ISAS-INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Thesis submitted for the degree of Doctor Philosophie

Ankrd2: a link between the sarcomere and the nucleus in skeletal muscle

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SUMMARY

Ankrd2 is an ankyrin repeat protein mainly expressed in skeletal muscle and heart and localized in the sarcomeric I-line, adjacent to the Z-line. Z-lines define the boundaries of each sarcomere, the contractile units of the myofibril and are composed by a highly intricate network of protein-protein interactions. Nowadays it has clearly emerged that several sarcomeric components are moving between this district and other subcellular compartments, such as the nucleus. Hence the sarcomere and in particular the Z-line are considered to be central nodes in which signals arrive and from which signals depart. Since Ankrd2 is detectable both in the sarcomere and in the nucleus, it is supposed to shuttle between these two compartments to transmit a still unknown signal. Moreover Ankrd2 expression increases after skeletal muscle stretch and during the myoblasts differentiation into multinucleated myotubes. Therefore it has been hypothesized an Ankrd2 involvement in hypertrophy or in myogenesis.

To better understand the biological role of Ankrd2 I focused my work on discovering its interacting partners and basing on the similarity between Ankrd2 and the cardiac ankyrin repeat protein (CARP), I tested if Ankrd2 was also able to bind the ubiquitous transcription factor YB-1, like CARP does. I have noticed that the interaction occurs, even if the biological meaning is not yet clear. Moreover, since YB-1 interacts with the tumor suppressor p53, which has also an important role in skeletal muscle differentiation, I considered interesting to look if Ankrd2 binds with it and the interaction between them was confirmed by different binding experiments. In addition I set up luciferase reporter genes assays in order to determine if Ankrd2 affects the

transactivation activity of p53 on specific promoters (p21 and MDM2), concluding that it exerts an enhancing effect. These results strongly support the hypothesis of Ankrd2 as a protein that enter the nucleus to regulate the activity of specific transcription factors, in particular involved in skeletal muscle differentiation. Interestingly I have shown that Ankrd2 has a speckled intra-nuclear pattern perfectly overlapping that of PML. These two proteins are not only co-localized into distinct nuclear structures called PML nuclear bodies but they also bind each other. Since nuclear bodies comprise a very wide set of proteins with different biological functions, the meaning of Ankrd2 localization in these structure is not easy to understand and we can just speculate about a possible Ankrd2 regulatory role exerted in combination with other PML NBs components, such as p53. At last since Ankrd2 accumulates in the cytoplasm in differentiated myotubes I looked for interacting partners among Z-line proteins and I detected an interaction between Ankrd2 and Telethonin, thus opening the exiting possibility of Ankrd2 as a shuttling signalling molecule moving from the Telethonin-MLP-Titin stretch sensor complex towards the nucleus.

ABBREVIATIONS

ALP	Actinin-associated LIM protein
Ankrd2	Ankyrin repeat domain 2 protein
AP	Alkaline phosphatase
APL	Acute promyelocytic leukemia
Arpp	Ankyrin repeat, PEST sequence and Proline rich protein
ATM	Ataxia teleangectasia mutated kinase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bHLH	basic-helix-loop-helix
BSA	Bovine serum albumin
cAMP	Cyclic adenosine mono phosphate
CARP	Cardiac ankyrin repeat protein
CBP	Creb binding protein
Cdk	Cyclin dependent kinase
CK	Casein kinase
CMD	Congenital muscular dystrophy
Co-IP	Co-immunoprecipitation
CSD	Cold shock domain
DARP	Diabetes related ankyrin repeat protein
DCM	Dilated cardiomyopathy
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's modified eagle medium
ECL	Enhanced chemiluminescence
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked- immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated kinase

FATZ	γ-filamin, Alpha-actinin and Telethonin binding protein
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
FL	Full lenght
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HA	Hemagglutinin
HCM	Hypertrophic cardiomyopathy
HMERF	Hereditary myopathy with early respiration failure
HRP	Horseradish peroxidase
Ig	Immunoglobulin
ip	intra–peritoneal
kDa	Kilo Dalton
IPTG	Isopropyl-β-D-thiogalactoside
IVTT	*In vitro* transcribed and translated
LB	Luria Broth
LGMD	Limb girdle muscular dystrophy
LPS	Lipopolysaccharide
LUC	Luciferase
MARP	Muscle ankyrin repeat protein
MCK	Muscle creatine kinase
MDM2	Mouse double minute 2
mdr1	multi drug resistance 1
MHC	Myosin heavy chain
MLC 2v	Ventricular myosin light chain 2
MLP	Muscle LIM protein
MRF	Myogenic regulatory factor
MURF	Muscle specific RING finger protein

NB	Nuclear body
NBT	Nitro blue tetrazolium
ND10	Nuclear domain 10
Ni-NTA	Nitrilo-tri-acetic acid
NLS	Nuclear localization signal
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PAGE	PolyAcrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDVF	Polyvinyldifluoride
PKC	Protein kinase C
PML	Promyelocytic leukaemia protein
POD	PML oncogenic domain
pRB	Retinoblastoma protein
RAR	Retinoic acid receptor
RBCC	RING B-box coiled-coil
sAnk1	small ankyrin 1
SDS	Sodium dodecyl-sulphate
SUMO	Small ubiquitin-like modifier
TB	Terrific broth
TGFβ	Transforming growth factor β
TID	Telethonin interacting domain
TNF	Tumor necrosis factor
VSMC	Vascular smooth muscle cell
wt	wild type
YB-1	Y-box binding protein 1
ZASP	Z-band alternatively spliced PDZ domain protein

Chapter 1

INTRODUCTION

1.1 WHAT ARE MUSCLES AND WHAT DO THEY DO?

Do we need muscles? Bones alone are not enough: they need help from muscles and joints. Without muscles our bodies would collapse. Muscles carry out many different functions: they are needed to keep organs in place, to pump blood, to enable us to have flexible joints and last but not least to move. Mammals have three basic types of muscle: skeletal, cardiac and smooth muscle. **Skeletal Muscle** is the most common of the three types of muscle in the body. It is attached to bones and produces all of the movements of the body; unlike smooth and cardiac muscle it is under voluntary control. Skeletal muscle is used in complex coordinated activities, such as walking or head movements; it generates rapid movement by contracting quickly. However, although it can contract (shorten or tighten) quickly and powerfully, it tires easily and has to rest between work. Another major function of skeletal muscle is to hold objects immobile. Skeletal muscle fibres are long, thin and multinucleated, crossed with a regular pattern of fine red and white lines, giving the muscle its distinctive striped appearance. Both skeletal and cardiac muscles show this striped appearance in the light microscope and are therefore called striated muscle. **Cardiac muscle** is found only in the heart. It is self activating and has single nucleate cells that are electrically connected via special

junctions. Cardiac muscle resembles skeletal muscle in many respects, but it is specialized for the continuous, involuntary rhythmic contractions needed in pumping blood. Although it appears striated it is not under voluntary control. **Smooth muscle** surrounds the blood vessels and all the internal organs. Its contraction and relaxation helps to propel food along the gastrointestinal tract and it also controls the diameter of blood vessels. It is non-striated, involuntary and single nucleate. Smooth muscle takes longer to contract than skeletal muscle; it contracts and relaxes slowly and thus can create and maintain tension for long periods of time, as it does not tire easily (reviewed in Alberts et al. 2002, Lodish et al 1995).

All the movements that muscles, either voluntary (skeletal) or involuntary (cardiac and smooth), produce are coordinated and controlled by the brain and central nervous system. The involuntary muscles are controlled by structures deep within the brain and the upper part of the spinal cord called the brain stem. The voluntary muscles are regulated by the parts of the brain known as the cerebral motor cortex and the cerebellum. When you decide to move, the motor cortex sends an electrical signal through the spinal cord and peripheral nerves to muscles, causing them to contract. The motor cortex on the right side of the brain controls the muscles on the left side of the body and vice versa. The cerebellum coordinates the muscle movements ordered by the motor cortex. Sensors in the muscles and joints send messages back through peripheral nerves to inform the cerebellum and other parts of the brain where and how an arm or leg is moving and its position. This feedback results in smooth, coordinated motion.

In this introduction I present an overview of skeletal muscle from its cytology to a brief summary of the major proteins composing its highly specialized cytoskeleton. I will

also give a short description of some of the transcription factors that play a role in skeletal muscle function. Some of these factors interact with Ankrd2, the skeletal muscle protein that represents the subject of this study.

1.2 SKELETAL MUSCLE

1.2.1 Myogenesis

The formation of skeletal muscle involves a series of steps in which multipotential mesodermal precursor cells become committed to a muscle cell fate and then proliferate as myoblasts until they encounter an environment lacking mitogens, at which point they exit the cell cycle and differentiate (McKinsey et al. 2001).

Skeletal, cardiac, and smooth muscle are each derived from mesodermal precursor cells in different regions of the embryo. Although these three different muscle cell types express many of the same muscle-specific genes, each type is unique with respect to the spectrum of muscle genes they express, their morphology, their ability to divide, and their contractile properties. Therefore, if any shared myogenic program exists it must be modified by different regulatory factors to generate the diversity of three muscle cell types.

During vertebrate embryogenesis, the myogenic precursor cells of limb muscles originate in the somitic dermomyotome, an epithelial layer located in the dorsal compartment of the somite, and then move to the limb buds. However adult myogenic cells are derived mainly from muscle satellite cells that are specialized myogenic cells found during late fetal development. In mice the myogenic precursors in the dermomyotome express Pax3, Pax7 and low levels of the myogenic determination

factor Myf5. The paired-domain transcription factors Pax3 and Pax7 act upstream of the primary myogenic basic helix–loop–helix (bHLH) transcription factors Myf5 and MyoD, in myogenic specification. When appropriate stimuli activate the myogenic determination genes MyoD and Myf-5 the muscle precursor cells are committed to become **myoblasts** and migrate into the adjacent embryonic connective tissue, or mesenchyme and express other muscle specific genes such as myogenin and MRF4, that after a period of proliferation, induce the fusion of myoblasts into multinucleated and highly specialized skeletal muscle cells called **myotubes** or **myofibres** (reviewed in Chen and Goldhamer 2003). A typical myofibre is cylindrical, large (measuring 1-40 mm in length and 10-50 μm) and multinucleated (containing as many as 100 nuclei). Muscle fibres are the basic contractile units of skeletal muscle and are individually surrounded by a layer of connective tissue and grouped into bundles to form skeletal muscle (reviewed in Alberts et al. 2002, Lodish et al. 1995).

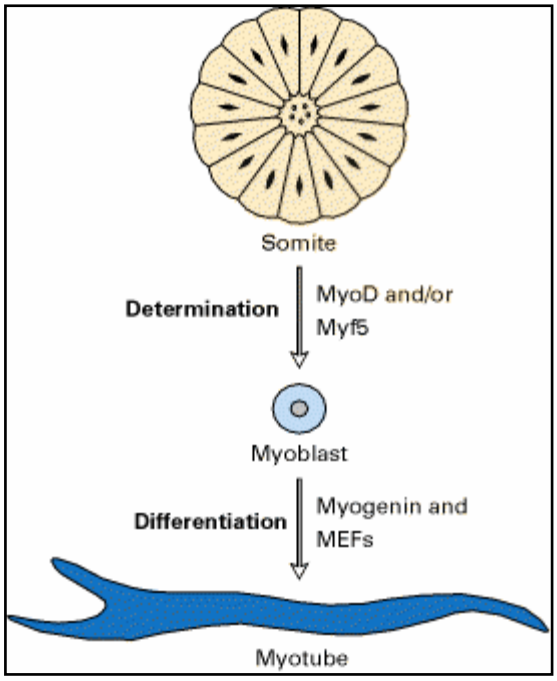


Figure 1. Schematic representation of the process of skeletal muscle differentiation. (Adapted from Lodish et al. 1995, *Molecular Cell Biology*).

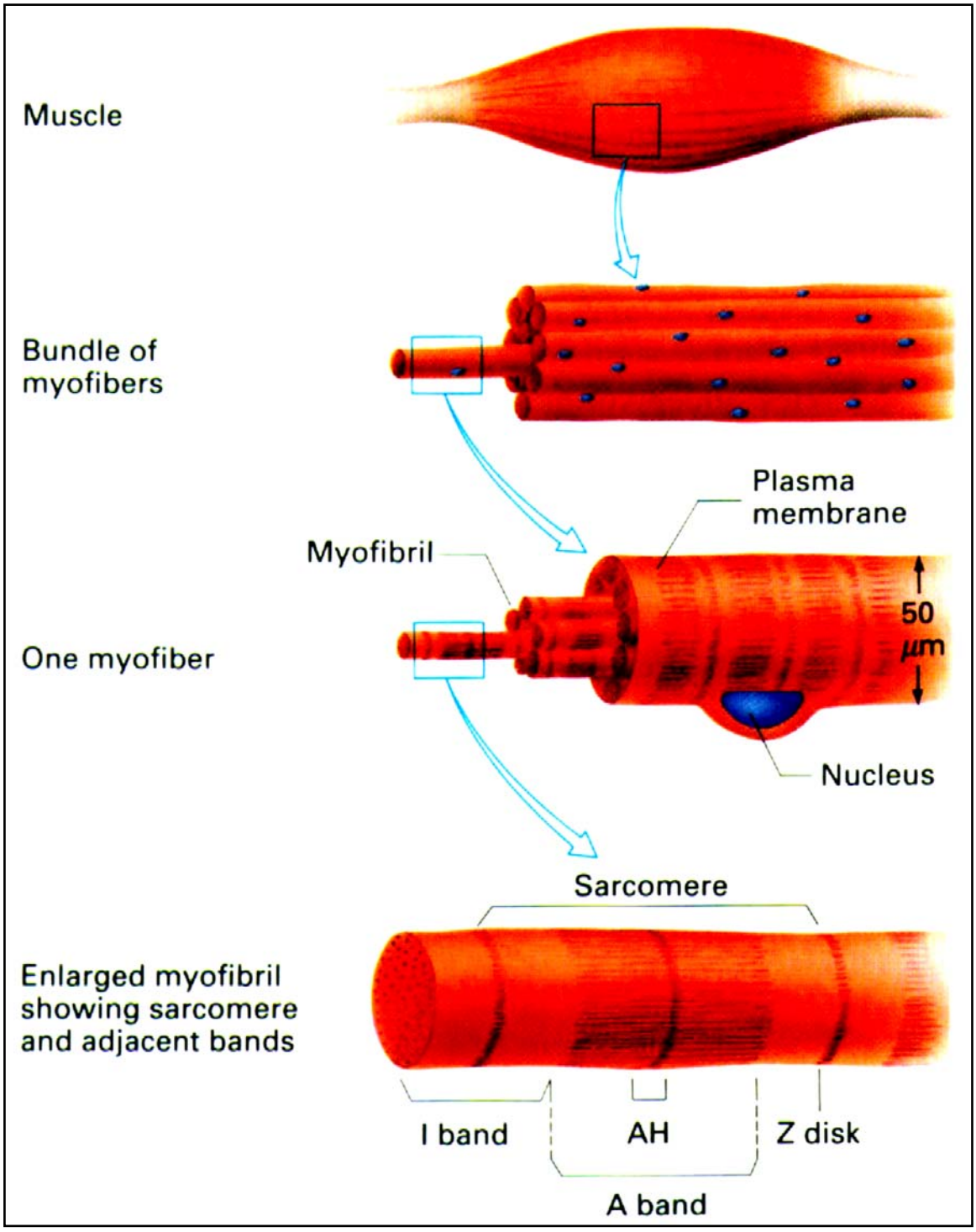


Figure 2. General structure of skeletal muscle. Skeletal muscle tissue is composed by bundles of multinucleated muscle cells, or myofibres. Each muscle cell is packed with bundles of actin and myosin filaments, organized into myofibrils that extend the length of the cell. Packed end to end in a myofibril is a chain of sarcomeres, the functional units of contraction. The internal organization of the filaments gives skeletal muscle cells a striated appearance. (Adapted from Lodish et al. 1995, *Molecular Cell Biology*).

1.2.2 Differentiation

Skeletal muscle differentiation is a tightly regulated process that requires the coupling of muscle-specific gene expression with the terminal withdrawal from the cell cycle. The MyoD family of basic helix-loop-helix (bHLH) skeletal muscle specific transcription factors plays a pivotal role in initiating skeletal muscle differentiation. MyoD and Myf-5 are the most important members of this protein family and are expressed in proliferating and undifferentiated cells, whereas the expression of other bHLH transcription factors such as myogenin and MRF-4 occurs only during differentiation or in adult mature skeletal muscle, respectively. These four myogenic bHLH proteins are known as myogenic regulatory factors (MRFs). The MRFs are able to bind DNA both in the form of homodimers as well as in the form of heterodimers with ubiquitously expressed transcription factors called E proteins. Their binding sites, called E boxes, share the consensus sequence CANNTG. The binding of the bHLH/E protein to DNA is essential for the activation of the muscle specific differentiation program (reviewed in Lassar et al. 1994).

The differentiation process starts during embryogenesis, when the appropriate environmental stimuli are encountered. In the myotome of the embryo, the MRF's present in determined myoblasts (MyoD and Myf-5) initiate a cascade of events that leads to the activation of other transcription factors, the MEF2 family, which are necessary for the transcription of myogenin and other skeletal muscle specific genes. Myogenin itself can also activate MEF2, creating a positive regulatory loop that ensures the maintenance of appropriate levels of these proteins in differentiating skeletal muscle (reviewed in Lassar et al. 1994, Weintraub et al. 1991). Amongst the earliest muscle genes to be expressed in the myotome are desmin, Titin and α-actin. The expression of

the myosin heavy chain gene occurs almost a day after the accumulation of the α-actin protein. Committed myoblasts initiate their transformation into differentiated myotubes by first expressing all the major structural proteins. Then, the other muscle genes will be activated following a strict temporal regulation during the embryonic, fetal and post-natal development (reviewed in Buckingham 1992).

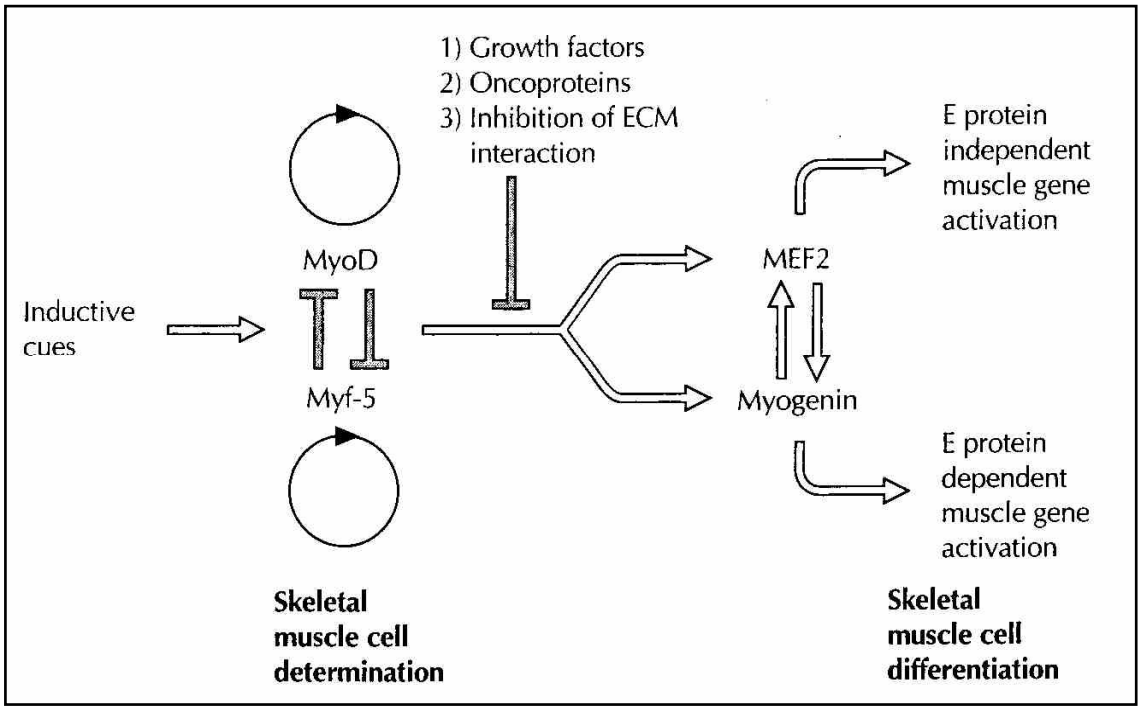


Figure 3. Skeletal muscle development. Schematic representation of the regulatory cascades that controls the skeletal muscle differentiation in vertebrates. (Adapted from Lassar et al. 1994. *Curr Opin Cell Biol* 6: 788-794)

At the same time a system will intervene to allow the cell to exit from the cell cycle thus permitting tissue specific gene expression, cell fusion and the formation of multinucleated myotubes (reviewed in Lassar et al. 1994). These events involve both muscle specific transcription factors and ubiquitous cell cycle regulatory proteins. In fact MyoD, that represents the major coordinator of skeletal muscle differentiation, is

also able to induce the expression of p21, a potent inhibitor of Cyclin-Dependent Kinases (Cdks), thus forcing the cell cycle withdrawal (Halevy et al. 1995). This event inhibits the Retinoblatoma protein (pRb) phosphorylation thus promoting its activation and allowing it to sequester the E2F transcription factor, thus blocking cell cycle progression (Wiman 1993). Moreover MyoD and pRb can directly interact with each other (Gu et al. 1993).

Many other cell cycle regulatory proteins are involved in this complex picture. In fact the p53 tumor suppressor protein is essential in the process of skeletal muscle differentiation, since p53-impaired cells fail to differentiate (Soddu et al. 1996, Mazzaro et al. 1999) even though cell cycle withdrawal takes place in a p53-independent manner. Indeed the p53 tumor suppressor is crucial to elevate un-phosphorylated pRb levels to a threshold sufficient to terminally maintain the cell in G_0/G_1 (Porrello et al. 2000) and to activate together with MyoD the expression of late muscle differentiation markers (Puri et al. 1997, Magenta et al. 2003).

The last step in the differentiation process is represented by myoblasts fusion. In fact cell fusion starts when cells leave the cell cycle. This event correlates with fibronectin secretion onto the extracellular matrix, to which differentiating cells attach using the $\alpha5\beta1$ integrin. After that, myoblasts start aligning into chains. This step is mediated by several cell membrane glycoproteins, including several cadherins and CAMs. Ultimatelty cell fusion occurs, mainly through the action of a set of metalloproteinases called meltrins and multinucleated differentiated myofibres are finally formed (reviewed in Gilbert 1997).

1.2.3 Fibre type specification

As the myofibre matures, it is contacted by a single motor neuron and expresses characteristic proteins for contractile function, principally different myosin heavy chain (MHC) isoforms and metabolic enzymes. Individual adult skeletal muscles are composed of a mixture of myofibres with different physiological properties, ranging from a slow contracting/fatigue resistant/MHC I expressing type to a fast contracting/non fatigue resistant/MHC II expressing type. The proportion of each fibre type within a muscle determines its overall contractile property (reviewed in Chargé and Rudnicki 2003).

Slow-twitch, or high-oxidative muscle fibres, contract more slowly, are smaller in diameter, have a better developed blood supply, have more mitochondria and are more fatigue resistant than fast-twitch muscle fibres. Slow fibres contain large amounts of myoglobin, a pigment similar to haemoglobin that acts as an oxygen reservoir when the blood cannot supply an adequate amount. Conversely, fast-twitch fibres possess large glycogen deposits and are well adapted to anaerobic metabolism. Even if they contract faster than slow fibres, they do so for a short period and fatigue relatively easily.

Fibre type specification programs of gene expression can be detected at early stages of myogenic development in the embryo, but remain plastic in adults, in whom they are subjected to modification as a function of contractile load, hormonal shifts or systemic diseases. Motor nerve activity has a crucial role in determining skeletal muscle fibre type composition. Specifically a brief burst of neural activity, interspersed between long periods of neuronal quiescence, promotes the acquisition of fast-twitch, glycolitic fibre characteristics, whereas extended periods of tonic motor nerve activity stimulates a shift to the slow twitch, oxidative myofibre phenotype. Neural stimulation provokes changes

in the intracellular concentration of several potential signalling molecules including calcium, cyclic AMP, nitric oxide as well as immediate early gene products (c-fos) and molecular chaperons (Chin et al. 1998).

As a consequence of more frequent neural stimulation slow fibres maintain higher levels of intracellular free calcium than fast fibres and this activate calcineurin, a calcium regulated serine/threonine phosphatase. Activation of calcineurin in skeletal myocytes selectively up-regulates slow-fibre-specific gene promoters. Conversely, inhibition of calcineurin in animals promotes slow to fast fibre transformation (Chin et al. 1998).

These data suggest a key role for calcineurin in activation of the slow muscle fibre phenotype. Moreover, a transgenic mouse over expressing calcineurin exhibits an increase in slow muscle fibre, demonstrating that calcineurin activation is sufficient to induce the slow fibre gene regulatory program (Naya et al. 2000).

1.2.4 Stem cells and muscle regeneration

Differentiated skeletal muscle cells are no longer able to divide; however, adult muscle tissue is capable of repair and regeneration when damaged. This feature is due to the persistence in the adult of a small population of stem cells that have been termed "satellite cells", since they are located beneath the basal lamina of mature skeletal muscle fibres.

Satellite cells are quiescent, non-fibrillar, mono-nucleated, small cells which express early myogenic transcription factors, such as Pax 3 and Pax 7 and they originate from the dermomyotome in the embryo (Relaix et al. 2005). They are responsible for skeletal muscle post-natal growth, repair and maintenance and are activated to proliferate upon

muscle injury. In fact, skeletal muscle tissue is always subjected to damage as a result of weight bearing, exercise or traumas. Hence it requires a system which can ensure an ever-available source of new healthy cells: the self-renewable stem cell. Once activated, the satellite cells re-enter the cell cycle and divide giving rise to a new myoblast population, which finally will fuse and differentiate into myofibres in order to repair the damaged tissue.

The stem cell population constitutes of about 32% skeletal muscle cells at birth, but then declines with age until only 1-5% in the adult. These data refer to the mouse, but the same situation occurs also in humans. Moreover the frequency of satellite cells varies also between muscle fibre type, since a higher number of stem cells have been observed in association with slow fibres rather than fast fibres. However, recent observations support the possible contribution to skeletal muscle repair of other stem cell populations, resident both in muscle and in bone-marrow. This information has added further complexity to understanding the muscle regeneration process (Chargé and Rudnicki 2003, Chen and Goldhamer 2003).

1.3 SARCOMERES

1.3.1 Myofibrillogenesis

Myofibrillogenesis is the process by which proteins in developing muscle cells form sarcomeres. Cultured skeletal myoblasts fuse and grow to form long, multinucleated cells. A three-step model for myofibrillogenesis (the formation of myofibrils) has been proposed that consists of premyofibril to nascent myofibril and then to mature myofibril (Rhee et al. 1994, Sanger et al. 2002). Premyofibrils are composed of minisarcomeric structures and arrange the initial stages for building sarcomeres (Rhee et al. 1994). The boundaries of the minisarcomere are composed of Z-bodies containing sarcomeric Alpha-actinin. Mini-A-bands composed of non-muscle myosin II filaments are present between these Z-bodies. Actin filaments overlap in these minisarcomeres, resulting in a continuous Actin pattern when stained with the fluorescent F-Actin staining reagent phalloidin. In the second proposed step of myofibrillogenesis, Titin and overlapping muscle myosin II filaments are recruited to premyofibrils, thus forming nascent myofibrils. Mature myofibrils are recognized by the absence of non-muscle myosin II, the alignment of muscle myosin II filaments to form A-bands, and the fusion of the Z-bodies to form Z-bands (Sanger et al. 2002). Additional support for this model of myofibrillogenesis was recently reported that demonstrates that myofibrils can be formed in the presence of taxol, and after the removal of taxol (Siebrands et al. 2004).

The bulk of the cytoplasm of mature myofibres is made up by myofibrils, which are the contractile elements of the muscle cell. They are cylindrical structures 1 to 2 μm in diameter and are often as long as the muscle cells itself. Each myofibril consists of a chain of tiny contractile units, or sarcomeres, each about 2.2 μm long, which give the vertebrate myofibril its striated appearance. This is readily observable by light

microscopy as alternating light and dark bands. This unique feature is a direct result of the precise alignment of the filament system of the sarcomere that is the basic contractile unit of myofibrils. Closer examination reveals that the dark bands (A-bands) are bisected by a dark line (M-line), while the light bands (I-bands) are bisected by a different dark line, the Z disk or Z-line**.** Electron microscopy and biochemical analysis have shown that each sarcomere contains two types of filaments: thick filaments composed of myosin, and thin filaments, containing Actin that together are responsible for muscle contraction. The I-band is a bundle of thin filaments. Biochemical studies show that a thin filament is basically an Actin filament plus two additional proteins (tropomyosin and troponin), involved in regulating actomyosin interactions. Actin filaments are capped at their ends by tropomodulin and CapZ. Thin filaments overlap with myosin-containing thick filaments in the A-band and the bare zones of the thick filaments corresponds to the M-line (reviwed in Lodish et al. 1995, Alberts et al. 2002, Clark et al. 2002). A third filament system organizes the thick and thin filaments in their three dimensional arrays that gives muscle much of its elastic properties. This system is made up of single molecules of the giant proteins Titin, and Nebulin. Titin is the third most abundant muscle protein and is the largest protein identified to date (Maruyama et al. 1977). Titin molecules span the entire sarcomere and their N-terminal and their C-terminal ends overlap in the Z-line and in the M-line respectively, thus forming a continuous filament system in the myofibrils (Labeit et al. 1995, Gregorio et al. 1998). Nebulin is another giant protein whose C-terminal region is partially inserted into the Z-line, whereas its N-terminal end extends to the pointed ends of thin filaments (reviewed in Clark et al. 2002).

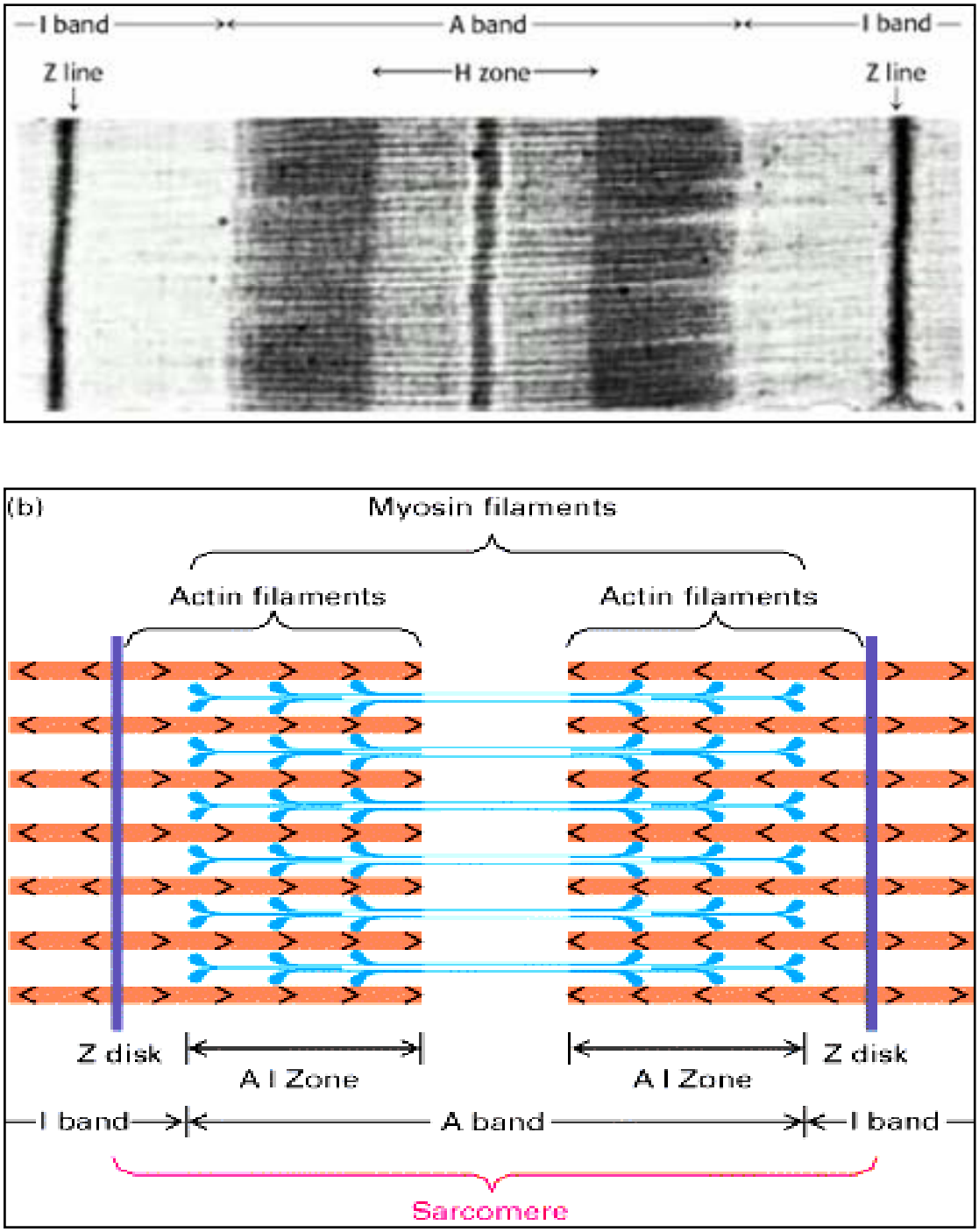


Figure 4. Sarcomere organization. The picture above is an electron micrograph which clearly shows dark and light striation of skeletal muscle sarcomeres. The other figure is a schematic representation of a sarcomere showing the thick and thin filament organization. (Adapted from Lodish et al. 1995, *Molecular Cell Biology)*.

