A Novel Role for the Transcriptional Co-activator VITO-1 in Skeletal Muscle Gene Regulation

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1. Introduction

1.1 Transcriptional regulation in Eukaryotes

In eukaryotes, RNA polymerase II transcribes protein-coding genes. Eukaryotes have three nuclear RNA polymerases (Table. 1), each with distinct roles and properties (Carter and Drouin 2009). RNA pol II is located in the nucleoplasm and is responsible for transcription of the vast majority of genes including those encoding mRNA, small nucleolar RNAs (snoRNAs), some small nuclear RNAs (snRNAs), and microRNAs. Gene transcription is a remarkably complex process. The synthesis of tens of thousands of different eukaryotic mRNAs is carried out by RNA pol II (Kettenberger, Armache et al. 2003). During the process of transcription, RNA pol II associates transiently not only with the template DNA but with many different proteins, including general transcription factors. The initiation step alone involves the assembly of dozens of factors to form a pre-initiation complex. Transcription is mediated by the collective action of sequence-specific DNA-binding transcription factors along with the core RNA pol II transcriptional machinery, an assortment of co-regulators that bridge the DNA-binding factors to the transcriptional machinery, a number of chromatin remodeling factors that mobilize nucleosomes, and a variety of enzymes that catalyze covalent modification of histones and other proteins. There are two other important eukaryotic polymerases – RNA polymerase I and RNA polymerase III (Hurwitz 2005). RNA polymerase I reside in the nucleolus and is responsible for synthesis of the large ribosomal RNA precursor. RNA polymerase III is also located in the nucleoplasm and is responsible for synthesis of transfer RNA (tRNA), 5S ribosomal RNA (rRNA), and some snRNAs (Hurwitz 2005). Plants have a fourth nuclear polymerase, named RNA polymerase IV, which is an RNA silencing-specific polymerase that mediates synthesis of small interfering RNAs (siRNAs) involved in heterochromatin formation (Onodera, Haag et al. 2005). For RNAP II (protein-coding genes), initiation requires several transcription factors that assist binding to promoter sites. Promoter sites recognized by RNAP II (and associated protein

factors) are several conserved elements that are located upstream from the transcription start point (the +1 base).

Name	transcribed	
RNA Polymerase I (Pol I, Pol A)	nucleolus	Larger ribosomal RNA (rRNA) (28S, 18S, 5.8S)
RNA Polymerase II (Pol II, Pol B)	nucleus	Messenger RNA (mRNA) and most small nuclear RNAs (snRNAs)
RNA Polymerase III (Pol III , Pol C)	nucleus (and possibly the nucleus-nucleoplasm interface)	Transfer RNA (tRNA) and other small RNAs (including the small 5S rRNA)

Table 1. The three nuclear RNA polymerases present in Eukaryotes

The basal level of transcription requires a set of basal transcription factors that bind to DNA or other proteins. Firstly, a protein recognizes and binds to the TATA sequence at the promoter. TATA binding protein (TBP) bends the DNA and auxiliary factors binds to the TBP. This protein complex promotes the binding of RNA polymerase and its association with the proteins. The complete pre-initiation complex denatures the nearby DNA helix. RNA polymerase II and its associated proteins move along the unwound DNA and produce a messenger complex complimentary to the DNA template.

The consensus sequences of the conserved elements are a) –30 = TATAAAA [TATA homology or Goldberg-Hogness box] b) –80 = GGCCAATCT [CAAT box] c) GGGCGG [GC box] and often present but occur in different positions and in ATTTGCAT [octamer box] different copy numbers. The TATA homology is found in all eukaryotic promoters known to date. The remaining “consensus” sites are found but not necessarily in the same promoter. All “consensus” sites affect binding efficiency of RNA polymerase/transcription factors (Smale and Kadonaga 2003). RNAP I and RNAP III

utilize some of the same transcription factors as RNAP II but the promoters are quite different. RNAP III utilizes internal promoters that are present within the transcriptional units (Veras, Rosen et al. 2009). The level of transcription is increased above the lower basal levels by activators bound to specific enhancer sequences. Activators can act to stabilize the pre-initiation complex and make it easier for the TBP or the RNA polymerase to bind to the DNA. Stabilization of the complex by activator proteins may also allow binding of subsequent RNA polymerase molecules. Activator proteins increase transcription while inhibitor proteins decrease transcription. Together, these regulatory proteins modulate transcription by determining where, when and how much transcription occurs in a particular type of cells.

1.1.1 Protein-coding gene regulatory elements

Expression of protein-coding genes is mediated in part by a network of thousands of sequence-specific DNA-binding proteins called transcription factors (Lee and Young 2000). Transcription factors interpret the information present in gene promoters and other regulatory elements, and transmit the appropriate response to the RNA pol II transcriptional machinery. Information content at the genetic level is expanded by the great variety of regulatory DNA sequences and the complexity and diversity of the multi-protein complexes that regulate gene expression. Many different genes and many different types of cells in an organism share the same transcription factors. What turns on a particular gene in a particular cell is the unique combination of regulatory elements and the transcription factors that bind them (Lee and Young 2000).

1.1.2 Transcription factors

mRNA and eventually proteins are most often produced when and where they are needed in an organism. Some genes, such as those for housekeeping proteins and processes like glycolysis are expressed all of the time. These genes undergo what is known as constitutive transcription. Other proteins are only produced at specific times in certain cells. In these cells, genes undergo a process called regulated transcription. Transcription is regulated by proteins called transcription factors. These factors are proteins produced

in the cytoplasm and eventually migrate into the nucleus where they interact with DNA and activate transcription. These transcription factors only interact with specific genes, those genes whose transcription they control. Most eukaryotic species have over 1000 transcription factors.

Regulated transcription begins when a signal is received by the cell. The signal, often a protein begins the signal transduction cascade that lets the cell know that certain proteins are now needed. The most common activation event for a protein in a signal pathway is the addition of a phosphate group. That activated protein in turn interacts with another protein. This interaction leads to the phosphorylation of the next protein in the pathway. There is often a series of proteins activated by phosphorylation. At the end of this cascade, the final protein will be activated and then enter the nucleus. It must make this journey because transcription only occurs inside the nucleus. After entering through the nucleopore, the protein interacts with the specific transcription factor responsible for activating mRNA production. As with the earlier proteins, the transcription factors are modified through phosphorylation. The activated transcription factor will next bind to an enhancer region. The enhancer is a region of DNA upstream of the transcription start site that binds the transcription factor. The transcription factor then binds to the DNA and moves to interact with the rest of the transcription protein complex located at the transcription start site. When the formation of this complex is complete, transcription of the gene will begin. It is the sequence of events that ensures the gene required for specific tissue only a t a specific time are expressed appropriately.

The regulation of gene activity at the transcriptional level generally occurs via changes in the amounts or activities of transcription factors. The genes encoding the transcription factors themselves may be transcriptionally induced or repressed by other regulatory proteins, or the transcription factors may be activated or deactivated by proteolysis, covalent modification, or ligand binding (Spiegelman and Heinrich 2004). Transcription factors influence the rate of transcription of specific genes either positively or negatively (activators or repressors, respectively) by specific interactions with DNA regulatory elements) and by their interaction with other proteins (Latchman 1997). Transcription factors mediate gene-specific transcriptional activation or repression. Transcription

factors that serve as repressors block the general transcription machinery, whereas transcription factors that serve as activators increase the rate of transcription by several mechanisms like stimulation of the recruitment and binding of general transcription factors and RNA pol II to the core promoter to form a pre-initiation complex (Abrahem and Pelchat 2008), inducing a conformational change or post-translational modification (such as phosphorylation) that stimulates the enzymatic activity of the general transcription machinery and interaction with chromatin remodeling and modification of complexes to permit enhanced accessibility of the template DNA to general transcription factors or specific activators. These different roles can be promoted directly via protein–protein interaction with the general transcription machinery or through interactions with transcriptional co-activators and co-repressors (Latchman 1997).

1.1.2.1 Transcription factors are modular proteins

Transcription factors are modular proteins (Yang 1998) consisting of a number of domains (Fig. 1). Recognition of this feature triggered the development of a powerful technique for analyzing protein–protein interactions *in vivo* – the yeast two-hybrid assay. The three major domains are a DNA-binding domain, a transactivation domain, and a dimerization domain.

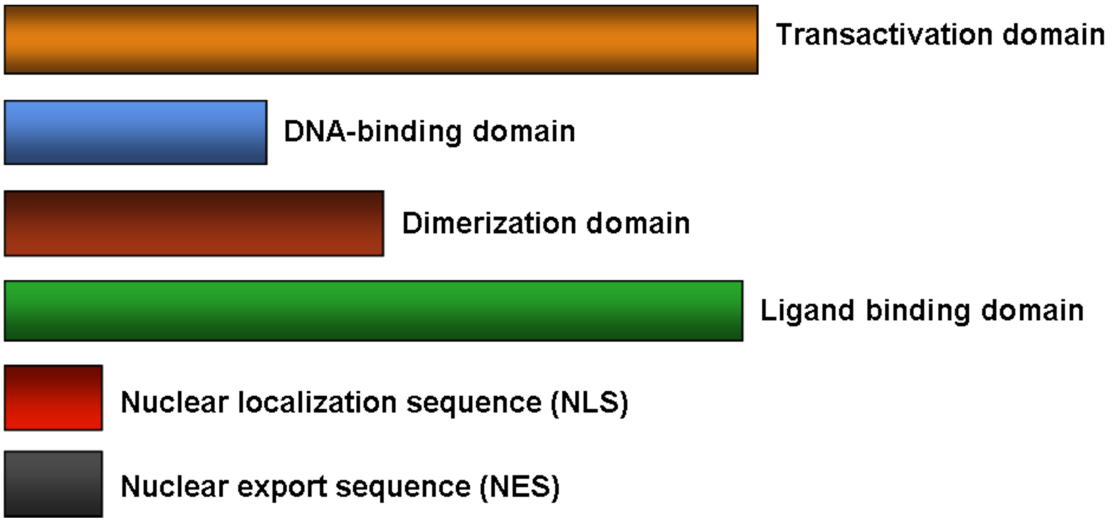


Figure 1. Transcription factors are composed of separable, functional components*. They typically include a DNA-binding domain, a transactivation domain, a dimerization domain, nuclear localization sequence (NLS), and some also have a nuclear export sequence (NES). Some transcription factors also have ligand-binding (regulatory) domains, such as hormone-binding domains, which are essential for controlling their activity.*

In addition, transcription factors typically have a nuclear localization sequence (NLS) (Kalderon, Richardson et al. 1984; Kalderon, Roberts et al. 1984), and some also have a nuclear export sequence (NES) like in the case of nuclear factor of activated T cells (NFAT) (Beals, Clipstone et al. 1997; Klemm, Beals et al. 1997). Some transcription factors also have ligand-binding (regulatory) domains, such as hormone-binding domains, which are essential for controlling their activity. Many transcription factors become activated or inactivated as a result of ligand binding (Grove and Walhout 2008).

1.1.2.2 Transactivation domain

The transactivation domain of a transcription factor is involved in activating transcription via protein–protein interactions. Transactivation domains may work by recruiting or accelerating the assembly of the general transcription factors on the gene promoter, but their mode of action remains unclear. Some transcription factors do not contact the general transcription machinery directly but instead bind co-activators that in turn contact the general apparatus. Unlike the well-defined DNA-binding domains, transactivation domains are structurally more elusive (Biochemistry of signal transduction and regulation – Gerhard Krauss 2008). They are often characterized by motifs rich in acidic amino acids, so-called “acid blobs” (Pandit, Bednarski et al. 2003) In addition to acid blobs, there are other distinct motifs. For example, transcription factor Sp1 contains a nonacidic transactivation region with multiple glutamine-rich motifs (Courey and Tjian 1988; Kadonaga, Courey et al. 1988). Other motifs associated with transactivation include proline-rich regions and hydrophobic β-sheets.

1.1.2.3 Dimerization domain

The majority of transcription factors bind DNA as homodimers or heterodimers. Accordingly, they have a domain that mediates dimerization between the two identical or similar proteins. In contrast to our detailed knowledge of protein–DNA interactions, far less is known about the exact molecular characteristics of these protein–protein contacts. As described above, two dimerization domains that are relatively well characterized structurally are the basic helix-loop-helix (bHLH) and basic region leucine zipper (bZIP) motifs. Several TFs bind DNA as obligatory dimers, including members of bZIP, bHLH and nuclear hormone receptor (NHR) families (Wolberger 1999); (Newman and Keating 2003); (Lamb and McKnight 1991).

1.1.3 Transcriptional Co-activators and Co-repressors

Gene transcription is a multistep process involving a very large number of proteins functioning in discrete complexes. As described above, transcription factors bind to DNA in a sequence-specific manner. They mark a gene for activation or repression through the recruitment of co-activators or co-repressors. Co-activators and co-repressors are proteins that increase or decrease transcriptional activity, respectively, without binding DNA directly. Instead they bind directly to transcription factors and either serve as scaffolds for the recruitment of other proteins containing enzymatic activities, or they have enzymatic activities themselves for altering chromatin structure. Co-activators and co-repressors have been much harder to study compared with transcription factors. In general, assays for protein–protein interactions are more difficult to perform than techniques for studying DNA–protein interactions. In addition, techniques for determining which co-activator is docking on a particular transcription factor *in vivo* were not available until recently.

Most known co-activators are very large proteins that harbor multiple activation domains and receptor-interacting domains (L'Horset, Dauvois et al. 1996; Ding, Anderson et al. 1998; Onate, Boonyaratanakornkit et al. 1998; Voegel, Heine et al. 1998). Interestingly, some co-activators share significant sequence homology. More interestingly, some co-activators are able to acetylate histones (Tsukiyama and Wu 1997). Acetylation of the conserved lysine residues in the N-terminal domain of histones results in the loosening of

the nucleosome structure, making the DNA more accessible to transcription factors Co-activators, in the broadest sense, can be divided into two main classes namely Chromatin modification complexes: multi-protein complexes that modify histones post-translationally, in ways that allow greater access of other proteins to DNA and Chromatin remodeling complexes: multi-protein complexes of the yeast SWI/SNF family (or their mammalian homologs BRG1 and BRM) and related families that contain ATP-dependent DNA unwinding activities (Chiba, Muramatsu et al. 1994) and (Ichinose, Garnier et al. 1997). On the other hand co-repressors have the opposite effect on chromatin structure, making it inaccessible to the binding of transcription factors or resistant to their actions (Jenster 1998). Deacetylating histones and thereby compacting nucleosomes into a tight and inaccessible structure is a potent mechanism for shutting down gene expression. Inhibitors of HDAC, such as trichostatin A, therefore relieve repression by unliganded receptors (Wong, Patterton et al. 1998).

