two obscurin interactions at the edge of the Z-disk.

The findings that obscurin may participate very early in the organization of the nascent SR membrane, probably through interactions with ankyrin 1.5 must be properly placed in the sequence of events as ankyrin 1.5 is in mature muscle an M-band associated protein as well. However, the localization of ankyrin 1.5 during muscle development is unknown to date and therefore the exact timing of the interaction with obscurin in early stages of muscle development is an open question for which more experimentation will help to find the answer in the future.

The implications of obscurin in many signalling pathways and the complex question of their timing might be explained by isoform specific interactions. One cardiac splice variant of obscurin was described by Young et al (Young et al., 2001), in which one Ig domain replaces 6 other in the middle of the protein giving rise to two obscurin isoforms of different length. Further splicing isoforms were recently reported by Russell et al (2002), in which several C-terminal variants contain one or two Ser/Thr kinases in the non modular end-sequence, although it is not yet entirely clear whether these kinases are encoded by the same gene as the initial obscurin transcript (Young et al., 2001). The localisation and function of these kinases is currently unknown, however, the existence of multiple spliced obscurin forms suggests that they may be employed by the muscle cell to simultaneously accomplish distinct functions in selectively restricted areas of the myofibril.

At closer examination, the interaction with the three ligands suggests that obscurin not only shifts between distances over 1μm but also that its position with respect to sarcomers might be reversed. At the level of the Z-disk, the C-terminus and the middle of obscurin bind inside the sarcomere to filamin C/titin, while at the M-band, its C-terminus is pointing away from sarcomeres towards the SR, where it binds ankyrin 1.5.

With its complex interactions, obscurin appears to be a versatile multifunctional protein with roles in various signalling pathways during muscle differentiation, mediated by specific ligand interactions. The mechanisms that determine obscurin translocation as well as the reasons that reside behind the dynamic ligand exchange between different sarcomeric sites remain to be identified.

4.3 CONCLUSIONS

The aim of this thesis was to study the function of the giant protein obscurin by investigating its interactions with new ligands in the region of the M-band and the Z-disk. Investigating in detail a previously discovered interaction with Tctel, a model was developed describing a novel role of obscurin in which, during its own transport to the sarcomeric sites upon synthesis, the giant protein functions as motor-cargo adaptor in the microtubule-dependent transport of vesicles involved in SR maturation. In a collaborative project, a novel interaction of obscurin with the sarcomeric, muscle-specific filamin C was investigated and provided some insight into the transient functioning of obscurin at the Z-disk during muscle formation.

The results of this work are only at the beginning of understanding obscurin signalling roles along the complex way of muscle formation. Future experimentation will be required to address important questions that was not possible to be covered by this study. Obscurin participates in vesicular transport during myofibrillogenesis, but the identification of the cargo molecules transported by the dynein-obscurin complex will delineate exactly for which signalling pathway this process is required. Mechanisms that control the ligand exchange at obscurin C-terminus end as well as defining isoform specific interactions are key issues that need clarification if it is to more fully understand obscurin functioning during muscle development or to get a better picture of its comparative roles in cardiac and skeletal muscle. Apart from the present panel of interactions that ought to be better dissected, the molecular architecture of obscurin and the increasing number of ligands suggest that the list of interactions may not stop here. It is very likely that new partners are still to be discovered that may open new avenues in investigating the role of obscurin in the complex signalling network of the muscle.

5 SUMMARY

Obscurin is a novel muscle protein recently discovered in our research laboratory, which joins the family of giant muscle proteins along with titin and nebulin (Young et al., 2001). In contrast with the later two, which are integral components of the sarcomere, obscurin was proposed to be positioned around myofibrils rather than inside them, due to an unusual change in location from the Z-disk to the M-band during myofibrillogenesis. Why such a surprising behaviour for a giant protein? Its complex domain pattern suggests that obscurin is very likely to play many roles in muscle architecture and signalling, many of which are possibly only transient. Its interaction with titin at the Z-disk has an unclear role so far. At the M-band, obscurin was reported to interact with small ankyrin 1.5 and mediate the anchorage of the sarcoplasmic reticulum in perfect alignment with the sarcomeric apparatus (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003). Discovery of new obscurin ligands and characterisation of their interactions in the cellular context may help understanding the specific roles of obscurin during muscle development.