1.3.2 Principal Z-line proteins

In this paragraph, to complete the overview on skeletal muscle, I will briefly outline the principal proteins found in the Z-line of striated muscle and their related myopathies. This is important because Ankrd2, the subject of this study, which is located in the I-band adjacent to the Z-line, directly interacts with a Z-line component. Therefore Ankrd2 could be considered to all intents and purposes as part of the protein interaction network of the Z-line.

As already mentioned, the Z-lines define the lateral boundaries of each sarcomere where the thin, Titin and Nebulin filaments are anchored. How the filaments are attached is not known, but it is thought that the attachment must involve the Actin capping protein CapZ and α-actinin. Due to this anchoring function Z-lines represent the primary conduits of the force generated by contraction in the sarcomere. Moreover Z-lines of adjacent myofibrils are perfectly aligned, in order to allow a coordinate contraction all along the myofibre (reviewed in Clark et al. 2002). Ten years ago, apart from Actin, α-actinin was the only well characterized Z-line protein; however, rapid advances in molecular biology have revealed that the Z-line is actually a complex protein network and that many protein-protein interactions are occurring between Z-line components, probably in a highly dynamic way. In fact no protein in the Z-line can be considered isolated from the other components and most of the proteins known to date have at least one if not more binding partners, creating an highly complex and dynamic structure (Faulkner et al. 2001).

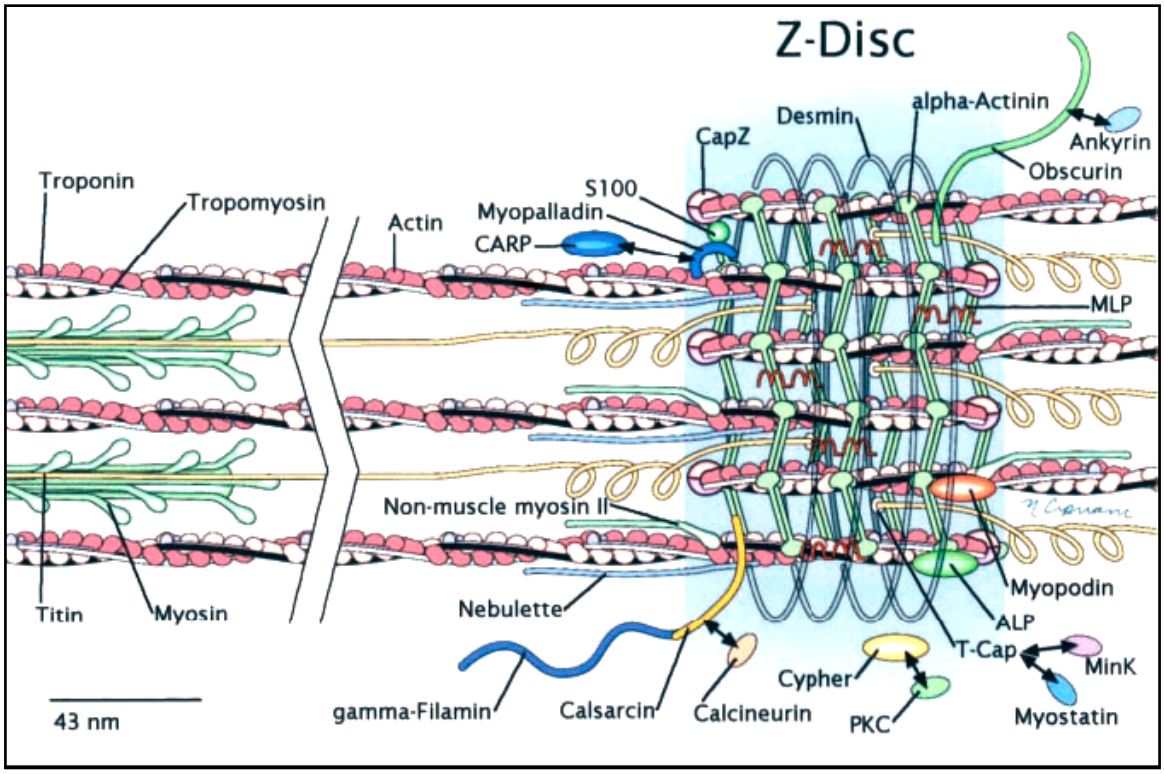


Figure 5. Cartoon of the network of proteins making up the Z-line. Z-line is a very complex network of interactions. For clarity in this picture stoichiometric relationships are not shown. (Adapted from Pyle et al. 2004, *Circ Res* 94: 296-305).

Obviously, many Z-line proteins have a structural role, defining Z-line architecture and are stably found in the Z-line. However more recently the idea has emerged that some proteins of the Z-line are implicated in mechanical sensing and in signalling pathways in order to maintain muscle homeostasis. These proteins do not have a fixed location, but can be found both in the Z-line and in other sarcomeric and cellular compartments. So it is not surprising that signal transduction components are constituents of the Z-line and that also a large number of Z-line associated proteins have a dynamic distribution and may shuttle between the Z-line and other subcellular locations. Thus the Z-line is not simply the structural border of the sarcomere, but likely plays an important role in

signalling and in regulating muscle response to environmental stimuli (reviewed in Clark et al. 2002).

Although we know some of the interactions occurring among Z-line proteins, very little is known about the overall molecular organization. In particular the most difficult point to understand is how these interactions occur and are able to generate and maintain the complex Z-line molecular architecture. The importance of Z-line proteins in the maintenance of structural and functional integrity is underlined by their involvement in several muscle pathologies such as cardiomyopathies and muscular dystrophies. Hence a greater knowledge of Z-line proteins and of their interactions is essential not only for understanding their role in the structure and function of muscle but also for discovering the causes and hopefully the remedies for these diseases (Faulkner et al. 2001).

1.3.2.1 Actin

Thin filaments are mainly composed of **Actin.** This protein is responsible for diverse cellular functions such as motility, cytokinesis and contraction. Actin filaments form two twisted α-helices that associate with the regulatory proteins tropomyosin and troponin. CapZ stabilizes and prevents depolymerization of Actin filaments by binding their plus (+) ends. Moreover it attaches Actin filaments to the Z-line and also binds Alpha-actinin 2 (Casella et al. 1987, Papa et al. 1999). Early in myofibril assembly, thin filament components associate with nascent Z-lines to form the first identifiable structures called I-Z-I complexes (Holtzer et al. 1997). Several Actin isoforms exist, each encoded by separate genes whose expression patterns are regulated developmentally and in a tissue specific manner. Their sequences and molecular structures are very similar. Two muscle-specific isoforms, cardiac and skeletal actins,

are co-expressed at varying levels depending on the species, muscle fibre and developmental stage. Vascular and visceral actins are expressed in smooth muscle and also in striated muscle fibres transiently during development. Two non-muscle actins, the cytoplasmic isoforms, are co-expressed with other Actin isoforms in many tissues.

Clinical investigations have revealed that specific point mutations in the Actin sequence result in various forms of human muscle myopathies such as **Actin myopathy** and **nemaline myopathy** characterized by myofibrillar structural abnormalities and muscle weakness (Nowak et al. 1999, Ilkovski et al. 2001), **familial dilated cardiomyopathy (DCM)** and **heart failure**, characterized by impaired force transmission and **hypertrophic cardiomyopathy (HCM)** (Olson et al. 1998, Olson and Williams 2000)**.** Thus single amino acid substitutions in the Actin molecule result in distinct clinical manifestations, depending on the particular functional domain affected.

1.3.2.2 Alpha-actinin

The major component of the Z-line is **Alpha-actinin,** a member of the spectrin superfamily. It is an Actin cross-linking protein which has a globular N-terminal Actin-binding domain, a central rod domain composed of four spectrin-repeats and a C-terminal domain that displays similarity to calmodulin. The rod domains of Alpha-actinin monomers interact to establish antiparallel dimers that cross-link Actin and Titin filaments from neighbouring sarcomeres (Djinovic-Carugo et al. 1999). In higher vertebrates there are four Alpha-actinin isoforms: Alpha-actinin 1 and 4 are non-muscle isoforms, whereas Alpha-actinin 2 and 3 are expressed in the Z-line of skeletal muscle even if they are not co-expressed in all striated muscles. In fact Alpha-actinin 3 is found only in fast muscle fibres, where it can form heterodimers with isoform 2, but not in

slow muscle fibres (reviewed in Faulkner et al. 2001, Clark et al. 2002). Since many Z-line proteins interact with Alpha-actinin those that bind to the same region must do so competitively. In addition to Actin and Titin, Alpha-actinin binds MLP (Muscle LIM Protein) (Pomies et al. 1999), ALP (Xia et al. 1997), FATZ (Faulkner et al. 2000, Frey et al. 2000, Takada et al. 2001), Myotilin (Salmikangas et al. 1999), ZASP (Zohu et al. 1999, Faulkner et al. 1999, Passier et al. 2000) and Myopalladin (Bang et al. 2001b).

1.3.2.3 ZASP/Cypher

Z-band alternatively spliced PDZ motif protein (ZASP) (Faulkner et al. 1999), also known as Cypher (Zohu et al. 1999) and as Oracle (Passier et al. 2000) is a member of the ALP-Enigma family, a newly emerging group of cytoskeletal proteins defined by an N-terminal PDZ domain and one or three C-terminal LIM domains. Both PDZ and LIM domains are typically involved in protein-protein interactions. The ZASP PDZ domain binds the C-terminal domain of α-actinin 2 and this interaction is thought to mediate ZASP localization to the Z-line. Instead the LIM domains can bind all the six PKC isoforms α, -β1, -δ, -ε, -γ and ξ (Zhou et al. 1999).

ZASP is up regulated on differentiation in human primary muscle cells and in adult striated muscle tissue. ZASP has many alternatively spliced forms and to date a total of six isoforms has been identified: the shorter one, ZASP1/Cypher2 has only an N-terminal PDZ domain whereas Cypher/ZASP variants 2-5 have also three C-terminal LIM domains. These isoforms can be divided into skeletal or cardiac specific and are regulated both at the transcriptional and the translational level during the different stages of embryonic and post-natal development (Huang et al. 2003, Vatta et al. 2003).

ZASP has been genetically ablated in the mouse. The Cypher knock out (-/-) mouse exhibits several defects and dies post-natally from functional failure in multiple striated muscles. From ultrastructural analysis it has been observed that skeletal muscle defects are post-natal, indicating that ZASP/Cypher is not necessary for sarcomerogenesis or Z-line assembly, but rather for stabilizing the structure once muscle contraction begins. Moreover, the Cypher -/- mouse develops a severe congenital myopathy and **dilated cardiomyopathy** (Zhou et al. 2001). More recently, several mutations in the ZASP gene sequence, resulting in the alteration of conserved amino acids, have been found in dilated cardiomyopathy patients, thus strengthening the hypothesis of a crucial role for ZASP in conserving the striated muscle cytoarchitecture (Vatta et al. 2003).

1.3.2.4 FATZ

Another protein mainly expressed during skeletal muscle differentiation is **FATZ (γ-filamin, Alpha-actinin and Telethonin binding protein of the Z-disk)** (Faulkner et al. 2000), also known with the name Calsarcin (Frey et al. 2000) or Myozenin (Takada et al. 2001). Two other members that display a high sequence similarity with FATZ have been discovered: Calsarcin1 (FATZ 2) and Calsarcin3 (FATZ 3) (Frey et al. 2000, Frey and Olson 2002). FATZ is directed and tethered to the Z-line through its association with Alpha-actinin, γ-filamin and Telethonin, three Z-line components. More recently, FATZ was also found to interact with ZASP (Frey and Olson 2002) and FATZ2 to bind and negatively regulate Calcineurin, a phosphatase involved in the hypertrophy pathway. Mice lacking FATZ 2 show an excess of slow skeletal muscle fibres and an activated hypertrophic gene program (Frey et al. 2004).

1.3.2.5 Telethonin

Telethonin (T-cap) is another FATZ binding partner (Faulkner et al. 2000) that was independently identified by three distinct groups (Valle et al. 1997, Mues et al. 1998, Gregorio et al. 1998) as one of the most abundant transcripts expressed in skeletal muscle. Telethonin is located in the Z-line and besides FATZ binds the N-terminal Z1-Z2 domains of Titin (Gregorio et al. 1998), minK (Furukawa et al. 2001), Myostatin (Nicholas et al. 2002) and Ankrd2 (Kojić et al. 2004).

As already mentioned, the giant protein Titin forms the third filament system of the sarcomere together with Nebulin. The interaction between Titin and Telethonin appears to be critical for sarcomeric structure, since over expression of Telethonin and the N-terminal region of Titin leads to Z-line disruption (Gregorio et al. 1998). Moreover Telethonin may be phosphorylated by the Titin kinase, located in the Titin C-terminal domain and activated by calcium/calmodulin binding, during myocyte differentiation. Since the Titin kinase domain is situated in the M-line, whereas Telethonin is in the Z-line, it has been proposed that during myofibrillogenesis the cytoskeleton undergoes reorganization and the Titin C-terminal becomes transiently located in close proximity to Telethonin, thus allowing its phosphorylation (Mayans et al. 1998). The direct binding of cardiac muscle Telethonin with the potassium channel (I_{KS}) β-subunit minK has been reported. This interaction suggests a T-tubule-myofibril linking system that may contribute to a stretch-dependent regulation of K^+ flux (Furukawa et al. 2001).

Interestingly Telethonin has been linked to a form of muscular dystrophy, since mutations in the Telethonin gene are responsible for a form of **autosomal recessive Limb-Girdle Muscular Dystrophy (AR LGMD) type 2G**. LGMDs are a genetically heterogeneous group of disorders that affect mainly the proximal musculature. Two

different mutations in Telethonin were identified in three families with LGMD 2G, both giving rise to premature stop codons resulting in truncated Telethonin. Interestingly, the C-terminal truncation eliminates the domain of Telethonin that is phosphorylated by Titin kinase. Telethonin is the first sarcomeric protein found to cause an AR LGMD (Moreira et al. 2000). Additional protein studies on these patients have shown normal expression of Dystrophin, Sarcoglycan, Dysferlin, Calpain3 and Titin. Immunofluorescence analysis for Alpha-actinin 2 and Myotilin showed a normal cross-striation pattern, suggesting that at least part of the Z-line of the sarcomere is preserved. Ultra structural analysis confirmed the maintenance of the integrity of the sarcomeric architecture. Therefore, mutations in the Telethonin gene do not seem to alter the sarcomere integrity (Vainzof and Zatz 2003), suggesting a signalling rather than structural role for Telethonin in striated muscle.

This hypothesis is also supported by the finding of an interaction between Telethonin and the growth factor Myostatin, a member of the transforming growth factor-β (TGF-β) family and a negative regulator of muscle cell growth. Telethonin binds to Myostatin and inhibits its secretion thus allowing muscle cells to proliferate (Nicholas et al. 2002).

1.3.2.6 Nebulin and Nebulette

Nebulin is the other large protein that together with Titin forms the third filament system of the myofibril. Only the C-terminal part of the protein is located in the Z-line, while the rest extends until the end of the thin filaments. Since Nebulin assembles early during myofibrillogenesis, it has been speculated that this protein acts as a template for the formation of Actin filaments (Kruger et al. 1991). In fact Nebulin is known to bind Actin filaments (Wright et al. 1993) and Alpha-actinin (Nave et al. 1990). Peculiarly

Nebulin is not present in the heart, where a smaller similar form, called **Nebulette** can be observed. Nebulette is highly homologous to the COOH-terminal region of Nebulin and it also extends into the Z-line. It has been suggested that this homology between Nebulin and Nebulette reflects conserved roles for their COOH-terminal ends in Z-line integration (Moncman and Wang 1995).

1.3.3 Other sarcomeric proteins

In this paragraph I will briefly describe a set of muscle proteins that can not easily be classified as Z-, I- or M-line components since they move between these sarcomeric regions or between the sarcomere and other sub-cellular compartments. In fact cytoskeleton-associated proteins often display dynamic distributions within cells and can participate in signal transduction cascades. Many of these proteins have been reported to reside also in the nucleus where they regulate gene expression in response to changes arising in structure or function of the contractile machinery. In particular these factors enable cells to adopt an efficient response to stress, stretching and pressure load in order to maintain muscle homeostasis (reviewed in Clark et al. 2002).

1.3.3.1 MLP

The Muscle Lim Protein is supposed to be a scaffold or linker protein mainly localized at the periphery of the Z-line (Arber et al. 1997) and at sites of membrane-cytoskeletal linkage, such as the intercalated disc (Ehler et al. 2001). The ultrastructural analysis of MLP -/- murine cardiomyocytes revealed a misalignement of the Z-line (Arber et al. 1997) and morphological defects at costameres and intercalated discs (Ehler at al. 2001) suggesting that MLP plays a primary role in maintaining the stability of these structures.

The genetic ablation of MLP in the mouse results in DCM (Arber et al. 1997) and recently a point mutation in the human MLP gene has also been associated with this pathological condition. This single nucleotide mutation resulted in a severe charge change at position 4 in the MLP protein (W4R) that lies within the N-terminal Telethonin interacting domain (TID) of MLP, thus disrupting the binding between the two proteins and causing Telethonin mis-localization and Z-line disruption.

Since MLP binds Telethonin, it was suggested that, together with Titin, they constitute a stretch sensor machinery (Knöll et al. 2002). MLP has also been implicated in the communication with the nuclear compartment especially in response to hypertrophic signals (Ecarnot-Laubriet et al. 2000) and to interact with MyoD (Kong et al. 1997).

1.3.3.2 Titin

Besides Telethonin, the giant protein **Titin** has several other binding partners all along the entire length of the sarcomere. Since Titin spans the entire sarcomere, it can be divided in three principal regions: Z-line Titin, I-line Titin and M-line Titin.

Z-line Titin represents the N-terminal part of the molecule, contains several Ig-like repeats and binds Alpha-actinin (Ohtsuka et al. 1997) and Telethonin (Gregorio et al. 1998). Titin, together with Telethonin and MLP constitutes a stretch sensor machinery in cardiomyocytes (Knöll et al. 2002). The difference in number of alternatively spliced Ig-like repeats in Titin isoforms varies among different muscle types and has been suggested to affect the number of cross-links between Alpha-actinin 2 and Actin and hence to cause variations in Z-line thickness (Gautel et al. 1996). Moreover Z-band Titin interacts with small ankyrin 1 (sAnk1) (Kontrogianni-Kostantopoulos and Bloch 2003), suggesting the existence of a complex implicated in the organization of the

sarcoplasmic reticulum around the myofibril and with Obscurin (Bang et al. 2001a). In addition to Titin's structural and elastic properties, mounting evidence indicates that this giant molecule participates in multiple myofibril signalling pathways. In fact **I-line** region of Titin interacts with all the MARP family members CARP, Ankrd2 and DARP (Miller et al. 2003) and with Calpain protease 94 (Sorimachi et al. 1995). Remarkably, all I-band ligands of Titin and their associated binding partners are also found in the nucleus where they can participate in transcriptional and cell cycle regulation. It seems likely that this dual localization (I-band and nucleus) is not a mere coincidence but instead reflects a dual function for these proteins: being part of a Titin-based stretch sensing complex in the I-band and regulating transcription in the nucleus. Furthermore, such dual localization may also provide a communication pathway between the I-band and nucleus that links stretch sensing to gene expression (Granzier and Labeit 2004).

The C-terminal part of **Titin** is in the M-line. This region contains a Serine/Threonine kinase activity that phosphorylates Telethonin in developing muscle (Mayans et al. 1998). However, the upstream elements controlling the Titin kinase activation, its range of cellular substrates and its role in mature muscle are largely unknown. The elastic properties of the Titin molecule and the mechanical deformation of the M-band during stretch and contraction suggest that the signalling properties of the Titin kinase might be modulated by mechanically induced conformational changes (Lange et al. 2005).

Moreover M-line Titin binds the RING finger protein MURF-1 (Centner et al. 2001) and nbr-1, which interacts with the MURF-2 binding protein p62 (Lange et al. 2005). Nbr-1 acts as a cytoskeleton-associated kinase scaffolding protein that assembles large sarcomeric "signalosomes" through interactions with multiple elements, linking the Titin kinase to p62 and MURF-1 (Lange et al. 2005).

Titin is involved in muscle disease; in fact mutations in the Titin gene, that affect the binding sites for Telethonin and Alpha-actinin, have been found in patients with **dilated cardiomyopathy** and are thought to be correlated with the disease (Itoh-Satoh et al. 2002). More recently, homozygous mutations in the Titin gene previously known to be responsible for autosomal dominant tibial muscular dystrophy, a form prevalent in Finland, were found to cause **LGMD type 2J** (Vainzof and Zatz 2003) and a point mutation in the kinase domain that disrupts the binding with nbr-1 leading to hereditary myopathy with early respiration failure (HMERF) (Lange et al. 2005).

1.3.3.3 Myopalladin

Myopalladin is a 145 kDa protein that interacts with Nebulin and Alpha-actinin in the Z-line and with CARP within the I-band. This interactions appear to be crucial for the sarcomeric integrity since the over expression of Myopalladin leads to the disruption of all the sarcomeric structure. These observations suggest that Myopalladin could link the regulatory mechanisms of the Z-line to muscle gene expression through its interaction with CARP (Bang et al. 2001b).

1.3.3.4 MURFs

The MURFs (Muscle Specific RING-finger proteins) are a family of RING/B-box proteins expressed in striated muscle. Three MURF genes encode three highly similar proteins that can form homo- and heterodimers: MURF-1, MURF-2 and MURF-3 (Centner et al. 2001). MURF-1 has been suggested to act as an ubiquitin ligase, thereby controlling proteasome-dependent degradation of muscle proteins. Among its targets there are M-line Titin, Nebulin, Myotilin and Telethonin. Other functions have been

ascribed to MURF-1 such as the regulation of gene expression, structural scaffold of the M-line lattice and the regulation of myocardiocytic contractility (Witt et al. 2005).

MURF-2 co-localizes both with MURF-1 in the M-line and with MURF-3 in microtubules (McElhinny et al. 2004). MURF-3 is a microtubule associated protein differentially expressed during myogenesis and up-regulated during differentiation, probably to stabilize microtubules (Spencer et al. 2000). MURF-3 also localizes in the Z-line of skeletal muscle. Since it binds MURF-1, MURF-2 and Z- and M-line proteins such as Titin, it probably functions as a link between the sarcomeric and the microtubules compartments (Spencer et al. 2000).

1.3.3.5 Calpain 3

Muscle-specific Calpain3/p94 is a Ca^{2+} dependent cysteine protease present both in the M- and in the I-line, where it binds to Titin (Sorimachi et al. 1995). Calpain3/94 is thought to have a regulatory function in the modulation of transcription factors and to be involved in the disassembly of sarcomeric proteins (Vainzof and Zatz 2003). The importance of its function is suggested by the observation that mutations in the Calpain3/94 gene, with consequent loss of function, lead to **LGMD type 2A** (Richard et al. 1995).

1.3.3.6 NF-AT3

NF-AT3 is a transcription factor localized both in the Z-line and in the nucleus. In normal conditions NF-AT3 is tethered to the Z-line by calcineurin. NF-AT3 resides in the Z-line because of its interaction with calcineurin. When a signal activates calcineurin, it de-phosphorylates NF-AT3 provoking its translocation into the nucleus.

Both NF-AT3 and Calcineurin are found **hypertrophy** pathways (reviewed in Olson et al. 2000).

1.3.3.7 CARP and the MARP family

CARP (Cardiac Ankyrin Repeat Protein) is a sarcomeric protein which translocates from the I-line to the nucleus. It is a nuclear co-factor downstream of the homeobox gene Nkx2.5 pathway that was firstly isolated from rat neonatal heart. CARP forms a physical complex with the ubiquitous transcription factor YB-1, thus acting as a negative regulator of HF-1 dependent pathways for ventricular muscle gene expression (Zou et a. 1997). At the same time another group identified CARP as a gene constitutively expressed in the heart, whose mRNA level was extremely reduced in response to doxorubicin (adriamycin) treatment. For these characteristics they termed it Cardiac Adriamycin Responsive Protein (Jeyaseelan et al. 1997). They also reported that CARP expression was restricted to fetal cardiac muscle, with low levels in the adult heart and only barely detectable in skeletal muscle. However CARP expression has also been observed in human fetal skeletal muscle and in experimentally denervated skeletal muscle. In particular CARP was found to be strongly induced in regenerating myofibres of Congenital (CMD) and Duchenne Muscular Dystrophy (DMD) patients (Nakada et al. 2003). Moreover, even if Zou and colleagues (Zou et al. 1997) described CARP as a nuclear protein, it can also be detected in the cytoplasm, precisely in the sarcomeric I-Z-I areas of regenerating skeletal muscle (Nakada et al. 2003). In fact CARP interacts with the I-band protein Myopalladin and therefore may have a role in the maintenance of sarcomeric integrity (Bang et al. 2001b). Other authors reported that CARP is induced by pressure overload in cardiac hypertrophy (Kuo et al. 1999), by TGF-β in vascular

smooth muscle cells (VSMCs) and that its over expression inhibits DNA synthesis (Kanai et al. 2001). These features are typical of early response genes. CARP is highly homologous to the human C-193 gene (Chu et al. 1995) and to MARP (Muscle Ankyrin Repeat Protein) a gene induced by denervation in adult rat skeletal muscle and also detected in heart and large blood vessels (Baumeister et al. 1997).

Ankrd2 that will be discussed in more detail in the next section is the gene with the highest homology to C-193/CARP/MARP. Ankrd2 interacts with the Z-line protein Telethonin and with the ubiquitous transcription factors p53, PML and YB-1. It is also thought to shuttle between the I-line and the nucleus in response to a still unidentified signal (Pallavicini et al. 2001, Kojić et al. 2004).

Since Ankrd2 and C-193/CARP/MARP are very similar both in sequence and in behaviour, it is possible that they play similar roles in skeletal muscle and in heart, respectively. An exciting hypothesis is that Ankrd2 and its homologues could be involved in the control and maintenance of an adequate muscle mass, respectively in skeletal muscle and heart. In fact CARP is regulated by cardiac pressure overload, hypertension and heart failure leading to a hypertrophic response. Similarly, in mouse skeletal muscle Ankrd2 is up-regulated after muscle stretching, indicating a possible involvement in the signaling pathway leading to muscle hypertrophy, triggered by stretch and overload (Pallavicini et al. 2001). Recently another protein that shows homology to CARP and Ankrd2 has been described and named DARP (Diabetes-related Ankyrin Repeat Protein). This protein is expressed in the nuclei of heart, skeletal muscle and brown adipose cells and is involved in energy metabolism being induced in animals recovering from starvation (Ikeda et al. 2003).

CARP, Ankrd2 and MARP have been grouped together into the MARPs family of Muscle Ankyrin Repeat Proteins (Miller et al. 2003).

It is relevant to notice how the expression of each MARP seems to be induced upon different stress stimuli: injury and hypertrophy (CARP), stretch and denervation (Ankrd2) and recovery following starvation (DARP). This suggests that these proteins are involved in different muscle stress response pathways. The MARPs have a Titin binding site. It has been postulated that they could be part of a Titin-N2A based complex together with Calpain3 and Myopalladin. This complex might represent a stress sensing system that sends signals to the nucleus in order to regulate gene expression (Miller et al. 2003).

1.4 ANKRD2

1.4.1 Ankrd2/ARPP

The human ortholog of the mouse Ankrd2 gene, already described by Kemp and colleagues in the year 2000 (Kemp et al. 2000), was independently discovered in 2001 by two laboratories using different approaches. Pallavicini and colleagues named the human gene and its product Ankrd2 (ANKyrin Repeat Domain 2) as the murine counterpart, whereas Moriyama and colleagues called it Arpp (Ankyrin Repeat, PEST sequence and Proline rich region). However, Ankrd2 and Arpp are the same gene/protein that for the purpose of this thesis will be referred to solely as Ankrd2

1.4.2 Gene organization

The human Ankrd2 gene has been mapped on the chromosome region 10q23.31-23.32 using the radiation hybrid technique (Pallavicini et al. 2001) or in 10q24 using fluorescent in situ hybridization (FISH) (Moriyama et al. 2001). The mouse gene, discovered by Kemp and colleagues (Kemp et al. 2000) and described also by Tsukamoto and colleagues (2002) as mouse Arpp (mArpp) has subsequently been located by FISH on mouse chromosome 19C3-D1 (Fujiwara et al. 2004). Recently also the Carp (Cardiac Ankyrin Repeat Protein) gene, very similar to Ankrd2, was positioned on the same human chromosome at 10q23.1, suggesting the possibility that some of the ankyrin repeat protein subfamily genes are clustered in the human genome (Moriyama et al. 2001). The human Ankrd2 gene has an open reading frame (ORF) of 999 base pairs and encodes a 333 amino acid protein (Moriyama et al. 2001, Pallavicini et al. 2001), while the mouse gene has an ORF of 996 base pairs and generates a product of 332 amino acids (Kemp et al. 2000). Both of them encode a protein of the

predicted molecular weight of about 37 kDa. The human and mouse gene organization appears to be very similar, with 9 exons, four of which (exons 5, 6, 7 and 8) encode ankyrin repeats. A possible fifth ankyrin repeat has been found only in the human gene (Pallavicini et al. 2001). A bioinformatic analysis was performed on the genomic sequence in order to characterize the transcription initiation site and the promoter region. Two ATG starting codons have been identified at 12 bases of distance one from the other, but maintaining the same reading frame (Pallavicini et al. 2001). A similar analysis was carried out by Kemp and colleagues (Kemp et al. 2000) indicated the presence of the additional twelve coding bases also in the mouse. The human and mouse Ankrd2 promoter sequence covers the 1157 base pairs 5' flanking region. A TATA box was found at 29 bp upstream of the transcriptional start site. Three putative binding motifs for MyoD and one for NF-kB were found, indicating that Ankrd2 gene expression may be regulated both by the MyoD and the NF-κB pathways (Pallavicini et al. 2001). Recently has been discovered that the Ankrd2 gene is under the control of MyoD during myogenic differentiation (Bean et al. 2005). In addition several E boxes and consensus sequences for other transcription factors (c-Myb, E47, GATA-X, Nkx 2.5) have also been detected (Miyazaki et al. 2002).

1.4.3 Expression

The human Ankrd2 transcript is 1159 nucleotides in length (Pallavicini et al. 2001), whereas the mouse is shorter in the 3' untranslated region and is 1101 nucleotides long (Kemp et al. 2000). The relative abundance of human Ankrd2 mRNA was predicted to be 0.055% as calculated from the percentage of Ankrd2 ESTs present in the muscle EST database (Pallavicini et al. 2001). The major sites of expression of the human

Ankrd2 gene as detected by northern blotting are adult skeletal muscle and heart (Moriyama et al. 2001, Pallavicini et al. 2001). However a lower level is detectable in other tissues such as kidney and prostate using RT-PCR analysis (Pallavicini et al. 2001). The fact that in northern blotting analysis only a single band is visible suggests the presence of a single Ankrd2 splicing product in striated muscle (Pallavicini et al. 2001). The expression of Ankrd2/Arpp in the foetal heart is significantly lower than in the adult where it is confined to the left and right ventricles, the interventricular septum and apex, but is not detectable in the left and right atria and in the aorta. This peculiar expression pattern suggests that this gene may be both developmentally and tissue regulated and that it probably has a specific role in the heart (Moriyama et al. 2001).

The Ankrd2 protein distribution among different human tissues was analyzed by western blot analysis using four different antibodies, a monoclonal antibody recognizing an epitope in the C-terminal region of the protein and three polyclonal antibodies raised against different segments of Ankrd2. The results confirmed the pattern observed for the RNA, since Ankrd2 was visible as a 42 kDa band mainly in skeletal muscle and to a lesser extent in the heart and in kidney (Pallavicini et al. 2001). The distribution of the Ankrd2 protein was also analysed by immunohistochemistry comparing normal adult skeletal muscle tissue with dystrophic using three different anti-Ankrd2 antibodies. An antibody against Alpha-actinin 3 was used as a marker of fast fibres. Results indicate that in the normal tissue Ankrd2 is preferentially expressed in slow fibres, whereas in the dystrophic muscle it was expressed in fast fibres together with Alpha-actinin 3 (Pallavicini et al. 2001).