1.2 Transcription Enhancer Factors (TEFs)

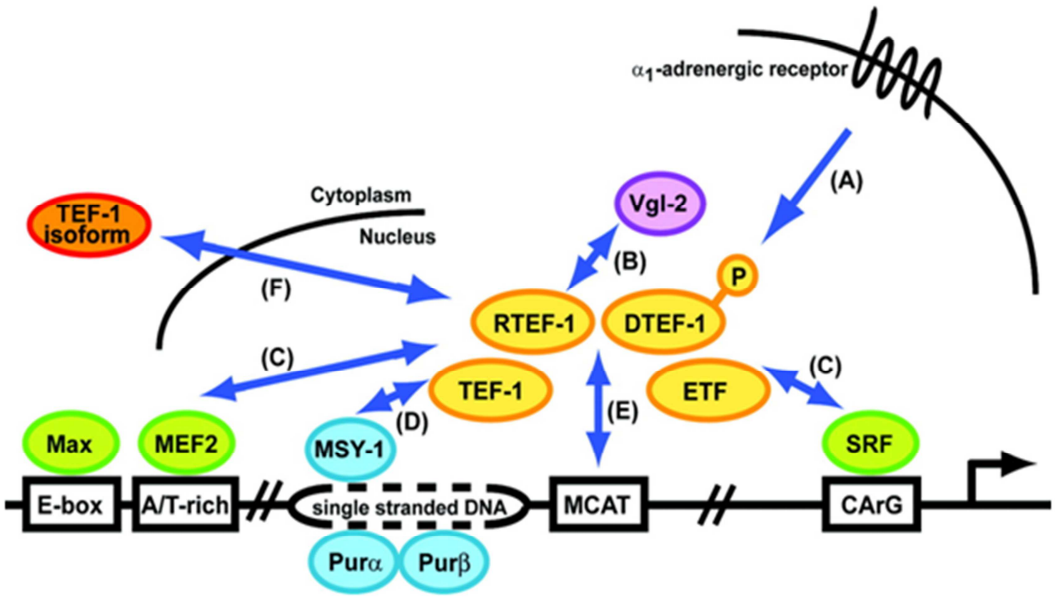
Transcription enhancer factors (TEFs) are essential for cardiac, skeletal, and smooth muscle development and uses its N-terminal TEA domain (TEAD) to bind M-CAT elements. The MCAT element was originally identified as a muscle-specific cytidine-adenosine-thymidine sequence, 5'-CATTCCT-3', in the chicken cardiac troponin T promoter (Cooper and Ordahl 1985). MCAT element has been found in a number of cardiac, smooth, and skeletal muscle-specific genes, including cardiac troponin T (Mar and Ordahl 1988), β-myosin heavy chain (β-MHC),(Rindt, Gulick et al. 1993) smooth muscle α-actin (SM α-actin), (Swartz, Johnson et al. 1998) and skeletal α-actin (Karns, Kariya et al. 1995). It has been shown to play a key role in the transcriptional regulation of these genes, although it is also present in the promoter regions of some non-muscle genes.

The proteins that bind to the MCAT element belong to the TEF-1 family of transcription factors (Xiao, Davidson et al. 1991), (Farrance, Mar et al. 1992). This family shares a highly conserved DNA binding domain called the TEA domain and consists of four

members (Table. 2) including TEF-1 (NTEF-1/Tead1), RTEF-1 (TEF-3/ETFR-2/FR-19/Tead4), ETF (TEF-4/Tead2), and DTEF-1 (TEF-5/ETFR-1/Tead3).

Name	Alternative Name	Percent Identity to TEF-1	Percent Identity to TEF -1 within the TEA Domain
TEF -1	NTEF -1, Tead1	…	…
ETF	ETEF -1, TEF -4, Tead2	64%	100%
RTEF -1	TEF -3, ETFR -2, FR -19, Tead4	74%	100%
DTEF -1	TEF -5, ETFR -1, Tead3	70%	99%

Table 2. The TEF Family Members



- Yoshida, T. et al, 2008, Arterioscler Thromb Vasc Biol

Figure 2. Regulation of TEF-1 family members

TEF-1 family member-dependent transcription of MCAT element-containing muscle-specific genes is modulated by: (A) phosphorylation of TEF-1 family members; (B) interaction with cofactors; (C) interaction with SRF/MEF2/Max; (D) flanking sequence

and its binding factors; (E) accessibility of TEF-1 family members to MCAT elements; and (F) alternative splicing of TEF-1 family member.

The TEA domain is also referred to as the ATTS domain, because it appears in yeast, vertebrate, plant, and fly transcription factors AbaA, TEC1, TEF-1, and Scalloped (Burglin 1991), (Campbell, Inamdar et al. 1992). AbaA regulates development of the asexual spores in *Aspergillus nidulans* and terminates vegetative growth, TEC1 is involved in the activation of the Ty1 retro transposon in yeast *Saccharomyces cerevisiae*, and the *Drosophila* gene Scalloped plays an important role in sensory neuron and wing development. Conservation of the TEA domain in multiple organisms indicates its critical role in regulation of gene transcription. TEF-1, RTEF-1, and DTEF-1, respectively, initiate translation at an isoleucine (AUU), leucine (UUG), and isoleucine (AUA) codon that lies upstream of the first methionine codon (Xiao, Davidson et al. 1991; Stewart, Richard et al. 1996; Jiang, Wu et al. 1999). ETF, however, uses the methionine (AUG) codon for the initiation of translation.(Yasunami, Suzuki et al. 1995) Second, TEF-1 family members bind to the double-stranded form of the MCAT element, but not to the single-stranded MCAT element (Carlini, Getz et al. 2002). The tissue distribution of TEF-1 family members has been examined by a number of studies. TEF-1, DTEF-1, and RTEF-1 are widely expressed in multiple tissues including the skeletal muscle, pancreas, placenta, lung, and heart. In contrast to these three factors, ETF is selectively expressed in a subset of embryonic tissues including the cerebellum, testis, and distal portion of the forelimb and hind limb buds as well as the tail bud, but it is essentially absent from the adult tissues (Yasunami, Suzuki et al. 1995). ETF has also been shown to be expressed from the 2-cell stage during development (Kaneko, Cullinan et al. 1997).

1.2.1 Role of Transcription Enhancer Factors

1.2.1.1 Cardiac development and hypertrophy

TEF-1 plays a vital role in cardiac development. TEF-1 knockout mice exhibited an enlarged pericardial cavity, bradycardia, a dilated fourth ventricle in the brain, and died by embryonic day (E) 12.5. Histological examination revealed that the ventricular wall in

the heart of TEF-1 knockout mouse embryos was abnormally thin with a reduced number of trabeculae. These results indicate that TEF-1 is required for cardiac morphogenesis and that other TEF-1 family members cannot compensate for TEF-1 function during embryogenesis. Cardiac hypertrophy occurs in a number of pathophysiological conditions such as hypertension, valvular disease, myocardial infarction, and cardiomyopathy. At the cellular level, it is characterized by an increase in cell size and protein synthesis and by reactivation of the fetal cardiac genes including β-MHC and skeletal α-actin (Simpson, Kariya et al. 1991). Stimulation of α_1-adrenergic signaling has been shown to induce cardiac hypertrophy and activate transcription of the β- MHC gene and the skeletal α – actin gene in cultured neonatal rat cardiomyocytes. Interestingly, α_1-adrenergic receptor-mediated induction of these genes is abolished by mutation of the MCAT element within the promoters (Kariya, Karns et al. 1994). In addition, RTEF-1, but not TEF-1, potentiates the α_1-adrenergic response of the β-MHC and skeletal α-actin promoters (Stewart, Richard et al. 1996). In addition, MCAT elements might contribute to the induction of fetal cardiac genes in other in vivo models of cardiac hypertrophy.

1.2.1.2 Smooth Muscle Development

Studies provide evidence that the MCAT element plays a critical role in smooth muscle development (Creemers, Sutherland et al. 2006; Gan, Yoshida et al. 2007). Transcriptional regulation of Myocardin which is exclusively expressed in SMCs and cardiomyocytes was recently identified to contain an MCAT element as well as a MEF2 binding site and multiple FoxO binding sites. Mutation of the MCAT element selectively abolished LacZ expression in SMCs, but not in the heart, whereas mutation of either the MEF2 binding site or FoxO binding sites dramatically reduced LacZ expression in both SMCs and cardiomyocytes. These results suggest that the MCAT element behaves as a SMC-specific upstream signaling pathway for the induction of the myocardin gene. However, it remains undetermined which TEF family members bind to this MCAT element, and how the MCAT element functions selectively in SMCs.

1.2.1.3 Skeletal Muscle Hypertrophy and Regeneration

The MCAT element has been shown to be involved in the regulation of muscle-specific genes in skeletal muscle like α-actin (Carson, Yan et al. 1995) and β-MHC (Rindt, Gulick et al. 1993). Denervation-induced decrease in β-MHC expression is also mediated by the proximal MCAT element in rat soleus (Huey, Haddad et al. 2003) which indicates the regulation of muscle-specific genes by MCAT element in skeletal muscle. However, a series of studies by Tsika and colleagues showed that induction of the β-MHC gene in overloaded skeletal muscle was not mediated by the MCAT elements, but by the association of TEF-1 proteins with the A/T-rich element located between two MCAT elements (Tsika, Wiedenman et al. 1996; Vyas, McCarthy et al. 1999; Karasseva, Tsika et al. 2003). Additional work is required to reconcile the discrepancy. Zhao et al. (Zhao, Caretti et al. 2006) showed that the MCAT element and ETF were implicated in a process of skeletal muscle regeneration. They showed that cardiotoxin-induced degeneration/regeneration of skeletal muscle occurred abnormally in fibroblast growth factor receptor 4 (FGFR4) knockout mice as compared with wild-type mice. By 14 days after cardiotoxin injection, much of the skeletal muscle exhibited impaired regeneration and was replaced by fat and calcifications in FGFR4 knockout mice. They found that ETF expression was induced in skeletal muscle during regeneration in vivo and that ETF induced the promoter activity of the FGFR4 gene via a MCAT element in C2C12 myoblasts. These results suggest the importance of the MCAT element and ETF in muscle regeneration, although evidence is indirect. Further studies are needed to determine whether knockout of ETF also exhibits abnormal skeletal muscle regeneration in vivo.

1.2.2 Cofactors of TEF-1 Family Members

Ectopic expression of TEF-1 does not induce target gene transcription in cell lines in which the endogenous TEF-1 protein is absent. Overexpression of TEF-1 in cells that express TEF-1 family members results in the transcriptional repression of MCAT element-containing genes (Xiao, Davidson et al. 1991). Because these observations are consistent with a squelching phenomenon of co-activator activity, the presence of

cofactors for TEF-1 family members has long been predicted. Recently, multiple cofactors for TEF-1 family members have been identified. They include the p160 family of nuclear receptor co-activators (SRC1, TIF2, and RAC3) (Belandia and Parker 2000), a Src/Yes-associated protein YAP65 (Vassilev, Kaneko et al. 2001), TAZ, (Mahoney, Hong et al. 2005). Vgll-2 / VITO-1 (Mielcarek, Gunther et al. 2002) and Vgl-4 (Chen, Mullett et al. 2004). Of these, Vgl-2 is expressed in a tissue-specific manner and contributes to the cell-specific transcription of MCAT element-containing genes.

VITO-1 (also called as Vgl-2) interacts with TEF-1 and RTEF-1 (Maeda, Chapman et al. 2002), and regulates the binding activity of TEF-1 family members to MCAT elements (Chen, Maeda et al. 2004; Gunther, Mielcarek et al. 2004). Co-transfection assays have shown that RTEF-1 and Vgl-2 cooperatively increase the promoter activity of the skeletal α-actin gene in cultured cells. Of importance, suppression of Vgl-2 by antisense morpholino decreases MHC expression in C2C12 myocytes and chicken limb muscles in vivo. As such, VITO-1 is a key cofactor of TEF-1 family members regulating muscle-specific gene transcription in skeletal muscle (Gunther, Mielcarek et al. 2004). In contrast, Vgl-4 does not exhibit cell type–specific expression patterns and functions differently from Vgl-2 (Chen, Mullett et al. 2004). Vgl-4 is relatively widely expressed in multiple tissues including the heart, brain, kidney, small intestine, lung, and placenta. Vgl-4 physically interacts with TEF-1 and MEF2. Overexpression of Vgl-4 interferes with the basal and α_1-adrenergic agonist-induced activity of the skeletal α-actin promoter in neonatal cardiomyocytes. In addition, α_1-adrenergic signaling elicits nuclear export of Vgl-4 in cardiomyocytes. These results suggest that Vgl-4 acts as a repressor of TEF-1 family member-dependent gene transcription under normal conditions, and that it translocates to the cytoplasm and modifies the transcriptional activity of these genes after stimulation.