The present study describes the interaction of obscurin with two new ligands. Tctel binds at the level of a unique obscurin Ig-like domain and mediates the attachment of the giant protein to the dynein motor complex on microtubules. This might represent a mechanism by which obscurin is transported from its synthesis place to the sarcomeric sites. Moreover, there are indications that vesicles of the endo- and sarcoplasmic reticulum (ER/SR) might be as well transported by this complex and a model is proposed in which obscurin acts as a motor-vesicle adaptor molecule.

In the second part of this study it is presented the interaction of obscurin with the Z-disk associated actin bundling protein filamin C, a muscle-specific filamin isoform. The assembly of highly organised sarcomeres involves massive cytoskeletal rearrangements and the interaction described might have a role in controlling these events. As the binding sites of titin and filamin C on obscurin are theoretically allowing the two proteins to bind simultaneously, it seems possible that these interactions may represent a collaborative action of these proteins in signalling events possibly required for the transition from stress fibre like structures to anti-parallel thin filaments anchored in the Z-disk. The mechanisms that control this rearrangement are not known to date. In contrast, the obscurin binding sites for small ankyrin 1.5 and filamin C are overlapping and mutually exclusive, therefore,

tight regulation mechanisms must exist to control the ligand exchange and specify the sarcomeric localisation of obscurin.

The new functional interactions of obscurin presented in this study are only at the beginning of their investigation, but they may provide already some insight into the complex signalling roles of this protein during muscle development. Future studies will hopefully lead to a better understanding of the entire panel of interactions on obscurin that may not be complete to this date and will reveal their significance for the process of striated muscle formation.

ZUSAMMENFASSUNG

Obscurin ist ein neuartiges Muskelprotein, das in unserem Labor entdeckt wurde, und Mitglied der Familie riesiger Muskleproteine wie Titin und Nebulin sind (Young et al., 2001). Im Gegensatz zu diesen beiden Proteinen, die integrale Bestandteile des Sarkomers sind, wurde für Obscurin eine Lokalisation um, anstatt im Sarkomer vorgeschlagen, im Einklang mit einer ungewöhlichen Relokalisation von der Z-Scheibe zur M-Bande während der Myofibrillogenese. Dieses Verhalten ist für ein so großes Protein sehr ungewöhnlich. Das komplexe Domänenmuster dieses modularen Proteins läßt vermuten, daß es verschiedene und distinkte Funktionen in der Architektur und Signaltransduktion des Muskels wahrnimmt, von denen viele eventuell nur transient sind. Die Interaktion mit Titin an der Z-Scheibe hat eine bislang unklare Funktion. In der M-Bande wurde eine Interaktion mit der muskelspezifischen kleinen Ankyrin Isoform 1.5 berichtet, und es wurde vorgeschlagen, das Obscurin dort für die exakte Alinierung und Verankerung des sarkoplasmatischen Retikulums mit dem sarkomerischen Apparat verantwortlich ist (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003). Die Identifizierung neuer Obscurin Liganden, und die Charakterisierung ihrer Funktionen im zellulären Kontext, können dazu beitragen, die spezifischen Funktionen von Obscurin während der Muskelentwicklung zu verstehen.

In dieser Arbeit werden die Interaktionen von Obscurin mit zwei neuen Liganden untersucht. Tctel bindet eine singuläre Obscurin Immunglobulin-artige Domäne und vermittelt die Interaktion mit dem Dynein Motorkomplex auf Mikrotubuli. Dies könnte einen Mechanismus darstellen, über den Obscurin vom Ort seiner Synthese zu den Stellen der sarkomerischen Integration transportiert wird. Weiterhin bestehen Hinweise, daß Vesikel des endo- und sarkoplasmatischen Retikulums (ER/SR) durch diesen Komplex transportiert werden können, und es wird ein Modell vorgeschlagen, in dem Obscurin die Funktion eines Motor-Vesikel Adaptors wahrnimmt.