In the mouse the situation appears to be quite different, since the Ankrd2 gene is expressed only in skeletal muscle, both in the embryo and in the adult, but not in the

heart. Muscles particularly rich in slow type 1 fibres express very high levels of Ankrd2 unlike muscles with fast fibres (Kemp et al. 2000). Other authors reported that the mouse Arpp/Ankrd2 protein is expressed also in neurons of the cerebellum and cerebrum, in the islets of Langherans and in the esophageal epithelium (Tsukamoto et al. 2002).

From gene expression studies at the mRNA level Ankrd2 appears to be up-regulated after 7 days of passive stretch of a mouse tibialis anterioris muscle (Kemp et al. 2000), to be markedly induced by denervation (Tsukamoto et al. 2002) and to be mainly expressed upon muscle cell differentiation (Kemp et al. 2000). The mouse Ankrd2 gene expression was also studied *in vitro*. Interestingly, even if it is expressed in proliferating C2C12, the Ankrd2 mRNA level dramatically increases during the period of maximum myoblast fusion and early maturation. At 14 days Ankrd2 mRNA diminished to levels similar to those observed in pre-fusion cultures. This expression pattern is reminiscent of myogenic factors such as Myogenin and MyoD (Kemp et al. 2000). The same behaviour was observed at the protein level performing western blots both on mouse C2C12 and human CHQ5B cell extracts. These observations lead to the conclusion that Ankrd2 is up-regulated during myogenesis (Pallavicini et al. 2001).

On the whole these data suggest that Ankrd2 might be involved both in the differentiation process and in stretch induced hypertrophy. However, very recent reports indicate a possible role in stretch-induced fast/slow muscle fibre type switching rather than in hypertrophy (Mckoy et al. 2005).

1.4.4 Intracellular localization

By immunofluorescence analysis on human CHQ5B myoblasts Ankrd2 localizes both in the nuclei, in form of speckles and in the cytoplasm, with a diffused pattern. As the differentiation into multinucleated myotubes progresses, Ankrd2 seems to change localization and to accumulate mainly in the cytoplasm (Pallavicini et al. 2001).

Also in the TE1 esophageal carcinoma cell line Ankrd2 has been detected in the nucleus and in the cytoplasm (Moriyama et al. 2001). Moreover, from skeletal muscle tissue analysis, the sarcomeric localization has been determined to be in the I-band, near the Z-line, both for the human and mouse counterparts. These observations suggest that the Ankrd2 protein may function by moving between the sarcomere and the nucleus (Tsukamoto et al. 2002).

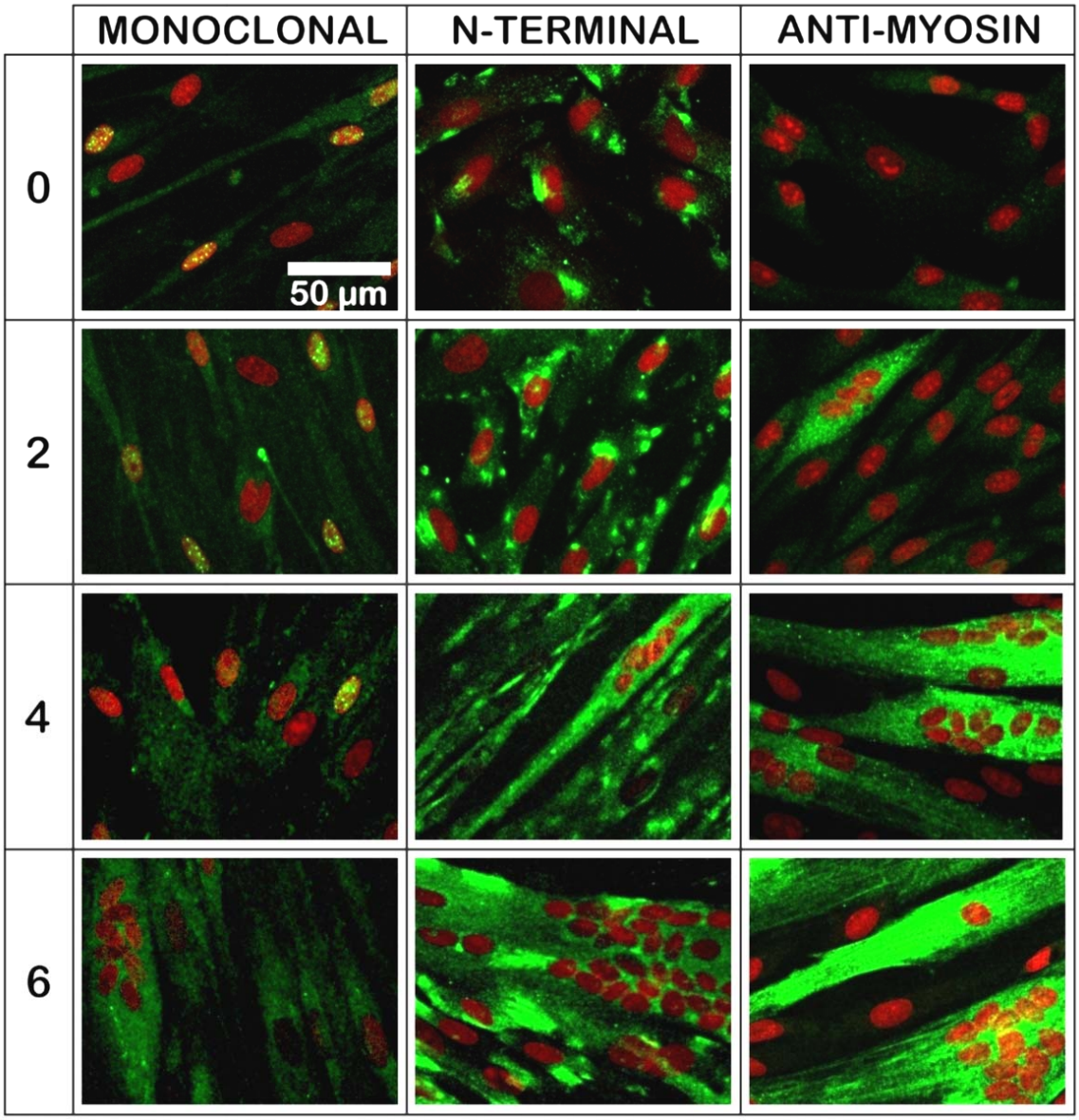


Figure 6. Time course of Ankrd2 expression in primary human muscle cells during differentiation as detected by indirect immunofluorescence. A C-terminal mouse monoclonal antibody (1:40 dilution) and an N-terminal mouse polyclonal antibody (1:40 dilutions) directed against Ankrd2 were used in the first and the second column; an anti myosin monoclonal antibody (MF 20, 1:100 dilution) was used as a marker of differentiation (third column). The days after the addition of differentiating medium are noted at the side of the figure. During differentiation the number of fluorescing cells as well as the intensity of fluorescence augments indicating that the level of Ankrd2 expression is increased. In undifferentiated myoblasts the Ankrd2 protein is visible in the nuclei in the form of speckles only when the monoclonal antibody is used. This pattern has never been detected with the N terminal polyclonal antibody, which instead detects Ankrd2 in the cytoplasm stronger than the monoclonal does. Therefore it is possible that the nuclear Ankrd2 could have the N terminal part hidden so that it could only be detected by the C terminal monoclonal antibody whereas the cytoplasmic Ankrd2 could have the C terminal region (in which the epitope recognized by the monoclonal antibody has been mapped) inaccessible, thus that it can be only recognized by the N terminal polyclonal.
(Adapted from Pallavicini et al. 2001, *Biochem Biophys Res Commun* 285: 378-386).

1.4.5 Protein structure

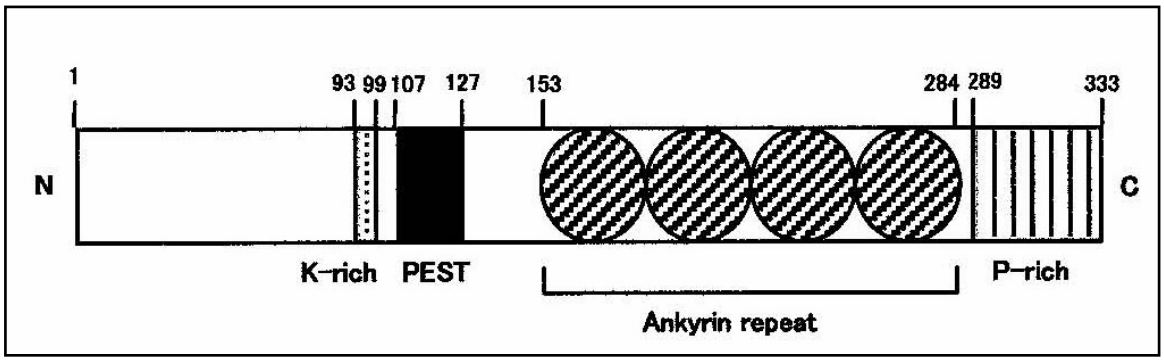


Figure 7. Schematic representation of the Ankrd2 protein. (Adapted from Moriyama et al. 2001, *Biochem Biophys Res Commun* 285: 715-723)

Human and mouse Ankrd2 proteins are extremely similar and their size and structure is conserved (Moriyama et al. 2001). The major structural characteristic of these proteins is the presence in the middle portion of four ankyrin repeat motifs (Kemp et al. 2000, Moryama et al. 2001, Tsukamoto et al. 2002). A possible fifth ankyrin repeat is present only in the human protein (Pallavicini et al. 2001). Ankyrin repeats are variations of 33 amino acids that appear in tandem arrays within a variety of functionally diverse proteins (Bork 1993). Proteins containing ankyrin repeats are also found in many different cellular sites both cytoplasmic and nuclear. Some transcription factors and transcription factors inhibitors have been shown to contain ankyrin repeats. In particular, ankyrin repeat domains are involved in protein-protein interactions (Kemp et al. 2000). In addition Ankrd2 contains other putative domains; there is a lysine-rich sequence similar to a nuclear localization signal (KKRK) and a PEST-like sequence (Kemp et al. 2000, Moriyama et al. 2001, Pallavicini et al. 2001). The Ankrd2 protein has many consensus phosphorylation sites for casein kinase I (CKI), casein kinase II

(CKII), protein kinase C (PKC), extracellular signal regulated kinase (ERK), cAMP-dependent protein kinase, calmodulin-dependent protein kinase II and cGMP-dependent protein kinase (Moriyama et al. 2001, Pallavicini et al. 2001).

1.4.6 Homologies

BLAST searches using the predicted murine Ankrd2 ORF revealed sequence identity between Ankrd2 protein and many other ankyrin repeat containing proteins. However, as already mentioned, the greatest sequence identity was observed between Ankrd2 and three closely related proteins found respectively in mouse, rat and human: muscle ankyrin repeat protein, MARP (Baumeister et al. 1997), cardiac ankyrin repeat protein, CARP (Zou et al. 1997) and C-193 (Chu et al. 1995). At the amino acid level MARP, CARP and C-193 share 90-95% of sequence identity. They also have high sequence identity (62-64%) with Ankrd2 over the ankyrin repeat region and 48-52% within the 97 amino acids flanking the repeats. The remaining 140 amino acids spread over the sequence toward the C-terminus and the N-terminus remain unique to Ankrd2 with no significant homology to MARP, CARP, C-193 or any other sequence in the database (Kemp et al. 2000). Also human Ankrd2, that displays 89% similarity at the amino acid level with mouse Ankrd2, shows 43% identity with MARP, CARP and C-193 (Pallavicini et al. 2001, Moriyama et al. 2001). Given the high similarity between Ankrd2 and C-193/CARP/MARP it is likely that they may be functionally related. This hypothesis is supported by a strict preservation of all the recognizable structural and functional domains between the two proteins. Since C-193/CARP/MARP is found essentially in the heart and Ankrd2 is found primarily in skeletal muscle, it could be that they have parallel functions, with respective specializations for the tissue in which they

are expressed (Pallavicini et al. 2001). Moreover, all these genes could be regulated by the NF-κB pathway since they carry a NF-κB box in their promoters. In addition also the relative protein products share several striking similarities both with NF-κB and with its inhibitor protein I-κB (Pallavicini et al. 2001).

1.4.7 Protein interactions

Many protein interactions (p53, PML, Telethonin and YB-1) have been recently discovered for Ankrd2, thus supporting the hypothesis of a transcription co-factor able to shuttle between the sarcomere and the nucleus of skeletal muscle cells in response to some stimuli, perhaps stress signals (Kojić et al. 2004).

Nowadays it does not seem strange that a cytoskeletal protein can also be found in other sub-cellular compartments such as the nuclei. In fact several sarcomeric associated proteins have been reported to reside in the nucleus, where they could participate in the response of the cell to changes in the structure or function of the contractile machinery. These molecules may serve as molecular messengers that enable muscle cells to mount efficient physiological response to muscle stress, load requirements and/or stretch (Clark et al. 2002).

1.5 TRANSCRIPTION FACTORS INTERACTING WITH ANKRD2

1.5.1 p53

The p53 tumor suppressor gene encodes a nuclear phosphoprotein involved in several biological functions which include regulation of cell cycle checkpoints, maintenance of genomic stability, apoptosis, differentiation and senescence (reviewed in Levine 1997). Since it exerts its function mostly as a transcription factor and activates genes involved in cell cycle arrest, DNA repair and apoptosis, mutations or inactivation of p53 are frequently associated with a large number of human cancers. For these reasons, p53 has been defined as the "guardian of the genome" (Lane 1992). As previously mentioned p53 acts as a transcription factor which regulates the expression of a vast number of genes, that can be categorized into genes involved in apoptosis (Scotin, Apaf-1, Bax, Fas, Puma, Noxa, Killer/Dr5, Igfbp3, Perp), cell cycle arrest (B99, cyclin G, p21/waf1/cip1, 14-3-3-σ, Gadd45), p53 auto-regulation (Mdm2, p73, Pirh2) and angiogenesis/metastasis/invasion (Maspin, Tsp1) (Vousden et al. 2002, Vogelstein et al. 2000).

Specific co-factors and post-translational modifications of p53 will determine whether the p53 response will be directed towards apoptosis, growth arrest or involved in other processes such as angiogenesis, differentiation or senescence (Vousden et al. 2002).

The human p53 gene encodes a protein of 393 amino acids that has been divided functionally and structurally into four domains. The N-terminal region of p53 is rich in acidic residues and contains a transcriptional trans-activation domain, which has been shown to form a direct contact with several basal transcription factors such as TBP, p300 and CBP. This region also contains the site for binding to MDM2 (murine double

minute 2), the principal p53 regulator. A proline-rich region links the N-terminal domain to the central core and has been shown to be important for the growth suppressor functions of p53. The central region of the protein (amino acids 102-292) constitute the DNA-binding domain and the zone in which most frequently missense mutations have been found. Finally the C-terminal region is essential for oligomerization (p53 is a tetramer in solution) and also contains three nuclear localization signals and a nuclear export signal that regulate the sub cellular localization of p53. Many post-translational modifications occur both in the N- and in the C-terminal region, including phosphorylations and acetylations (reviewed in Levine 1997, Fisher 2001).

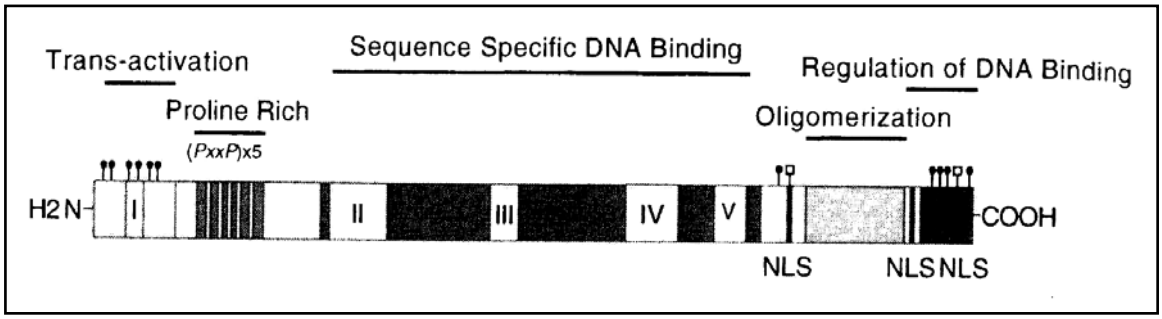


Figure 7. Schematic representation of the human p53 protein. The important functional domains (trans-activation, proline-rich, sequence-specific DNA-binding, oligomerization, regulation of DNA binding), conserved box regions I-V, sites of phosphorylation within the N- and C-terminal regions (filled circles) and sites of acetylation within the C-terminus (open squares), are indicated. (Adapted from Fisher 2001, Tumor suppressor genes in human cancer).

CREB-binding protein (CBP) and p300 are ubiquitous transcriptional co-activators that interact with a plethora of transcription factors, among which there is p53. CBP/p300 binds the transactivation domain and stimulates the p53 transactivation activity.

Moreover CBP/p300 acetylates p53 in two different sites in the C terminal region, thus increasing p53 activity (reviewed in Coutts et al. 2005).

The p53 protein is also both mono- and poly-ubiqutinated by the MDM2 protein (Li et al. 2003) with help from p300/CBP (Grossman et al. 2003). Protein modification by ubiquitin conjugation is a general intracellular mechanism that consists in the attachment of poly-ubiquitin chains to lysine residues in order to target proteins to proteasomes for degradation. p53 can also be modified by SUMO-1 (Gostissa et al. 1999).

The activity of p53 in controlling cell growth needs to be tightly restrained in normal unstressed cells to allow cell proliferation and development. In fact in normal growing cells p53 levels are kept low through its proteasome-mediated degradation, promoted by Mdm2 (reviewed in Momand et al. 2000).

However p53 levels rapidly increase following a variety of stress signals, including DNA damage, hypoxia, oncogene activation, heat shock, metabolic changes, viral infection or cytokine treatment (Fisher 2001). This is due to p53 stress-induced post-translational and conformational modifications that provoke Mdm2 detachment and consequently p53 protein stabilization. Accumulation of high p53 protein levels in the cell principally ends in cell cycle arrest or in apoptosis, in order to protect the organism from the propagation of cells carrying damaged DNA with potentially oncogenic mutations (reviewed in Momand et al. 2000).

During the past decade, Mdm2 has emerged as the principal cellular antagonist of p53 by limiting the p53 tumor suppressor function. Actually Mdm2 itself is the product of a p53 inducible gene. Thus the two molecules are linked to each other through an auto-

regulatory negative feedback loop aimed at maintaining low cellular p53 levels in the absence of stress (Barak et al. 1993, Wu et al. 1993).

MDM2 is a phosphoprotein that interacts with the N-terminal region of p53, directly blocking its trans-activation domain, but that also harbours an E3 ubiquitin ligase activity towards the C-terminal domain of p53, thus targeting this protein for degradation in nuclear and cytoplasmic 26S proteasomes (Honda et al. 1997). The E3 ubiquitin ligase activity resides in the MDM2 C-terminal RING finger domain.

MDM2-mediated p53 ubiquitination takes place in the nucleus in a complex with the p300/CREB-binding protein (CBP) transcriptional coactivator proteins that serve as a scaffold. Whereas MDM2 catalyzes p53 mono-ubiquitination, which in itself is not a substrate for proteasome degradation, p300/MDM2 complexes mediate the final p53 poly-ubiquitination (reviewed in Moll & Petrenko 2003).

The disruption of the complex between p53 and MDM2 is the crucial event during p53 induction and leads to the accumulation of high p53 levels and in fact several stress pathways and signalling molecules act by targeting MDM2 for degradation. In addition proteins like c-abl, DNA-PK, Akt/PKB and CK2 can modify MDM2 thus influencing its ability to bind and regulate the activity of p53, ARF, p300 (Meek and Knippschild 2003).

Moreover several kinases regulate MDM2 activity, hence p53 stability: MDM2 phosphorylation by ATM (Ataxia Teleangiectasia-Mutated kinase) impairs its ability to promote p53 degradation; AKT phosphorylates MDM2 allowing it to enter the nuclear compartment and to bind p53 and ARF binds the RING finger domain of MDM2 inhibiting its E3-ligase activity.

MDM2 has a homologue known as MDMX (MDM4) which binds p53 and MDM2 (Shvarts et al. 1996).

MDMX is not an E3 ubiquitin ligase but cooperates with MDM2 in negatively regulating and in degrading p53, by stabilizing MDM2 (reviewed in Marine and Jochemsen 2005).

Other proteins have been recently discovered to show E3 ligase activity and to target p53 for degradation independently by MDM2: COP1 (Dornan et al. 2004), PIRH2 (Leng et al. 2003) and ARF-BP1/Mule (Chen et al. 2005).

p53 has many other binding partners such as the isoform 3 of the Promyelocytic Laeukemia Protein (PML 3/PML IV) (Fogal et al. 2000) and YB-1 (Okamoto et al. 2000), that also are Ankrd2 binding partners and that will be better described in the following sections.

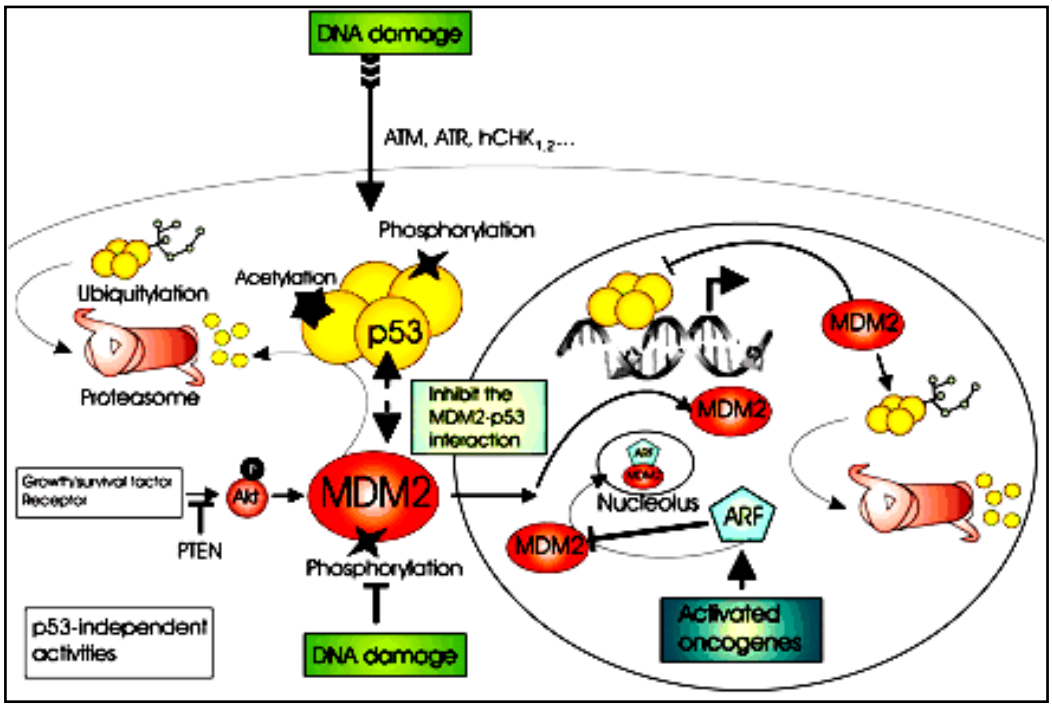


Figure 8. Regulation of p53 by MDM2. p53 and MDM2 form an autoregulatory feedback loop. p53 stimulates the expression of MDM2 and MDM2 inhibits p53 activity stimulating its degradation in the nucleus and in the cytoplasm. (Adapted from Moll and Petrenko 2003, *Mol Cancer Res* 1: 1001-1008).

1.5.1.1 The p53 family

p63 and p73 are two genes related to p53 that share significant sequence homology with p53 especially in the central DNA binding domain (Lohrum et al.1999). They have some functions in common with p53; both p63 and p73 have the ability to transactivate most of the same set of target genes and also to induce cell cycle arrest and apoptosis (Lohrum et al. 1999). The most important roles for p63 and p73 would appear to be in developmental processes (Mills et al. 1999, Yang et al. 2000). Both p63 and p73 exist as multiple isoforms and their expression differentially regulates p53, MDM2 and MDMX functions (Melino et al. 2003). p63 and in particular p73, are important players in the p53 network since both are necessary for efficient p53 apoptosis and transactivation of certain target genes (Flores et al. 2002).

1.5.1.2 p53 in skeletal muscle differentiation

The p53 oncogenic suppressor protein regulates cell cycle checkpoints and apoptosis, but increasing evidence also indicates its involvement in differentiation and development (Almog and Rotter 1997). However the effect of p53 on differentiation is apparently in contrast with the normal development of the p53 null mice (Soddu et al. 1996). The apparent conflict may be explained by the presence of a redundant activity in the animal, in this case the compensatory effect of the p53 related proteins p63 and p73 that are able to substitute for the absence of p53 during embryogenesis and development in the p53-/- mice. However this redundant function cannot be activated in the context of already determined cell lines (Tamir and Bengal 1998, White et al. 2002). A correlation between increased expression of endogenous p53 protein and some physiological differentiative processes such as hematopoiesis (Kastan et al. 1991) and

spermatogenesis (Almon et al. 1993) was observed. The role of p53 in skeletal muscle cell differentiation has been partially elucidated. The p53 protein is required for C2C12 myoblasts and for primary mouse satellite cell differentiation, but is not essential for cell cycle withdrawal and for p21 upregulation (Soddu et al. 1996, Mazzaro et al. 1999). In fact p21, a likely determinant of the growth arrest associated with muscle terminal differentiation, can be upregulated by MyoD independently of p53 (Halevy et al. 1995). More recently it was understood that p53 is necessary for differentiation because it is essential to increase the expression levels of retinoblastoma protein (pRb) (Porrello et al. 2000). pRb is a nuclear phosphoprotein sharing homology with two other Rb-like proteins p107 and p130 (Classon et al. 2002). It is a regulator of the G1 cell cycle checkpoint and binds to members of the E2F transcription family that are necessary for S-phase entry, the key member being E2F-1 (Chellappan et al. 1991, Bagchi et al. 1991). When Rb is hyperphosphorylated by the G1/S cyclin dependent kinases it will release the E2Fs which otherwise are bound to hypophosphorylated Rb thus allowing transcription of genes needed for cell cycle progression and replication of DNA (Nevins et al. 1997 and 2001). Thus the hypophosphorylated form of pRb is needed for cell cycle withdrawal. p53 impaired myoblasts maintain the capacity to hypophosphorylate pRb and to stop in G1, but they are unable to increase pRb expression and hence have strongly reduced MyoD activity and differentiation potential. In conclusion, p53 in the presence of differentiation promoting conditions modifies the Rb gene transcription and contributes to the increase of pRb levels. Therefore the upregulated pRb can cooperate with MyoD to transcribe late markers of differentiation (Porrello et al. 2000). p53 also works together with MyoD to activate the muscle creatine kinase (MCK) promoter in skeletal muscle cells (Tamir and Bengal, 1998). Other components play a role in

muscle differentiation in combination with p53, pRb and MyoD. For example Guo and colleagues have proposed a model in which pRb and Mdm2 regulate muscle cell differentiation by modulating Sp1 activity (Guo et al. 2003). Sp1 is a ubiquitous transcription factor that in skeletal muscle cells acts in combination with MyoD to activate a muscle specific gene program. The interaction of Sp1 with Mdm2 inhibits the activation of muscle specific promoters; pRb competes with Mdm2 for Sp1 binding and may displace Mdm2, restoring Sp1-dependent muscle specific gene expression. Moreover, a trimeric complex forms between pRb-Mdm2-p53. Mdm2 binds both p53 and pRb. This situation stabilizes the p53 protein, blocking its degradation (Yap et al. 1999).

1.5.2 PML and the Nuclear Body

The promyelocytic leukaemia protein (PML) gene was originally cloned as the t(15;17) chromosomal translocation partner of the retinoic acid receptor α (RARα) in acute promyelocytic leukaemia (APL). This translocation leads to the production of a PML-RARα chimeric oncoprotein, which is thought to interfere with both the PML and the RARα pathways (Kakizuka et al. 1991). The expression of the fusion protein is sufficient for transformation in APL cells and the development of leukaemia. APL is characterized by a block in differentiation of promyelocytes and unlike normal cells in APL cells PML is dispersed into hundreds of tiny dots in the nucleus and the cytoplasm (Weis et al. 1994). PML belongs to a family of proteins that is characterized by the presence of the RBCC (Ring B-box coiled-coil) motif. This domain consists of a C_3HC_4 zinc finger motif (RING finger) and one or two additional cysteine-rich zinc binding regions (B boxes) followed by a predicted leucine coiled-coil region. The RBCC

domain mediates protein-protein interactions and is responsible for PML multimerization, localization in the nuclear body and heterodimerization with PML-RARα (reviewed in Zhong et al 2000, Salomoni and Pandolfi 2002). The PML gene has nine exons and is subjected to alternative splicing. Several PML spliced forms have been described. All of the PML isoforms differ in the C terminal region but share the N terminal RBCC domain and the SUMO-1 modifications sites. The PML isoforms can be divided into several groups designated as PML I-VII on the basis of sequence differences at the C-terminus (Jensen et al. 2001).

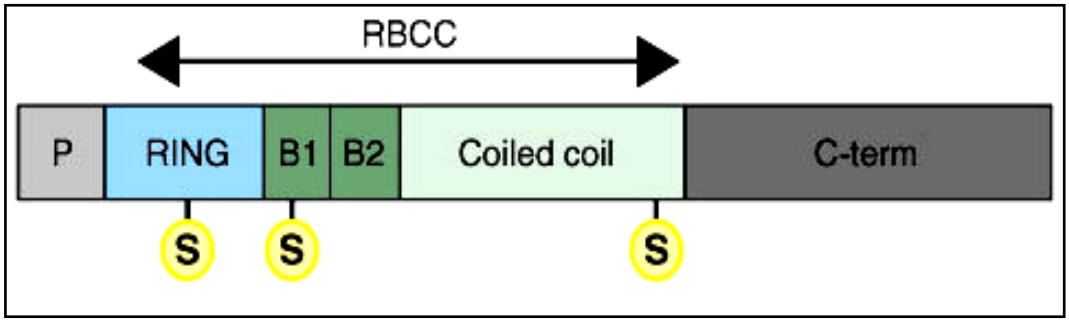


Figure 9. Schematic structure of PML. The principal PML structural and functional domains are indicated. In yellow are shown the SUMOylation sites. (Adapted from Zhong et al. 2000, *Nat Cell Biol* 2: 85-90).

The ubiquitin-like protein SUMO-1 can covalently bind to PML. Unmodified PML is associated with the soluble nucleoplasmic fraction whereas the SUMOylated PML fraction is tightly associated with the nuclear body, suggesting that PML needs to be SUMOylated in order to localize in the nuclear body. PML is specifically required for the proper formation of the nuclear body (Zhong et al. 2000). PML nuclear bodies (NBs) are speckled macromolecular nuclear domains present in almost all mammalian cells. They are also called ND10 (nuclear domain 10), PODs (PML oncogenic domains)

or Kremer bodies. PML NBs are disrupted in APL cells and also as consequence of various viral infections. Cells typically contain 10-30 nuclear bodies with diameters between 0.2 and 1 μm, although their number and size change during the cell cycle (reviewed in Zhong et al. 2000). They are intimately associated with the nuclear matrix, although they contain neither chromatin nor nascent RNA (Boisvert et al. 2000), and they are dynamic structures, of which the PML protein is the major regulator, controlling nuclear body organization and function (Dyck et al. 1994, Lallemand-Breitenbach et al. 2001). A large number of proteins are described to localize to PML nuclear bodies. These include SUMO-1, Sp100, Sp140, CBP, BLM, Daxx, pRB and p53 (reviewed in Zhong et al. 2000). Thus PML NBs could be considered as dynamically composed multiprotein complexes because many of their components are present only transiently in these structures (Gostissa et al. 2003). The real function of PML NBs is still unknown, but they seem to be involved in many different biological processes such as DNA repair, immune response, apoptosis, growth regulation, senescence and transcriptional regulation. Many transcription factors and co-factors are present in the NBs, so it is possible that they are recruited to these structures or to their proximity either to take part in transcription or to be modified (Zhong et al. 2000). A striking observation is that p53 is recruited to the PML NB by direct binding with the carboxy-terminal end of PML IV (PML-3). This interaction and the recruitment to NBs increases p53 transactivating functions, even if the biological outcome of this interaction can be different, depending on cell type and stimulus (Fogal et al. 2000).