1.3 VITO-1 (Vestigial like 2, Vgl-2)

VITO-1 is a homolog of Drosophila vestigial and human TONDU protein identified using a subtractive hybridization approach (Mielcarek, Gunther et al. 2002). VITO-1 is characterized by the presence of a scalloped interaction domain (SID) which has been

described in both Drosophila vestigial and TONDU proteins. It is presumed that the interaction and regulation of VITO-1 with its target genes are mediated through this SID domain. VITO-1 is expressed in the differentiating somites and branchial arches during embryogenesis and is exclusively expressed in skeletal muscle in the adult (Maeda, Chapman et al. 2002). The expression of VITO-1 starts from E8.75 in the somatic myotome during mouse embryonic development. In addition, transient domains of VITO-1 expression were found in the branchial arches, Pharyngeal pouches and clefts, cranial pharynx and Rathkes pouch during embryogenesis. In adult tissues its expression was restricted to skeletal muscles (Mielcarek, Gunther et al. 2002). VITO-1 was identified as the first known TEFs co-activator, which modulates transcription enhancer factors activity in a tissue specific manner. VITO-2 starts to be expressed from E8.0 in the prospective area of the midbrain followed by its expression in the myotome of the somites from E9.5. In contrast to VITO-1, VITO-2 is ubiquitously expressed in adult tissues and its expression domains overlap with TEF-3. Analysis of VITO-1/2 expression in delta1 and Myf-5 knockout embryos indicated that VITO-2 but not VITO-1 is under control of the Notch pathway and that the VITO family of genes is a direct target of Myf-5 during specification of the myogenic precursor cells or myoblasts. However, VITO-1 was still detectable in the branchial arches of embryos lacking Myf-5 indicating that VITO-1 in the brachial arch is not expressed in muscle precursor cells. It should be noted that VITO-1 and VITO-2 strongly stimulate TEF-1 and TEF-3 mediated transcriptional activation (Gunther S, Mielcarek et al 2004). Functional analysis of VITO-1 shows that VITO-1 is able to enhance MyoD mediated conversion of 10T1/2 and 3T3 fibroblasts cells into myotubes but are not able to activate trans-differentiation of these cell lines by itself indicating that it plays a supportive role, which enhances and modifies a cellular decision imposed by other target genes (Gunther, Mielcarek et al. 2004). VITO- 1 together with MyoD and TEF-3 induced MyHC expression in 293T cells, which are not able to differentiate into myotubes thus indicating an important role in control of muscle gene regulation. Disruption of VITO-1 using siRNA approach resulted in an inhibition of myogenin expression and an obstruction of C2C12 myotubes formation (Mielcarek, Gunther et al. 2002). VITOs act as specific modulators of TEF proteins without the

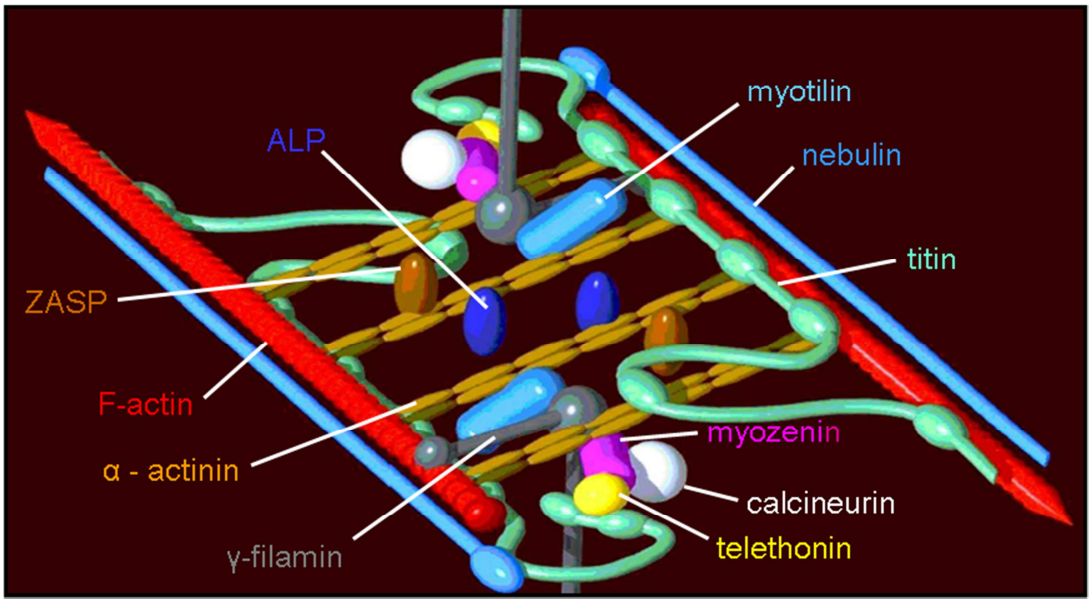
ability to activate transcription directly as they lack the transactivation domain or nuclear localization signal (NLS).

1.4 Contractile proteins in skeletal and cardiac muscle are organized in sarcomeres

In general all muscles use actin and myosin for contraction, but only in skeletal and cardiac muscles are these proteins organized into defined structures called sarcomeres, which are the fundamental contractile units of striated muscle. The sarcomere is composed of ordered thick (myosin) and thin (actin, tropomyosin, troponin) filaments that slide past each other during contraction. The Z-disc is the boundary of individual sarcomeres where thin filaments are anchored (Stromer 1998). Some of the key features of Z-discs from electron microscopy studies are: 1) anchoring points for actin filaments; 2) placed in a precise and complicated context of intracellular sarcoplasmic cisternae and T-tubules; 3) seen as dense, protein-rich formations. However, despite these features being known for some time, most Z-disc proteins have only recently been discovered and characterized at the molecular level (Faulkner, Lanfranchi et al. 2001).
At a molecular level in the sarcomere, the mechanical force produced by the interaction of myosin and actin within the sarcomere requires a suitable structure able to collect it. The emerging structural complexity of the Z-disc is an extraordinary example of intricate molecular architecture, while the mechanisms of its assembly and adaptation remain mostly unknown (Faulkner, Lanfranchi et al. 2001).

1.4.1 The Z-disc is a complex integration of several proteins

As shown in Figure 3, EM studies show that within the Z-disc the actin filaments are organized in a square pattern (Yamaguchi, Robson et al. 1982). Furthermore, for several proteins it was possible to determine the approximate localization using EM immunolabeling; for instance, telethonin maps near the edge of the Z-disc (Gregorio, Trombitas et al. 1998; Mues, van der Ven et al. 1998), whereas titin, the giant protein that spans across half a sarcomere, has its N-terminus located near the Z-disc and it’s C-terminus near the M-band at the center of the sarcomeres (Labeit, Gautel et al. 1992).



- Georgine Faulkner el al IUBMB Life, 51: 275–282, 2001

Figure 3. Schematic representation of the Z-disc proteins embedded in muscle fibers

The Z-disc has been schematically represented as a single pair of anti-parallel F-actins (red rods) joined by α-actinin 2 (yellow/brown). The N-terminal of titin (green) is interacting with telethonin (yellow) and with the C-terminal of nebulin (blue rod) before crossing the Z-disc where a variable number of z-repeats (green ovals) organize the binding of α-actinin 2. Titin also interacts with the central portion of α-actinin 2, before proceeding towards the center of the sarcomere (not shown). FATZ (Myozenin) is interacting with α-actinin 2, telethonin, calcineurin (white), and γ-filamin (gray). The latter is also binding F-actin and myotilin (light blue), as well as sarcoglycans on the sarcolemma (not shown). ALP and ZASP (dark blue and brown) are shown attached to the spectrin-like repeats of α-actinin 2. It should be noted that in some cases, where there is more than one protein partner, competition rather than simultaneous binding could occur

The above figure gives a very simplified representation of Z-disc proteins, in which everything has been squeezed between two antiparallel F-actins. The actual 3D organization of the Z-disc is certainly more complex. Alpha-actinin connects two anti-parallel F-actins at the “sides” of the square pattern in which F-actins are arranged.

However, an alternative pattern could be obtained by diagonally connecting F-actins (Schroeter, Bretaudiere et al. 1996), on the basis of a computational analysis from EM data. Furthermore, from EM data it appears that during contraction the structure of the Z-disc undergoes conformational modifications that may play an active role in the mechanism of contraction (Jarosch 2000). However, the molecular basis of these modifications remains uncertain and it is unknown whether they are accompanied by topological alterations.

1.4.2 Proteins of the Z-discs

Actin, one of the best-studied proteins belongs to a highly conserved family of cytoplasmic proteins present in two physical forms-globular actin and F-actin polymers. Six genes code for different isoforms: α-skeletal, α-cardiac, α-vascular smooth, and γ -enteric smooth actin are found in muscle cells whereas the non-muscle cytoskeletal isoforms β- and γ -actin are components of the microfilament network. In the sarcomere, F-actin forms thin filaments that are both structurally and functionally polarized. CapZ is a heterodimeric protein, which in muscle is composed principally of two subunits (α 2 and β1). It attaches actin filaments to the Z-disc and binds to the (C) end of the actin filaments to prevent de-polymerization, thus rendering the thin filaments stable. CapZ also binds to the spectrin-like repeats of α -actinin 2 (Papa, Astier et al. 1999), a Z-disc protein that binds actin. Alpha-actinins belong to a family of proteins that cross-link F-actin as antiparallel homodimers. There are four different isoforms of α -actinin in cells, isoform 2 that is found in skeletal and cardiac muscle (Beggs, Byers et al. 1992) is a major component of the Z-disc. It is found in all fiber types where it links overlapping antipolar F-actin thin filaments from adjacent sarcomeres (Yamaguchi, Izumimoto et al. 1985). The α -actinin 3 isoform (Beggs, Byers et al. 1992) is not found in heart but only in skeletal muscle in a subset of type 2 fast muscle fibers where it can form heterodimers with the α -actinin 2 isoform (Chan, Tong et al. 1998). Several Z-disc proteins have been reported to bind α -actinin 2, including nebulin (Nave, Furst et al. 1990), ALP (Xia, Winokur et al. 1997), Myozenin (FATZ) (Faulkner, Pallavicini et al. 2000), myotilin (Salmikangas, Mykkanen et al. 1999), titin (Ohtsuka, Yajima et al. 1997; Sorimachi, Freiburg et al. 1997; Young and Gautel 2000), and ZASP (Faulkner, Pallavicini et al.

1999). Titin (connectin) is one of the largest known proteins having a molecular weight greater than 3,000 kD. It is an elastic filament that is anchored in the Z-disc and extends to the M-line at the center of the sarcomere where actin and myosin fibers overlap (Trinick 1996). The N-terminal region of titin (30 kD) is located at the periphery of the Z-disc and the following 60-kD region extends across the Z-disc. The Z-disc region is composed of two Ig-like repeats (Z1 and Z2) followed by a series of alternatively spliced z-repeats (zr1–zr7). The difference in number of alternatively spliced repeats in titin isoforms has been suggested to affect the number of cross-links between α -actinin 2 and F-actin filaments and hence to cause variation in Z-disc thickness (Gautel, Goulding et al. 1996). There appear to be two types of binding sites for titin in α -actinin 2: 1) the C-terminal CaM domain that interacts with several z-repeats of titin; 2) the zq-Z4 region that binds to two spectrin-like repeats (R2 and R3 The latter binding is independent of dimer formation (Young and Gautel 2000), whereas the binding of titin z-repeats to α -actinin 2 does not occur if the α -actinin 2 is present in the form of a dimer. This auto-inhibition can be released by phosphatidylinositol-bisphosphate binding to the actin-binding domain of the dimer. This mechanism has been proposed as a means of controlling α -actinin 2 interactions with titin during sarcomere formation (Young and Gautel 2000).

Nebulin is a large inextensible protein (800 kD) that is anchored at the Z-disc by its C-terminal region and spans the length of the thin filament ending at the edge of the H-zone (Kruger, Wright et al. 1991; Wright, Huang et al. 1993). It is found in skeletal, but not heart, muscle whereas nebulette (107 kD) a smaller protein with a very high similarity to the C-terminal region of nebulin is only found in heart muscle (Moncman and Wang 1995; Millevoi, Trombitas et al. 1998). As nebulin has the same length as the thin filaments in skeletal muscle, it has been suggested to be a “protein ruler” regulating the length of these filaments (Kruger, Wright et al. 1991). Nebulin can bind strongly to actin in vitro and may have many actin binding sites along its length (Wright, Huang et al. 1993). It has also been shown to bind α -actinin 2 and its N-terminal domain binds tropomodulin.

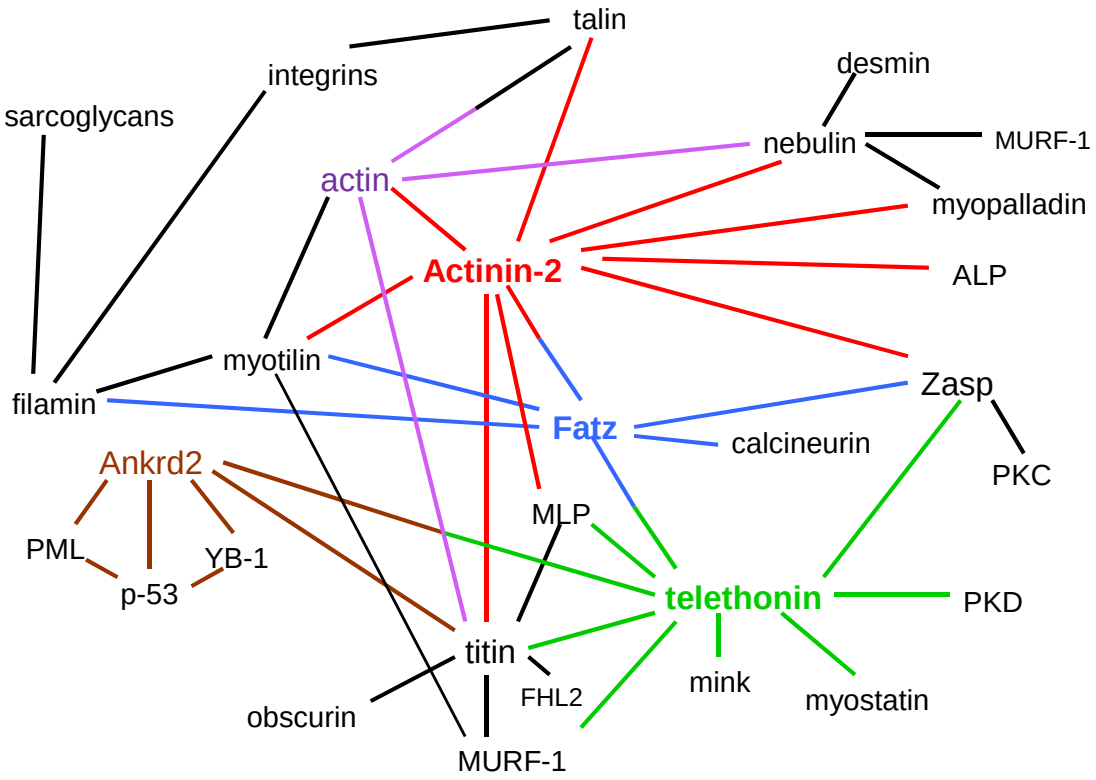


Figure 4. The complexity of Z-disc proteins and their interaction with other partners

Telethonin (T-cap) has been identified as one of the most abundant transcripts expressed in striated muscle (Valle, Faulkner et al. 1997). It co-localizes with actin in the Z-disc, not myosin as originally thought. Two other Z-disc proteins interact with telethonin, namely Myozenin (FATZ) and titin. Telethonin binds to the Z1 and Z2 Ig-like N-terminal repeat region of titin (Mues, van der Ven et al. 1998). The C-terminal region of titin can function as a kinase that has been shown to phosphorylate a serine (S157) of telethonin in early differentiating myocytes (Mayans, van der Ven et al. 1998). The activation of this titin serine kinase occurs when tyrosine (Y170) on titin is phosphorylated and calcium/calmodulin binds to the C-terminal of titin. Interestingly, the titin kinase site is in the C-terminal of titin that is located at the edge of the M-band, whereas telethonin, its substrate, is located in the Z-disc approximately 1 µm from this site. Therefore, it is difficult to explain how they can interact. However, both telethonin and the C-terminal domain of titin have been found to co-localize in stress fiber-like structures

differentiating myocytes; thus, it has been proposed that during myofibrillogenesis when the cytoskeleton undergoes reorganization, the titin C-terminal could be transiently in close proximity to telethonin thus allowing phosphorylation (Mayans, van der Ven et al. 1998). Recently, the absence of full-length telethonin has been found to be responsible for a form of autosomal recessive limb-girdle muscular dystrophy (AR LGMD) type 2G (Moreira, Wiltshire et al. 2000). 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 gave 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 associated with an AR LGMD (Moreira, Wiltshire et al. 2000).