Im zweiten Teil dieser Arbeit wird eine neue Interaktion von Obscurin mit dem Z-Scheiben assoziierten Aktin-bündelnden Protein Filamin-C charakterisiert, einer muskelspzifischen Filamin-Isoform. Der Aufbau hochorganisierter Sarkomere erfordert den massiven Umbau des Zytoskeletts, einschließlich des Aktins, und diese Interaktion könnte eine Rolle in der Kontrolle dieser Vorgänge haben. Da die Bindungsstellen von Titin und Filamin es zumindest theoretisch zulassen, daß beide Proteine gleichzeitig

binden, scheint es möglich, daß diese Interaktionen ein kollaboratives Wechselspiel dieser Proteine darstellen, das für strukturelle und Signalprozese beim Umbau der Aktin Stressfasern zu antiparallen Aktinfilamenten mit Verankerung an der Z-Scheibe verantwortlich ist. Die Mechanismen, die diesen Vorgang steuern, sind bislang unbekannt.

Im Gegensatz dazu sind die Bindungsstellen des Ankyrin 1.5 und Filamin C überlappend und gegenseitig ausschließend, weshalb eine strenge Kontrolle dieser Wechselwirkungen existieren sollte, um den Austausch von Liganden und die sarkomerische Lokalisation von Obscurin zu regulieren.

Die neuen funktionellen Interaktionen von Obscurin, die in dieser Arbeit vorgestellt werden, sind erst der Anfang iher detaillierten Untersuchung, doch sie sollten bereits wichtige Einblicke in die komplexen Signalübertragungsfunktionen dieses Proteines während der Muskelentwicklung geben. Zukünftige Studien werden hoffentlich zu einem detaillierten Verständnis der gesamten Palette von Wechselwirkungen des Obscurins führen, und ihre Bedeutung für die Entwicklung des quergestreiften Muskels erhellen.

LIST OF ABBREVIATIONS USED

3-AT	3 aminotriazole
ABD	Actin-binding domain
ADP, ATP	Adenosine-5'-diphosphate, Adenosine 5'-triphosphate
CaM	Calmodulin
cDNA	Complementary deoxyribonucleic acid
CH	Calponin homology
C-terminal	Carboxy terminal
CV	column volume(s)
DH	Dbl homology
DIC, DHC, DLC	Dynein intermediate chain, heavy chain, light chain
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxid
DTT	Dithiotreitol
ECL	Enhanced chemiluminescence
EDTA	Ethilendiaminetetraacetic acid
EGTA	Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid
EMBL	European Molecular Biology Laboratory
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinases
F-actin	Filamentous actin
FCS	Fetal calf serum
Fn3 domain	Fibronectin 3-like domain
FPLC	Fast Performance Liquid Chromatography
Gal	Galactose
GdmCl	Guanidinium hydrochloride
GDP, GTP	Guanine-5'-diphosphate, Guanine-5'-triphosphate
GEF	Guanine-nucleotide exchange factor
GFP, EGFP	Green fluorescent protein, enhanced green fluorescent protein
Glc	Glucose
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIS3	Histidine synthesis gene 3
Ig domain	Immunoglobulin like domain
IP3	Inositol triphosphate

IPTG	Isopropyl β D thiogalactoside
IQ	Protein motif named after the conserved Isoleucine (I) and Glutamine (Q) which it contains
kDa	kilo Dalton
MLCK	Myosin light chain kinase
NP40	Nonidet P-40 commercially not available anymore, Equivalent of Igepal (Octylphenyl - polyethanol glycol)
N-terminal	Amino terminal
P/S/N	Penicillin/Streptomycin/Neomycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFA	para-Formaldehyde
PH	Plecstrin homology
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium Dodecyl Sulphate
SFLS	Stress-fibre like structures
SH3	Src homology 3
SR	Sarcoplasmic reticulum
TCA	Trichlor acetic acid
Tev	Tobacco Etch Virus
Tris	Tris[hydroxymethyl] amino methane
xg	Number of gravitational acceleration fields
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
β-Gal	β-galactosidase

All amino acid abbreviations followed IUPAC-IUB Commission instructions (1968).

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PUBLICATIONS

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