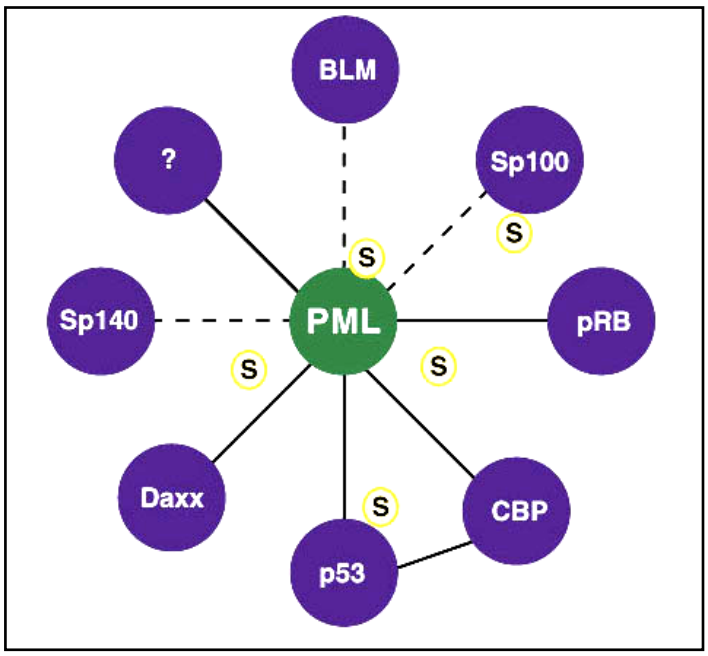


Figure 10. Schematic representation of the PML nuclear body. PML nuclear bodies are distinct nuclear structures formed around the SUMO modified PML protein. They contain several different components some of which are indicated in the picture. SUMO modification is indicated by a yellow circled S. (Adapted from Zhong et al. 2000, *Nat Cell Biol* 2: 85-90).

It is noteworthy that some authors have suggested another function for PML nuclear bodies based on the observations that these nuclear structure are not present in all cell types, that they are not essential for cell survival and that they contains a large number of proteins with different biological functions. They think that PML nuclear bodies could function as nuclear depots (Negorev and Maul 2001). Recently PML III has also been detected in the centrosome and its deficiency is thought to cause centrosome amplification. Centrosome disregulation causes genome instability and is often associated with aneuploidy in most human cancers. Many other cell cycle proteins, including p53 have been detected in the centrosome. All these observations support the hypothesis of PML as a tumor suppressor gene that has a role in cell growth control (Xu et al. 2005).

1.5.3 YB-1

The human Y-box binding protein (YB-1) belongs to the Y-box protein family which is widely distributed from bacteria to mammals. Y-box proteins are evolutionary conserved and function as transcription factors, regulators of RNA metabolism and protein synthesis. They also play developmental roles during embryogenesis (Jurchött et al. 2003). YB-1 was first described as a DNA-binding protein that interacts with a specific DNA sequence, called the Y-box, in MHC class II promoters (Didier et al. 1988). High levels of YB-1 mRNA were detected in the heart, skeletal muscle, liver, lung, adrenal gland and brain (Spitkovsky et al. 1991). YB-1 is mainly localized in the cytoplasm, particularly in the perinuclear region, but accumulates in the nucleus when genotoxic stress occurs (Koike et al. 1997). In addition YB-1 changes its intracellular localization in a cell cycle dependent fashion. In fact YB-1 is visible in the cytoplasm in G1 phase and accumulates in the nucleus at the G1/S phase transition. This phenomenon is associated with the transcriptional activation of cyclin A and B genes. These observations suggest a role for YB-1 in cell proliferation control (Jurchött et al. 2003). YB-1 consists of three domains: a glycine-rich N-terminal domain, a highly conserved nucleic acid-binding domain and a C-tail domain containing alternating regions of basic or acidic amino acids. The C-terminal domain is thought to function either as a nucleic acid-binding domain or a protein-protein interaction domain. Moreover YB-1 contains the Cold Shock Domain (CSD), which is highly conserved in prokaryotes and eukaryotes and that binds DNA and also RNA (Izumi et al. 2001). In fact YB-1 has been identified as the major component of messenger ribonucleoprotein particles (mRNPs) in mammalian cells (Raffetseder et al. 2003). YB-1 interacts both with single and double stranded DNA by binding to an inverted CCAAT sequence (Y-

box) (Didier et al. 1988), that is present in the promoters of many genes. Since Y-box elements are present in the promoters of several genes whose activity is associated with cell division, YB-1 may have a role in promoting cell proliferation (Okamoto et al. 2000). It has also been reported that YB-1 possesses an intrinsic 3'→5' DNA exonuclease activity (Izumi et al. 2001). A role in DNA repair has also been postulated for YB-1. In fact it is well known that YB-1 is a stress inducible protein that preferentially binds to apurinic DNA and to cisplatin modified nucleotides (Ise et al. 1999). Therefore, YB-1 could be considered a multifunctional ubiquitous transcription factor that is able to regulate gene expression both at the transcriptional and translational level. Another intriguing characteristic of this protein is its direct interaction with p53. It is possible that YB-1 interacts with p53 in the region with DNA damage to function in the DNA repair machinery (Okamoto et al. 2000). In addition the same authors reported that the binding of YB-1 to p53 increases p53 DNA binding activity. In contrast Lasham and colleagues assert that YB-1 is a negative regulator of p53 because it represses the p53 promoter and down-regulates endogenous p53 expression (Lasham et al. 2003).

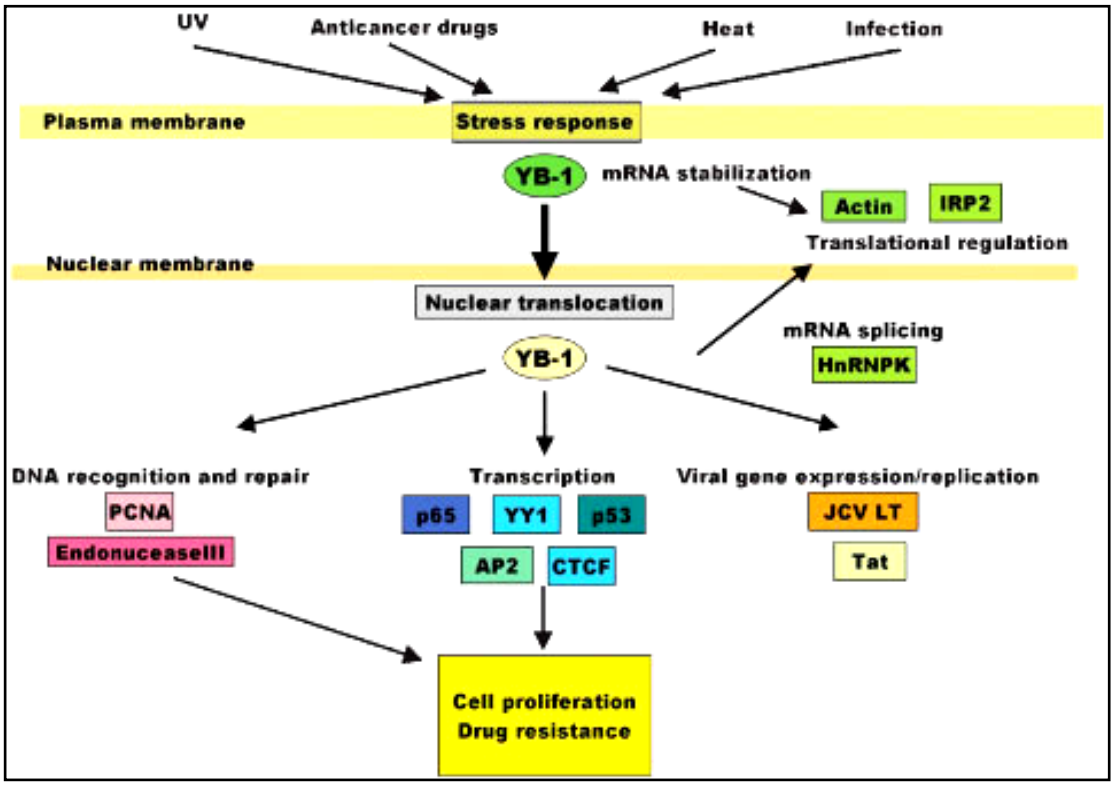


Figure 11. Cellular functions of YB-1. YB-1 is mainly localized in the cytoplasm. It interacts with cytoplasmic proteins and RNAs. Chemotherapy, UV irradiation and other stimuli can initiate nuclear translocation of YB-1. YB-1 complexed with other proteins has a variety of cellular functions. (Adapted from Kohno et al. 2003, *BioEssays* 25:691–698).

AIM OF THE STUDY

The subject of this study is the skeletal muscle ankyrin repeat protein Ankrd2 that was previously identified and partially characterized before my arrival in the Muscle Molecular Biology laboratory (ICGEB-Trieste) under Dr. Georgine Faulkner direction.

Hence this work represents the natural continuation of the project on Ankrd2 and has been mainly focused on the detection of possible Ankrd2 binding partners, in order to elucidate its biological function in muscle cells. To do this I have made use of different techniques to study protein-protein interactions, such as co-immunoprecipitation, in vitro binding, GST pull down and GST overlay. Moreover, in collaboration with the Dr. Giorgio Valle group at CRIBI (University of Padua) we have set up an alpha-screen analysis of the Ankrd2 protein interactions that I have discovered. At last I added also some functional study such as luciferase assays, to test the effect of Ankrd2 on the transactivation activity of p53 that I have found to be one of the Ankrd2 interacting partners and that is also involved in skeletal muscle differentiation.

Chapter 2

MATERIALS and METHODS

2.1 Bacterial strains and growth media

The following *E.Coli* strains were used for this work:

DH5α recA1$^-$ strain (F^-, recA1$^-$, endA1, gyrA96, thi-1, hsdR17 (rk^-, mk+), supE44, elA1) for the propagation of eukaryotic expression vectors; the K-12 derived *E.Coli* strain **M15[pREP4]** (Nal^S, Str^S, rif^S, lac^-, ara^-, gal^-, mtl^-, F^-, $recA^+$, uvr^+) for the expression and purification of His tag recombinant proteins; the **BL21 (DE3) pLys S** strain (F^-, ompT, $hsdS_B$ (r_B^- m_B^-), dcm, gal, (DE3), pLysS, Cm^r) (Promega) for the expression and purification of Glutathione-S-transferase (GST) recombinant proteins. Both the M15 and the BL21 strains allow high levels of expression of recombinant proteins after Isopropyl-β-D-thiogalactoside (IPTG) induction.

The bacterial cultures were grown in one of the following media:

Luria Broth (LB): 10 g baktotryptone, 5 g yeast extract, 5 g NaCl (for 1 L).

Terrific Broth (TB): 12 g baktotryptone, 24 g yeast extract, 4 ml glycerol (for 1 L). Before use KH_2PO_4 at 17mM final concentration and K_2HPO_4 at 72 mM final concentration should be added.

2.2 Plasmids

2.2.1 Ankrd2

Ankrd2-pCMV-3B: the human full-length Ankrd2 cDNA was cloned into a eukarytotic expression vector, pCMV-tag-3B (Stratagene), for the in vivo expression of Ankrd2 recombinant protein tagged at the N-terminal with c-myc (EQKLISEEDL). The Ankrd2 cDNA cut at restriction sites *BglII- EcoR1* was inserted into the *BamH1-EcoR1* site of the vector. Epitope-tagged proteins are very useful especially for protein-protein interaction studies, such as co-immunoprecipitations, since in an epitope-tagged protein, the added sequence is a short peptide (3-14 amino acids) usually with no function of its own but with the important property to be recognized and bound by a single, tag-specific antibody.

Ankrd2-pcDNA3.1: the human full length Ankrd2 cDNA was cut by *EcoR1* and cloned *EcoR1-EcoR1* into pcDNA3 (Invitrogen). The correct orientation was checked using *BamH1* digestion.

Ankrd2-pGEX-6P: the human full-length Ankrd2 cDNA (FL, 5-333aa) was cloned into the pGEX-6P-3 vector (GE Healthcare) for the expression and purification of a GST-Ankrd2 fusion protein from bacterial cells to be used in protein-protein interaction studies. The cDNA was first amplified by PCR and then cloned using the *EcoRI* restriction enzyme.

N-Ankrd2-pGEX-6P, CA-Ankrd2-pGEX-6P, C-Ankrd2-pGEX-6P-: also the cDNAs corresponding to different fragments of the Ankrd2 gene (N terminal 13-360; CA 361-999; C 838-999) were inserted into the pGEX-6P-3 vector using the *EcoR-SalI* restriction sites. These cDNAs correspond to the following protein regions: N-terminal protein (N, 5-120aa) and two C-terminal proteins (CA, 121-333aa; C, 179-333aa).

Ankrd2-pQE30: the human full-length Ankrd2 cDNA has been cloned into the pQE30 (QIAGEN) vector using the *KpnI-SalI* restricton sites. This vector allows the production of a protein with a six histidine (6xHis) tag at its N-terminal region that was used for polyclonal antibody production and in the GST-overlay assays.

2.2.2 Telethonin

Telethonin-pcDNA3-HA: full length human Telethonin cDNA was cloned into a pcDNA3-HA vector using the *BamH1-EcoR1* restriction sites. The pcDNA3-HA vector was a modified pcDNA3 vector (Invitrogen) with an HA tag (YPYDVPDYA) cloned into the *HindIII-BamHI* sites. The N-terminal HA tagged Telethonin protein was used in protein-protein interaction studies, such as co-immunoprecipitations.

Δ38-Telethonin-pcDNA3-HA: the partial Telethonin cDNA sequence lacking the first 38 amino acids and thus called Δ38-Telethonin (39-167aa) was cloned into the pcDNA3-HA vector using the *BamH1-EcoR1* restriction sites. The resulting HA tagged Δ38-Telethonin protein was used in protein-protein interaction experiments.

Telethonin-pcDNA3.1: the full-length Telethonin cDNA was inserted into the eukaryotic expression vector pcDNA3.1 (Invitrogen) using the *BamHI-NotI* restriction sites. The recombinant protein was expressed in eukaryotic cells or produced by *in vitro* transcription/translation for protein-protein interaction studies.

Telethonin-pQE30: the full-length Telethonin cDNA was inserted into the pQE30 vector (QIAGEN) using the *BamHI-HindIII* restriction sites. The His-tagged recombinant protein was used for antibody production and for GST-overlay assays.

2.2.3 PML

PML-pcDNA3-HA containing full length PML isoform IV (PML 3) was kindly supplied by Dr. G. Meroni, TIGEM, Naples. This construct expressed the HA tagged recombinant protein that was used in co-immunoprecipitation experiments for protein-protein interaction studies.

PML-pcDNA3.1 and PML-pGEX: these vectors were kindly provided by Dr. L. Banks, ICGEB, Trieste.

2.2.4 YB-1

All of the YB-1 constructs used in this thesis were kindly provided by Dr. K. Kohono, University of Occupational and Environmental Health, Kitakyushu, Japan.

FLAG-YB-1-pcDNA3: the full-length YB-1 cDNA with a FLAG tag at the N terminal was cloned into a pcDNA3 vector for expression in eukaryotic cells and protein-protein interaction studies.

YB-1-pGEX-4T and YB-1 deletion mutants-pGEX-4T: the full length YB-1 cDNA and five different YB-1 cDNAs deletion mutants were cloned into the pGEX plasmid to produce the following GST fusion proteins: GST-YB-1 (1-324aa), GST-Δ1-YB-1 (51-324aa), GST- Δ2-YB-1 (1-205aa), GST- Δ3-YB-1 (129-324aa), GST- Δ4-YB-1 (1-129aa) and GST- Δ5-YB-1 (51-129aa). All these proteins were used in GST pull down experiments and also used by our collaborators (the laboratory of Prof. G. Valle, University of Padua) for alpha-screen protein–protein interaction experiments.

2.2.5 p53

p53-pEGFP: the wild type (wt) full length p53 cDNA cloned into the pEGFP vector was kindly provided by Dr. C.Kühne (ICGEB, Trieste) and was used in co-immunoprecipitation experiments.

p53-pcDNA: the wt-p53-pGEX plasmid was digested with *BamHI-EcoRI* and the resulting insert ligated into pcDNA3. This construct was then used to produce *in vitro* transcribed and translated protein for *in vitro* binding experiments.

p53-pGEX2T: this construct was kindly supplied by Dr. L.Banks (ICGEB, Trieste). The wt-p53 human sequence is cloned in the *BamHI-EcoRI* restriction sites. The GST-p53 recombinant protein was used in GST pull down experiments.

p53-pQE30: this construct was prepared by digesting the wt-p53-pTrcA vector with *BamHI-HindIII* and then ligating the resulting p53 cDNA into the same sites in pQE30. The His-tagged p53 was used in GST-pull down experiments.

2.2.6 Other plasmids

p21-pGL2, Mdm2-pGL2: these constructs contain the promoters of the $p21^{WAF1/CIP1}$, and of the Mdm2 p53 inducible genes cloned upstream of the firefly luciferase gene and were used as reporter genes in transactivation experiments.

2.3 Commercial antibodies

2.3.1 Primary Antibodies

Anti-p53 (DO-1) mouse monoclonal antibody (isotype IgG_{2a}) (sc-126, Santa Cruz Biotechnology, Inc.): p53 (DO-1) reacts with an amino terminal epitope mapping between amino acid residues 11-25 of wt and mutant p53 of human origin; **anti PML (N-19) goat polyclonal antibody** (sc-9862, Santa Cruz Biotechnology, Inc.): this antibody is raised against a peptide mapping near the amino terminus of PML of human origin; **anti NF-kB p65 (F-6) mouse monoclonal IgG_1 antibody** (sc-8008, Santa Cruz Biotechnology, Inc.): this antibody is raised against a recombinant protein corresponding to amino acids 1-286 mapping to the amino terminus of NF-kB p65 of human origin; **anti SUMO-1 (FL-101) rabbit polyclonal antibody** (sc-9060, Santa Cruz Biotechnology, Inc.): SUMO-1 (FL-101) is raised against a recombinant protein corresponding to amino acids 1-101 representing full-length SUMO-1 of human origin; **anti c-myc mouse monoclonal clone 9E10, ascites fluid** (M5546, SIGMA): monoclonal anti c-myc (mouse IgG1 isotype) recognizes an epitope located in the amino acid sequence EQKLISEEDL (residues 410-419) of the human oncogene product *c-myc*; **anti FLAG M2 mouse monoclonal antibody** (SIGMA): the M2 monoclonal antibody recognizes the FLAG sequence (DYKDDDDK) at the N-terminus, Met-N-terminus or C-terminus of FLAG fusion proteins; **anti HA (F-7) mouse monoclonal antibody** (sc-7392, Santa Cruz Biotechnology, Inc.): the anti HA-probe is a mouse monoclonal antibody raised against a peptide mapping to an internal region of the influenza hemagglutinin (HA) protein (YPYDVPDYA); **anti GFP antiserum** (Invitrogen): the GFP antiserum is a rabbit polyclonal antibody that allows detection of

the wt and mutant *Aequorea victoria* green fluorescent protein (GFP) as well as the GFP fusion protein; **anti GST polyclonal antibody** (GE Healthcare): goat anti GST polyclonal antibody; **anti His-RGS mouse monoclonal antibody** (QIAGEN) for highly specific detection of 6xHis tagged proteins. .

2.3.2 Secondary antibodies

anti-mouse IgG AP-conjugated (Sigma A3562): anti-mouse immunoglobulins conjugated with alkaline phosphatase (AP) developed in goat; **anti-rabbit IgG AP-conjugated** (SIGMA A3687): anti-rabbit immunoglobulins conjugated with alkaline phosphatase (AP) developed in goat; **anti-goat IgG AP-conjugated** (SIGMA A4187): anti-goat immunoglobulins conjugated with alkaline phosphatase (AP) developed in rabbit; **anti-mouse FITC** (Sigma F4018): anti-mouse fluorescein isothiocyanate (FITC) immunoglobulins; **anti-goat Alexa 546** (Molecular Probes, A21085): fluorescent donkey anti-goat IgG Alexa 546 that reacts with IgG heavy chains and all classes of immunoglobulin light chains from goat. Alexa 546 dye to which these antibodies are conjugated absorbs light at 556 nm and emits light at 573 nm; **anti-mouse HRP:** anti mouse immunoglobulins conjugated with the horseradish peroxidase used in chemiluminescence detection (ECL GE Healthcare Biosciences).

2.4 Antibody production

2.4.1 Polyclonal antibodies production

In order to produce polyclonal antibodies female mice belonging to the Balb C strain were immunized by intra-peritoneal (ip) injection of the Ankrd2 protein prepared as follows: a His-tag recombinant Ankrd2 protein was produced and its purity tested by SDS-PAGE. Then 40 μg of protein were diluted in PBS to give a final volume of 100 μl and mixed with Freund's incomplete adjuvant (SIGMA).

Blood was taken from the carotid artery before immunization and sera prepared (pre-immune sera). Every two weeks mice were injected ip with protein, after three injections a blood sample was taken, thereafter boost were given every 2 weeks and samples taken on alternate weeks.

Sera was prepared by allowing the blood to coagulate (37°C for 30 minutes) and then centrifuging at 14000 rpm at 4°C for 30 minutes using an Eppendorf centrifuge. The supernatants were recovered in a clean tube and centrifuged again for 10 minutes at 4°C to obtain completely clear sera. To avoid the growth of bacteria and fungi sodium azide was added to the sera to a final concentration of 0.02%. Samples were aliquoted and conserved at –20°C. The efficiency of the immune response was controlled by western blot analysis on 10-60 μg of human skeletal muscle protein extract (Clontech).

2.4.2 Monoclonal antibodies production

The protocol used to produce monoclonal antibodies was similar to that outlined in Harlow and Lane (ref CSH 1988). Balb C female mice were immunized as outlined above and when a good immune response was obtained sacrificed to obtain their

spleens. These spleen cells were dissociated and fused in the presence of polyethylene glycol with the NP1 myeloma cells. The NP1 myeloma cells are a natural mutant derived from NS1 cells and do not secrete light chain. Both the NS1 and NP1 cells were originally obtained from Prof. C Milstein as a gift to Prof. G Valle (Padua). After fusion the hybridoma cells were selected using RPMI medium supplemented with HAT (Sigma) and the clones tested by ELISA against Ankrd2 protein. Positive clones were subcloned, tested by ELISA and then grown to obtain a monoclonal cell line. The monoclonal antibody used in this thesis was produced in this way.

Ascites fluid was produced as outlined by Harlow and Lane.

2.4.3 List of antibodies produced

The following antibodies have been produced with the procedures described in the previous paragraphs:

anti-Ankrd2 mouse polyclonal antibody: this antibody was raised against the full length human Ankrd2 cDNA sequence (5-333aa) inserted into the His-tag expression vector pQE30 (QIAGEN).

anti-Ankrd2 mouse monoclonal antibody clone 2F10 (ascites fluid): the monoclonal antibody was produced using the C-terminal region of Ankrd2 (aa 297-333). The position of the epitope was pin-pointed to the last 36 amino acids of the C-terminal of Ankrd2. This was done using GST fusion proteins for different regions of the Ankrd2 protein.

anti-Telethonin mouse polyclonal serum: polyclonal antibodies against His-tagged telethonin were raised in mice.

All the antibodies produced in our laboratory were tested by immunoblot analysis of human skeletal muscle tissue extract (Clontech).

2.5 GST recombinant proteins production and purification

GST-recombinant proteins were produced and purified using the Glutathione S-transferase Gene Fusion System (GE Healthcare) for the expression, purification and detection of fusion proteins produced in *E.Coli*. The pGEX plasmids are designed for inducible, high-level intracellular expression of genes or gene fragments as GST fusion proteins. Full length Ankrd2 (FL, 5-333 aa) and three Ankrd2 deletion mutants (N, 5-120 aa, CA, 121-333 aa and C, 179-333 aa), PML IV and YB-1 cDNAs were cloned into the pGEX plasmids. For protein production and purification, these vectors were transformed into the *E.Coli* strain BL21(DE3)-pLys S (Promega) that is engineered to contain the pLys plasmid encoding the T7 lysozyme. Then a fresh colony was grown in LB in the presence of ampicillin (100 μg/ml) (SIGMA) at 37°C with shaking until the bacterial culture reached the OD_{600} of 0.6. At this point the GST-recombinant protein expression was induced by the addition of 0.5-1 mM IPTG and the bacteria grown for a further 3-4 hours with mixing at room temperature or at 37°C to produce the native or the denatured protein respectively. Then the cells were collected by centrifugation at 6000 rpm for 15 minutes, resuspended in lysis buffer and kept on ice for 20 minutes. The composition of the lysis buffer varied depending on the protein to be purified. At this point the suspension was sonicated until it appears clear and then centrifuged at maximum speed to get rid of the cell debris. The supernatant was recovered and incubated for 1 hour with the Glutathione Sepharose-4B beads (GE Healthcare), at 4°C with mixing. During this step the recombinant proteins should bind to the glutathione

conjugated beads. Afterwards, the solution was briefly centrifuged and the recovered resin was washed three times for five minutes with mixing at 4°C in lysis buffer. After a final wash in PBS (Phosphate buffered saline), the GST-recombinant proteins were eluted from the beads by the addition of 200 µl of 20 mM Glutathione (SIGMA) for 10 minutes at room temperature, for recovering native protein. As an alternative, proteins can be kept bound to the resin for use in GST pull down experiments. To do this, after the washing steps resin is washed once in cold PBS and resuspended in PBS plus complete protease inhibitors (Roche). The levels and purity of proteins were determined by SDS-PAGE and subsequent Coomassie Brilliant Blue R (Coomassie Blue 0.4%, Acetic Acid 10%, Methanol 40%) staining.

2.6 His-tagged proteins production and purification

Six-Histidine tagged proteins were produced using the QIA*express* system (QIAGEN). His-tagged proteins were used in overlay experiments and for antibody production, since the 6xHis affinity tag is very poorly immunogenic. Ankrd2, p53 and Telethonin cDNAs were cloned into pQE30 plasmids in order to have the 6xHis tag at the N terminal of the protein. pQE vectors allow a tight regulated expression of 6xHis tagged proteins. Expression from the promoter/operator region is extremely efficient and can only be prevented by the presence of high levels of *lac* repressor. For this reason to obtain 6xHis tagged proteins is essential to transform a particular *E.Coli* strain, M15, which contains multiple copies of the plasmid pREP4 carrying the *lacI* gene, encoding the *lac* repressor. Expression from pQE vectors is rapidly induced by the addition of IPTG, which inactivates the repressor and clears the promoter. Thus, fresh colonies were grown in LB with both ampicillin (100 µg/ml) (SIGMA) and kanamycin (25

μg/ml) (SIGAMA) selection up to an OD_{600} of 0.6. At this point 1-2 mM IPTG was added and the culture was grown at 37°C or at room temperature (native protein protocol) for 4-5 hours. Then cells were harvested by centrifugation at 6000 rpm for 15 minutes, the pellet resuspended in the appropriate lysis buffer and kept on ice for 30 minutes. The temperature at which the purification is carried out and the composition of lysis, washing and elution buffers differ depending if you require the protein in a denatured or native form. The suspension was sonicated until it became transparent and centrifuged 10 minutes at 14000 rpm to eliminate the cellular debris. The supernatant was transferred to a fresh tube, mixed with 1 ml of Ni-NTA (QIAGEN) resin and incubated for 1 hour at room temperature (denatured) or 4°C (native). The Ni-NTA (nitrilo-tri-acetic acid) resin is a metal chelant adsorbent attached to Sepharose CL-6B which binds with high affinity the 6xHis tail. Any host proteins that bind non-specifically to the NTA resin can be easily washed away under relatively stringent conditions, without affecting the binding of the 6xHis proteins.

Then the resin was collected by centrifugation and washed three times for 10 minutes. The desired recombinant protein was eluted by the addition of 1 ml of the appropriate elution buffer. Protein levels and purity were checked by SDS-PAGE followed by Coomassie Blue staining.

2.7 Cell culture

2.7.1 Human Primary Skeletal Muscle Cells (CHQ5B)

CHQ5B primary human myoblasts (22 divisions) were kindly provided by Dr. V. Mouly (URA, CNRS, Paris, France). CHQ5B human myoblasts were isolated from the quadriceps of a newborn (5 days post-natal) without any sign of neuromuscular

disorders and the protocols used for this work were in full agreement with the current legislation on ethical rules. This strain of cells can achieve 55-60 divisions before reaching proliferative senescence. Growth conditions: medium composed by 64 % v/v DMEM (Gibco-Invitrogen) and 16 % v/v Medium 199 (Gibco-Invitrogen) supplemented with 20 % Foetal Bovine Serum (Gibco-Invitrogen) and gentamycin 50 μg/ml. Cultures must not be allowed to become confluent, as this will deplete the myoblastic population in the culture. To obtain differentiated cells, the growth medium was replaced with DMEM supplemented with 0.4 % v/v Ultroser G and 50 μg/ml gentamycin. Myotubes can be detected two days after the addition of this medium and continue to develop for at least another four days.

2.7.2 C2C12

C2C12 are mouse myoblasts that on the addition of differentiating medium or at confluency differentiate rapidly, forming contractile myotubes. Growth conditions: DMEM supplemented with 10 % v/v Foetal Calf Serum and 50 μg/ml gentamycin. Differentiation conditions: DMEM supplemented with 0.4 % Ultroser G and 50 μg/ml gentamycin.

2.7.3 COS-7

COS-7 cells are derived from African Green Monkey kidney cells transformed by SV40 and containing T antigen. Growth conditions: DMEM supplemented with 10 % Foetal Calf Serum and 50 μg/ml gentamycin.

2.7.4 Saos-2

The Saos-2 human osteogenic sarcoma cell line was kindly provided by Dr. G. Del Sal (University of Trieste, Italy). This line was established from the primary osteogenic sarcoma of a Caucasian woman in 1973 (ATCC HTB 85). Cells are adherent, epithelial-like and grow in monolayers. They are null for p53 and pRB (p53 -/-, pRB -/-). Growth conditions: DMEM supplemented with 20 % Foetal Calf Serum and 50 μg/ml gentamycin.

2.8 *In vitro* transcription and translation

The TNT Coupled Reticulocyte Lysate System (Promega) was used to produce *in vitro* transcribed/translated (IVTT) proteins such as Ankrd2, p53, PML, Telethonin and YB-1. This system allows the production of both radioactive and non-radioactive proteins.

Each transcription/translation reaction is performed using 25 μl of TNT Rabbit Reticulocyte Lysate, 2 μl of TNT Reaction buffer, 1 μl of T7 RNA polymerase, 2-5 μl of the appropriate radiolabeled aminoacid (in this case Pro-mix [^{35}S] cell labelling mix, GE Healthcare Biosciences was used), 1 μl of the amino acid mixture minus methionine/cysteine, 1 μl of RNasin ribonuclease inhibitor, DNA template (0.5-1 μg) and nuclease free water to a final volume of 50 μl. The reaction is then performed at 30°C for 90 minutes.

Samples were tested for the presence of the desired protein by SDS-PAGE and then exposing dried gel using SR Packard phosphor screen.

2.9 Total cell extracts and nuclear extracts preparation

Total extracts from CHQ5B and C2C12 cells were prepared as follows: cells were harvested in PBS with protease inhibitors and then resuspended in 150 µl of E1A lysis buffer (50 mM Hepes pH 7, 250 mM NaCl, 0.1 % NP40 and protease inhibitors Complete, EDTA Free, Roche).

CHQ5B nuclear and cytoplasmic extracts were prepared using the Active Motif Nuclear Extract Kit, following the instructions provided by the supplier.

2.10 Immunofluorescence

Undifferentiated and differentiated primary human muscle cells (CHQ5B) were grown on collagen-coated (Rat Tail Collagen Type I, Becton Dickinson Labware) coverslips and then fixed with 3% paraformaldehyde for 20 minutes at room temperature or with cold methanol (-20°C) for 4 minutes. After that coverslips were treated with 0.1 M glycine for 5 minutes and then 0.05 % Tween-20 and 1 % BSA (Bovine Serum Albumin, SIGMA) for 1 hour to permeabilize cells and to block any non-specific signal. All the washing steps and the antibody dilutions for these experiments were performed in PBS with 0.1 % BSA and 0.05 % Tween-20. The samples were incubated with the following primary antibodies for 90 minutes: anti Ankrd2 monoclonal antibody (2F10) ascites fluid (1:50 dilution) and anti PML (N-19) goat polyclonal antibody (sc-9862 Santa Cruz). The secondary antibodies were anti mouse or anti goat fluorescein isothiocyanate (FITC) conjugated (Sigma, F 4018) and anti goat Alexa 546 conjugated (Molecular Probes, A21085). All commercial immunochemicals were diluted as recommended by the suppliers. The nuclei were stained with propidium iodide (3.5µg/ml, sigma P4170). In the end, after several washings, the coverslips were

mounted on glass slides using Vectashield mounting medium (Vector Labs Inc.) and examined by confocal microscopy. An LSM 510 laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany) was used for confocal microscopy both at 40x and 100x magnification.

2.11 Transfections and co-immunoprecipitations

Sub-confluent COS-7 (7.5 x 10^5 cells) were seeded onto 100 mm plates (Falcon) and transfected with the following expression vectors (2 μg) Ankrd2-c-myc-pCMV, p53-pEGFP, PML-HA-pcDNA3, Telethonin-HA-pcDNA3and YB-1-FLAG-pcDNA3 using the FuGENE 6 reagent (Roche Molecular Bio-chemicals) as described in the manufacturer's protocol. Then 48 hours after transfection cells were harvested and lysed in 300 μl of E1A buffer (50 mM Hepes pH 7, 250 mM NaCl, 0.1 % v/v NP40 and protease inhibitors (Complete-EDTA free, Roche Molecular Biochemicals). Samples were briefly sonicated (10 seconds) at maximum power and then centrifuged for 10 minutes at 14000 rpm at 4°C in an Eppendorf centrifuge to get rid of cell debris. Supernatants were recovered and protein concentration was determined using the Biorad Protein Assay (Biorad). Extracts were subjected to SDS-PAGE and transferred onto a PDVF membrane to be tested in western blot for the presence of the desired protein products, using appropriate antibodies.