Myozenin also known as FATZ or Calsarcin (γ-filamin, α-actinin, and telethonin-binding protein of the Z-disc) is a novel protein expressed in skeletal muscle, which is up-regulated during differentiation. The C-terminal region of myozenin binds to two of the spectrin-like repeats (R3-R4) of α-actinin 2 (Faulkner, Pallavicini et al. 2000). The same region of myozenin also binds γ-filamin, as detected by yeast-two-hybrid experiments, whereas the region binding telethonin remains undefined. Frey and colleagues (Frey, Richardson et al. 2000) found that an interaction may occur between myozenin and calcineurin. Calcineurin has been implicated in the transduction of signals that control the hypertrophy of cardiac muscle and slow fiber gene expression in skeletal muscle. How myozenin can bind all of these different proteins is not clear, especially because it has no canonical protein-protein interaction domains. However, Takada and colleagues (Takada, Vander Woude et al. 2001) have shown that α-actinin 2 and γ-filamin compete with each other for binding to the C-terminal region of Myozenin. Myozenin could have a structural role in Z-disc assembly via its ability to bind different Z-disc proteins as well as a possible role in signaling pathways via its binding to calcineurin.

ZASP (Cypher/Oracle) is a Z-band protein that has recently been characterized in human skeletal muscle, as an acronym for “Z-band alternatively spliced PDZ motif protein.” (Zhou, Ruiz-Lozano et al. 1999), (Passier, Richardson et al. 2000). ZASP1 (Cypher2) binds α-actinin 2 via the interaction of its N-terminal PDZ domain with the C-terminal

CaM domain of α-actinin 2 (Faulkner, Pallavicini et al. 1999; Zhou, Ruiz-Lozano et al. 1999). The LIM domains of Cypher1, one of the alternatively spliced forms of ZASP (Cypher), can bind equally well all six isoforms of protein kinase C (PKC)-α, -β1, -δ, - ε, - γ, -ξ (Zhou, Ruiz-Lozano et al. 1999). Therefore, it has been proposed that Cypher1 could function as an adapter to couple PKC–mediated signaling to the sarcomere. Another interesting hypothesis is that the major form in skeletal muscle ZASP1 (Cypher2) that lacks LIM domains may inhibit the LIM domain forms in a dominant-negative manner. ALP (Actinin-associated LIM protein) is another PDZ/LIM domain protein of the Z-band that is up-regulated on differentiation. In contrast to ZASP, its N-terminal PDZ domain binds to the spectrin-like repeats of α-actinin 2 (Xia, Winokur et al. 1997). There are two isoforms of ALP derived by alternative splicing; one is found in heart and the other in skeletal muscle. The skeletal muscle isoform has a central region of 111 amino acids that is spliced out and replaced by a different region (63 amino acids) in the heart isoform (Pomies, Macalma et al. 1999).

Filamins (or actin-binding proteins) belong to a family of dimeric proteins that cross-link actin filaments. There are three isoforms, filamin (ABP-280) that is ubiquitous, γ -filamin (ABP-L, filamin2) that is specific for striated muscle (Thompson, Chan et al. 2000; van der Ven, Obermann et al. 2000), and β-filament. All of these isoforms have an N-terminal actin binding domain followed by 24 Ig-like repeats and a C-terminal domain necessary for dimer formation. Interestingly, γ-filamin is the only isoform with a unique 78-amino acid insertion in the Ig-like domain 20, and this insertion has been shown to be responsible for targeting γ-filamin to Z-disc of striated muscle (van der Ven, Wiesner et al. 2000). The N-terminal of γ-filamin is located at the periphery of the Z-disc and recently this region has been found to bind to myotilin, an α-actinin-binding protein. During a search for proteins binding to sarcoglycans, γ-filamin was found to bind specifically to γ- and δ- but not α- and β-sarcoglycans (Thompson, Chan et al. 2000). In normal muscle γ-filamin is found mainly in the Z-disc but also at low levels in the sarcolemma, whereas in muscle with mutations in γ-sarcoglycan, δ-sarcoglycan, or dystrophin, there is an increase in the γ -.filamin level in the sarcolemma. Although γ -filamin is not expressed in undifferentiated muscle cells, it is very quickly up-regulated

during differentiation and may therefore be involved in the formation of the Z-disc (van der Ven, Wiesner et al. 2000).

Myozenin can also bind γ-filamin, α-actinin 2, and telethonin (Faulkner, Pallavicini et al. 2000), therefore, this would be another case of γ-filamin forming a link between γ- and δ-sarcoglycans and indirectly α-actinin 2. Mutations in telethonin can lead to LGMDs, respectively type 1A (Hauser, Horrigan et al. 2000) and type 2G, and that telethonin has been suggested to be involved in signal transmission in myofibrillogenesis (Mayans, van der Ven et al. 1998; Mues, van der Ven et al. 1998).

In conclusion, the complex z-disc proteins found in sarcomeres mediate multiple protein-protein interactions, thereby playing a major role in integrating structure and signaling in this complex three-dimensional network

1.4.3 The Z-discs act as Stretch sensing sensors in transmitting signals

It is known from previous studies that in heart, active muscle contraction is produced by membrane depolarization that stimulates Ca^{2+} efflux from the sarcoplasmic reticulum (SR), initiating discrete pulses of Ca^{2+} transients called sparks. These coalesce and raise Ca^{2+} levels throughout the cytoplasm surrounding the contractile filaments (Maier and Bers 2002). Contraction is switched on by the binding of Ca^{2+} to the thin filament troponin-tropomyosin complex, producing a conformational change that enables cross-bridge formation. Calcium uptake back into the SR through an ATP-dependent pump relaxes the muscle. This pump (SERCA) is partially inhibited by phospholamban (PLB), which can be reversed by cAMP-dependent protein kinase (PKA)-mediated PLB phosphorylation. The release of this inhibition is mediated by the β-adrenergic signaling pathway, which increases the rate of force production and relaxation under exercising conditions and is blunted in heart failure. It is during the relaxed phase of the heart cycle that inflowing blood passively stretches the chambers of the heart. Stretch induces changes in cardiomyocyte biology that are implicated in heart failure, but the mechanism by which stretch is sensed and signals are transduced is unknown. New understanding of the Z disc elements of contractile units is beginning to elucidate the mechanism of stretch sensing and its relation to cardiac adaptation and disease (Epstein and Davis 2003).

Cardiac architecture at the cellular level is determined by myocytes, which contain the bundled myofibrils with repeating series of contractile units, the sarcomeres bounded by Z discs. Titin spans the distance from Z disc to M line and holds myosin-containing thick and actin-containing thin filaments in longitudinal register. Human titin mutations are associated with dilated cardiomyopathy in which the ventricular cavity enlarges as the walls stretch and become thinner (Gerull, Gramlich et al. 2002). Titin contains distinct motifs with different elasticity that sequentially unfold as the muscle is stretched and which provide much of the cardiac cell's passive tension when it is stretched (Granzier and Labeit 2002). Mutations in many sarcomeric protein-encoding genes have been identified as causing hypertrophic cardiomyopathy, an inherited predisposition to increased ventricular wall thickening and corresponding decrease in size of the ventricular cavity. Some of the same genes and other cytoskeletal genes have been shown to cause dilated cardiomyopathy. The Z disc transmits the tension between sarcomeres and its architecture is believed to be partly organized by unique N-terminal titin Z repeats that bind α-actinin. Additional Z disc-associated proteins are being reported, and mutations in many of these are associated with disease in humans or mice.

The Starling Law of the Heart suggests that a primary stretch sensor and responder operate on a beat-to-beat time scale. The work done by Chien and colleagues (Knoll, Hoshijima et al. 2002) describes a primary stretch sensor, which when disrupted, leads to cardiac dilation and failure. The study focuses on their previously reported mouse model of dilated cardiomyopathy produced by knocking out MLP, a two LIM domain protein that binds to α-actinin in the Z disc (Arber, Hunter et al. 1997). Hearts from MLP null mice appear normal at 2 weeks of age but dilate and display contractile dysfunction by 4 weeks. The fact that MLP expression is restricted to striated muscle strongly suggested that this was due to a primary defect at the level of the cardiac myocytes. A striking finding is seen when neonatal cardiomyocytes from these mice are cultured on deformable membranes and stretched. Under these conditions, the cells fail to upregulate brain natriuretic peptide (BNP) and atrial natiuretic factor (ANF) mRNA, both reliable in vivo indicators of mechanical stress (Epstein and Davis 2003). Despite

the uncoupling of this response from mechanical load, the hormonally activated portion of the pathway remains functional in the null MLP cells, since treatment with endothelin or the α-adrenergic agonist phenylephrine triggers BNP mRNA synthesis. T-cap and MLP are shown to interact, and the authors observe the loss of T-cap from the Z discs of some muscle fibers in the MLP null mouse heart. Consistent with this is the detection of T-cap in the soluble fraction of homogenates. These findings to the hypothesis that a titin/T-cap/MLP complex is critical to the sensing of muscle length and those distortions of the architecture and associated structures uncouple the normal response to stretch (Granzier and Labeit 2002).

Transcriptional control of skeletal muscle-specific gene expression is achieved by combined action of various transcription factors that are either specifically expressed in the skeletal muscle lineage or show a more widespread expression pattern. Transcriptional regulation in heart and skeletal muscles is basically governed by 3 major families namely the bHLH, MADs box transcription factors and the Transcriptional enhancer factor (TEFs) which play important roles for the development of muscle tissues and for the regulated expression of muscle specific genes. It seems rather easy to anticipate how tissue-specific regulation of muscle cell-specific genes might be achieved by transcription factors that are themselves tissue-specific such as the MyoD family of transcription factors. Vito1 is a 33 kD protein expressed only in skeletal muscle but not in other tissues. Skeletal actin and cardiac TroponinT genes depend on SRF and TEF transcription factors, respectively. In such cases muscle specific transcription might be achieved by the assembly of larger transcriptional complexes, which utilize DNA-binding proteins as docking stations for tissue-specific bridging or co-activating factors that might confer additional activities to DNA-bound proteins. Recently a new family of transcriptional co-regulators (VITO gene family) (Mielcarek et al., 2002) was identified, which bind to different TEF transcription factors (Gunther et al., 2004). Vito1 was found to be an essential co-factor of TEF1 thereby activating TEF1, which results in the activation of its downstream target genes leading to muscle-specific gene regulation.

The aim of this study was: 1) to find the specific role of individual components of the TEF/VITO complex for muscle specific gene activation. 2) Does the TEF/VITO/ complex contain additional proteins and do they affect DNA binding specificity and/or transactivation? 3) Identification and investigation of new interacting partners of VITO-1 and establish their biological significance in skeletal muscle gene regulation.

2. Materials and Methods

2.1 Materials

2.1.1 Basic materials

Bacterial / Yeast Plates
Cell culture Plates
Filters Minisart NML
Whatmann filter paper
Cover slips
Cover slides
Glass wares
Polyacrylamide gel
Nitrocellulose membranes
Plastic Pipettes
Protein G beads
Cell scraper
Surgical instruments

2.1.2 Chemicals / Reagents

In general the basic reagents and chemicals were ordered from the following companies: Roth (Hamburg/Karlsruhe, Germany), BD BioSciences, Boehringer Mannheim (Mannheim), Invitrogen (Karlsruhe), Merck (Darmstadt), Molecular Probes (Göttingen), New England Biolabs (Schwalbach), Pharmacia (Frieberg), Promega (Mannheim), Qiagen (Hilden), Serva Feinbiochemika (Heidelberg), Sigma-Aldrich (Deisenhofen), Stratagene (Heidelberg).

2.1.3 Radiochemicals

Radioactive labeling of translated proteins were used in TNT Coupled Reticulocyte Lysate Systems and was purchased from Promega

- (35S)methionine (> 1000Ci/mmol at 10 Ci/ml)

2.1.4 Specific reagents

- DAPI (4',6-diamidino-2-phenylindole)	Invitrogen (Karlsruhe)
- Dimethylsulfoxid	Sigma-Aldrich(Deisenhofen)
- Dubelcco's Modified Eagle Medium (DMEM)	Invitrogen (Karlsruhe)
- Fetal Bovine Serum	Invitrogen (Karlsruhe)
- Fluoromount	Sigma-Aldrich(Deisenhofen
- Horse serum	Invitrogen (Karlsruhe)
- IPTG (Isopropyl-β-D-tiogalactopyranosidase)	Roth (Karlsruhe)
- NP-40	Roth (Karlsruhe)
- Penicillin-Streptamycin-Glutamine	Invitrogen (Karlsruhe)
- (Y2H) All the yeast reagents used	BD Biosciences - Clontech
- PFA (paraformaldehyde)	Merck (Darmstadt)
- Prestained Protein Ladder 10-180 kDa	Fermentas (Lithuania)
- Protease inhibitor coctail EDTA-free	Roche (Karlsruhe)
- RNasin®	Invitrogen (Karlsruhe)
- Triton X-100	Roche (Karlsruhe)
- Trypsin 2,5%	Invitrogen (Karlsruhe)
- Tween-20	Roche (Karlsruhe)
- X-Gal (5-bromo-4-chloro-3-indolyl	
- β-D-galactopyranoside)	Roth (Karlsruhe)

2.1.5 Instruments / Equipments

Confocal Laser scanning microscope
Normal Microscope
Cell culture laminar flow hood
Cell culture incubator
PCR machine
Spectrophotometer
pH meter
Yeast incubator
Versadoc
Centrifuge
Microcentrifuge
Vortex
Sonicator
Protein measurement
Vacuum / drier for gels

2.1.6 MATERIALS COMPANY

Biodyne® Nylon Membrane Pall (Dreieich, Germany)
Bio-Traces® Nitrocelulose Pall (Dreieich, Germany)
Blotting Paper 3MM Whatman International (Maidstone,
England)
Cell culture plates Nunc (Roskilde, Denmark), Falcon
Glass slides and cover slides Roth (Karlsruhe, Germany)
Glassware Schütt (Göttingen, Germany)
Filters Minisart NML (0.2 and 0.45 μm) Sartorius (Göttingen, Germany)
Filters
Schleicher & Schüll (Hannover,
Germany)

NAP-5™ columns (Sephadex® G-25) Pharmacia Biotech (Sweden)

Plastic ware Nunc (Wiesbaden, Germany)

Pro-Bond Ni-chelating Resins Invitrogen (Karlsruhe, Germany)

Round cover slides Roth (Karlsruhe, Germany)

X-ray developer Kodak (Frankfurt/Main, Germany)