Tagged proteins were immunoprecipitated from the extracts by the addition of specific antibodies for 2 hours at 4°C. Then protein A-Sepharose (GE Healthcare) was added and samples kept in agitation for 1 hour at 4°C. After five washings in E1A buffer the beads were centrifuged and following the addition of protein sample buffer, boiled 5 min and subjected to SDS-PAGE and western blot. The antibodies used for

immunoprecipitation were anti c-myc clone 9E10 mouse monoclonal ascites fluid (M5546, SIGMA), anti HA (F-7) mouse monoclonal antibody, sc-7392 (Santa Cruz) and anti FLAG (M2), F3165 mouse monoclonal antibody (SIGMA).

2.12 Western blotting

Protein extracts or complexes from immunoprecipitations were separated by SDS-PAGE and then blotted onto Immobilon-P membrane (Millipore) to perform western blot.

The transfer of protein samples from the gel onto the membrane was done in the appropriate apparatus using a specific buffer (20 % methanol, 10 % Tris-glycine) running at 20 V over night.

The membrane was then blocked for at least 1 hour in a solution of 10 % low fat milk in PBS containing 0.05 % Tween-20. The primary antibodies were diluted in 5 % low fat milk in PBS with 0.05 % Tween-20 and incubated for at least 90 minutes with agitation. Primary antibodies used for western blot in this work are the following: anti Ankrd2 mouse polyclonal and monoclonal (1:200-1:400 dilution), anti GFP rabbit polyclonal (R970-01 Invitrogen; 1:1000 dilution), anti p53 DO-1 mouse monoclonal (sc-126, Santa Cruz; 1:500 dilution), anti YB-1 (A-16) goat polyclonal (sc-18057, Santa Cruz; 1:100), anti PML (N-19) goat polyclonal (sc-9862, Santa Cruz; 1:100), anti c-myc 9E10 mouse monoclonal (M5546 Sigma, 1:200), anti FLAG M2 (F3165) mouse monoclonal (Sigma, 1:500) and anti HA (F-7) mouse monoclonal (Santa Cruz, 1:200), anti His-RGS mouse monoclonal (QIAGEN, 1:2000).

The secondary antibodies were diluted in 5% low fat milk in PBS with 0.05% Tween 20 and incubated 45 minutes with agitation. Both Alkaline Phosphatase (AP)- and

Horseradish Peroxidase (HRP)- conjugated secondary antibodies were used, according to the detection method employed, NBT/BCIP colorimetric assay or ECL respectively.

2.13 GST-pull down

Equal amounts of GST fusion proteins still bound to glutathione-Sepharose 4B resin (GST, GST-FLAnkrd2, GST-N terminal Ankrd2, GST-CA Ankrd2, GST-C terminal Ankrd2 and GST-YB-1), produced as described above, were incubated with mixing for two hours or overnight at 4°C with protein extract (300μg) from COS-7 cells transfected with YB-1-FLAG-pcDNA3 or p53-c-myc-pCMV. After washings, the samples and a control of 20 % of the total cell lysate used in each of the binding reactions were separated by SDS-PAGE, transferred to Immobilon P membrane and then incubated with the appropriate antibody, anti p53 (DO-1) mouse monoclonal (Santa Cruz) or anti FLAG M2 mouse monoclonal (SIGMA). As secondary antibody goat anti mouse alkaline phosphatase conjugated was used and the blot developed using NBT/BCIP.

2.14 *In vitro* binding

Equal amounts of GST proteins bound to glutathione-Sepharose 4B resin (GST, GST-Ankrd2 and GST-YB-1) were incubated for three hours at room temperature with the following *in vitro* transcribed and translated (IVTT) ^{35}S radiolabeled proteins: Telethonin, Ankrd2, p53, PML and YB-1. Then the resins were briefly centrifuged and washed several times before being subjected to SDS-PAGE together with 30 % of the total amount of IVTT protein used in each of the binding reactions. The gels were dried and exposed either to BioMax autoradiography film or to SR Packard phosphor screens;

the analyses were done on a Packard Cyclone Phosphor Imager (Packard Instrument Co.).

2.15 GST-overlay

Ankrd2, p53 and Telethonin His-tagged proteins (4 μg) were subjected to SDS-PAGE and then blotted onto Immobilon-P membrane (Millipore). The membrane was blocked in 10 % low fat milk in PBS with 0.05 % Tween-20 to avoid non-specific interactions and then incubated either with the GST protein alone (4μg) and with the GST-PML or GST-Ankrd2 recombinant proteins (4μg).

After five washings the membrane was incubated first with a primary antibody against GST (anti GST goat polyclonal, GE Healthcare, 1:1000) for 90 minutes and then with a secondary antibody anti goat alkaline phosphatase conjugated (Sigma) for 1 hour. The membrane was finally developed using NBT/BCIP (Roche).

2.16 Reporter gene assay

The Dual-Luciferase Reporter Assay System (Promega) was used to carry out transactivation experiments using p21 and Mdm2 promoters cloned upstream of the Firefly (*Photinus pyralis*) luciferase reporter gene.

The FuGENE 6 reagent was used to transfect the Saos-2 human osteosarcoma cell line with p21promoter-LUC or Mdm2 promoter-LUC and pRL-CMV or pRL-TK as control plasmid carrying the Renilla (*Renilla reniformis*) luciferase reporter gene. The firefly luciferase activity deriving from the activation of the p21 or Mdm2 promoters was normalized against Renilla luciferase activity. Together with the luciferase expressing vectors also different amounts of p53-pcDNA3, Ankrd2-pcDNA3, YB-1-pcDNA3-

FLAG, pRB-pCMV and PML-pcDNA3 were transfected. Results were plotted as a histogram using the mean of at least three independent experiments. The luciferase activity was measured in a Turner Design luminometer (Promega).

2.17 Electrophoretic Mobility Shift Assay (EMSA)

The Electrophoretic Mobility Shift Assay (EMSA), also known as Band Shift is a technique commonly used to study DNA-protein interactions. In order to determine if Ankrd2 could act as a NF-κB inhibitor and affect NF-κB binding to DNA, the NF-κB consensus sequence (GGGRNNYYCC) was labelled with γ^{32}ATP (New England BioLabs) using the T4 polynucleotide kinase (New England Biolabs) for 1 hour at 37°C and subsequently incubated with human myoblast extract, previously treated for 24 hours with 10 ng/ml of TNF-α, as a source of NF-κB. In a different reaction the GST-Ankrd2 recombinant protein was added to the mixture, to test its ability to affect the NF-κB activity. Both GST and an inhibitor of NF-κB (MAD3) were used as controls. In addition two super shift reactions were performed with two antibodies (Santa Cruz) directed against the NF-κB subunits (p65 and p50), in order to confirm the presence of NF-κB. All the reactions were then separated on 6% polyacrylamide gels and the results analyzed with a Packard Cyclone Phosphor Imager (Packard Instrument Co.).

2.18 Alpha-screen

The Alpha-screen method relies on hydrogel coated Donor and Acceptor beads providing functional groups for conjugation to biomolecules. In the Alpha-screen assays, a signal is generated when a donor and an acceptor bead are brought into proximity by an interaction between the two conjugated biomolecules. The laser

excitation at 680 nm of a photosensitiser (Phthalocyanine) present on the Donor bead results in the production of singlet oxygen. The short lifetime of singlet oxygen in aqueous solution (~4 μsec) allows diffusion over a distance up to ~200 nm. The singlet oxygen migrates to react with a thioxene derivative in the Acceptor bead generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead emitting light at 520− 620 nm. This reaction cascade results in amplification of the signal, as a signal resulting from the 60,000 singlet oxygen molecules generated by each Donor bead can allow detection down to the attomolar level. In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead, resulting in a very low background signal. The half−life of the decay reaction is 0.3 sec, which makes the fluorescence signal very long lived and allows the technology to operate in time−resolved mode ensuring a reduced background by minimizing the effect of auto−fluorescence. Also a long excitation wavelength of 680 nm combined with a shorter emission wavelength of 520−620 nm reduces interference from biological or assay components.

The main reason for which the alpha-screen method was employed is to evaluate the strength of protein−protein bindings, to study the interactions occurring when more than one binding partner of a protein is present and also to eliminate the suspicion that there is an intermediary protein present as can be the case in experiments using cell lysates and in vitro transcripted translated protein. The alpha-screen was set up in collaboration with the Genome Research Group, CRIBI, Padua University to which my laboratory supplied all the soluble proteins that will be used in the alpha-screen assays to test interactions that we already know to be positive as well as to check new once.

The CRIBI group has a Fusion−alpha microplate analyzer (Packard BioScience) which is a universal multiplate analyzer designed to measure top and bottom fluorescence intensity, time−resolved, fluorescence, absorbance and luminescence. At present the protocol used is to biotinylate the protein to be used as Donor (usually a His tagged protein) and bind it to Streptavidin−Donor beads. The proteins used as Acceptors (GST−tagged proteins) are bound to the Glutathione−Acceptor beads, the opposite combination is also possible as both GST and His detection kits are available. The advantages of the Alpha-screen is that it is suitable for automation and High Throughput Systems (HTS) since the beads are very small (200 nm in diameter) therefore suitable for automated liquid handling requirements. Moreover the Alpha-screen uses small amounts of reagents and several multiwell formats 96, 384 and 1536; therefore it is possible to test multiple reactions at the same time.

2.19 Real Time RT-PCR

Real time RT-PCR is a highly sensitive technique that allows the quantification of rare transcripts and small changes in gene expression. This method allows the determination of the initial concentration of a specific target in the sample being examined. The real time RT-PCR principle is based on the detection and quantification of a fluorescent signal that is generated during each PCR cycle by a fluorescent reporter and that is directly proportional to the amount of the product synthesized. Since the quantity of product generated during each cycle is proportional to the initial amount of template the most abundant mRNA species in the sample will be detected at earlier cycles during the PCR than the rarest ones. The fluorescent signal can be produced with different methods. The simplest one, which has also been employed in this case, is to use the

SYBR Green I DNA intercalating agent (Finnzymes). SYBR Green I emits a fluorescent signal when it binds to the DNA double helix minor groove.A The Gene Amp 5700 machine (Applied Biosystem) was used for the real time PCR experiments. This instrument cannot analyze the data in real time, but only at the end of each PCR reaction. The software automatically elaborates the data calculating the final fluorescence value with respect to an endogenous invariant control that usually is constituted by a housekeeping gene (in this case GAPDH). An important parameter is the threshold cycle (Ct) that is the first cycle at which a significant increase of the fluorescence level is detectable. Hence the most abundant transcripts will have lower Ct values than the rarest ones.

For this thesis the real time RT-PCR experiments were performed in collaboration with the group of Dr. G. Valle (CRIBI- Padua University) in order to determine the presence and the relative abundance of the putative alternative Ankrd2 transcripts analyzing the mRNA extracted from human skeletal muscle and heart both at the foetal (Invitrogen) and at the adult (Ambion) stage. These mRNAs were used in an initial reverse transcription step to synthesize the first-strand cDNAs to be amplified in the following real time PCR reactions. The reverse transcription was performed using the Super Script II (Invitrogen).

For the real time PCR amplification of the Ankrd2 forms listed below the AmpliTaq Gold polymerase and the following pairs of primers were used:

For the "Normal" Ankrd2 form

ANKRD2-7-FOR	GGTGGAGATTGTGGAGCACT	Tm 65.62°C
ANKRD2-9-REV	GGTCTCTCGCCCACTATCAT	Tm 64.58°C
ANKRD2-E1S-FOR	CGGTTATGGACGGCACCAT	Tm 67.25°C
ANKRD2-E2-REV	CTTCTCATCCTCCAGCACCA	Tm 65.05°C

For the Ankrd2 form lacking exon 7 (form starting from the ATG in the middle)

ANKRD2-6/8-FOR	CCAATGTGAGGGATAAGGAAGGG	Tm 65.83°C
ANKRD2-9-REV	GGTCTCTCGCCCACTATCAT	Tm 64.58°C
ANKRD2-E1M-FOR	CAGTGAGCTCATGGCAAAGG	Tm 65.00°C
ANKRD2-E2-REV	CTTCTCATCCTCCAGCACCA	Tm 65.05°C

For the Ankrd2 form lacking exon 7 and 8

ANKRD2-6/9-FOR	CAATGTGAGGGATAAGGCAGGA	Tm 65.40°C
ANKRD2-9-REV	GGTCTCTCGCCCACTATCAT	Tm 64.58°C

For the long Ankrd2 form

ANKRD2-E1L-FOR	AGCATGCAGCCAGCAGT	Tm 66.30°C
ANKRD2-E1-REV	TAATAGGCCAGGAGTTGGGG	Tm 64.54°C

For the endogenous control (GAPDH)

GAPDH-FOR	ACATCATCCCTGCCTCTACTG	Tm 64.70°C
GAPDH-REV	ACCACCTGGTGCTCAGTGTA	Tm 66.80°C

Real-time RT PCR cycle

Step	Temperature	Time	Cycle
First denaturation	95°C	3'00"	1X
Denaturation	95°C	15"	40X
Annealing*	62°C	1'10"	
Final estension	72°C	2'00"	1X
Cooling	4°C	∞	1X

*NOTE: Cycle with combined step of annealing/extension at 62°C.

Chapter 3

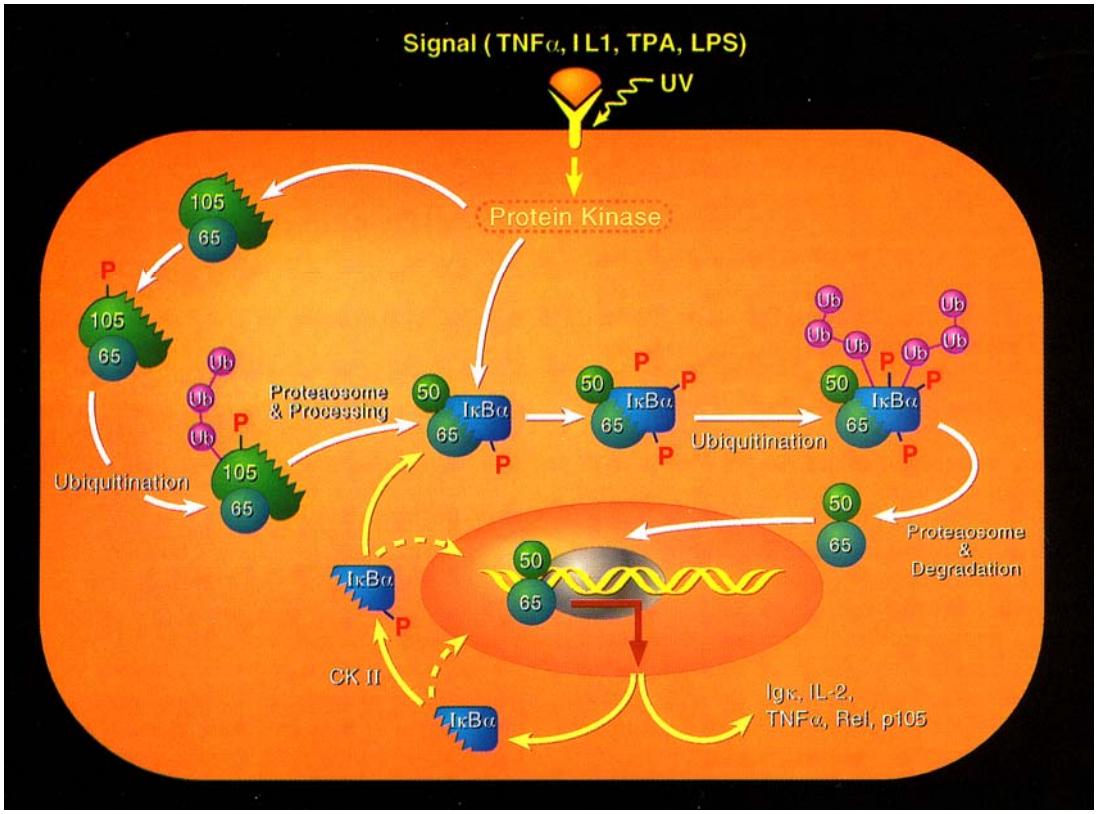
RESULTS

3.1 Ankrd2 does not behave like IκB

The Ankrd2 promoter contains an NF-κB box, suggesting that it could be regulated through the NF-κB pathway. Moreover Ankrd2 shows a striking similarity with NF-κB inhibitor, IκB since both have ankyrin repeats and PEST sequence (Pallavicini et al. 2001).

The NF-κB family of transcription factors comprises proteins that are sequestered in the cytoplasm in an inactive state in association with inhibitory proteins called IκB, which through its ankyrin repeats, binds and masks their nuclear localization signal (NLS) thereby preventing NF-κB nuclear translocation. Upon stimulation with signalling molecules, such as tumor necrosis factor alpha (TNFα) or lipopolysaccharide (LPS), IκB is phosphorylated and targeted for rapid degradation in the 26S proteasome and NF-κB is released and translocated to the nucleus, where it regulates gene transcription. NF-κB is involved in the activation of immune response genes, but also in cellular proliferation and apoptosis. Many NF-κB proteins exist that can form homo- and heterodimers, which than bind DNA (consensus sequence GGGRNNYYCC) and promote transcription. The classic NF-κB heterodimer is composed by p50/p65

subunits. Also several IκB isoforms are present in the cell that differentially regulates the various NF-κB dimers. NF-κB p50/65 is specifically inhibited by IκBα (reviewed in Verma et al. 1995) (Fig. 1a).



Picture adapted from: Verma et al. 1995, *Genes Dev* 9: 2723-2735

Figure 1a: NF-κB pathway. The p50 subunit is initially synthesized as a p105 precursor. In response to external signals a protein kinase is activated and phosphorylates both p105 and IκBα that become substrates for ubiquitination hence for degradation in the proteasome. The p65/p50 complex (NF-κB) then translocates to the nucleus activating genes containing κB sites in their promoter, including IkBα. IκBα is phosphorylated by CKII and form a complex with p65/p50 (NF-κB) thus retaining it in the cytoplasm until a new signal is provided.

I performed an Electrophoretic Mobilty Shift Assay (EMSA), also known as band shift, to test if Ankrd2 behaved like IκB by inhibiting NF-κB binding to its DNA consensus sequence. The basis of the band shift assay is that protein-DNA complexes remain intact when gently fractionated by gel electrophoresis and migrate as distinct bands, but more slowly than the free DNA fragment. Band shift experiments can also be used to visualize protein-protein interactions between a DNA binding protein and other non DNA binding proteins, also through the use of specific antibodies. The binding of a second protein to a protein-DNA complex to form a triple complex is visualized by a further retardation of mobility (super shift).

An NF-κB consensus oligonucleotide reconstructing the NF-κB binding site (Santa Cruz) was labelled with γ^{32}-ATP using T4 polynucleotide kinase and then incubated with a cell extract from TNFα-treated human CHQ5B myoblasts as a source of NF-κB (NF-κB) or with GST-Ankrd2 recombinant protein.

As controls the following reactions were prepared:

- a super shift reaction employing an anti p65 specific antibody (Santa Cruz), in order to ensure that the shift is really due to NF-κB
- a reaction with NF-κB and MAD3, an NF-κB inhibitor belonging to the IκBα protein family
- a reaction with the GST-recombinant protein alone
- a reaction with a 100% excess of cold oligonucleotide

After electrophoresis and autoradiography it emerged clearly that the NF-κB present in the cell extract can bind to the consensus oligonucleotide and that Ankrd2 is not behaving like IκB (or MAD3) since it does not interfere with this interaction (Fig. 1b).

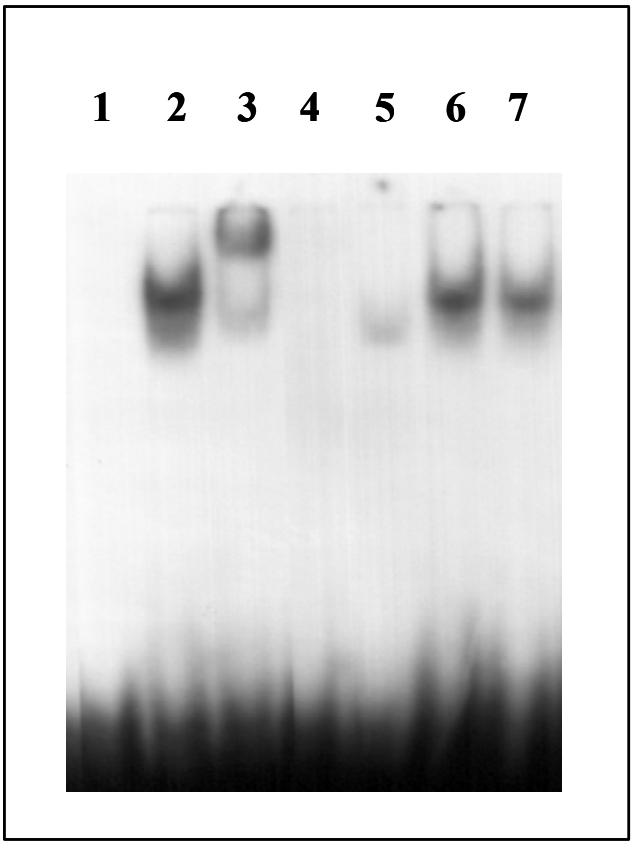


Figure 1b: Electrophoretic Mobility Shift Assay (EMSA). Ankrd2 does not behave like IκB, since it does not inhibit NF-κB binding to DNA. When a ^{32}P labelled oligonucleotide forms a complex with a protein it migrates slower than if it was free and therefore it is visible as a higher band (band shift). Moreover the addition of specific antibodies against the proteins bound to the oligonucleotide will produce an extra shift (super shift). In this experiment a ^{32}P labelled oligonucleotide was used that contains the consensus sequence for NF-κB binding (GGGRNNYYCC). Skeletal muscle myoblasts (CH5QB) treated with a NF-κB inducer (TNF-α) were used as a source of NF-κB. MAD3 that is an inhibitor of NF-κB activity belongs to the IκBα family. Lane 1: ^{32}P oligo alone (control). No shift visible; Lane 2: NF-κB + ^{32}P oligo. A shift is visible; Lane 3: NF-κB+^{32}P oligo+ anti p65 antibody (Santa Cruz): a super shift can be seen. Lane 4: ^{32}P oligo + specific oligo without label (in 100x excess with respect to the labelled oligo) + NF-κB. No bands are visible. Lane 5: ^{32}P oligo + NF-κB+ MAD3. The binding of NF-κB to the DNA is strongly reduced. Lane 6: ^{32}P oligo+ NF-κB + GST (control). No changes are visible. Lane 7: ^{32}P oligo + NF-κB + GST-Ankrd2. No difference with respect to NF-κB alone is detectable.

3.2 Ankrd2 binds YB-1 *in vivo* and *in vitro*

Ankrd2 is also structurally and functionally similar to Cardiac Ankyrin Repeat Protein (CARP) (Moriyama et al. 2001, Pallavicini et al. 2001), which is known to bind the ubiquitous transcription factor YB-1 (Zou et al. 1997).

From our data the binding occurs both *in vivo,* as can be seen from the results of the co-immunoprecipitation experiments and *in vitro,* see data from the GST-pull down and the *in vitro* binding experiments. For co-immunoprecipitation experiments COS-7 cells were transfected with cMyc-tagged Ankrd2 either alone or with FLAG-tagged YB-1. Cell lysates of untransfected and transfected COS-7 cells were immunoprecipitated with antibody against the FLAG-tag, then subjected to SDS-PAGE, immunoblotted and probed with antibody to Ankrd2. A signal indicating an interaction between YB-1 and Ankrd2 could be detected (Fig. 2a).

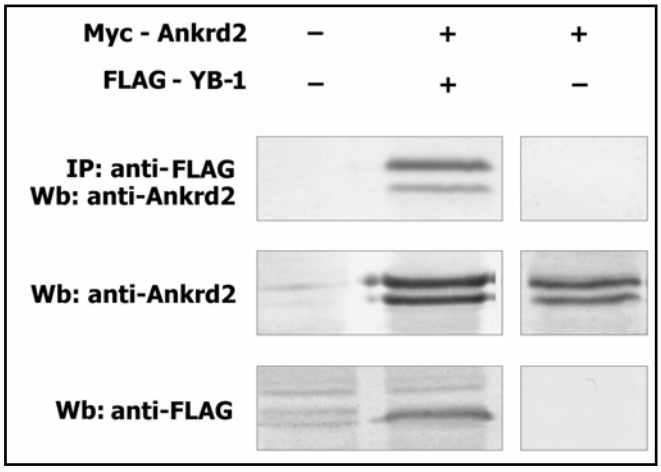


Figure 2a: Co-IP of Ankrd2 and YB-1 from COS-7 cells: untransfected (first column) and transfected with pCMV-cMyc-Ankrd2 plus pcDNA3-FLAG-YB-1 (second column) or pCMV-cMyc-Ankrd2 alone (third column). The cell lysates were immunoprecipitated with anti FLAG (M2) mouse monoclonal antibody (SIGMA), separated by SDS-PAGE and then immunoblotted using polyclonal anti Ankrd2 antibody (first row). As controls cell lysates were tested with anti Ankrd2 polyclonal antibody (second row) and with anti FLAG (M2) mouse monoclonal antibody (third row).

In GST pull down experiments (Fig. 2b) cell lysates from COS-7 cells transfected with FLAG-tagged YB-1 were incubated with either GST alone or with GST-Ankrd2 recombinant protein both attached to glutathione sepharose-4B. Results confirm the data obtained from the co-immunoprecipitation experiments.

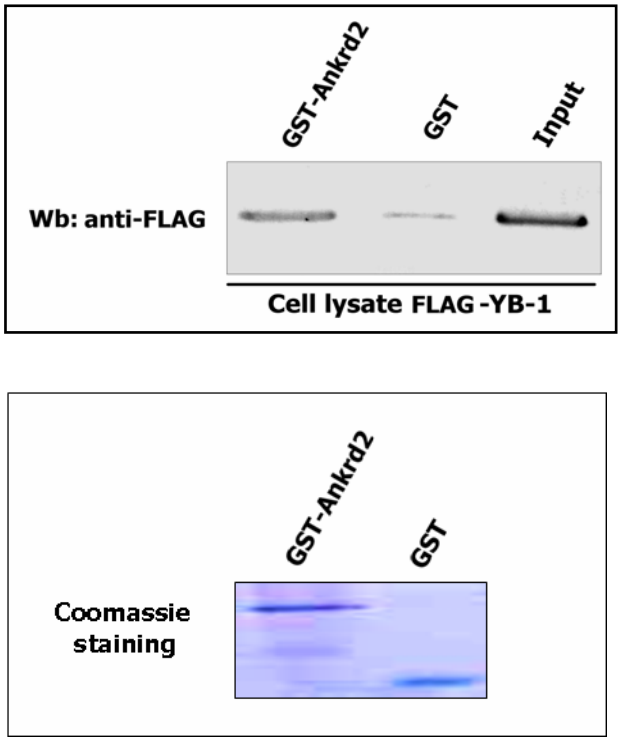


Figure 2b: Upper panel: GST pull down between Ankrd2 and YB-1. GST-Ankrd2 (first column) and GST alone (second column) attached to glutathione-sepharose-4B were mixed with lysates from COS-7 cells transfected with pcDNA3-FLAG-YB-1. The resins were washed, subjected to SDS-PAGE, immunoblotted using anti FLAG antibody directly conjugated with alkaline phosphatase (AP). The last column shows 20% of cell lysates from FLAG-YB-1 transfected cells used in each of the binding reactions. **Lower panel:** SDS-PAGE followed by Blue Coomassie staining showing that equal amounts of GST-Ankrd2 and GST were used in the GST pull down.

In vitro binding assays between an *in vitro* transcribed and translated (IVTT) [^{35}S]-labelled YB-1 and a GST-Ankrd2 or a GST-YB-1 recombinant protein bound to glutathione sepharose-4B indicate that an interaction between YB-1 and Ankrd2 exists and that YB-1 could form homodimers (Fig. 2c). This type of assay was also done in the opposite combination using an IVTT [^{35}S]-Ankrd2 protein and GST-YB-1 or GST-Ankrd2 recombinant proteins, confirming again that Ankrd2 and YB-1 can bind each other. Interestingly Ankrd2 seems to be able to dimerize (Fig. 2d).

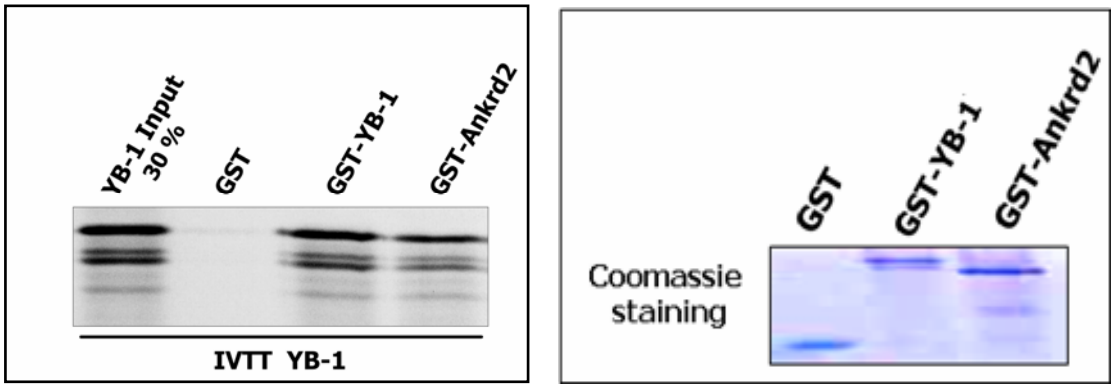


Figure 2c: *In vitro* binding of GST-Ankrd2 and radiolabeled IVTT YB-1. **Upper panel:** the first column shows 30% of the amount of radiolabeled IVTT YB-1 used in each of the reactions. GST, GST-YB-1 and GST-Ankrd2 all bound to glutathione-sepharose 4B were incubated for three hours at room temperature with radiolabeled IVTT YB-1, washed and then subjected to SDS-PAGE. **Lower panel:** a blue Coomassie staining shows that the same amount of GST, GST-YB-1 and GST-Ankrd2 has been used in the *in vitro* binding assay.

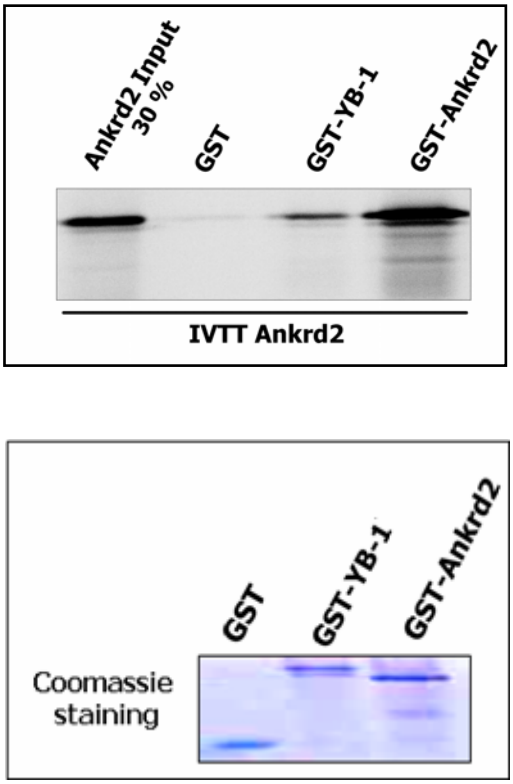


Figure 2d: *In vitro* binding of GST-YB-1 and radiolabeled IVTT Ankrd2. **Upper panel:** the first column shows 30% of the amount of radiolabeled IVTT-Ankrd2 used in each of the binding reactions. GST, GST-YB-1 and GST-Ankrd2 all bound to glutathione-sepharose 4B were incubated for three hours at room temperature with radiolabeled IVTT Ankrd2, washed and then subjected to SDS-PAGE. Besides the binding between Ankrd2 and YB-1, is also evident that Ankrd2 could dimerize. **Lower panel:** equal amount of the GST recombinant proteins have been used in the *in vitro* binding experiment.

3.3 YB-1 binding site is located in the N-terminal Ankrd2 region

The GST pull down approach has also been used to map the regions of Ankrd2 that are involved in the binding with YB-1. The binding site for YB-1 would appear to be in the N-terminal region of Ankrd2 since only the GST constructs of full length Ankrd2 (FL, 5-333 amino acids) and N-terminal Ankrd2 (N, 5-120 amino acids) bind FLAG-YB-1. The C-terminal-GST Ankrd2 constructs (CA, 121-333 amino acids; C, 279-333 amino acids) did not bind YB-1-FLAG (Fig. 3).