X-ray Film Kodak (Frankfurt/Main, Germany)

2.1.7 Kits used

Yeast transformation kit	BD Biosciences
Yeast plasmid isolation kit	BD Biosciences
Qiagen mini prep kit	Qiagen
Nucleobond AX 500 Maxi kit	Macherey-Nagel
DNA Cycle sequencing kit	Abi, Weitersadt
TnT® Coupled Reticulocyte Lysate System	Promega
QIAEX II Gel Extraction Kit	Qiagen
Nucleofector ™ electroporation kit	Amaxa Biosystems
FuGENE 6 transfection kit	Roche

2.1.8 Bacterial Strains used

BL21 (DE3	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]
BL21 (DE3) pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3) pLysS(cm^R)
DH10B (Invitrogen)	F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-
JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 $mcrB^+$ Δ(lac-proAB) e14- [F' traD36 $proAB^+$ $lacI^q$ lacZΔM15] hsdR17($r_K^- m_K^+$)
TOP10 (Invitrogen)	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-
XL1-Blue (Stratagene)	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17($r_K^- m_K^+$)

2.1.9 Cell lines used

Cell line	**Organism**	**Origin tissue**	**Morphology**	**ATCC-Nr**
HEK-293	Homo sapiens	Embryonic kidney	epithelial	CRL-1573
C3H-10T1/2	Mus musculus	Embryonic mesenchymal	fibroblast	CCL-226™
C2C12	Mus musculus	muscle	myoblast	CCL-1772™
COS-1	Cercopithecus aethiops	kidney	fibroblast	CRL-1650™

Table 3. Cell lines used

2.1.10 Vectors used

Vector	Size	Selection markers	Source
pGBKT7	7.3 kb	E.coli – Kanamycin Yeast – W (Trp)	Clontech
pGADT7	8.0 kb	E-coli – Ampicillin Yeast – L (Leu)	Clontech
pEGFP-C2	4.7 kb	Kanamycin	Clontech
pDsRed2-N1	4.1 kb	Kanamycin	Clontech
pCS2	4.1 kb	Ampicillin	Kind gift from Prof. T. Braun
pCDNA3.1	5.4 kb	Ampicillin	Kind gift from Prof. T. Braun
HA-N1	4.1 kb	Kanamycin	Kind gift from Prof. M.S. Gautel
pCR®2.1-TOPO ®	3.9 kb	Kanamycin/ampicillin	Invitrogen
pRSETA	2.9 kb	Ampicillin	Invitrogen
pT7T3-Pac	2.9 kb	Ampicillin	Pharmacia

Table 4. Vectors used during this project

2.1.11 Sequencing primers

Primers	Sequences
T7	TAA TAC GAC TCA CTA TAG GG
T3	ATT AAC CCT CAC TAA AGG GA
Sp6	ATT AGG TGA CAC TAT AG
M13 forward	GTA AAA CGA CGG CCA G
M13 reverse	CAG GAA ACA GCT ATG AC
5`- GFP	CAA GTC CGG CCG GAC TCA GAT C
5`- pDsRed 2.1	CCA AAA TGT CGT AAC AAC TC
5`AD LD-Insert screening amplimer	CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC
3`AD LD-Insert screening amplimer	GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG AT
pGBKT7 (T7)	TAA TAC GAC TCA CTA TAG GGC GA
3`DNA-BD sequencing primer	AAA AGC AAA ATT TTG GAT TTC TCA G
GST	GAC CAT CCT CCA AAA TCG GAT CTG
Vito1	GAG TAG TAG GCT AGT TTC TGG
Vito1 –SID	CTC CAG ATG TGT CCT CTT CAC

Table 5. Primers used for sequencing plasmids during this study

2.1.12 Antibodies used

Primary antibody

C-myc	anti-mouse (Santacruz)
Hemagglutinin (HA)	anti-mouse (Abcam)
α – actinin	anti-rabbit (Sigma)
α – sarcomeric actinin	anti-rabbit (Sigma)
α – tubulin	anti-mouse (Sigma)
α – sarcomeric actin	Sigma
telethonin (T-cap)	BD Bioscience
Myozenin1	Eurogentec
eGFP	anti-mouse (Invitrogen), anti-rabbit (Abcam)

Secondary antibody

Alexa fluor 488	anti-mouse, rabbit (Invitrogen)
Alexa fluor 594	anti-mouse, rabbit (Invitrogen)
Cy3	anti-mouse (Rockland)
Horse radish peroxidase (HRP)	anti-mouse (Pierce)
HRP	Anti-rabbit (Pierce)

2.2 Materials for Y2H Screen

2.2.1 Testing for GAL1-HIS3 Auto-Activation

Yeast media containing additives such as 3-amino triazole (3-AT) should be produced by adding the appropriate amount of a concentrated filter-sterilized solution after autoclaving and cooling the medium to at least 60°C.

2.2.2 Testing for Colony β-Galactosidase Activity

Z Buffer: NaH2PO4·H2O 13.79 g/L, KCl 0.75 g/L, MgSO4·7H2O 0.246 g/L, Titrate with 10 N NaOH to pH 7.0. Z buffer/β-ME: This should be made fresh by adding 270 µL of β-mercaptoethanol (β-ME)/100 mL of Z buffer. X-GAL: 20 mg/mL, dissolve 1.0 g of X-GAL in 50 mL of N, N-dimethyl-formamide and store at −20°C. Z buffer/β-ME/X-GAL: This should be made fresh by adding 270 µL of β-ME and 1.67 mL of X-GAL solution to 100 mL of Z buffer

2.2.3 Preparation of Yeast Lysates for Western Blotting

Extraction buffer: 50 m*M* HEPES, pH 7.4, 200 m*M* NaCl. 10 m*M* EDTA (ethylenediaminetetraacetic acid) , 2 m*M* NaVO4, 10 m*M* NaF, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 2 µg/mL E-64 (trans-Epoxysuccinyl-L-leucyl-amido (4-guanidino)butane; *N*-(trans-Epoxysuccinyl-L-leucyl-amido) 4-guanidinobutylamide; L-trans-3-Carbonxyoxiran-2-carbonyl-L-leu-cylagmatine), 2.5 µg/mL pepstatin A, 1 m*M* phenylmethylsulfonyl fluoride (PMSF). Acid washed glass beads, 425–600 microns (Sigma, cat. no. G-8772). Sodium dodecyl sulfate (SDS) loading buffer: 3% (w/v) SDS, 62.5 m*M* Tris, pH 6.8, 720 m*M* β-mercaptoethanol, 10% (v/v) glycerol, 0.125% (w/v) bromophenol blue.

2.2.4 AD: cDNA Library Amplification

LB (Luria Bertani) Medium + ampicillin (600 mL), tryptone 6 g, yeast extract 3 g, NaCl 6 g, distilled water 600 mL. Titrate to pH 7.0 with 10 *N* NaOH. For plates: add 10 g

Difco Bacto Agar to 600 mL volume in each flask prior to sterilization. When cooled to 60°C add 300 µL of a 100 mg/mL stock of ampicillin, mix, and pour plates. Sterile saline: 150 m*M* NaCl, dissolve 8.7 g/L and autoclave. TE (Tris EDTA) Buffer: 10 m*M* Tris, pH 8.0, 1 m*M* EDTA. Make 100 mL by adding 1 mL of 1.0 *M* Tris Cl, pH 8.0, and 0.2 mL of 0.5 *M* EDTA, pH 8.0 to 98.5 mL of double-distilled H2O.

2.2.5 Library Transformation Efficiency Test

All solutions for transformation can be referred to (*R. Daniel Gietz – Yeast Protocol*) or Yeast protocols handbook BD Biosciences

2.2.6 Isolation of AD: cDNA Plasmid

Yeast Lysis buffer, 20 m*M* Tris, pH 8.0, 10 m*M* EDTA, 100 m*M* NaCl, 1% (w/v) SDS, 2% (v/v) Triton X-100. Make 100 mL by adding 2 mL of 1.0 *M* Tris, pH 8.0, 2 mL of 0.5 *M* EDTA, pH 8.0, 2 mL of 5.0 *M* NaCl, 5 mL of 20% (w/v) SDS, and 2 mL of Triton X-100.

2.2.7 Transforming chemically Competent *E.coli* and Selection of LEU+ Colonies

M9 salts (10X): Na2HPO4 60 g, KH2PO4 30 g, NaCl 5 g, NH4Cl 10 g, per L of distilled water and autoclave. M9 Leucine prototrophy medium (M9-L): 60 mL 10X M9 salts, 540 mL distilled water, 10 g Difco Bacto-agar. Autoclave this solution and allow to cool to 60°C, then add the following amounts (each solution is sterile) 0.6 mL 1.0 *M* MgSO4, 0.6 mL 0.1 *M* CaCl2 0.5 mL thiamine (4 mg/mL), glucose (20% [w/v]), 0.15 mL FeCl3 (0.01 *M*), 0.6 mL vitamin B1 (2 mg/mL). Depending on the genetic markers found in the *E. coli* strain, add the appropriate amino acids. *E. coli* strain KC8 (genotype; *hsdR*, *leuB600*, *trpC9830*, *pyr::Tn5(kan* r), *hisB463*, *lacDX74*, *strA*, *galU*, *galK*) add the following; 6 mL histidine (2 mg/mL), 6 mL uracil (2 mg/mL), 6 mL tryptophan (2 mg/mL).

2.3 Methods

2.3.1 Two-Hybrid Screen Preparation

2.3.1.1 Construction of the DNA Binding-Domain Bait Gene Fusion Plasmid

The first step is to construct the DNA binding-domain bait gene fusion plasmid. Our favorite gene encoding the protein of interest (considered the bait), VITO-1 is cloned into a suitable Y2H vector (here pGBKT7) in-frame with the chosen DNA binding domain.

Plasmid	DNA binding domain	Restriction sites DNA sequence and frame	Yeast selection marker
pGBKT7	*GAL4* BD	NdeI SfiI/NcoI EcoRI SmaI BamHI SalI PstI	TRP1
		CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA C	
pAS2	GAL4 BD	NdeI SfiI/NcoI SmaI BamHI SaiI PstI	TRP1
		CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA CCT GCA GCC	CYH2
pGBT9	GAL4 BD	EcoRI SmaI BamHI SalI PstI	TRP1
		GGA TTC CCG GGG ATC CGT CGA CCT GCA G	
pBTM116	*LexA* BD	EcoRI SmaI BamHI SalI PstI	TRP1
		GGA TTC CCG GGG ATC CGT CGA CCT GCA G	

Table 6. Two-Hybrid System DNA Binding Domain Plasmid Vectors

There are many different DNA binding-domain vectors available and are listed in (James 2001). An important consideration is to match the DNA binding-domain plasmid used to

the appropriate yeast strain. A few of the DNA binding domain vectors used more frequently are listed in Table 6.

2.3.1.2 Choosing the Right Y2H Reporter Strain

Yeast strains for the *GAL4* system include reporter constructs that utilize the *HIS3*, *ADE2*, *LEU2*, *URA3*, *CYH2*, *lacZ*, and *MEL1* genes. The *HIS3* reporter allows direct selection of Y2H positives; however, some strains contain "leaky" derivatives, which require the addition of the chemical 3-amino triazole (3-AT) to the medium to quench background expression of the *HIS3* gene product (Durfee, Becherer et al. 1993). The *HIS3* reporter gene also selects for the optimal ratios of fusion proteins to produce reporter gene expression for growth on selective medium (Aspenstrom and Olson 1995). In some strains, the *ADE2* reporter can be used to select for co-activation giving rise to a more stringent screen. The *lacZ* and the *MEL1* reporters can be used to verify positives through co-expression as well as generate quantitative measurements of gene expression. Another important quality of a Y2H yeast strain is its transformation characteristics. The ability to generate large numbers of transformants using current transformation protocols is essential for Y2H screening.

2.3.1.3 Y2H Reporter Strains

Yeast Promoter Constructs Used To Regulate Reporter Gene Expression					
Plasmid or host strain[a]	**Reporter gene**	**Origin of UAS**	**UAS regulated by**	**Origin of TATA sequence**	**Expression level[b] Induced (uninduced)**
HF7c	lacZ	$UAS_{G\ 17\text{-}mer(x3)}$[c]	GAL4	CYC1	low
	HIS3	GAL1	GAL4	GAL1	high (tight)
Y190	lacZ	GAL1	GAL4	GAL1	high
	HIS3	GAL1	GAL4	HIS3 (TC+TR)	high (leaky)
Y187	lacZ[d]	GAL1	GAL4	GAL1	high
SFY526	lacZ	GAL1	GAL4	GAL1	high
PJ69-2A	HIS3	GAL1	GAL4	GAL1	high (tight)
	ADE2	GAL2	GAL4	GAL2	high (tight)
AH109	HIS3	GAL1	GAL4	GAL1	high (tight)
	ADE2	GAL2	GAL4	GAL2	high (tight)
	lacZ	MEL1	GAL4	MEL1	low

Table 7. Yeast two hybrid reporter strains used to regulate the expression of reporter gene

2.3.1.4 Testing for GAL1-HIS3 Auto-Activation

The activation of reporter genes by the BD:bait plasmid in the absence of an activation domain plasmid is defined as auto-activation. Transform the BD:bait plasmid into the appropriate reporter yeast strain using the rapid transformation protocol (Gietz and Woods 2006) then proceed to test both reporter genes for auto-activation. To test for auto-activation of the *GAL1-HIS3* reporter, yeast cells containing the BD:bait plasmid should be plated, not streaked, onto SC-H (synthetic complete medium minus histidine) medium containing increasing concentrations of 3-AT (1, 5, 10, 25, 50 m*M*). As well, these yeast cells should be plated onto SC-W (synthetic complete medium minus tryptophan) medium to select for the BD:bait plasmid as a control of growth. The addition of 3-AT into the medium is used to suppress the "leaky" nature of this reporter in most strains. The concentration of 3-AT needed to eliminate background growth is plasmid and strain-dependent.

Grow the yeast transformant containing the verified BD:bait plasmid overnight in SC-W medium to select for maintenance of this plasmid. Alternatively, a 10-µL blob of cells can be scraped from a freshly grown SC-W plate and resuspended in 1 mL of sterile water. Titer the liquid culture using a spectrophotometer (OD_{600} 0.1= ~ 1×10^6 cells/mL) or a hemocytometer Plate at least 500 cells/plate onto a pair of SC-W plates as well as pairs of SC-H plates containing 0, 1, 5, 10, 25, and 50 m*M* 3-AT and incubate at 30°C for up to 5 d. Examine the SC-H + 3-AT plates for growth. Most BD:bait plasmids will not produce colonies on the medium once the appropriate concentration of 3-AT is reached. The SC-W control plates should contain approximately 500 colonies. If growth cannot be inhibited completely even at a 50 m*M* concentration, its better considering either to clone a different gene fragment into the BD:bait plasmid, or cloning our gene of interest into another BD vector.

2.3.1.5 Testing for Colony β-Galactosidase Activity

In addition to testing for *GAL1-HIS3* auto-activation, it is prudent to also test for *GAL1-lacZ* auto-activation in those strains that contain this reporter gene. This can be

accomplished by using the pair of SC-W plates that were plated for the *GAL1-HIS3* auto-activation test above

1. Carefully place a sterile 75-mm circle of Whatman #1 filter paper on top of the colonies or patches growing on selective medium. Ensure that the filter paper makes good contact with the colonies. Mark the orientation of the filter paper relative to the plate using an 18-gauge needle to punch through the filter in an asymmetric pattern.

2. Remove the filter from the plate with sterile forceps after it has become fully absorbed to the colonies and immerse into liquid nitrogen for 10–15 s.

3. Carefully remove the filter from the liquid nitrogen and thaw by placing on a piece of plastic wrap colony-side up. Repeat the freeze-thaw cycle twice more.

4. Place another 75-mm sterile Whatman no. 1 filter into an empty petri plate (100 × 15 mm) and dispense 1.25 mL of Z buffer/β-ME/X-GAL onto the filter.

5. Place the filter, colony-side up, onto a filter paper soaked with Z buffer/β-ME/XGAL taking care that the filters line up to distribute the solution evenly.

6. Place the lid on each plate and transfer to a plastic bag and incubate at 37°C.

Strong activation of the *lacZ* gene will give a blue color within 1–2 h. If color does not develop, continue to incubate the filters overnight. Note the time needed for color production. A faint blue color after overnight incubation is considered minimal *lacZ* activation.

2.3.1.6 Preparation of Yeast Lysates for Western Blotting

Prior to screening, the steady-state expression of the BD:bait fusion protein should be assayed by Western blotting. This may be accomplished if the appropriate reagents are available, such as a specific antibody for the product of our gene of interest. Some vectors, such as pGBKT7 and pGADT7, contain the myc and HA tag that can be recognized by the commercially available monoclonal antibody (MAb). The $Gal4_{BD}$

antibody (Santa Cruz Biotechnology Inc. or Invitrogen) can also be used. In addition to indicating the expression levels of the fusion protein, Western blotting can verify the in-frame cloning strategy, because the size of the fusion protein detected should compare to the predicted value. Yeast lysates are prepared for electrophoresis following a modified method of (Rocchi, Tartare-Deckert et al. 1996). Inoculate the yeast strain containing the BD:bait plasmid into 50 mL of SC-W medium. Incubate at 30°C with shaking until a titer of 1.0×10^7 cells/mL is reached. This may take 16–24 h. Alternatively a 10-mL overnight culture can be used to inoculate 50 mL to 2.5×10^6 cells/mL and incubate until a titer of $1–2 \times 10^7$ cells/mL is reached. This will take 4 to 6 h or longer in SC-W medium. Collect the yeast cells by centrifugation at 5000*g* for 5 min and wash the cells with 1/2 volume of sterile water. Determine the volume of the cell pellet by adding a specific volume of water and then measuring the total volume of the cell slurry. Transfer the slurry to a 1.5-mL microfuge tube. Re-suspend the cells in 2 volumes of ice-cold Extraction buffer. Add 1 volume of glass beads and place each sample onto ice. Vigorously vortex each sample for 30 s and return to ice to cool. Repeat six times for each sample. Centrifuge samples at 14,000*g* at 4°C for 1 min to pellet unbroken cells and cell debris. Transfer the supernatant to another 1.5-mL microcentrifuge tube and cool each sample in ice slurry for 1 min. Centrifuge each sample again at 14,000*g* at 4°C for 1 min to further clarify extract. Carefully remove supernatant, mix 1:1 with SDS loading buffer, and heat in a boiling water bath for 2 min. These extracts can be used for Western blot analysis and stored at −70°C until needed.

2.3.1.7 Library Transformation Efficiency Test

Library transformation efficiency test is accomplished by transforming increasing amounts of library plasmid DNA into the two-hybrid yeast strain containing the BD:bait plasmid at a 1 x transformation scale. This experiment will allow you to use the library plasmid DNA efficiently, as well as target a specific number of transformants for Y2H screening. If the DNA concentration used for the transformation is too high, multiple AD:cDNA library plasmids will be transformed into a single yeast cell, making subsequent analysis of two-hybrid positives more difficult.

1. Using the "high-efficiency transformation protocol" (Gietz and Woods 2006), transform increasing amounts of the AD:cDNA library plasmid DNA into your Y2H yeast strain containing the BD:bait plasmid at the 1X transformation scale (e.g., 0.1 µg, 1 µg, 2 µg , 5 µg, and 10 µg of AD:cDNA library plasmid DNA). Plasmid DNA preparations containing RNA can be estimated for concentration from agarose gels. Incubate the plates for 3–4 d at 30°C.

2. Count the colonies on each set of plates to determine the Transformation Yield (total number of transformants) as well as the Transformation Efficiency (transformants/µg) for each transformation.

2.3.1.8 The Library Screen

Once the transformation yield test has been completed, a large-scale library screen can be performed as outlined in (Gietz and Woods 2006). Typically a 30x or 60x transformation scale-up is used. It is recommended to use at least 50–100 large (150 × 15 mm) Petri plates containing SC-W-L-H + 3-AT medium. Freshly made plates should be allowed to dry for a few days at room temperature to eliminate excessive condensation. Media should also be stored in the dark to prevent a reduction in plating efficiency owing to exposure to fluorescent lighting. Using the appropriate amount of AD:cDNA library plasmid DNA, transform the Y2H yeast strain containing the BD:bait plasmid using the methods outlined in (Gietz and Woods 2006) and plate onto medium that selects for reporter gene activation. Plating a 30X or 60X transformation onto 100 large plates can take up to 30 min. Spread the plates out on a counter top, 10 at a time, and dispense 400 µL of transformed cells onto each plate. Using a sterile glass spreading wand, the inoculum was carefully spread onto the surface of the entire plate. Incubate plates in loosely taped Petri plate bags to reduce drying during growth for 4–21 d at 30°C.

2.3.1.9 Picking Y2H Positives

Transformation plates were checked for colonies after 4 d of incubation at 30°C. Continue to check the plates and pick positives every day for the first week and then every 2 d for up to 3 wk. When colonies become visible they were patched to fresh

selection plates (SC-W-L-H + 3-AT) in a grid pattern. These patched plates were incubated at 30°C until sufficient growth occurs. Colonies that do not produce growth on the patched plate after 5–7 d can be eliminated. When picking positives, colonies that are actively growing were selected. To be certain, observe the colony growth over a number of days. Depending on the strain and BD:bait plasmid, small colonies can usually be found in areas of the plate containing heavy inoculum. These types of colonies were avoided because they are usually not true positives. Positives were kept on medium that selects for reporter gene activation and all plasmids at all times (e.g., SC-W-L-H + 3-AT plates). This ensures that the BD:bait and AD:cDNA library plasmids encoding the interacting fusion protein are maintained. In cases where a yeast transformant contains multiple AD:cDNA library plasmids, this will ensure the maintenance of the correct plasmid. Yeast colonies were streaked to fresh plates weekly and/or cryo-preserved (positives) as soon as possible.

2.3.1.10 LacZ Reporter Gene Activity

A good indication of a true Y2H positive is co-activation of all reporter genes. The *lacZ* reporter can be used for this purpose. Once positives are patched and replicated, *lacZ* gene activation can be assayed. It is important to maintain positives on medium that selects for *GAL1-HIS3* reporter activation. This will optimize the expression of fusion proteins to give good levels of reporter gene activity.

2.3.1.11 Cryo-Preserving the *His*+ *lacZ* + Positives

Patched colonies that activate the *lacZ* reporter should be cryo-preserved. Streak the *His*+ *lacZ*+ positives onto fresh SC-W-L-H + 3-AT plates and incubate at 30°C for 24 to 48 h. Scrape a blob of fresh inoculum using an inoculating loop or a sterile toothpick and resuspend in 1 mL of sterile 25% glycerol in a 1.5-mL microcentrifuge tube or cryo-tube. Store at -70°C.

2.3.1.12 Characterizing Two-Hybrid Positives

Primary Y2H positives that activate both the *HIS3* and the *lacZ* reporter genes can now be subjected to further analysis. Owing to the in vivo nature of this system, unforeseen obstacles may be encountered that may one to return to a previous step.

2.3.1.13 Isolation of AD:cDNA Plasmid

To isolate the AD:cDNA library plasmid, nucleic acids are extracted from the yeast cells of each Y2H positive. A quick and effective method described in (Hoffman and Winston 1987) uses glass beads and phenol:chloroform to extract nucleic acids. Alternatively, a method (Cryer, Eccleshall et al. 1975) that uses lyticase to produce spheroplasts can also be used. These nucleic acid preparations will include both *TRP1* and *LEU2* plasmids and should be transformed into an *E. coli* host containing a *leuB* mutation to specifically select for the yeast *LEU2* gene harbored on the AD:cDNA library plasmid.

This protocol, modified from (Hoffman and Winston 1987), can be used to isolate DNA from yeast cells grown in either liquid culture or harvested from a plate. Inoculate individual Y2H positives from SC-W-L-H + 3-AT plates into 2 mL of SC-H or SC-W-L medium and incubate at 30°C overnight. Alternatively, scrape a 50-µL blob of cells from an SC-W-L-H + 3AT plate and resuspend in 500 µL of sterile water in a 1.5-mL microcentrifuge tube. Collect the yeast cells from the liquid culture by centrifugation at 13,000*g* for 30 s. Remove the supernatant and add 200 µL of yeast lysis buffer and gently resuspend the cell pellet using a micropipet tip to avoid the generation of bubbles. Add an approx 200 µL volume of glass beads and 200 µL of buffer-saturated phenol:chloroform (1:1 [v/v]). Vortex each sample vigorously for 30 s and then place on ice. Repeat twice, leaving samples 30 s on ice between treatments. Centrifuge tubes at 13,000*g* for 1 min. Remove the aqueous phase (~ 200 µL) to a fresh tube and precipitate the nucleic acids by adding 20 µL of 3.0 *M* sodium acetate, pH 6.0, and 500 µL of 95% ethanol. Incubate at −20°C for 30 min and collect the precipitate by centrifugation at 13,000*g* for 5 min at 4°C. Wash the pellet with 100 µL of 70% ethanol (room temp) and dry the pellet for 5 min at room temperature. Dissolve the pellet in 25 µL of TE buffer and store at −20°C.

2.3.1.14 Transformation of Chemically Competent E. coli – selection of LEU+ colonies

Transformation efficiency is significantly affected by temperature. Falcon tubes and pipette tips should be prechilled at 4°C. Prepare chemical competent cells and thaw them in ice.Add 10µl of yeast plasmid solution to a prechilled 10-ml Falcon tube. Add 100µl of competent cells to the tube and mix well by gently tapping the tube. Incubate on ice for 30 min. Transfer the tube to a 42°C water bath and incubate for 45-50 sec. Chill on ice for 2 min. Add 1 ml of LB or (preferably) SOC medium with no antibiotic. Incubate at 37°C for 1 hr with vigorous shaking (250 rpm). Pellet cells by centrifuging at 2,500 rpm for 5 min in a table top centrifuge. Discard supernatant and resuspend pellet in 100µl of the selection medium and spread on the appropriate plates. Incubate plates at 37°c for 24 hr (LB/amp selection only), or for 36-48 hr (for nutritional selection on M9 medium). Typically, 10-100 colonies will be seen on the plate for a successful transformation using isolated yeast plasmid. A parallel transformation using the control pUC19n DNA should be used to calculate the transformation efficiency.

2.3.1.15 Isolating Plasmid DNA from the E. coli transformants

This can be done using a standard plasmid mini-prep procedure to isolate plasmid DNA from the E. coli transformants. (Sambrook et al., 1989)

2.3.3.16 Analysis of Isolated AD:cDNA Plasmids

The *LEU2* AD:cDNA library plasmids isolated from the *leuB E. coli* strain can now be characterized by restriction enzyme (AluI, HaeIII) digestion and agarose gel electrophoresis. Restriction enzymes that digest on the 5′ and 3′ ends of the cDNA are vector- and library-specific; check the AD:cDNA library plasmid information. This analysis will group the plasmids by insert size and restriction pattern. Restriction enzyme analysis should be carried out on 4–5 library plasmid isolates from each Y2H positive. If more than one type of library plasmid is isolated from a single Y2H positive, further analysis should be carried out with each unique isolate. Independent positives with

similar-sized inserts should not be considered duplicates until sequence information can be produced.

2.3.1.17 Reconstruction of Two-Hybrid System Positives

Plasmid DNA isolated from the *leuB E. coli* strain is transformed back into the Y2H yeast strain containing the BD:bait plasmid. Thus, a representative from each plasmid group is tested for activation of both the *HIS3* and *lacZ* reporter genes when in combination with the original BD:bait plasmid. This is accomplished using the high-efficiency transformation protocol mentioned above. Transformed cells are plated onto SC-W-L as well as SC-W-L-H + 3-AT media. Growth on SC-W-L confirms the presence of both the BD:bait and AD:cDNA library plasmids. Colony formation on SC-W-L-H + 3-AT demonstrates activation of the *HIS3* reporter gene. These His+ colonies can also be tested for activation of *lacZ* reporter using the β-galactosidase assay.

2.3.1.18 Failure of Y2H Positives to Reconstruct

The failure to obtain colonies on SC-W-L-H + 3-AT medium while generating colonies on SC-W-L medium suggests that the AD:cDNA library plasmid used in the transformation was not responsible for activation of the reporter genes in the original Y2H positive. There are two specific situations that are known to give rise to this. The first is the presence of multiple AD:cDNA library plasmids in the original Y2H positive, caused by transformation with high library plasmid DNA concentrations. The second is alteration of the BD:bait plasmid in the yeast strain.

2.3.1.19 Multiple Library AD:cDNA Plasmids

The presence of multiple AD:cDNA library plasmids in a single yeast Y2H positive is a relatively common occurrence if high plasmid DNA concentrations were used in the library transformation reaction. This situation will be immediately apparent if multiple restriction digestion patterns are identified among the 4–5 AD:cDNA library plasmids originally isolated. Each plasmid type should be tested for reconstruction. If each of the 4–5 AD:cDNA library plasmids have identical restriction patterns and do not reconstruct

reporter gene activation, it is likely that your Y2H positive contains multiple AD:cDNA library plasmids. An additional 10–20 *E. coli* colony should be isolated from the yeast DNA preparation and analyzed to identify others plasmids that may be responsible for reporter gene activation. Failure to identify other AD:cDNA library plasmids in a Y2H positive suggests that it may be nontypical. Depending on the numbers of positives from the screen, these nontypical positives can be retired for later analysis.