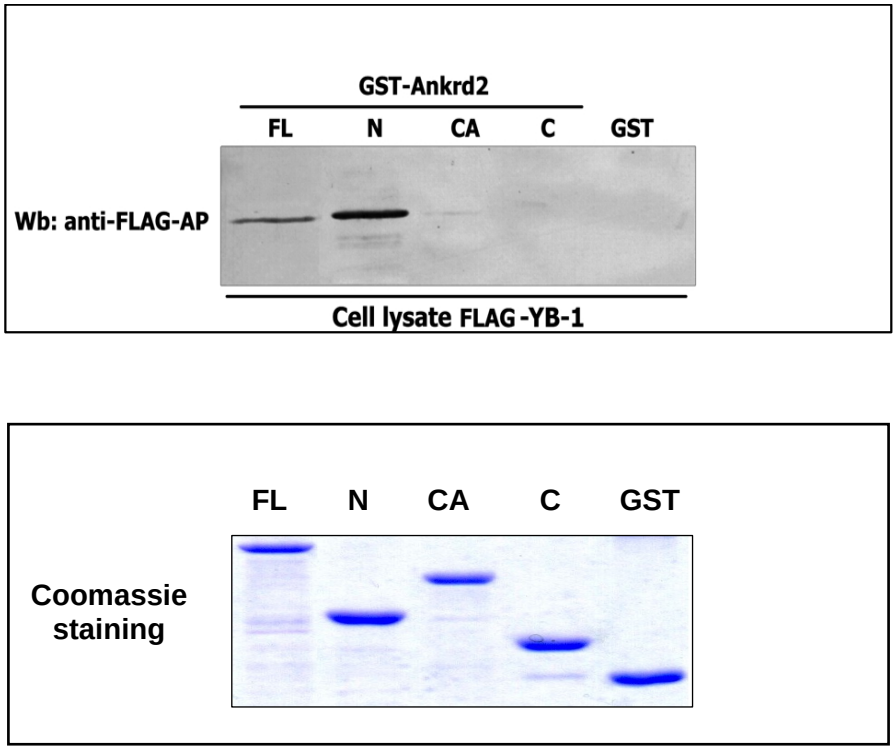


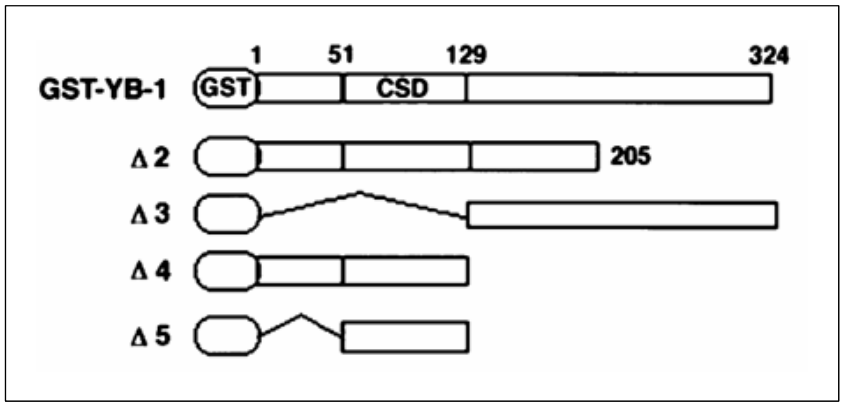
Figure 3: GST pull down. The N terminal region of Ankrd2 contains the binding site for YB-1. GST and GST-FL-Ankrd2 (5-333 aa), GST-N-Ankrd2 (5-120 aa), GST-CA-Ankrd2 (121-333 aa) and GST-C-Ankrd2 (279-333 aa) attached to glutathione-Sepharose 4B were mixed with lysate from COS-7 cells transfected with pcDNA3-FLAG-YB-1. The membrane was probed with an anti FLAG AP-conjugated antibody (SIGMA) and binding was only detected between YB-1 and GST-FL-Ankrd2 and GST-N-Ankrd2. The Coomassie blue staining in the lower panel shows that equal amounts of each GST recombinant protein have been employed in the experiment.

3.4 The C-terminal domain of YB-1 may contain the binding site for Ankrd2

In collaboration with the group of Prof. G.Valle at CRIBI (Department of Molecular Biology, Padua University, Italy) several assays have been performed using a quite new technique to detect protein-protein interactions, called Alpha Screen.

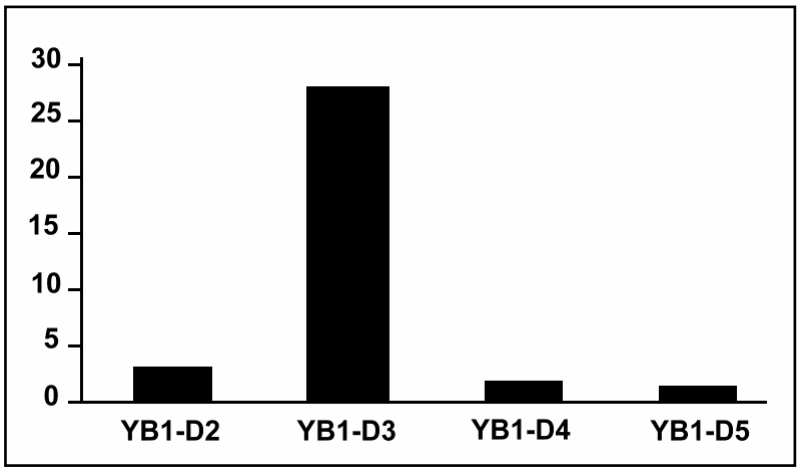
Alpha Screen is a bead based non-radioactive **A**mplified **L**uminiscent **P**roximity **H**omogeneous **A**ssay (Packard BioScience). If a biological interaction brings the beads together, a cascade of chemical reactions acts to produce a greatly amplified signal. On laser excitation, a photosensitizer in the "Donor" bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative on the "Acceptor" bead generating chemiluminiscence at 370 nm that further activates fluorophores contained on the same bead. The fluorophores subsequently emit light at 520-620 nm. In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result the background of this type of reaction is very low.

Using the Alpha Screen technique, not only an interaction between the full length Ankrd2 and YB-1 proteins was observed, but moreover the binding site on YB-1 was detected. Four GST-YB-1 deletion mutants were used that were kindly provided by Dr. Kohno, University of Occupational and Environmental Health, Kitakyushu, Japan. These were GST-Δ2YB-1, GST-Δ3YB-1, GST-Δ4YB-1 and GST-Δ5YB-1 (Fig. 4a). The interaction with Ankrd2 was detected only with GST-Δ3YB-1, indicating that probably the binding site is in the C-terminal part of YB-1 (Fig. 4b).



Picture adapted from: Okamoto et al. 2000, *Oncogene* 19: 6194-6202

Figure 4a: YB-1 deletion mutants. GST-YB-1 full length and four GST-YB-1 deletion mutants (Δ2, Δ3, Δ4 and Δ5) kindly provided by Dr. Kohno, University of Occupational and Environmental Health, Kitakyushu, Japan. CSD is the conserved cold shock domain. These constructs have been employed in the Alpha Screen assays to identify the region containing the binding site for Ankrd2



Results obtained from the work performed in collaboration with Dr. I Zara, Padova

Figure 4b: Alpha Screen analysis of YB-1 deletion mutants/Ankrd2 interaction. From the Alpha screen analysis done by Dr. I Zara, University of Padova it was seen that Ankrd2 strongly interacts only with the GST-Δ3-YB-1 mutant, it does not bind the other mutants employed. Therefore this suggests that the Ankrd2 binding site could be located in the last 119 amino acids of the C terminal of YB-1.

3.5 Ankrd2 co-localizes with PML in NBs

Previous immunofluorescence analysis performed on primary human myoblasts (CHQ5B) using either a mono- or a polyclonal antibody to Ankrd2, showed that Ankrd2 is distributed in the nuclei in the form of speckles (Pallavicini et al. 2001). Further experiments revealed that this nuclear pattern is visible in a large number of myoblasts (40-60%) and that the percentage of these nuclear structures progressively decreases during differentiation and becomes very low (1% or less) in totally differentiated human skeletal muscle myotubes (Fig. 5a). The nucleus is a very complex structure and it is compartmentalized into highly organized structural and functional domains. In fact many different sub-nuclear structures have been described and associated with particular biochemical functions. Among them PML nuclear bodies are one of the best studied (Zhong et al. 2000). In order to characterize the nuclear structures in which Ankrd2 was observed, we co-stained human primary myoblasts for promyelocytic leukaemia protein (PML), which is the major component of PML nuclear bodies and for Ankrd2, then we analyzed the samples by confocal microscopy (40X and 100X magnification) using a LSM 510 laser-scanning microscope (Zeiss). As can be seen in Fig. 5b, Ankrd2 and PML are perfectly co-localized in the nuclei of the myoblasts in PML nuclear bodies. This pattern is not dependent on the method used to fix the cells, since both paraformaldehyde and methanol fixed samples showed the same localization of the Ankrd2 protein. Interestingly, in differentiated myotubes few or no nuclear bodies are detected when cells are fixed with paraformaldehyde and PML appears to be diffusely distributed in the cytoplasm. However following methanol fixation PML can be detected both in PML NBs in the nuclei and also in the cytoplasm. No co-

localization occurs in the myotubes between Ankrd2 and PML except in the few cases where Ankrd2 is present in the NBs of these cells (1%) (Fig. 5a).

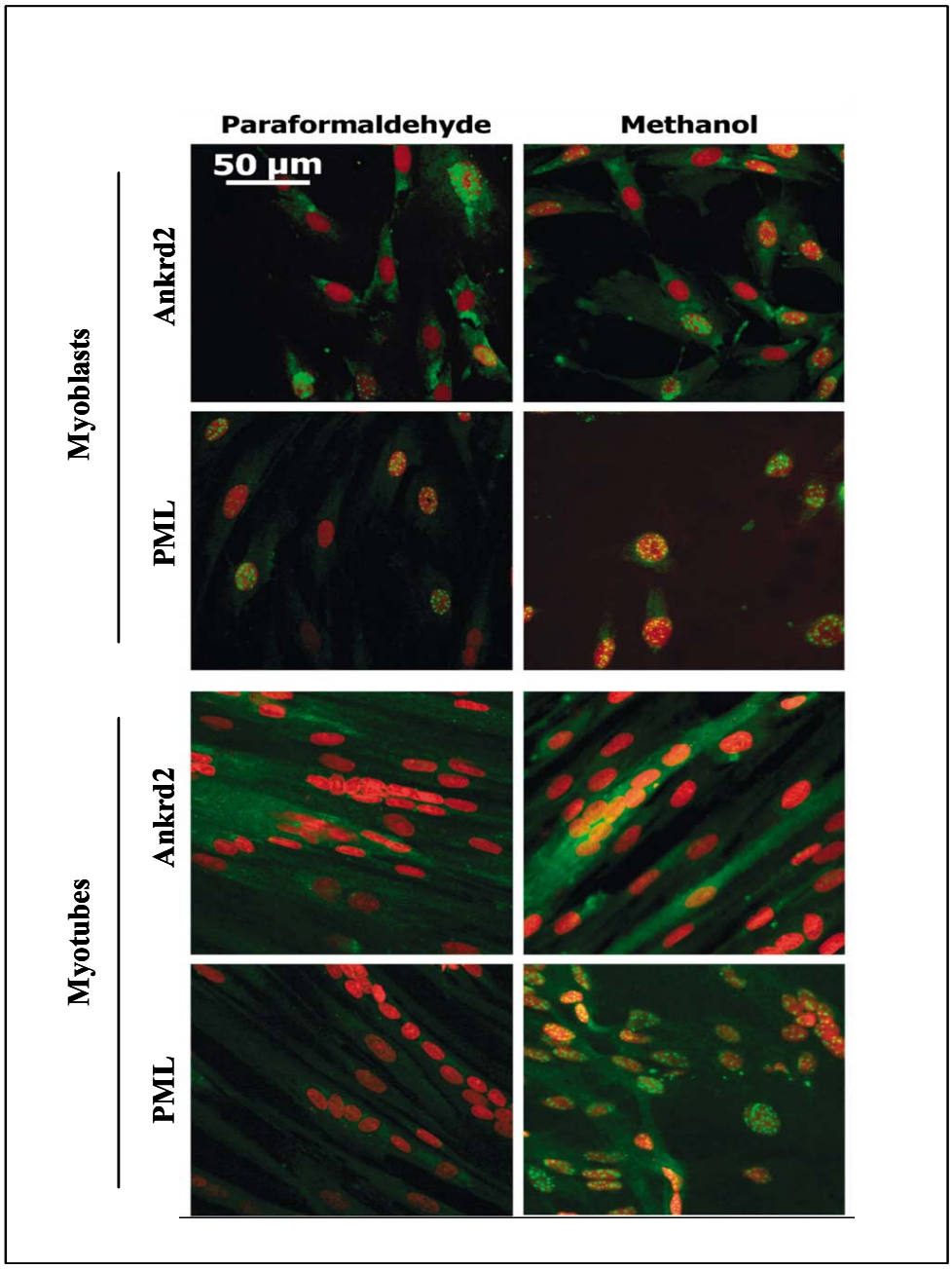


Figure 5a: Localization of PML and Ankrd2 in human myoblasts and myotubes (CH5QB cells). Both Ankrd2 and PML are visible in the nuclei of myoblasts with a speckled pattern after either paraformaldehyde or methanol fixation. In the multinucleated myotubes Ankrd2 is almost completely cytoplasmic and no differences are observed between the two methods of fixation. Differently PML is still present in the nuclear bodies, but is visible only when methanol fixation is used. The primary antibodies were used a mouse monoclonal against the C-terminal region of Ankrd2 and a goat polyclonal for PML (Santa Cruz). The secondary antibodies used were anti mouse conjugated with FITC (green) and anti goat conjugated with Alexa 546 (red). These pictures were captured using a LSM 510 laser confocal microscope (Zeiss) with 40x resolution.

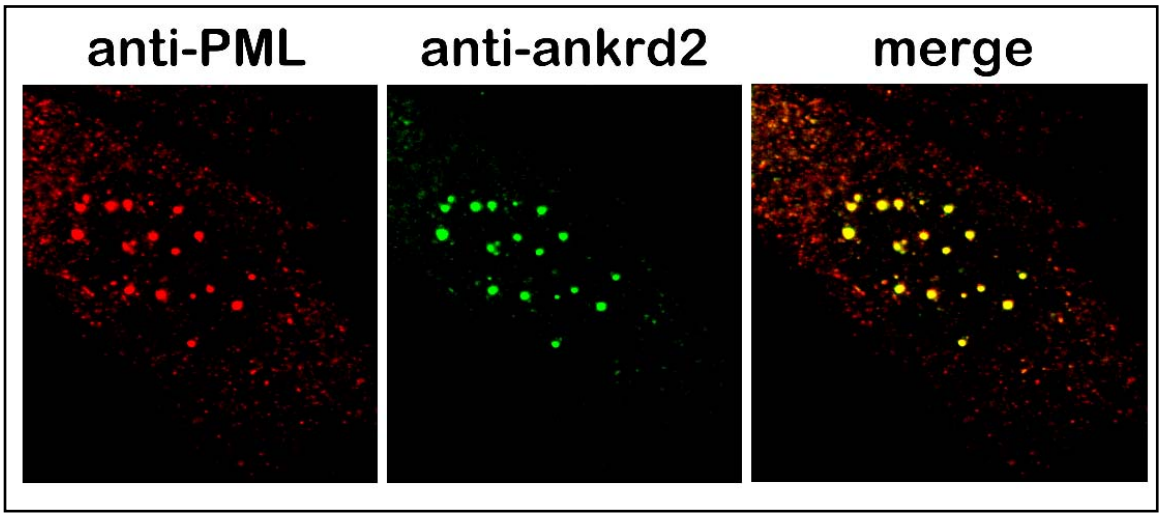


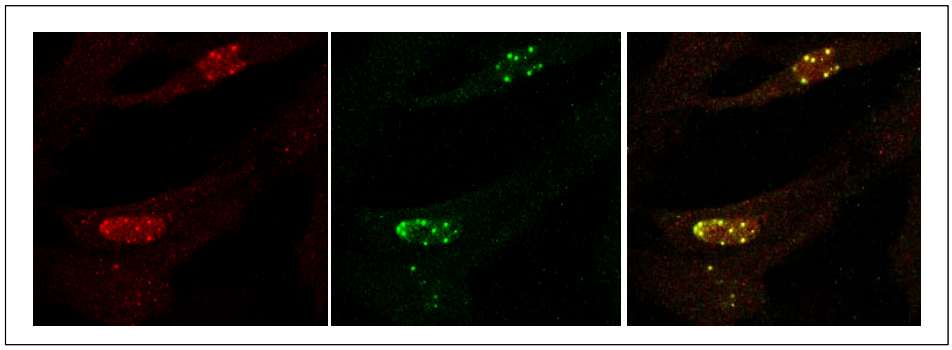
Figure 5b: Ankrd2 and PML co-localize. Ankrd2 and PML show a perfect co-localization in the nuclei of human myoblasts. The indirect immunofluorescence was performed using an anti-Ankrd2 mouse monoclonal antibody and an anti-PML goat polyclonal antibody (Santa Cruz). The secondary antibodies used were an anti mouse conjugated with FITC (green) and an anti goat conjugated with Alexa 546 (red). The two proteins are localized in distinct nuclear structures called PML nuclear bodies. The picture was acquired using a LSM 510 laser confocal microscope (Zeiss) with 100x objective.

3.6 Ankrd2 only partially co-localizes with SUMO-1

SUMO-1 is an ubiquitin-like protein that covalently binds PML. SUMO-modified PML is essential for nuclear body assembly, since only SUMOylated PML is found in these structures whereas un-modified PML localizes in the nucleoplasm. SUMOylation is supposed to target protein to specific cellular compartments. Moreover some PML NBs components such as Sp100 and p53 are SUMO-modified (Gostissa et al. 1999, Seeler and Dejean 2001, Hay 2005).

For this reason we tested by immunofluorescence if Ankrd2 and SUMO-1 co-localize in the PML NBs in the nuclei of human CHQ5B skeletal muscle myoblasts. We found only a partial co-localization of PML and SUMO-1 (Fig. 6a and 6b). This suggests that

probably Ankrd2 does not need to be modified by SUMO-1 in order to be targeted to PML nuclear bodies.



SUMO-1 **Ankrd2** **SUMO-1+Ankrd2**

Figure 6a: SUMO-1 and Ankrd2 only partially co-localize in PML NBs. Single (first and second panel) and double (third panel) immunofluorescence on human myoblasts (CHQ5B) were performed using an anti SUMO-1 rabbit polyclonal antibody (Santa Cruz) (red) and an anti Ankrd2 monoclonal antibody (green). The pictures were captured with a laser scanning confocal microscope (Zeiss) with 40x objective.

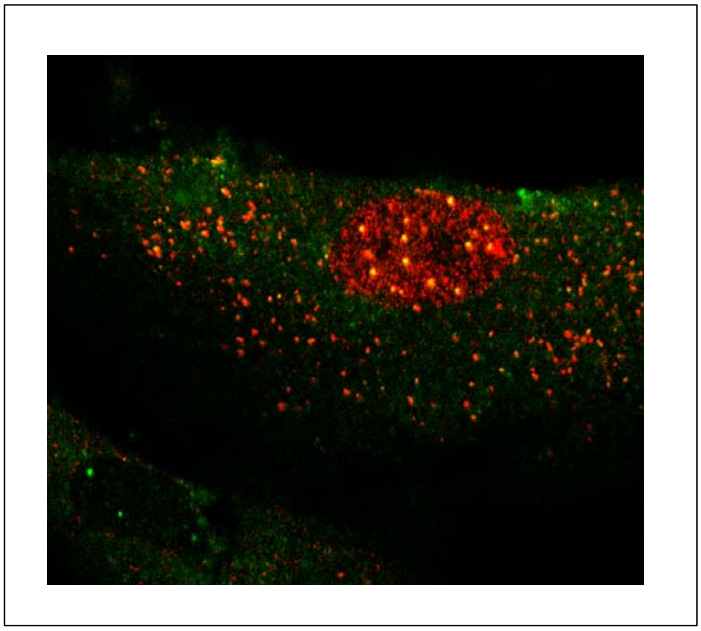


Figure 6b: SUMO-1 only partially co-localizes with Ankrd2. This picture is an enlargement of a scan done using the LSM 150 confocal microscope (63X magnification) on a human myoblasts treated both with anti SUMO-1 (red) and anti Ankrd2 (green) antibodies. This shows in more detail the partial co-localization of these two components in the PML NBs.

3.7 Ankrd2 interacts with PML isoform IV (PML3) *in vivo* and *in vitro*

Since from fluorescence experiments Ankrd2 co-localizes with PML in the PML NBs, it was reasonable to test if an interaction between the two proteins occurred. Both *in vivo* and *in vitro* techniques have been employed to evaluate this and in each case a binding between the two proteins was found. In all our experiments we used the PML isoform IV (PML 3), that recruits p53 into the nuclear body (Fogal et al. 2000).

In co-immunoprecipitation experiments COS-7 cells were transfected with expression vectors for HA-tagged PML IV and cMyc-tagged Ankrd2 or with cMyc-Ankrd2 alone. The cell lysates were immunoprecipitated with an anti-HA mouse monoclonal antibody (Santa Cruz), subjected to SDS-PAGE and finally blotted and tested for the presence of Ankrd2 with an anti-Ankrd2 mouse polyclonal antibody (Fig. 7a). Other co-immunoprecipitations were performed using different tags and different antibodies and the results confirmed these findings (data not shown).

Similar results were obtained from the GST overlay assay where an His-tagged Ankrd2 protein bound the GST-PML IV (Fig. 7b) and from *in vitro* binding experiments between a GST-Ankrd2 recombinant protein bound to glutathione sepharose-4B and an IVTT ^{35}[S]-labelled PML protein (Fig. 7c). A GST recombinant protein was always used as a control, to avoid false positive signals. The results confirm the existence of an interaction between Ankrd2 and PML IV.

So, not only PML co-localizes with the endogenous Ankrd2, but recombinant PML and Ankrd2 can be shown to interact both *in vivo* and *in vitro*. These interactions would appear to be direct, as in the overlay assay no other possible intermediary proteins were present.

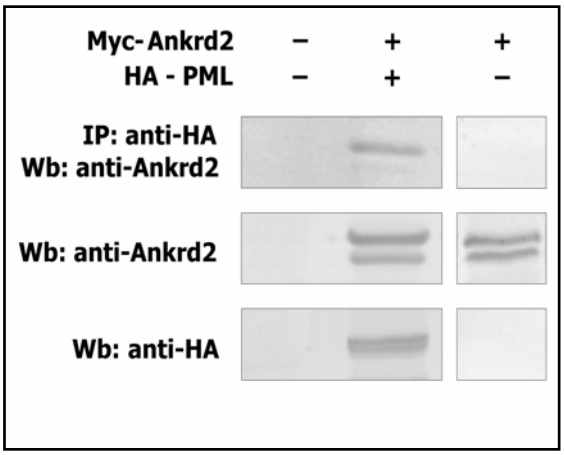


Figure 7a: Co-IP of Ankrd2 and PML IV from COS-7 lysates untransfected (first column) and transfected with pCMV-cMyc-Ankrd2 plus pcDNA3-HA-PML IV (second column) or pCMV-cMyc-Ankrd2 alone (third column). The cell lysates were immunoprecipitated with anti-HA mouse monoclonal antibody, separated by SDS-PAGE and then immunoblotted and probed with a polyclonal anti-Ankrd2 antibody (first row). As controls cell lysates were tested with anti-Ankrd2 polyclonal antibody (second row) and with anti-HA antibody (third row).

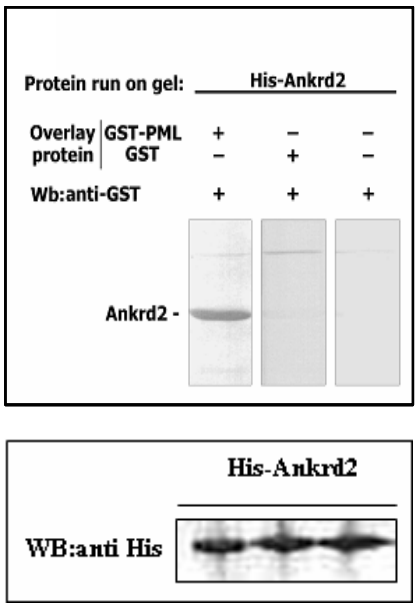


Figure 7b: GST overlay assay. His-Ankrd2 (4 µg) was separated by SDS-PAGE and transferred to Immobilon P membrane. Membrane strips were incubated with GST (4 µg) or GST-PML (4 µg), washed and then incubated with anti GST goat polyclonal antibody (Amersham). A signal indicating a direct interaction between Ankrd2 and PML is visible. In the lower picture a western blot performed with the anti histidine antibody (QIAGEN, 1:2000 dilution) is shown, indicating that the same amount of His-Ankrd2 was loaded.

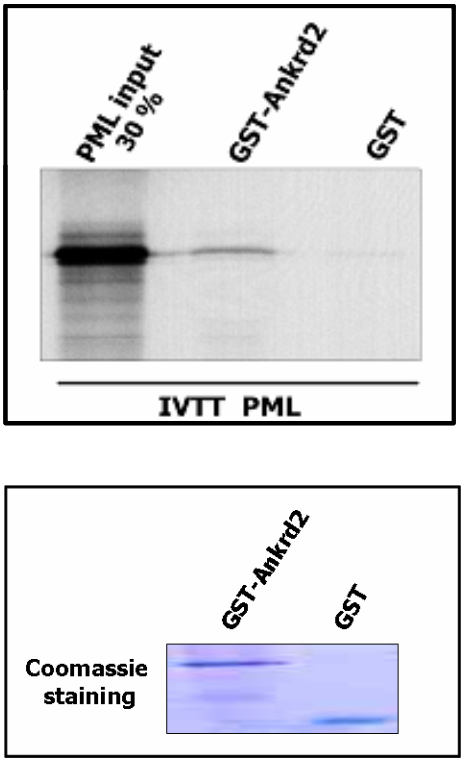


Figure 7c: *in vitro* binding. Upper panel: GST and GST-Ankrd2 bound to glutathione-Sepharose 4B were incubated with radiolabeled IVTT PML, washed and subjected to SDS-PAGE. In the second column is visible a band indicating that PML and Ankrd2 are binding. The first column shows 30% of the amount of radiolabeled IVTT-PML used in each of the binding experiments. **Lower panel:** the same amount of GST-Ankrd2 and GST recombinant proteins was used in the *in vitro* binding assay.

3.8 Ankrd2 interacts with p53 *in vivo* and *in vitro*

The tumour suppressor protein p53 is present in the PML NBs, since it is recruited in these structures by PML IV (Fogal et al. 2000). Moreover p53 is known to bind to the ubiquitous transcription factor YB-1 (Okamoto et al. 2000). Thus we considered relevant to test if Ankrd2 interacts with p53. We found a binding between Ankrd2 and the wild type p53, both using *in vivo* and *in vitro* techniques.

Co-immunoprecipitation (Fig. 8a) was performed using lysates from COS-7 cells transfected with cMyc-Ankrd2 and GFP-p53, to distinguish transfected p53 from the endogenous counterpart. An anti cMyc mouse monoclonal antibody (SIGMA) was used for immunoprecipitation. After SDS-PAGE, proteins were blotted and the presence of Ankrd2-bound p53 was detected with an anti-GFP rabbit polyclonal antibody (Invitrogen). This experiment was then repeated with similar results in the p53 -/- Saos-2 cell line to completely avoid any interference from endogenous p53 (data not shown).

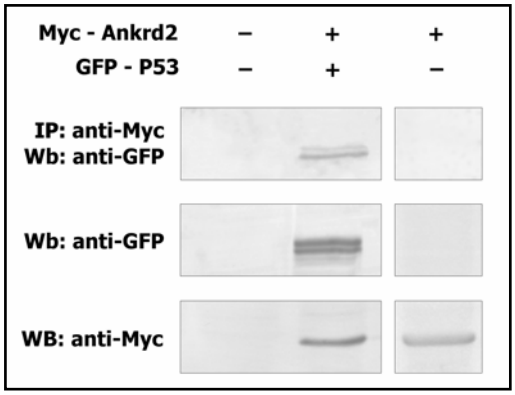


Figure 8a: Co-IP of Ankrd2 and p53 from COS-7 cell lysates untransfected (first column) or transfected with cMyc-Ankrd2-pCMV and p53-pEGEFP (second column) or with cMyc-Ankrd2-pCMV alone (third column). cMyc-Ankrd2 was immunoprecipitated with an anti cMyc mouse monoclonal antibody (SIGMA), then separated by SDS-PAGE and blotted using an anti GFP rabbit polyclonal antibody (Invitrogen) (first row). In the second and third rows are shown controls of cell lysates probed both with anti cMyc and anti GFP antibodies.

The interaction between Ankrd2 and p53 was detected also using GST-pull down (Fig. 8b) and *in vitro binding* (Fig. 8c) assays however only from the results obtained with GST-overlay experiments it is possible to assert that the interaction is direct rather than indirect (Fig. 8d).

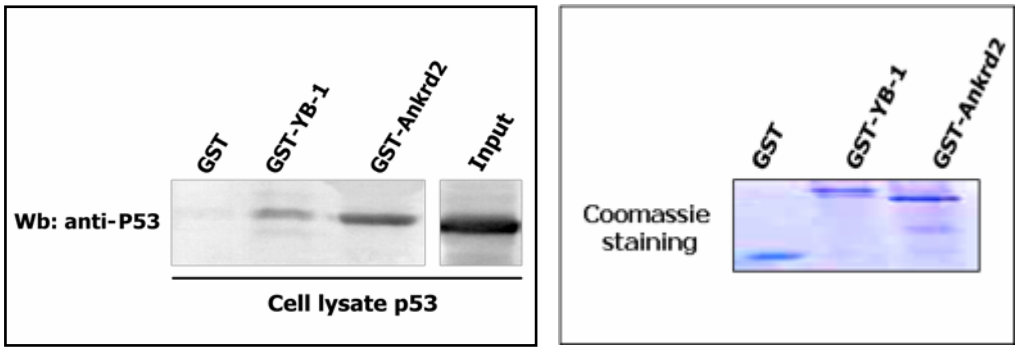


Figure 8b: GST pull down. Left panel: lysates of COS-7 cells transfected with p53-pcDNA3 were incubated with the following recombinant proteins attached to glutathione-Sepharose 4B: GST-Ankrd2, as well as GST and GST-YB-1 as negative and positive controls respectively. The reactions were then subjected to SDS-PAGE and the membrane probed with anti p53 mouse monoclonal antibody (DO-1, Santa Cruz). As expected p53 and YB-1 bind each other, whereas GST alone does not bind to p53. Interestingly Ankrd2 and p53 interact. **Right panel:** SDS-PAGE followed by Coomassie Blue staining showing that similar amounts of the GST proteins were employed in the pull down assay.

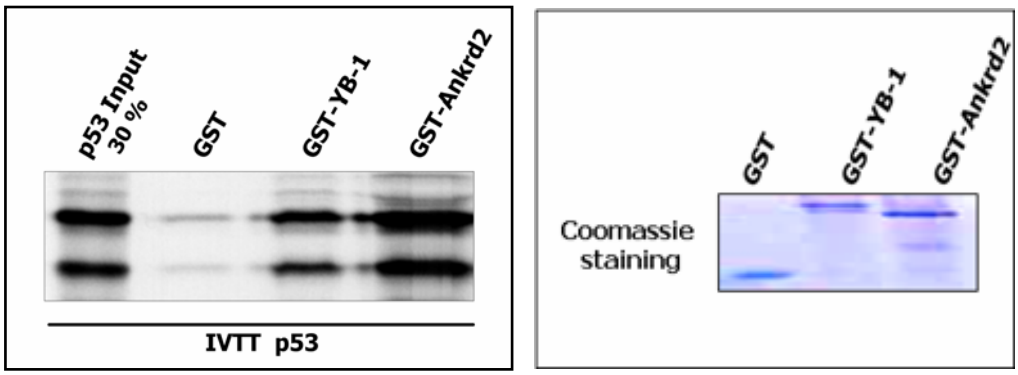


Figure 8c: Left panel: in vitro binding between IVTT p53 labeled with ^{35}S and the following GST recombinant proteins bound to glutathione-Sepharose 4B: GST as negative control, GST-YB-1 as positive control and GST-Ankrd2. The first column shows 30% of IVTT p53 used in each reaction. **Right panel:** a SDS-PAGE followed by Coomassie Blue staining shows that similar amounts of GST, GST-YB-1 and GST-Ankrd2 were used in the *in vitro* binding assay.