2.3.1.20 Sequencing Positives

Representative members of each group of AD:cDNA library plasmids that reconstruct should be sequenced to identify those positives that contain ORFs in-frame with the *GAL4* $_{AD}$ Double-stranded plasmid DNA can be sequenced using various commercial kits or companies. The primers used to sequence any *GAL4*-based Y2H vector can be found in Table 8.

Primers	Vectors
5' -TCA TCG GAA GAG AGT AG -3'	pGBKT7, pGBT9, pAS1, pAS2
5' -TAC CAC TAC AAT GGA TG -3'	pGADT7, pGAD10, pGAD424, pACT, pACT2

Table 8. Sequencing Primers

DNA sequence information can be analyzed using your favorite DNA analysis software package. Complete or partial THS vector sequence files can be found either at GenBank http://www.ncbi.nlm.nih.gov or the Vector database (http://seq.yeastgenome.org/vectordb/). The sequences from AD:cDNA library plasmids should be analyzed using the BLAST 2.2.9 algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) to identify previously cloned genes in the GenBank database. In addition, the amino acid sequence of the predicted in-frame ORF can be used to search for similarities in a protein database. Positives found to encode short fusion proteins of under 20 amino acids can usually be eliminated from further analysis; however, this decision should be made with reference to additional

criterion. For example, the Y2H was used to successfully identify short peptides that interact with a protein of interest (Yang, Wu et al. 1995).

2.3.1.21 ONPG Test

***Ortho*-Nitrophenyl-β-galactoside** (ONPG) is a colorimetric and spectrometric substance for detection of beta-galactosidase activity. This compound is normally colorless. However if β-galactosidase is present, it hydrolyses the ONPG molecule into galactose and ortho-nitrophenol. The latter compound has a yellow coloration that can be used to check for enzyme activity by means of a colorimetric assay. Beta-galactosidase is required for lactose utilization, so the intensity of the color produced can be used as a measure of the rate that Beta-galactosidase can hydrolyze lactose

Co-transform the two supposedly interacting plasmids which need to be assayed into an appropriate yeast strain like AH109. Prepare overnight cultures in the appropriate liquid SD selection medium. Vortex and transfer 2 ml of this to 8 ml of YPD medium and grow the cells at 30°C with shaking (250 rpm) until they reach an OD_{600} of 0.5-0.8. Transfer 1.5 ml culture onto three 1.5 ml tubes and centrifuge at 14,000 rpm for 30 sec. Resuspend the cell pellet in 1.5 ml of Z-buffer, centrifuge again and finally disperse the cells in 300 µl of Z-buffer. Note the concentration factor. Transfer 100 µl of cell suspension into a fresh tube and subject it to several freeze thaw cycles using liquid nitrogen for complete cell lysis. Set up a blank tube with 100 µl of Z buffer. Add 0.7 ml of Z buffer + β-mercaptoethanol to the reaction and blank tubes. Immediately add 160 µl of ONPG in Z buffer to the reaction and blank tubes and incubate at 30°C until the development of yellow color. Stop the reaction by adding 400 µl of 1 M Na_2CO_3. Record time elapsed in minutes. Centrifuge reaction tubes for 10 min at 14,000 rpm and measure the OD_{420} of the supernatant against the blank using the spectrophotometer. The ODs should be between 0.02–1.0 to be within the linear range of the assay.

Calculate the ß-galactosidase units using the formula **β-galactosidase units = 1,000 x OD420 /(t x V x OD600)** where: t = elapsed time (in min) of incubation, V = 100 µl x concentration factor and OD600 = A600 of 1 ml of culture

2.3.2 Rabbit Reticulocyte Lysate

Rabbit reticulocyte lysate is a highly efficient in vitro eukaryotic protein synthesis system used for translation of exogenous RNAs (either natural or generated in vitro). In vivo, reticulocytes are highly specialized cells primarily responsible for the synthesis of hemoglobin, which represents more than 90% of the protein made in the reticulocyte. These immature red cells have already lost their nuclei, but contain adequate mRNA, as well as complete translation machinery, for extensive globin synthesis.

Coupled *in vitro* transcription / translation

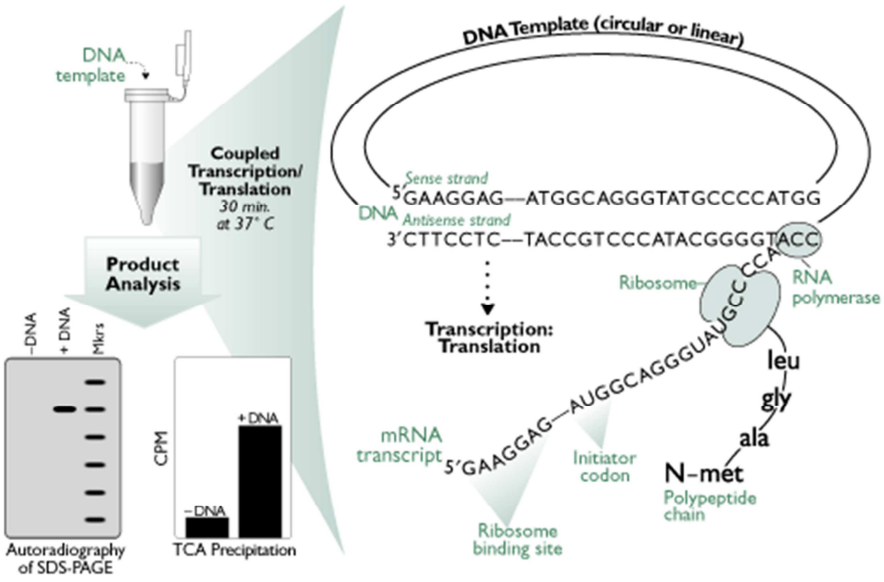


Figure 4. The principle of Coupled Invitro transcription/translation

This method is used for transcription and translation of genes cloned into appropriate vectors downstream from the SP6 (circular plasmid DNA), or T3 and T7 (linear DNA) RNA polymerase promoter.

TNT® Lysate Reactions

Component	Standard reaction Using [^{35}S]methionine
TNT® Rabbit Reticulocyte Lysate	25µl
TNT® Reaction Buffer	2µl
TNT® RNA Polymerase (SP6, T3 or T7; 1ml of appropriate polymerase[s])	1µl
Amino Acid Mixture, Minus Methionine, 1mM	1µl
[^{35}S]methionine (>1,000Ci/ mmol at 10mCi/ml)	2µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
DNA template(s) (0.5µg/µl)	2µl
Nuclease-Free Water to a final Volume of	50µl

Table 9. The components to be used for In vitro transcription/ translation reaction

Rapidly thaw the components mentioned in the table at 25°C and assemble the reaction components together on ice. Mix them gently and incubate the reaction mixture at 30°C for 90 minutes. Once the translation reaction is complete, denature the proteins by adding SDS sample buffer and heating at 100°C for 2 minutes. Load a small aliquot of the denatured proteins onto a SDS-PAGE. After electrophoresis, place the gel in a plastic box and cover it with fixing solution (50% methanol, 10% glacial acetic acid and 40% water) and agitate slowly in a shaker for 30 minutes. Now soak the gel in 7% acetic acid, 7% methanol, 1% glycerol for 5 minutes to prevent the gel from cracking and place them on a sheet of Whatmann® 3MM filter paper, cover with plastic wrap and dry at 80°C for 30-90 minutes under a vacuum using a conventional gel drier. Expose the gel on Kodak® X-ray film for 6-15 hours at room temperature with autoradiography.

2.3.2.1 Co-Immunoprecipitation (Co-IP from *in vitro* transcripted / translated proteins)

Co-IP is the technique of precipitating a protein antigen out of a solution using an antibody that specifically binds to that particular protein. This process can be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins and can be used to study protein-protein interaction. The SP6, T7 –

based coupled transcription and translation system, with rabbit reticulocyte lysate (TNT, Promega) and Easy Tag [^{35}S] methionine-L was used to produce radiolabled proteins and peptides. Briefly, equal amounts of ^{35}S labeled proteins (bait and prey) were mixed together in 200µl of interaction buffer (20 mM Tris (pH 8.3), 150 mM NaCL, 1% Nonidet P-40. 0.1% Tween 20, and 1 mg/ml bovine serum albumin or cocktail protease inhibitors).Each reaction was carried out in triplicate for 30 minutes on ice. Subsequently each reaction mixture was nonspecifically immunoprecipitated with rabbit IgG or mouse IgG for 1 h. Following pulling down of nonspecific immune complexes with protein A/G sepharose beads, supernatants were subjected to co-immunoprecipitation with specific primary antibodies (anti-myc or anti-HA, anti E2-2). Immnune complexes were pulled down with protein A/G beads. Beads were washed 5 x with interaction buffer, suspended in SDS sample buffer, heated at 75°C for 10 min, and loaded on SDS-PAGE for analysis. Following electrophoresis, the gels were fixed, dried and subjected to autoradiography either at -80°C overnight or 6-10 h room temperature.

2.3.3 Phenol: Chloroform Purification of DNA

20-30µgs of plasmid DNA to be purified was diluted in 100 µl water. To this, 60 µl of Phenol: chloroform mixture was added and vortexed. Centrifuge for 1 min at max speed and transfer the upper phase to a new eppi. Add 50 µl of chloroform to the upper phase and vortex. Centrifuge for 1 min at max speed and transfer the upper phase to a fresh eppi. Measure the upper phase volume. Add 2.5 volume (of upper phase) of ice cold ethanol (-20°C) and 0.1 volume of 3 M sodium acetate pH 5.2 and incubate for 20 min -1 h at -80°C. Remove and centrifuge for 5 min at max speed. Discard the upper phase. Add 50 µl of ethanol to the pellet, centrifuge, remove supernatant and air dry. Resuspend purified DNA pellet in 20-30 µl water and measure the concentration using nano drop or spectrophotometer.

2.3.4 Sequencing of plasmids

Sequencing was carried out using an automated DNA sequencer based on the principle of chain terminating inhibitors (Sanger, Nicklen et al. 1977) using ABI PRISM TM system.

Reaction mix:

Ready reaction premix (DNA polymerase, dNTPs, fluorescent marked ddNTPs) – 4 µl

5 x Big dye sequencing (Terminator) buffer – 2 µl

Primer - 10 pmol

Template – (200ng -1µg) depends on the template used

The reaction mixture were amplified in the thermocycler

25 x	95°C (Denaturation)	30 sec
	37 – 60°C (annealing - varies with the primers used)	30 sec
	60°C (extension)	4 min

Table 10. The thermocycling reaction performed using the PCR

Annealing temperature is calculated using the formula $Tm = 4(G + C) + 2(A + T)^{oC}$.

Preparation of reaction mix for sequencing:

To the reaction mixture add 5 µl of 125 mM EDTA, followed by 75 µl of 100% ethanol in an eppi. Mix them by inverting the tube several times. Incubate at room temperature for 15 min in dark. Centrifuge at 13000 rpm at room temperature for 15 min and carefully remove the supernatant. Add 200 µl of 70% Ethanol and incubate for 5 min at room temperature. Centrifuge, discard supernatant and air dry the pellet. The pellet is finally dissolved in 12 µl TSR sequencing buffer and denatured for 2 min by the automatic sequencer.

2.3.5 Western Blot

Western Blot is an analytical method that involves the immobilization of proteins on membranes before detection using monoclonal or polyclonal antibodies. Concentration of protein samples were measured using the standard Bradford assay. The proteins were dissolved in SDS sample buffer and denatured at 95°C for 3 min or 75°C for 10 min. The samples were loaded on a SDS - polyacrylamide gel (NuPAGE 4%-12% bis-tris, Invitrogen) and subjected to electrophoresis for 90 min. Depending on the experiment proteins can also be visualized without blotting by doing a Coomassie staining. The proteins were then wet transferred to a nitrocellulose membrane by blotting them for 2 hr at 30 V. The membrane is then washed with water and a red alert staining is performed to visualize the protein bands which confirm the proper transfer of the proteins onto the nitrocellulose membrane. The membrane is then washed for 30 min in TBS buffer followed by blocking the membrane with 3% milk powder prepared in TBST for 1 hr at room temperature with gentle agitation. The membrane is then incubated with the respected primary antibodies specific to the proteins (diluted in 3% milk powder-TBST) either 1 hr at room temperature or overnight at 4°C. Wash the membrane 5x 5 min in TBST and add the secondary antibody diluted 1:5000 – 1:10000 (HRP conjugated anti mouse IgG or anti rabbit IgG) and incubate the membrane for 45 min at room temperature. The membrane is then washed 5 x 5 min in TBST and the proteins were detected using chemiluminescent detection system (Super Signal ® West Femto, Pierce).

2.3.6 Cloning strategies

All plasmids were cloned either by subcloning into another factor in frame or by PCR amplification. After PCR amplification with Taq Polymerase (Eppendorf) or Expand High Fidelity Polymerase (Roche) and specific primers (see 3.1.7.1), fragments were cloned into the pGEM-T-vector (Promega), pCR TOPO 2.1 or pCR TOPO II (Invitrogen). After digestion with restriction endonuclease enzymes, positive clones were analyzed using agarose gel electrophoresis to verify the proper length of the insert and vector and finally were sequenced using primers from both sides. All sequencing reactions were prepared with ABI 310 Genetic Analyzer sequencer (Perkin Elmer). After

verification of the clones, fragments were cut out with restriction enzymes and subcloned into the target vectors. All sequence and alignments were prepared using data bases at **www.ncbi.nih.gov** BLAST

2.3.7 Cell culture

Frozen C2C12 or HEK 293 or 10T1/2 cells were rapidly thawed in 37°C bath and distributed into 10 ml of DMEM (Dulbecco's Modified Eagle's Medium) with 1% glucose, 1% penicillin/streptomycin and 10% FCS (Fetal calf serum) in a 100 mm tissue culture dish (Nunc). Incubate cells at 37°C in 10% CO_2. The medium is changed after all cells have adhered. Cells were routinely split 1:5 – 1:10 when they get dense, usually every 2 days. C2C12 cells should not be allowed to reach confluence as they will begin to differentiate, even in normal growth media. Cells are usually grown to about "70% - 80%" confluence. Cells were fed every alternate days..

To differentiate C2C12 cells, they were allowed to grow to about "90%" confluence. Switch dense cells into DM (differentiation medium) and feed every day. Ideally, the cells should be plated on collagen coated dishes before differentiating (for better adherence of the myotubes). In a healthy culture, one must see some spontaneous twitching of the myotubes. To get maximum differentiation of C2C12 cells, they can be treated with AraC (10 mM) when switched into DM to eliminate dividing cells. This keeps the myoblasts from crowding out the myotubes, and allows a myotube culture to be maintained for about 2 weeks.