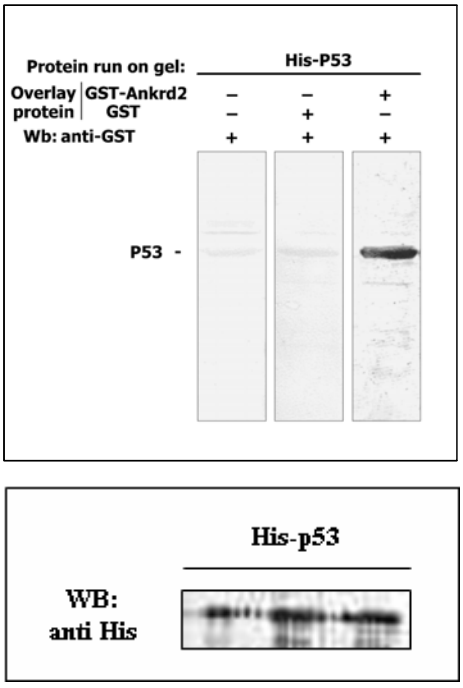


Figure 8d: overlay assay. p53-His (4 µg) was separated by SDS-PAGE and then blotted onto a membrane that was subsequently incubated with 4 µg of GST (second column) and GST-Ankrd2 (third column), then after washing probed with an anti GST antibody (Amersham). In the third column is a strong band showing that p53 and Ankrd2 have interacted. To avoid false positives p53-His was also tested with the anti GST antibody (first column).
The lower panel shows a western blot performed with an anti histidine mouse monoclonal antibody (1:2000 dilution) on the His-p53 recombinant protein to ensure that equal amounts of sample have been loaded.

3.9 Ankrd2 and PML interact independently from p53

Co-immunoprecipitations in the p53 -/- Saos-2 cell line were performed after transfection in order to check if p53, Ankrd2 and PML could form a trimeric complex. In this experiment the cells were transfected with both Ankrd2 and PML-HA and also with Ankrd2, PML-HA and p53 together. Cell lysates were immunoprecipitated with an anti-HA mouse monoclonal antibody and the subjected to SDS-PAGE separation and western blot analysis to search for the presence of Ankrd2. The results show that PML and Ankrd2 still bind each to each other even in the absence of p53. Hence we can deduce that the interaction of Ankrd2 to PML is independent of p53. Interestingly the binding between Ankrd2 and PML is impaired when p53 is added (Fig. 9). These data suggest a competition between PML and p53 for the same binding site on Ankrd2; however this point needs to be studied in more detail.

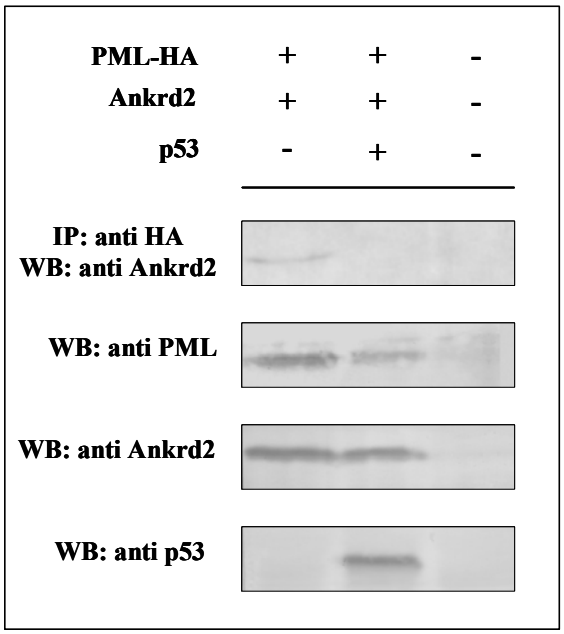


Figure 9: Co-immunoprecipitation. PML and Ankrd2 interact independently by p53. Saos-2 cells (p53-/-) were transfected respectively with PML-HA+Ankrd2 (first column) or with PML-HA+Ankrd2+p53 (second column). After immunoprecipitation of PML with an anti HA antibody (Santa Cruz), the sample was tested with an anti Ankrd2 monoclonal antibody in order to determine the occurrence of the interaction. It is clearly visible that Ankrd2 and PML interact in the absence of p53, whereas when p53 is also present in the cells the interaction is not occurring (first row). In the third column the data relative to non transfected cells are shown. In second, third and fourth row controls of transfections are shown relative to PML, Ankrd2 and p53 respectively.

3.10 Ankrd2 enhances p53 mediated transactivation of the p21 promoter

To evaluate if the interaction between Ankrd2 and p53 has a functional consequence, the effect of this binding was studied using a p53 responsive promoter cloned upstream of the firefly luciferase reporter gene: the p21 $^{WAFI/CIPI}$ promoter.

Co-expression of wild type p53 with increasing amounts of Ankrd2 (125 ng-1000 ng) leads to an enhancement of the p53-mediated transactivation of the p21 promoter in Saos-2 (p53-/-, pRb -/-) cells, suggesting that Ankrd2 might act as an enhancer of p53 activity.

Similarly, co-expression of wild type p53 with YB-1 (1000 ng) also produced an increase of the p21 promoter activation and in particular if Ankrd2 is also present (1000 ng) this enhancement appears to be augmented, suggesting an additive effect of YB-1 and Ankrd2 on p53 transcriptional activity (Fig. 10).

These results are in agreement with the work of Okamoto and colleagues who considered YB-1 as a positive regulator of p53 (Okamoto et al. 2000).

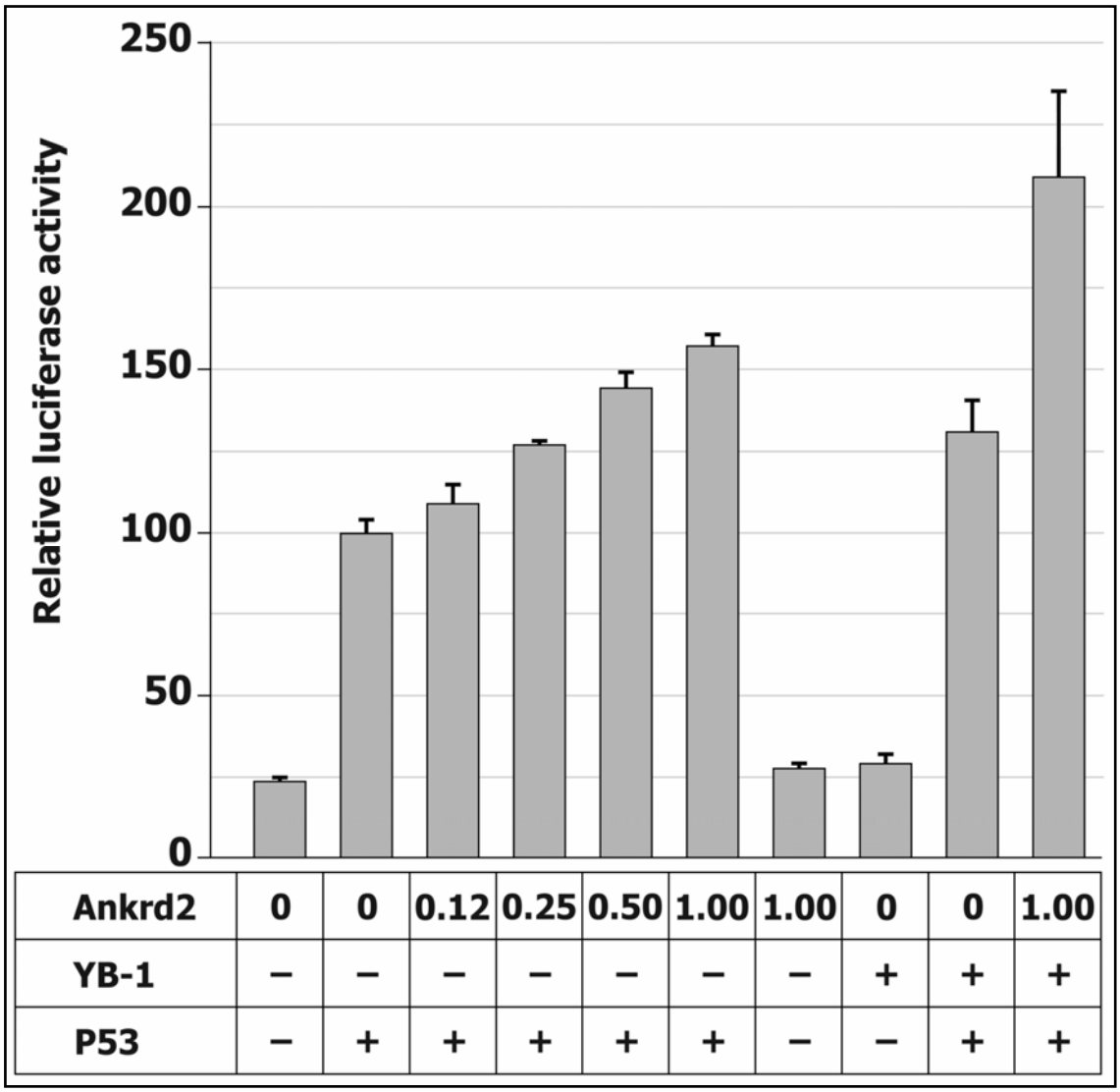


Figure 10: Ankrd2 enhances p53-mediated transactivation of the p21 promoter. The p53- mediated transactivation of the p21-luciferase reporter gene (1 μg) was assayed by transfecting Saos-2 cells with p53-pcDNA3 (10 ng) either alone or with YB-1-pcDNA3 (1 μg) or Ankrd2-pcDNA3 (from 0.125 to 1 μg) as shown in the histogram. The amount of DNA used in all of the transfections was kept constant by the addition of pcDNA3 without insert. The cells were co-transfected with the *Renilla* luciferase reporter gene (pRL-CMV; 0.05 μg) as a control. The *Firefly* luciferase was normalized against the *Renilla* luciferase using the Dual-Luciferase Reporter Assay System. The histogram shows the mean of at least three independent experiments; the bars indicate the standard deviation.

3.11 Ankrd2 enhances the p53 mediated transactivation of the MDM2 promoter

In normal growing cells, p53 activity needs to be tightly regulated and in fact in normal conditions the half life of the p53 tumor suppressor protein product is quite short. MDM2 is one of the major regulators of p53 activity, since it is an E3 ubiquitin ligase that by binding to the N-terminal p53 transactivation domain attaches ubiquitin molecules to the p53 protein, thus targeting it for proteasome degradation. However, even if MDM2 is a p53 antagonist, it also is a p53 induced gene. Therefore p53 and MDM2 are linked by an autoregulatory loop (Barak et al. 1993, Wu et al. 1993). For this reason we wanted to verify if Ankrd2 can act by modifying the transactivation activity of p53 on its antagonist's promoter (MDM2-promoter), thus as a result altering the stability of the p53 protein.

The experiment was set up using a similar protocol to that for p21 promoter transactivation assay, this time using the MDM2 promoter cloned upstream of the *Firefly* luciferase as a reporter gene. Saos-2 cells were transfected with p53-pcDNA3 (10-50 ng) alone or in conjunction with Ankrd2-pcDNA3 (10-100 ng). Luciferase activity was measured and normalized against the *Renilla* luciferase using the Dual-Luciferase Reporter Assay System. From the results (Fig.11) it can be seen that Ankrd2 is also able to enhance the p53 transactivation activity on the MDM2 promoter. This is particularly evident when 10 ng of p53-pcDNA3 are used to transfect cells, since the curve relative to promoter activation is still exponential and not in the plateau region, thus enabling any change in the promoter activity to be noted.

To exclude false positives, we also set up an experiment with p53 mutants deleted in the N-terminal region which contains both the p53 transactivation domain and the MDM2

binding site. No luciferase activity was detected with the p53 N-terminal deletion mutants on MDM2 promoter, indicating that in these experiments the N-terminal of p53 is necessary for transactivation (data not shown).

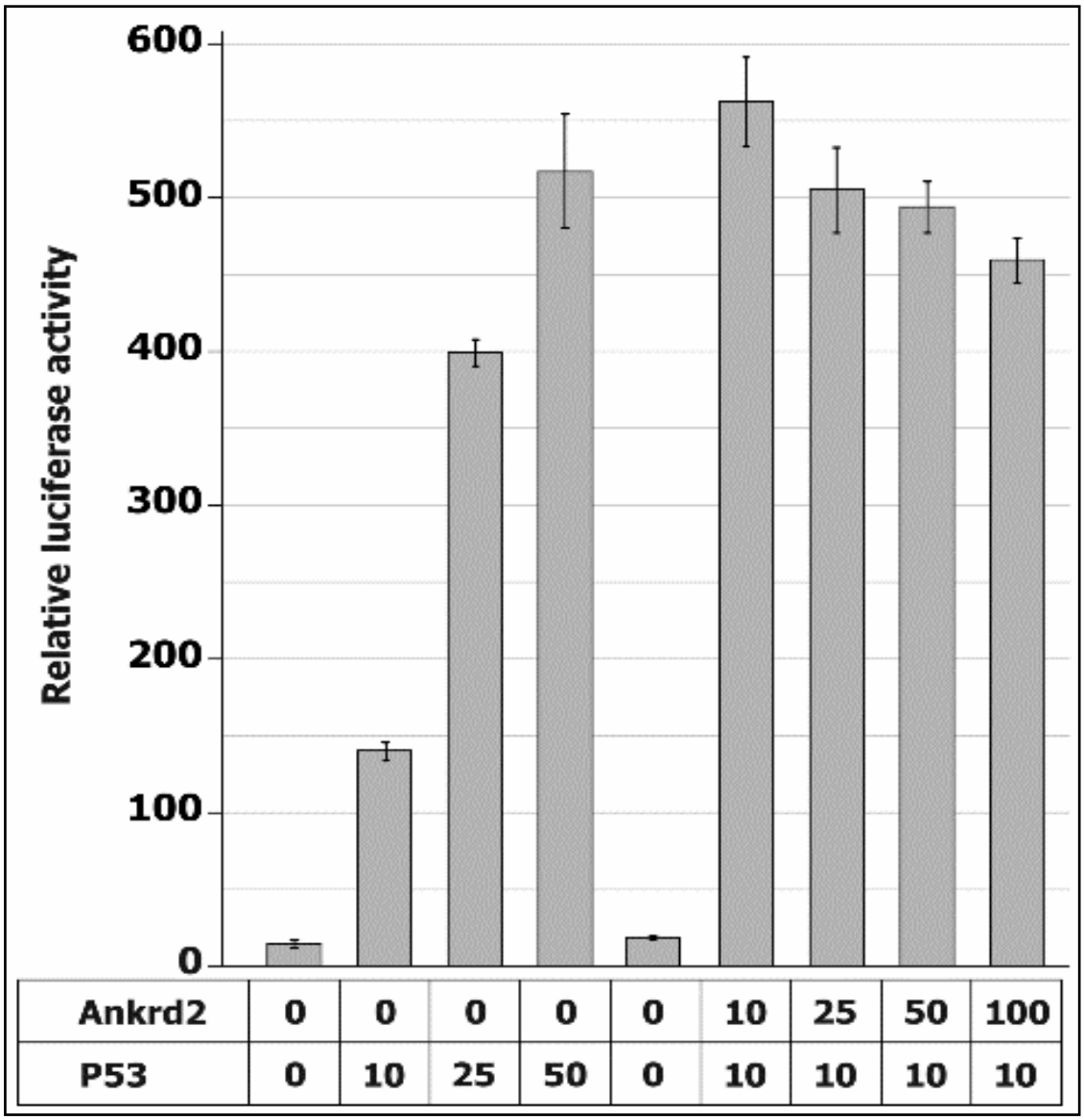


Figure 11: Ankrd2 enhances the p53-mediated transactivation of the Mdm2 promoter. The Saos-2 p53-/- cell line was transfected with the MDM2-promoter-Luc (300 ng) as a reporter gene and with different amounts of p53-pcDNA (10 ng-50 ng) alone or in combination with Ankrd2-pcDNA3 (10 ng-100 ng) as shown in the picture. The cells were also co-transfected with the *Renilla l*uciferase plasmid as a control for transfection efficiency and the firefly luciferase activity was normalized against it using the Dual-Luciferase Reporter assay.

From the histogram is evident that Ankrd2 can enhance the p53-mediated transactivation activity of the MDM2 promoter.

3.12 Ankrd2 binds the Z-line protein Telethonin *in vivo* and *in vitro*

Telethonin (T-Cap) is a 19 kDa Z-line protein, which binds titin (Gregorio et al. 1998), FATZ (Faulkner et al. 2000), minK (Furukawa et al. 2001) and myostatin (Nicholas et al. 2002) and which is mutated in Limb Girdle Muscular Dystrophy type 2G (Moreira et al. 2000).

We found that Ankrd2 is able to bind Telethonin both *in vivo*, by Co-IP experiments and *in vitro* by GST-overlay assay and *in vitro* binding experiments.

For Co-IP COS-7 cells were transfected with Myc-Ankrd2 either alone or with HA-Telethonin. Cell lysates of untransfected and transfected COS-7 cells were immunoprecipitated with antibody to the HA-tag (Santa Cruz) subjected to SDS-PAGE, immunoblotted and probed with antibody to Ankrd2. There was no cross-reaction between the anti-HA antibody and the proteins in the cell lysates other than HA tagged telethonin (Fig. 12a).

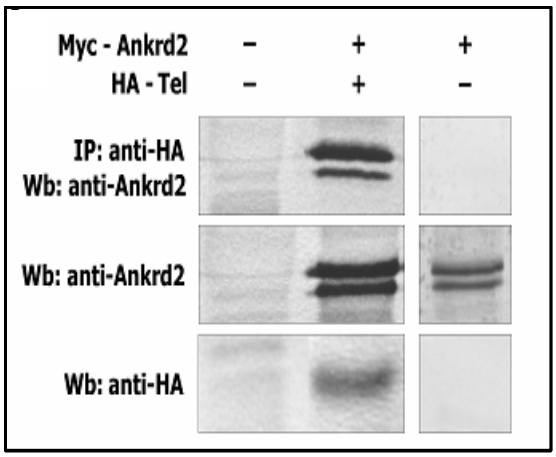


Figure 12a: Co-IP of Ankrd2 and Telethonin from lysates of untransfected COS-7 cells (first column) and cells transfected with cMyc-Ankrd2-pCMV and HA-Telethonin-pcDNA3 (second column) or with cMyc-Ankrd2-pCMV alone (third column). Cell lysates were immunoprecipitated with anti HA mouse monoclonal antibody and then separated by SDS-PAGE and immunoblotted using anti Ankrd2 mouse monoclonal antibody (first row). Cell lysates were immunoblotted using anti Ankrd2 antibody (second row) and anti HA antibody (third row).

The same result was obtained from *in vitro* binding experiments using *in vitro* transcribed and translated (IVTT) Telethonin labelled with [^{35}S] and a GST-Ankrd2 recombinant protein still bound to glutathione-Sepharose 4B (Fig. 12b).

In experiments using cell lysates and IVTT protein the presence of intermediary proteins taking part in the binding interactions cannot be excluded. However, in the GST overlay assay the binding appears to be direct, since there are no other proteins present. In this experiment we have used His-Telethonin recombinant protein subjected to SDS-PAGE and than blotted onto a PDVF membrane and GST-Ankrd2 recombinant protein as the overlay protein. As a control we used the GST protein alone. The detection was performed with an anti GST antibody (Amersham) (Fig. 12c).

The interaction Telethonin-Ankrd2 is very exciting since Telethonin, MLP and Titin form a stretch sensor complex that could function by sensing stretch from the membrane and sending a signal towards the nucleus (Knöll et al. 2002).

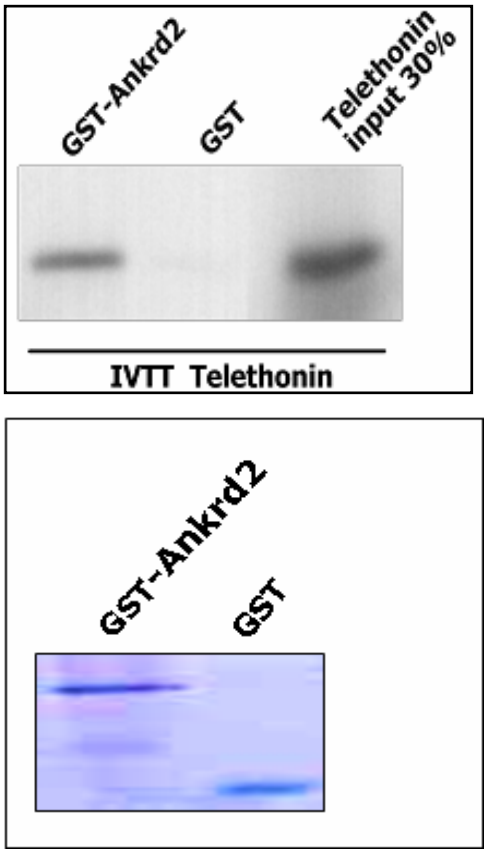


Figure 12b: Binding of GST-Ankrd2 and IVTT ^{35}S Telethonin. Upper panel: GST proteins (GST, GST-Ankrd2) bound to glutathione-Sepharose 4B were incubated for three hours at room temperature with IVTT ^{35}S Telethonin, washed and subjected to SDS-PAGE. The last column shows 30% of the total amount of radiolabeled IVTT-Telethonin used in each binding reaction. In the **lower panel** a blue Coomassie staining shows that equal quantities of GST-Ankrd2 and GST were used in the experiment.

Figure 12c: GST overlay assay. His-telethonin was loaded and separated by SDS-PAGE, then blotted on a PDVF membrane and incubated with GST (second column) or GST-Ankrd2 (third column). After that the membrane was washed and incubated with anti GST goat polyclonal antibody (Amersham). A band indicating that a binding took place appeared (third column), whereas the GST control was clean. Additionally also His-Telethonin alone was incubated with anti GST antibody to exclude possible cross-reactions (first column).

The bottom panel shows that equal amounts of His-Telethonin have been loaded. The western blot has been performed with an anti histidine mouse monoclonal antibody (QIAGEN, 1:2000 dilution).

3.13 The first 38 amino acids of Telethonin are not essential for Ankrd2 binding

In order to map which regions of Ankrd2 and Telethonin are involved in the binding, we have carried out co-immunoprecipitation experiments using the cMyc-Ankrd2 and a truncated HA-Telethonin protein lacking the first 38 amino acids (HA-ΔN-Telethonin). HA-ΔN-Telethonin was precipitated with an anti-HA mouse monoclonal antibody (Santa Cruz), subjected to SDS-PAGE and blotted. Then the membrane was probed for the presence of cMyc-Ankrd2 using an anti Ankrd2 polyclonal antibody. From the results of this assay it would appear that Ankrd2 binds HA-ΔN-Telethonin, indicating that the first 38 amino acids are not essential for the interaction (Fig. 13).

The co-immunoprecipitation has also been repeated using Flag-Ankrd2 and HA-ΔN-Telethonin, precipitating with anti HA and probing the blot with an anti Flag rabbit polyclonal antibody (SIGMA) obtaining the same result (data not shown).

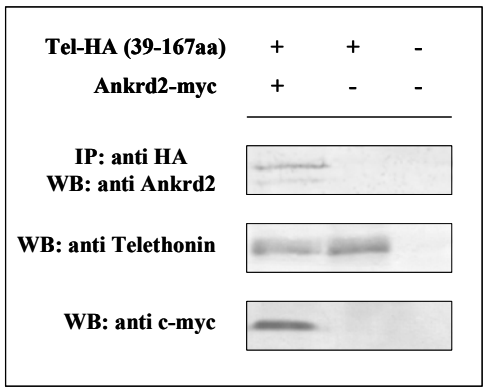


Figure 13: Co-immunoprecipitation. The first 38 amino acids of Telethonin are not needed to bind Ankrd2. COS-7 cells were transfected with a Telethonin deletion mutant lacking the first 38 amino acids (ΔN-Telethonin) (first column) with an HA tag and with Ankrd2-c-myc or with ΔN-Telethonin alone (second column). In the third column data relative to non transfected cells are shown. ΔN-Telethonin was immunoprecipitated with an anti HA antibody (Santa Cruz). After SDS-PAGE separation and immuno blotting with an anti Ankrd2 monoclonal antibody an interaction between the two proteins is still visible (first row). In second and third row control western blots for transfections are shown.

3.14 Do other possible isoforms of Ankrd2 exist?

Until now only one 37 kDa Ankrd2 protein (running around 42 kDa after SDS-PAGE separation) was thought to exist. Nevertheless recently two groups have reported that a higher molecular weight Ankrd2 isoform visible at 55 kDa after SDS-PAGE separation is detectable in mouse skeletal muscle and heart, in human heart (Mckoy et al. 2005) and in rat heart tissue (Miller et al. 2003). Also from the UCSC Genome Browser Database (www.genome.ucsc.edu) is evident that the human Ankrd2 gene has two other putative ATG start codons upstream of the ATG (*) from which the transcription of the 37 kDa form starts and that alternative mRNAs have also been found.

The furthest ATG (*) is positioned 339 bp upstream of ATG* and could give origin to a longer Ankrd2 isoform with an extension in the N-terminal region of 118 amino acids. This isoform has a predicted molecular weight of about 50 kDa and probably is the protein detected by different groups as a 55 kDa band on SDS-PAGE. We have analyzed the additional N terminal part with domain prediction programs such as Pfam and SMART but this region dose not seem to contain any known binding motifs.

The ATG in the middle (*) is 81 bp upstream of the normal ATG (*) and it also extends the N terminal part of the protein, but only by 27 amino acid residues. The predicted molecular weight of this form is 36 kDa, since from database information it appears to lack exon 7 thus reducing its molecular weight therefore in electrophoretic separation it would not be easily distinguishable from the 37 kDa form. Thus surprisingly this form originating from the intermediate ATG (*) is probably alternatively spliced, since it lacks the exon 7. This is particularly interesting, since exon 7 encodes an ankyrin repeat motif and ankyrin repeats are domains known to be involved in protein-protein

interactions. Thus it is feasible that the absence of this ankyrin repeat could eliminate the binding of particular proteins (Fig. 14).

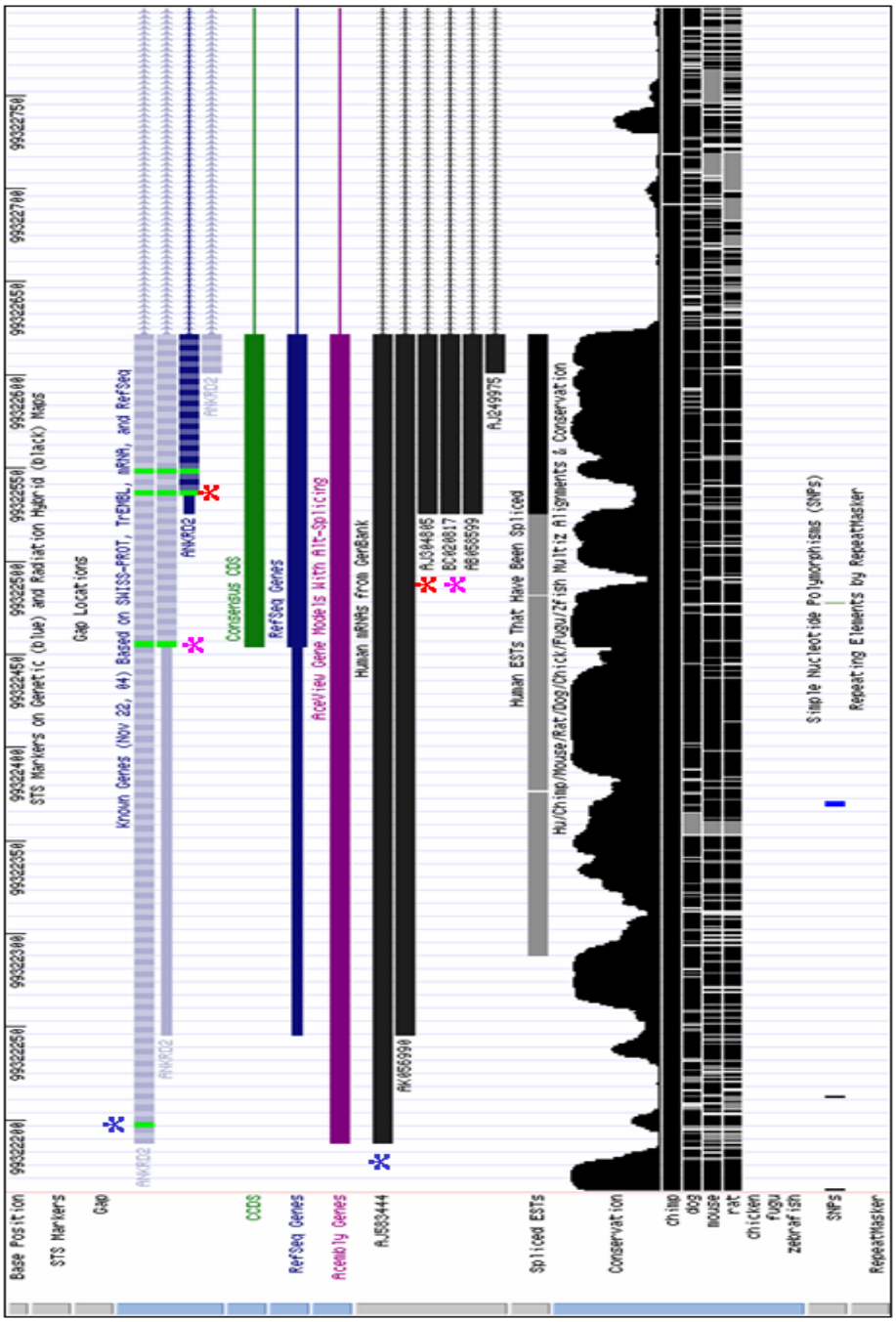


Figure 14: UCSC Genome Browser Database web page showing the human Ankrd2 gene with putative ATGs (in green) and the different Ankrd2 mRNAs originating from them. * indicates the furthest ATG giving origin to a longer Ankrd2 protein; * indicates the ATG in the middle from which starts the transcription of the Ankrd2 form lacking exon 7; * indicates the "classic ATG" from which originates the 37 kDa Ankrd2 form.

3.15 Looking for Ankrd2 isoforms by Western Blot Analysis

In order to determine the expression pattern of the Ankrd2 isoforms we performed a set of western blots on adult human tissues and on human skeletal muscle (CH5QB) cell nuclear and cytoplasmic extracts both in their undifferentiated state and after 7 days of differentiation with medium containing 0.4% of ultroser G.

Ankrd2 was detectable only in skeletal muscle, heart and kidney, and then the only isoform visible was the "classic" Ankrd2 (42 kDa) when nine different adult human tissues tested using an anti Ankrd2 mouse monoclonal antibody. However, after an accurate observation it would appear that in the adult skeletal muscle tissue after SDS-PAGE resolution two distinct bands very close one to the other are visible, that at a first glance seem to be just one. The meaning of these two bands it is not clear at the moment, but two explanations seem plausible: either they are two different post-translationally modified Ankrd2 forms, or that one band is the "normal" Ankrd2 (42 kDa) and that the lower band is the form lacking the exon 7. Unfortunately we cannot distinguish between Ankrd2 and the alternatively spliced form, since our antibodies recognize epitopes shared by both isoforms (Fig. 15a).

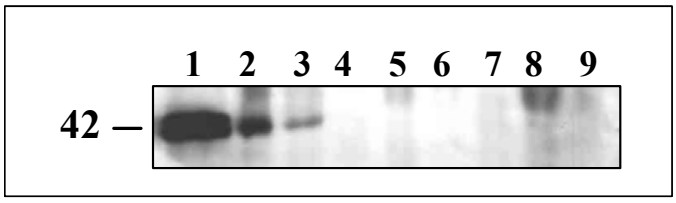


Figure 15a: Ankrd2 isoform expression in adult human tissues. Western blot analysis on nine different adult human tissues (Clontech) using an anti Ankrd2 mouse monoclonal antibody: 1) Skeletal Muscle; 2) Heart; 3) Kidney; 4) Liver; 5) Lung; 6) Brain; 7) Spleen; 8) Testis; 9) Ovary. As we expected Ankrd2 is mainly expressed in skeletal muscle and heart and to a lower extent in kidney.

From analyses of extracts of human skeletal muscle (CHQ5B) cells using a monoclonal antibody against Ankrd2, I observed that there is a form of Ankrd2 that migrates at about 55 kDa on SDS-PAGE. This "long" form is present only in the nuclear fractions of both undifferentiated (myoblasts) and differentiated (myotubes), whereas the "classic" Ankrd2 form, migrating about 42 kDa, is visible only in the cytoplasmic fraction of differentiated cells (Fig. 15b).

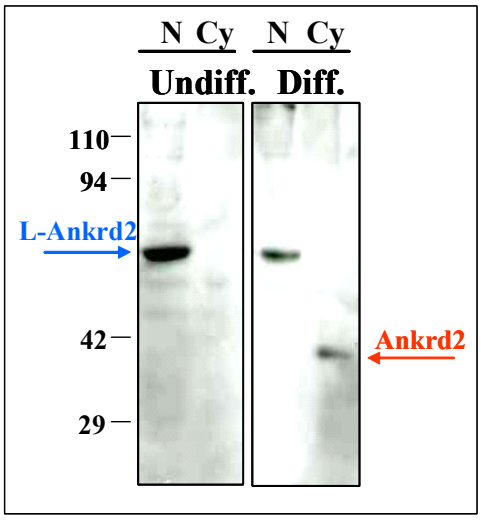


Figure 15b: Distribution of Ankrd2 isoforms in human skeletal muscle (CHQ5B) cell extracts both undifferentiated and after 7 days of differentiation. The long Ankrd2 (L-Ankrd2) form (55 kDa) is present only in the nuclear extracts (N) and it is indicated by the blue arrow, whereas "normal" Ankrd2 (42 kDa) is detectable only in the cytoplasmic extract of differentiated cells and it is indicated by the red arrow. For this experiment an anti Ankrd2 mouse monoclonal antibody was used.

On the whole these data suggest that the "normal" Ankrd2 protein is prevalently expressed in adult striated muscle tissues and in differentiated myotubes and that it is cytoplasmic, whereas the "long" isoform is visible only in the nuclei of both undifferentiated and differentiated cells.

One hypothesis could be that the Ankrd2 gene is subjected to developmental regulation and that different protein isoforms may act in different cellular compartments at different times during myogenesis.

Moreover we can speculate that the Ankrd2 isoform that binds PML and localizes in the NBs is the longer one.

3.16 Looking for Ankrd2 isoforms by Real Time RT-PCR analysis

In collaboration with the CRIBI laboratory of Prof. G.Valle at the University of Padua, we decided to perform a Real Time RT-PCR analysis on the messenger RNA from skeletal muscle and heart both adult (Ambion) and foetal (Invitrogen), in order to detect and quantify the different putative Ankrd2 isoforms with respect to the "normal" one. The house keeping GAPDH mRNA was amplified as a normalisation control in all of the reactions.

Unfortunately we were not able to amplify the long form (L-Ankrd2) of Ankrd2 from any of the tissues used. The reason for this is unknown and may reflect the fact that I have detected this form only in nuclear extracts from skeletal muscle (CHQ5B) cells originally obtained from Dr V. Mouly as isolated satellite cells from the quadriceps of a newborn child (5-d postnatal).

However the Ankrd2 form lacking the exon 7 (M-7 Ankrd2) has been detected both in adult and foetal skeletal muscle and heart, but it seems to be more abundant in the cardiac tissue. Surprisingly another alternatively spliced isoform that lacks both exon 7 and 8 (M-7/8 Ankrd2) has also been amplified from adult and foetal skeletal muscle. However it is important to underline that these alternatively spliced isoforms are expressed at very low levels if compared to the "normal" Ankrd2.

These data are very preliminary and more investigation is needed. Therefore at present we can just speculate that differently spliced Ankrd2 transcripts have distinct biological functions during development and in different cellular compartments.

In the following picture (Fig. 16) all of the putative Ankrd2 isoforms detected to date are indicated.

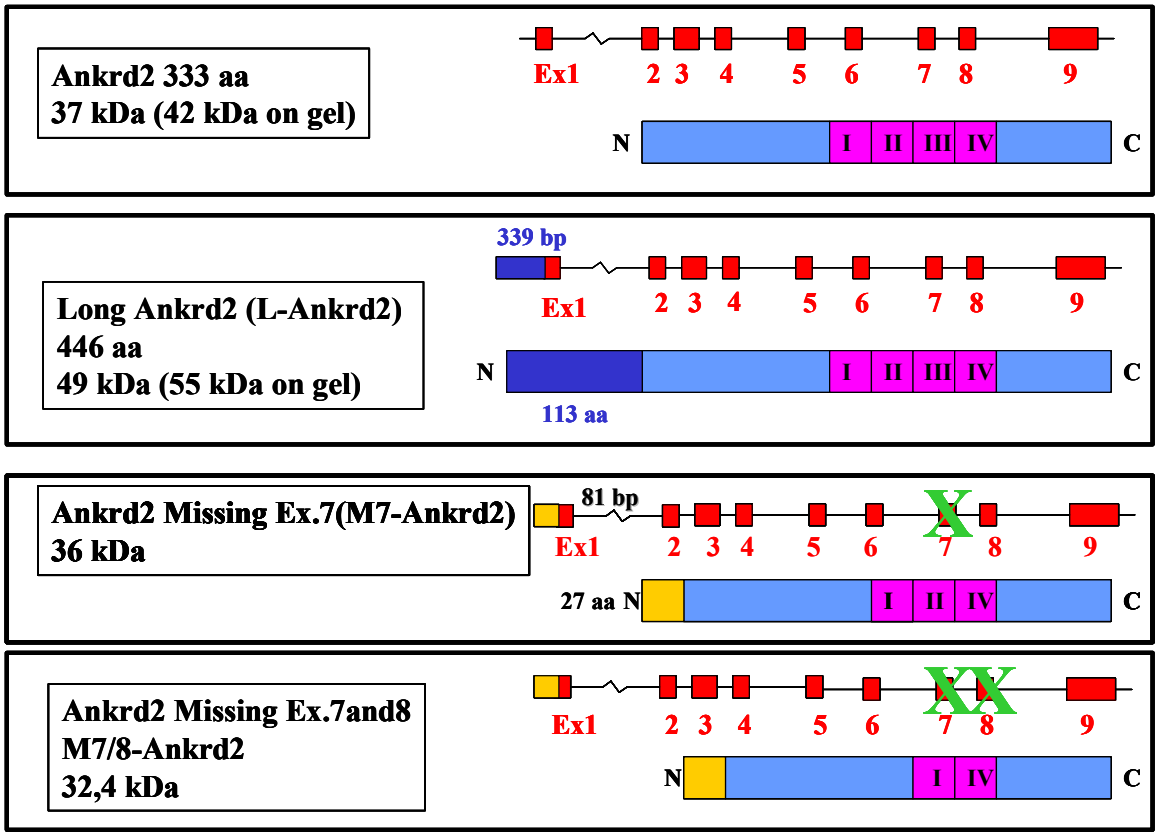


Figure 16: Ankrd2 isoforms. Four different Ankrd2 isoforms have been detected to date using both western blot analysis, real time PCR and informatic analysis. In this diagram are shown the genes (with the exons) and the relative protein products.

Chapter 4

DISCUSSION

The sarcomere is the basic contractile unit of striated muscle and its boundaries are defined by Z-lines. Z-lines are also the anchoring sites for thin, Titin and Nebulin filaments and hence they constitute the primary conduits of the force generated by contraction. The Z-line is a complex structure composed of a large number of proteins linked together by a dynamic network of interactions. However, even if many of the interactions occurring in the Z-line are known, the overall organization and the processes in which Z-line proteins participate are still not very clear.

Recently the concept that the Z-line has an important role in signalling and in muscle homeostasis not just as the structural border of the sarcomere has gained strength due to the numerous signal transduction components that have been localized in this region. Moreover a large number of Z-line associated proteins have been noted to have a dynamic distribution and shuttle between the Z-line and other subcellular compartments to transmit signals (reviewed in Faulkner et al. 2001, Clark et al. 2002). For example the transcription factor NF-AT3 exhibits dual localization at the Z-line and nucleus of skeletal muscle. NF-AT3 is probably tethered to the Z-line through its interaction with calcineurin, a Ca^{2+}-dependent phosphatase of the hypertrophy pathway that can also dephosphorylate NF-AT3 allowing it to enter the nucleus (Horsley and Pavlath 2002).

Also the muscle ankyrin repeat protein Ankrd2 shows dual localization in skeletal muscle myoblasts. In fact despite Ankrd2 being localized in the sarcomeric I-band, it is also present in the nucleus of myoblasts (Pallavicini et al. 2001, Tsukamoto et al. 2002). For this reason it is thought to be a factor that shuttles between these two cellular compartments in order to transmit a signal and regulate muscle cell response to environmental changes such as stress, load requirements or stretch. Therefore it was reasonable to hypothesize that Ankrd2 has one or more binding partners in the sarcomere and some others in the nuclear compartment.

NF-κB is another example of a factor that moves from the cytoplasm towards the nucleus to activate the transcription of specific genes. Ankrd2 displays a striking sequence and structural similarity with IκB, the specific NF-κB inhibitor, since they share both ankyrin repeats and PEST sequences (Pallavicini et al. 2001). NF-κB is kept in the cytoplasm in the inactive state by IκB. In response to CKII phosphorylation of IκB a cascade of events is triggered leading to IκB degradation followed by the release of NF-κB. Thus the active form of NF-κB translocates to the nucleus. There is also some evidence that IκB could enter the nucleus (Verma et al. 1995).

Based on this knowledge I decided to test if the Ankrd2 protein could behave like IκB by exerting an inhibitory effect on NF-κB binding to its DNA consensus sequence. From the band shift experiments that I undertook it is clear that Ankrd2 does not interfere with the capacity of NF-κB to bind DNA (see Results, Paragraph 3.1, Fig. 2) (unpublished data), thus Ankrd2 is not able to substitute the function of IκB.

However these results do not exclude the possibility that Ankrd2 may be regulated in a similar way to NF-κB or NF-AT3 by some factor that keeps it inactive in the cytoplasm in order to release it under specific conditions, after which Ankrd2 could enter the

nucleus. Ankrd2 is up-regulated on muscle cell differentiation and is almost completely cytoplasmic in the multinucleated myotubes (Pallavicini et al. 2001) therefore it is possible that there is a temporal regulation of Ankrd2 activity by an unknown factor that sequesters the protein in the cytoplasm in fully differentiated cells. Since Ankrd2 is visible only in the nuclei of undifferentiated myoblasts (Pallavicini et al. 2001) it is reasonable to deduce that the protein could be active during the early stages of muscle development.

Ankrd2 is also highly homologous to the Cardiac Ankyrin Repeat Protein (CARP) (Pallavicini et al. 2001, Moriyama et al. 2001). They have been grouped together into a conserved gene family called MARPs (Muscle Ankyrin Repeat Proteins) that also includes another member named DARP. The MARPs are all induced by different types of stress: Ankrd2 by stretch or denervation, CARP by injury and hypertrophy and DARP during recovering following starvation (Miller et al. 2003).

Amongst these proteins CARP is the better characterized and is also the most similar to Ankrd2 in sequence, structure and in its dual localization both in the I-line and in the nucleus. Moreover CARP forms a physical complex with the ubiquitous transcription factor YB-1, thus acting as a negative regulator of the HF-1 dependent pathway for ventricular gene expression. In fact YB-1 is a positive regulator of the HF-1 element of the ventricular light chain 2 (MLC-2v) promoter and is sequestered and inhibited by its interaction with CARP (Zou et al. 1997). I have shown that Ankrd2 is also able to interact with YB-1 (Results, Paragraph 3.2) and that the binding region is located in the N-terminal of Ankrd2 (Results, Paragraph 3.3) (Kojić et al. 2004) and in the C-terminal of YB-1 (Results, Paragraph 3.4) (unpublished data). Where this interaction takes place and its biological meaning is not known at present, but it is possible to speculate that

Ankrd2 and YB-1 act synergistically to regulate the expression of specific genes. In fact it is intriguing that even if YB-1 is an ubiquitous transcription factor, it is preferentially expressed in the heart during early development and in the dermomyotome where it probably controls the skeletal muscle gene program (Zou et al. 1997) and that Ankrd2 is found not only in skeletal muscle but also in specific heart compartments such as the ventricles, the inter-ventricular septum and the apex (Moriyama et al. 2001). An exiting observation is that the ventricular myosin light chain 2 gene (MLC-2v) has the same pattern of expression of Ankrd2: right and left ventricles and slow skeletal muscle fibres (Zou and Chien 1995). Hence it is plausible that YB-1 and Ankrd2 control the specific gene programs that regulate skeletal muscle and heart ventricular development through MLC-2v expression. Furthermore YB-1 also shuttles between the cytoplasm and the nucleus and it does it in a cell cycle related way, since it enters the nucleus only during G1/S phase transition (Jurchött et al. 2003). At the moment it is not known if also Ankrd2 localization depends on the cell cycle, but it is feasible that the interaction YB-1/Ankrd2 takes place in the nucleus in this precise phase of the cell cycle. If this supposition is true, YB-1 could represent the factor that brings Ankrd2 into the myoblast nucleus in G1/S phase and that localises with it in the cytoplasm of myotubes blocked in G1. Of course several experiments are needed to confirm this hypothesis.

Another intriguing characteristic of YB-1 is its direct interaction with the tumor suppressor protein p53 (Okamoto et al. 2000). The same authors also reported that YB-1 positively regulates p53 transactivation activity on the p21 promoter (Okamoto et al. 2000), similar to what we have shown (Results, paragraph 3.10) (Kojić et al. 2004), whereas others affirm that YB-1 is a negative p53 regulator and that interestingly

controls in the opposite way the expression of many p53 induced genes, such as *mdr1* and *c-fas* (Lasham et al. 2003).

I have demonstrated that Ankrd2 is involved in interactions with p53 (Results, Paragraph 3.8) and that it can also enhance the p53 transactivation activity both on the p21- (Kojić et al. 2004) and on the MDM2-promoter (unpublished data) (Results, Paragraphs 3.10 and 3.11). The relevance of this interaction is underlined by the involvement of p53 in the process of skeletal muscle differentiation. In fact beside its "classical" role as a tumor suppressor protein increasing evidence indicates that p53 has a crucial role in some differentiation processes, such as haematopoiesis (Kastan et al. 1991), spermatogenesis (Almon et al. 1993) and especially myogenesis (Almong and Rotter 1997). p53 is essential for skeletal muscle cells differentiation, since C2C12 mouse myoblasts in which a dominant negative p53 form was present failed to differentiate (Soddu et al. 1996). An apparent contradiction represented by the normal development of the p53 knock out mouse is easily explained by the compensatory effect of the p53 family members p63 and p73 that substitute for p53 during all the developmental stages in the animal (Tamir and Bengal 1998, White et al. 2002). However, even if p53 is essential for myogenesis it is not need to withdraw from the cell cycle and in order to induce high p21 levels, since MyoD itself can up-regulate the expression of p21 and force the cell to block G1 phase (Halevy et al. 1995). Only recently it was clarified that p53 is essential for an increase in the level of the hypo-phosphorylated form of the retinoblastoma protein (pRb), which is fundamental for cell cycle withdrawal. In addition pRb cooperates with MyoD to induce the transcription of skeletal muscle specific genes (Porrello et al. 2000). Moreover MyoD itself can induce pRb expression in association with CREB/p300 (Magenta et al. 2003). Thus the skeletal

muscle differentiation process appears to be a very complex system in which several unexpected components take part. Although the functional consequences of the binding between Ankrd2 and p53 are not clear it allows speculation about a role for Ankrd2 in myogenesis, since Ankrd2 has recently been found to be induced by MyoD (Bean et al. 2005). Since Ankrd2 increases the transactivation activity of p53 on the p21- and of MDM2-promoters (Results, Paragraph 3.10), it could also indirectly through p53 enhance the transcription of pRb hence allowing the cell to proceed through the process of differentiation.

MDM2 is the main p53 antagonist, since through its E3-ligase activity it targets p53 for rapid degradation in the 26S proteasome. Despite this, MDM2 itself is a p53-induced gene. Hence these two molecules are linked to each other in an auto regulatory feedback loop aimed at maintaining low cellular p53 levels in the absence of stress (Barak et al. 1993, Wu et al. 1993). The binding of p53 with Ankrd2 seems to lead to an increased MDM2 transcription (Results, Paragraph 3.11) (unpublished data), that should result in greater degradation of p53. What this means is not clear at the moment. However it is relevant that MDM2 exerts an inhibitory role in the process of skeletal muscle differentiation, since MDM2 amplification in rhabdomyosarcoma interferes with MyoD activity and consequently inhibits muscle cell differentiation (Fiddler et al. 1996). Moreover MDM2 competes with pRB for binding the transcription factor Sp1 and since pRb binding to Sp1 is necessary to induce the expression of muscle specific genes, MDM2 represents an inhibitor of skeletal muscle differentiation (Yap et al. 1999).

Surprisingly several proteins involved in skeletal muscle differentiation are also present in PML nuclear bodies: MDM2 (Gostissa et al. 2003), p53 (Fogal et al. 2000), pRb (Alcalay et al. 1998) and p300 (von Mikecz et al. 2000). PML nuclear bodies (PML

NBs) are alternatively called PML oncogenic domains (PODs), nuclear domains 10 (ND10) or Kremer bodies and they are distinct nuclear structures with a diameter of 0.2-1 μm present in almost all mammalian cell types. Each cell typically contains 10-30 PML NBs even if their number, size and composition change during the cell cycle (reviewed in Zhong et al. 2000). PML is the major component of NBs and it also regulates their composition, organization and function (Dyck et al. 1994, Lallemand-Breitenbach et al. 2001). It also needs to be SUMO-modified in order to assemble into NBs. Many different roles have been ascribed to NB's for instance DNA repair, growth regulation, apoptosis, senescence, transcriptional regulation and at last nuclear depot. In immunofluorescence microscopy PML NBs give the appearance of speckles distributed throughout the nuclei. Also Ankrd2 shows this typical nuclear pattern especially in human myoblasts (Pallavicini et al. 2001). Further experiments revealed that this nuclear pattern is visible in a large number of myoblasts (40-60%) and that the percentage of these nuclear structures progressively decreases during differentiation and becomes very low (1% or less) in totally differentiated human skeletal muscle myotubes. Therefore I wondered if Ankrd2 was localized in PML NBs and from double immunofluorescence analysis on human myoblasts (CH5QB) I found a perfect co-localization of Ankrd2 and PML in these nuclear structures (Results, Paragraph 3.5). No co-localization occurs in myotubes between Ankrd2 and PML except in the few cases where Ankrd2 is present in the NBs of these cells (1%) (Kojić et al. 2004). These observations are very exiting since they support the idea of Ankrd2 as a nuclear co-factor. At this point it was reasonable to verify if Ankrd2 and PML were interacting or not. The binding between these two proteins was detected and confirmed using different techniques (Results, Paragraph 3.7) (Kojić et al. 2004). In all experiments the PML

isoform IV (PML 3) was employed, since this is the most abundant isoform present in the nuclear bodies and it also recruits p53 into the nuclear bodies (Fogal et al. 2000). PML and MDM2 compete for the same binding sites on p53, so when PML binds p53 in the NBs it directly inhibits MDM2 binding hence p53 degradation. This should result in an increased stability of the p53 tumor suppressor protein (Kurki et al. 2003, Zhu et al. 2003). Probably p53 is recruited into PML nuclear bodies and its activity is then tightly modulated by PML IV and MDM2 levels. The presence of Ankrd2 in these structures opens the possibility that Ankrd2 is recruited by PML to be regulated or to be kept in contact with other factors that participate in the process of myogenesis, such as p53 or pRb and maybe to regulate transcription. Actually all these proteins interact with each other affecting their function and stability and often competing for the same binding sites. For example MDM2 binds both p53 and pRB in a trimeric complex. When both p53 and pRb are linked to MDM2 the p53 tumor suppressor is stabilized, whereas when only p53 and MDM2 interact, p53 is rapidly degraded (Yap et al. 1999). At present the regions of Ankrd2 involved in binding p53 and PML as well as the domains of p53 and PML that bind Ankrd2 are unknown. However I have observed that not only the binding between Ankrd2 and PML can occur in the absence of p53 but that this interaction is blocked in the presence of p53 (Results, Paragraph 3.9) (unpublished data). This could mean that Ankrd2 and p53 compete for the same site on PML or that p53 sequesters either Ankrd2 or PML proteins thus preventing interaction between them. However since to date the domains involved in these bindings have not been determined is not possible to answer these questions.

SUMO-1 is an ubiquitin-like protein that covalently binds PML. As I have already mentioned SUMO-modified PML is essential for nuclear body assembly, since only the

SUMOylated form of PML is found in these structures, whereas un-modified PML localizes in the nucleoplasm. Thus SUMOylation is thought to target protein to specific cellular compartments and moreover some PML NBs components such as Sp100 and p53 are SUMO-modified (Gostissa et al. 1999, Seeler and Dejean 2001, Hay 2005). For this reason I tested by immunofluorescence if Ankrd2 and SUMO-1 co-localize in PML NBs in the nuclei of human skeletal muscle myoblasts (CHQ5B) and I observed only a partial co-localization of Ankrd2 and SUMO-1 in these NBs. This suggests that probably Ankrd2 does not need to be modified by SUMO-1 in order to be targeted to PML nuclear bodies. However at the moment we cannot definitely exclude that Ankrd2 is not SUMO-modified (Results, Paragraph 3.6) (unpublished data).

Until now I have discussed a set of Ankrd2 interactions, which most probably occur in the nucleus or perhaps in the PML NBs. Nevertheless, as I have already said, Ankrd2 can also be found in the I-line, at the Z-line boundaries. Due to its dual localization pattern Ankrd2 could be considered a protein that shuttles between the sarcomere and the nucleus, possibly transmitting a signal.

We looked for sarcomeric partner/partners of Ankrd2 and identified the Z-line protein Telethonin as an Ankrd2 binding protein (Results, Paragraph 3.12) (Kojić et al. 2004). Telethonin (T-Cap) mRNA is one of the most abundant in skeletal muscle and the Telethonin protein is localized in the Z-line (Valle et al. 1997, Mues et al. 1998, Gregorio et al. 1998). It interacts with many other proteins, such as the Z-line protein FATZ (Faulkner et al. 2000), the β-subunit of the potassium channel minK (Furukawa et al. 2001), the muscle growth regulator Myostatin (Nicholas et al. 2002) and the giant protein Titin (Gregorio et al. 1998) which also phosphorylates Telethonin (Mayans et al. 1998). Mutations in Telethonin give rise to Limb Girdle Muscular Dystrophy type 2G

(LGMD 2G) (Moreira et al. 2000). Two different mutations in the Telethonin gene both gave rise to premature stop codons resulting in truncated forms of the protein in patients with LGMD type 2G. Interestingly in both the truncated forms of Telethonin the C-terminal domain that is normally a substrate for Titin kinase is ablated (Moreira et al. 2000). Moreover immunofluorescence analysis of skeletal muscle samples from LGMD 2G patients showes a normal striation pattern indicating that at least the structure of the Z-line is preserved. Thus is reasonable to think that these Telethonin mutations do not alter the sarcomeric integrity and that Telethonin could have a signalling rather than a structural role (Vainzof and Zatz 2003). In fact Telethonin is thought to be part of a muscle stretch sensor complex also composed of Titin and MLP which gauges the stretch signal from the sarcolemma and sends information to the nucleus or other cell compartments (Knöll et al. 2002). Keeping this in mind, the interaction of Telethonin with Ankrd2 becomes even more interesting, since Ankrd2 could be a shuttling molecule. In fact it is well known that Ankrd2 is up regulated after skeletal muscle stretch (Kemp et al. 2000) and now the connection between Ankrd2 and the stretch sensor machinery, represented by the binding with Telethonin, allows speculation that Ankrd2 could move from this site towards the nucleus in response to stretch stimuli.

However at the moment this is all speculation as it is still unknown which kind of response could be triggered when Ankrd2 enters the nucleus. During the differentiation process and in mature myotubes Ankrd2 could act by inhibiting proliferation, early skeletal muscle genes re-expression and by maintaining the differentiated state, as it has been reported for CARP (Kanai et al. 2001), that is considered an early marker of cardiac and skeletal muscle hypertrophy induced by p38 and Rac1 stress pathway kinases through their binding to the M–CAT element in the CARP promoter (Aihara et

al. 2000). In fact both CARP and p53 are over-expressed in mouse skeletal muscle following of high resistance contraction (1-6 hours). They are considered to be genes associated with antigrowth and stress response which act in terminally differentiated myotubes in order to counteract the proliferative function of genes activated by contraction thus maintaining the cells in G1 phase and allowing DNA repair after injury caused by exercise (Carson et al. 2002, Chen et al. 2002).

Recently it was reported that Ankrd2, which is preferentially expressed in slow type fibres (Pallavicini et al. 2001), is a stretch-response protein associated with the induction of a slow type fibre phenotype rather than hypertrophy (Mckoy et al. 2005), as was previously suggested (Kemp et al. 2001).

Finally new evidence indicates the existence of other Ankrd2 isoforms besides that of the 37 kDa form (approx. 42 kDa after SDS-PAGE separation), which represents the protein that has been studied until now in our and in other laboratories. In fact recent publications have reported the detection of a larger form of Ankrd2 detected after SDS-PAGE separation of mouse skeletal muscle, of human and murine heart (Mckoy et al. 2005) and of rat heart (Miller et al. 2003). It is noteworthy that data from the UCSC Genome Browser Database (www.genome.ucsc.edu) show that the human Ankrd2 gene has two other putative ATG start codons upstream of the ATG from which the transcription of the 37 kDa form starts and that alternative mRNAs have been detected. The furthest ATG is positioned 339 bp upstream of the ATG from which originates the 37 kDa Ankrd2. From this start codon a longer mRNA could be produced whose transcription would result in a heavier Ankrd2 isoform with an extension in the N-terminal region of 118 amino acids (Results, Paragraph 3.14). The predicted molecular weight of this isoform is about 50 kDa and probably this is the same protein detected by

different groups as a 55 kDa band on SDS-PAGE. However it is curious since the UCSC Genome Browser Database does not show other possible isoforms for rat or mouse Ankrd2. From the analysis using domain prediction programs such as Pfam and SMART of the extra N-terminal part I have seen that this region dose not contain any known binding motif. Nevertheless, I noticed an unusual abundance of tryptophan residues that could be indicative of a specific biological function.

The ATG positioned in the middle is 81 bp upstream of the normal ATG and it extends the N-terminal part of the protein by 27 amino acids. The predicted molecular weight of this Ankrd2 isoform is 36 kDa; therefore in electrophoretic separation it would not be easily distinguishable from the 37 kDa form and to date no reports are known on it. Surprisingly this form is probably alternatively spliced, since it lacks the exon 7. This is particularly interesting, since exon 7 encodes an ankyrin repeat motif and ankyrin repeats are domains known to be involved in protein-protein interactions (Results, Paragraph 3.14) (unpublished observations). Therefore it is possible that this shorter form is unable to bind to one of the interacting partners of the 37kDa form.

In order to deepen our knowledge on the probable isoforms of Ankrd2 I studied their distribution among nine different human tissues and on human undifferentiated and differentiated cell extracts. After SDS-PAGE separation I detected a heavier Ankrd2 isoform around 55 kDa mainly in the nuclear extracts of human myoblasts and at a lower extent in the nuclei of myotubes (CH5QB) and in the cytoplasm of myoblasts, but not in the cytoplasm of differentiated cells where only the 37 kDa form is present (42 kDa in SDS-PAGE). In skeletal muscle, heart and kidney tissues I saw only the 37kDa form (42 kDa in SDS-PAGE) as already published by Pallavicini and colleagues (2001). At a first glance the band that is visible in skeletal muscle tissue seems to be just one,

but after a more accurate observation two distinct bands very close to each other are distinguishable. These are probably the 37 kDa form of Ankrd2 (42 kDa in SDS-PAGE) and the spliced variant that lacks exon 7 or alternatively two different phosphorylation states of 37 kDa Ankrd2 (Results, Parapgraph 3.15) (unpublished data). To summarise the larger Ankrd2 protein (55kDa in SDS-PAGE) is localized in the nuclear compartment and is prevalently expressed in undifferentiated cells, whereas the 37 kDa Ankrd2 (42 kDa in SDS-PAGE) is mainly expressed on differentiation and is localized in the cytoplasm. From these observations I can hypothesize a temporal regulation of the different Ankrd2 isoforms and suggest that they might have distinct roles during development and in diverse cellular compartments. Unexpectedly another alternatively spliced Ankrd2 form has been amplified by Real Time PCR performed on adult and foetal skeletal muscle. This variant lacks both exon 7 and 8, both of which encode ankyrin repeat domains (Results, Paragraph 3.16) (unpublished data). The biological significance and the role of the two splice variants identified up to date are completely obscure. Nonetheless it is important to note that the level of expression of the spliced variant is significantly lower than that of our Ankrd2.

Chapter 5

CONCLUSIONS

Skeletal muscle tissue constitutes a large part of the human body and is responsible for locomotion, facial expressions, posture, respiratory movements and many other movements of the body. It is under voluntary control with the exception of reflexes that are involuntary. It is a highly specialized tissue in whose cells the force necessary for movements is generated. Contractile activity is the main feature of skeletal muscle as it is able to lengthen and shorten forcefully; however it has also a very well developed capacity to adapt to environmental modifications such as contractile load, exercise, neural activity, metabolic changes, injury and pathological conditions. Muscles act in order to maintain tissue homeostasis and adapt to the new conditions by triggering one of the following physiological responses: hypertrophy, fibre type switching and regeneration.

Several signalling pathways are responsible for the activation and the regulation of these tissue specific programs. The sarcomere, which is the basic contractile unit of the myofibril and in particular the Z-line are now considered as crucial nodes for signal transmission in muscle cells. In fact different groups have shown signalling molecules and transcription factors can be found in the sarcomere and they can move between it and other cellular compartments.

The skeletal muscle protein Ankrd2 is both localized in the sarcomeric I line and in the nucleus. Since it interacts with three transcription factors (p53, PML and YB-1) and with a Z-line component (Telethonin), it is thought to constitute a link between the sarcomere and the nuclear compartment (Kojić et al. 2004).

To date it is not completely clear which pathways Ankrd2 participates in and what is its specific function. Nevertheless we can propose a few hypothetical models to describe possible Ankrd2 functions as part of the following cellular pathways:

1)- STRETCH SENSING and HYPERTROPHY

The Ankrd2 protein is highly expressed after skeletal muscle stretch (Kemp et al. 2000) and communicates through Telethonin with the muscle stretch sensor complex constituted also by Titin and MLP (Knöll et al. 2002).Therefore it is plausible that the stretch signal is transmitted to Ankrd2 via the interaction with Telethonin and that thereafter Ankrd2 would move towards the nuclear compartment where it would regulate gene expression interacting with a set of transcription factors (p53, YB-1 and PML). Moreover we can speculate that Ankrd2 would behave very similarly to its homologous protein CARP; hence from this point of view Ankrd2 would act in order to maintain the cells in their differentiated state inhibiting the re-activation of an embryonic gene program and the triggering of the hypertrophy pathway (Chen et al. 2002).

2)- REGENERATION

It is known that skeletal muscle is constantly exposed to damage and that contraction and stretch represent a form of stress and are causes of tissue injury. However the skeletal muscle tissue has the ability to respond to damage by triggering the

regeneration process, thanks to the persistence in the adult of stem cells called satellite cells.

Ankrd2 is also considered a member of the MARP family of proteins involved in muscle stress pathways (Miller et al. 2003), stretch being the trigger for Ankrd2 activation. The tissue responds to stretch and exercise by increasing the expression of specific proteins amongst which there is also p53, which is well known to be stress induced (Chen et al. 2002). Hence, since Ankrd2 is a p53 interacting protein (Kojić et al. 2004) we can suggest that it enters a stress pathway activated by exercise and that it has a role in satellite cell activation and in tissue regeneration after stretch induced injury. This hypothesis is strengthened by the fact that we have observed Ankrd2 in CH5QB cells, which are derived from skeletal muscle satellite cells.

3)- DIFFERENTIATION

Ankrd2 is strongly expressed upon differentiation of skeletal muscle cells (Pallavicini et al. 2001). Recently it has also been demonstrated to be regulated by the muscle specific transcription factor Myo D (Bean et al. 2005). Moreover, Ankrd2 interacts with the tumor suppressor p53 (Kojić et al. 2004), which has also a crucial role in the skeletal muscle differentiation process (Soddu et al. 1996) and with YB-1, which through the interaction with CARP participates in the regulation of gene expression during the heart development (Zou et al. 1997). For these reasons we hypothesize that Ankrd2 could take part in myogenesis by acting as a cofactor that influences the activity of a set of transcription factors such as p53 and YB-1. It is also plausible that this gene regulation takes place after Ankrd2 recruitment into the PML nuclear bodies.

4)- FIBRE TYPE DETERMINATION

Initially Ankrd2 was found as a novel transcript upregulated in mouse tibialis anterior (TA) muscles (fast muscle) after 7 days of passive stretch immobilization in vivo (Kemp et al. 2000). However now the same group in light of further work using kyphoscoliotic mutant mice, which lack the hypertrophic response pathway proposed that expression of Ankrd2 in stretched fast muscle is associated with the stretched–induced expression of slow muscle phenotype rather than the hypertrophic response (McKoy et al. 2005). We can suppose that when the muscle tissue is stretched Ankrd2 helps it to adapt by increasing the percentage of slow fibres that are more resistant to fatigue.

Since this would take place in a fully differentiated tissue, able to contract, we can assign these functions to the 37 kDa Ankrd2 form, which is the most abundant in myotubes and adult tissue. Conversely, it could be that the nuclear Ankrd2 form, which is mostly detectable in the myoblasts, is the longest one. So we could speculate that only this form is recruited by PML into the NBs where it could play a role in transcriptional regulation through the interaction with transcription factors like p53 and YB-1.

Finally we can conclude that the Ankrd2 protein could have an important role in stress sensing signalling pathways or in skeletal muscle development. Hence it is reasonable to suppose that still uncharacterized pathological conditions in which muscle functionality is compromised could be due to mutations in the Ankrd2 gene.

Chapter 6

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COLLABORATIONS

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PUBLICATIONS

Snezana Kojic1†, Elisa Medeot1†, Ernesto Guccione, Helena Krmac, Ivano Zara, Valentina Martinelli, Giorgio Valle and Georgine Faulkner (2004) **The Ankrd2 Protein, a Link Between the Sarcomere and the Nucleus in Skeletal Muscle.** *J. Mol. Biol*. **339:** 313–325.

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