2.3.7.1 Culture of primary chicken myocytes

Chicken eggs were bought from the farmer and kept in the incubator at 37°C with timely rotations for the proper orientation of the embryos. Fertilized chicken embryos that were 11 – 13 days old were cleaned with 70% ethanol and placed on a small beaker with its round side facing upwards. The shell was carefully broken and the embryo was taken out with a forceps and decapitated. The thin outer skin near the breast muscle was carefully removed. Small pieces of tissue of the breast muscle was picked with sharp forceps and suspended in DMEM with 10% FCS. This procedure was repeated with all the embryos

and finally the muscle tissues were pooled together. Mince them into as small pieces as possible with the help of micro scissors. Transfer the tissue along with the medium to a 50 ml falcon tube and homogenize by pipetting up and down. Centrifuge at 1000 rpm for 5 min. Discard the supernatant carefully and add 0.25% trypsin without EDTA (Gibco) and incubate at 37°C for 15 min. Repeat this procedure if the cells are not completely dissociated. Pass the cells through a 100 micron nylon filter (BD Bioscience) and add primary culture medium (DMEM + 10% horse serum + 2% chicken embryo extract) and plate the cells densely. The medium is changed the following day and the cells are set for transfection using the $CaCl_2$ method.

2.3.7.2 Primary chicken Cardiomyocytes

Embryonic chick cardiomyocytes were isolated using a method (Budinger, Chandel et al. 1996) modified from (Barry, Pober et al. 1980). Briefly, hearts were removed from 11-day-old chick embryos and washed in Hanks' balanced salt solution lacking magnesium and calcium (Life Technologies). The ventricular tissue was minced and then dissociated using 4-6 cycles of trypsin (0.025%, Gibco) digestion with gentle shaking at 37°C. After 8 min, the trypsin digestion was stopped by transferring the cells to a trypsin inhibitor solution. The cells were then filtered (100-µm mesh), centrifuged for 5 min at 1,200 rpm at room temperature, and resuspended in growth medium with 6% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were preplated for 45 min on a petri dish in a humidified incubator (5% CO_2-95% air at 37°C) to promote early adherence of fibroblasts. The nonadherent cells were counted with a hemacytometer, and their viability was assessed using trypan blue (0.4%). Approximately 1×10^6 cells in nutritive medium were plated onto glass coverslips (25 mm) in 35 × 10 mm dishes. Cells were maintained in a humidified incubator for 2-3 days, at which time synchronous contractions of the monolayer of cells were noted though not always.

2.3.7.3 Cell stocks

Cells were trypsinized and then spun down. Cells were resuspended in DMEM (500 µl per 100 mm dish) containing 20% FCS. Now DMSO was added slowly and finally accomplished by adding to the resuspended cells an equal volume of DMEM containing 20% fetal calf serum and 20% DMSO. Cells were placed in a well-insulated box that has been pre-chilled in the freezer and put the box in the -70°C freezer overnight. Transfer the cells to liquid nitrogen the following day for long term storage.

2.3.8 Transfection

Transfection is the process of introducing nucleic acids into cells by non-viral methods. Cells were grown to 40-50% confluence and the medium was changed 2 – 4 hr before transfection. Cells were transfected either using FuGENE 6 transfection reagent (Roche) or $CaCl_2$ method. In the $CaCl_2$ method method, $CaCl_2$ was pipetted to the eppi containing DNA. Now 2 x HEPES buffer was added dropwise with simultaneous mixing. The mixture was incubated for 15-20 min at RT for precipitate formation. This precipitate was added to the cells slowly with constant mixing for even distribution. Cells were incubated at 37°C for 6-8 hr. Then cells were washed with serum free medium and exposed to glycerol shock buffer (3 parts glycerol 15%, 7 parts water, 10 parts 2 x HEPES) for 1-3 min. Wash the cells with serum free medium and finally add the respected growth medium and incubate at 37°C for 24-48 hr. Cells are later lysed for protein extraction or fixed to make immnunocytochemistry.

In the FuGENE system, the FuGENE reagent was directly added to the medium, mixed and incubated for 5 min at room temperature. Plasmid DNA was added to this mixture and incubated for 15 min at room temperature. Remove culture dish from the incubator and add the transfection reagent: DNA complex to the cells in a drop-wise manner. Swirl the dish to ensure even distribution. Cells were incubated for 24-48 hr for protein expression.

2.3.9 Immunocytochemistry

Immunocytochemistry is a common lab practice which uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. Cells were washed with PBS and fixed with 4% PFA (Para formaldehyde) for 15 min at room temperature. Wash them with PBS. Fixed cells were permeabilized with 0.01% triton X-100 for 15 min and washed again with PBS. Add blocking buffer (1% BSA in PBS) and incubate the cells for 30 min at room temperature. Add antigen specific primary antibody diluted in 1% BSA-PBS and incubate the cells for 1 hr at room temperature. Wash the cells 3-5 timed with PBS. Now add the biotinylated secondary antibody (anti rabbit IgG or anti mouse IgG) and incubate for 45 min. Add Dapi to a final dilution of 1:2000 and incubate the cells for 10 min. Wash the cells 3-5 times with PBS. Finally the cells were washed with water (to remove the salts) and air dried. The cover slips were mounted on glass slides with moviol for microscopic imaging of the expressed proteins.

3. Results

3.1 Expression of the transcriptional co-activator VITO1 in different cell types

Previous studies showed that VITO1 is a skeletal muscle specific gene expressed in the somatic myotome from E8.75 of mouse embryonic development and its transient domain expression during embryogenesis was found in the branchial arches, Pharyngeal pouches and clefts, cranial pharynx and Rathkes pouch (Mielcarek, Gunther et al. 2002). VITO-1 was identified as the first known co-activator of TEFs (Gunther, Mielcarek et al. 2004), which modulates transcription enhancer factors' activity in a tissue specific manner. To analyze and compare the subcellular localization in different cell types, VITO-1 was over-expressed in HEK 293, C2C12 and CH10T1/2 cell line.

3.1.1 Over-expression of VITO-1 in HEK 293 cell is predominantly cytoplasmic

Human Embryonic Kidney 293 cells, also often referred to as 293 cells, are a specific cell line originally derived from human embryonic kidney cells grown in cell culture. HEK 293 cells are very easy to grow and transfect very readily and have been widely-used in cell biology research for many years. They are also used by the biotechnology industry to produce therapeutic proteins and viruses for gene therapy.

To investigate the expression of VITO-1 in HEK 293 cell line, VITO-1 was ectopically expressed by transfection using FuGene 6 reagent. Different concentration of the plasmid DNA ranging from 0.5 to 2 µg was tested to optimize for higher transfection efficiency. Using 1 µg of VITO-1 dsRed along with 3 µl of Fugene reagent in serum free DMEM, HEK 293 cells were transfected and incubated for 12 hr in serum free medium. The medium was later changed to growth medium i.e. DMEM with 10% FCS and antibiotics

and the cells were further incubated until 24 hr. The cells were fixed with 4% PFA at two different time points, 24 hr and 48 hr. The nucleus was stained using Dapi. After 24 hrs of transfection, VITO-1 dsRed showed a cytoplasmic distribution (Fig. 5A). Even after 48 hrs, VITO-1 still remained in the cytoplasm showing no nuclear localization (Fig. 5C). Although VITO-1 is a co-activator of TEFs, this cytoplasmic distribution may be attributed to the absence of endogenous VITO-1 or TEFs.

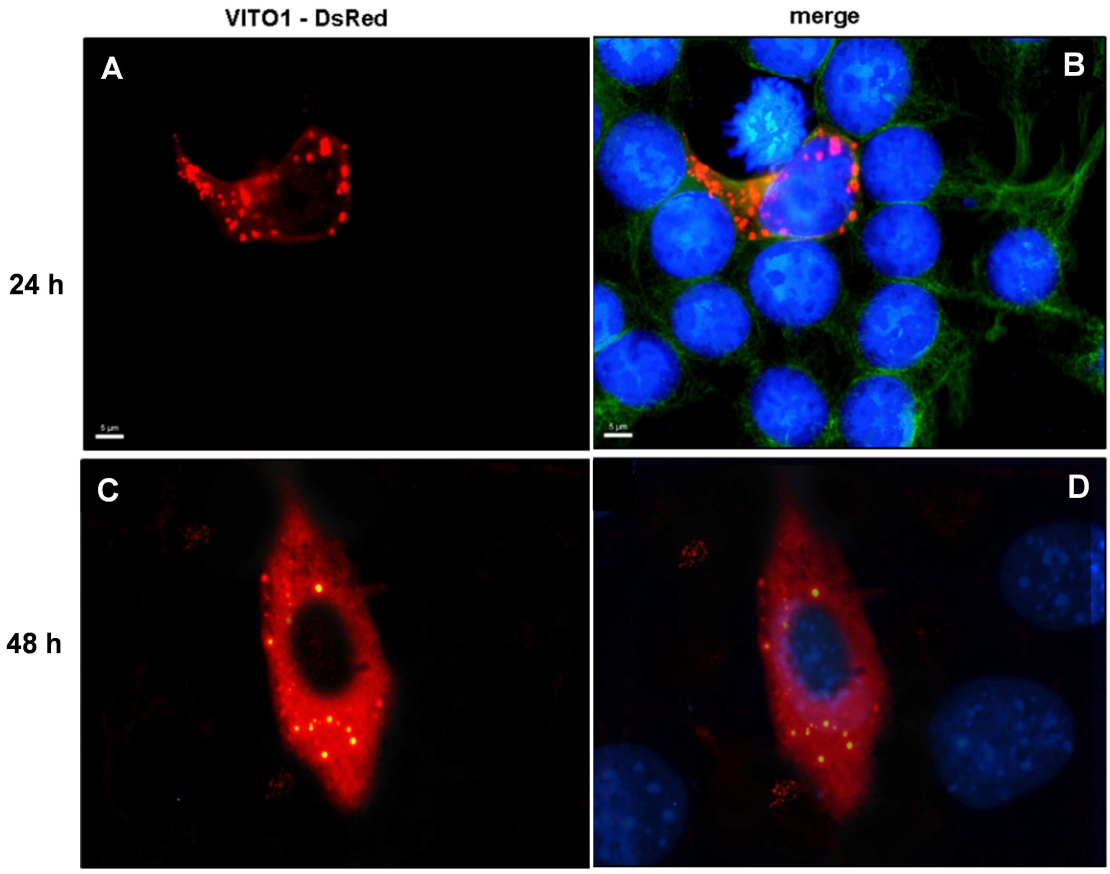


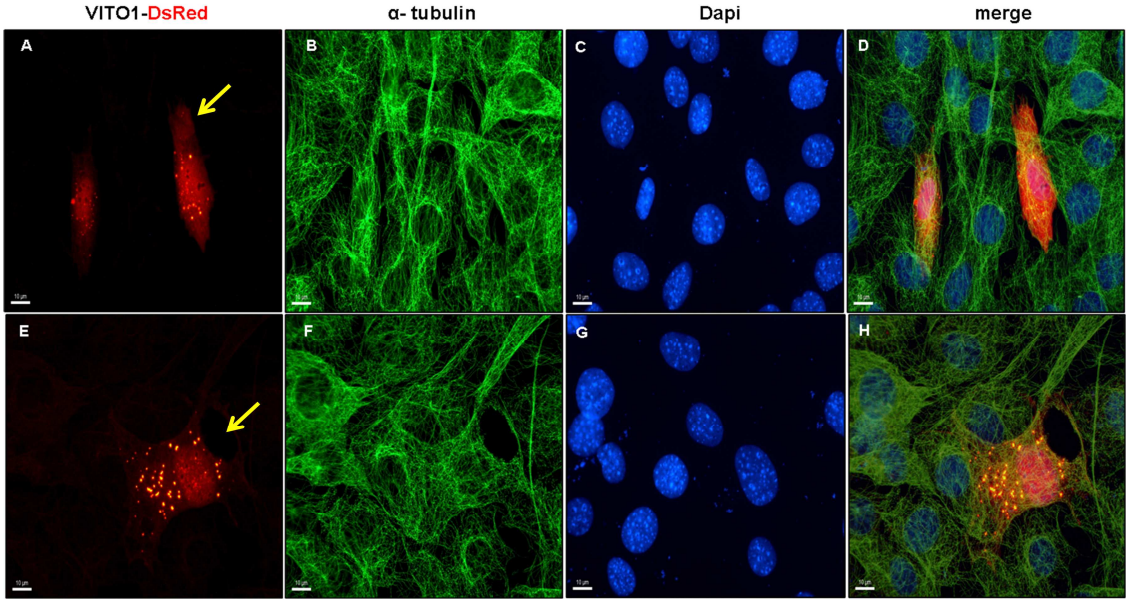
Figure 5. Subcellular localization of Vito1 (red) in HEK 293 cell line.

VITO-1 DsRed was transfected in HEK 293 cells using Fugene 6 reagent to see the expression pattern. (A) Cells expressing VITO-1 showed a cytoplasmic distribution after 24 hrs of transfection. (C) After 48 hrs of transfection, VITO-1 still remained in the cytoplasm. Nuclei were stained with dapi.

3.1.2 VITO-1 shows a predominant nuclear localization in C3H-10T ½ cell line

The clonal mouse embryo cell line, C3H/10T1/2, clone 8 (10T1/2) provides a unique opportunity to examine the molecular genetic regulation of both the developmental determination of vertebrate stem cell lineages and their subsequent differentiation. 10T1/2 is an apparently multipotential cell line that can be converted by 5-azacytidine (Constantinides, Jones et al. 1977) into three mesodermal stem cell lineages. Since these cells have the ability to differentiate into muscle cells on external stimulation, we decided to test the expression of VITO-1 in these undifferentiated cells.

VITO-1 DsRed was transfected using Fugene 6 and Calcium phosphate to test different concentrations of plasmid DNA to optimize the transfection efficiency. Since FuGene 6 yielded a better efficiency with 1.3 µg of DNA in 6 well plates, VITO-1 DsRed was transfected by this method and incubated for 18 hrs in Serum free DMEM without any antibiotics. Afterwards the medium was replaced with DMEM containing 10 % FCS and antibiotics (penicillin/streptomycin). Cells were fixed with 4% PFA and permeabilized using 0.05% Triton. To score for any cytoskeletal changes, microtubules were stained using mouse α – tubulin (1:500) as the primary antibody and detected by Alexa 488 (1:1000) as the secondary antibody (Fig. 6B, 6F). The nuclei were stained with dapi (Fig. 6C, 6G) and the cells were mounted on glass slides for microscopic analysis.



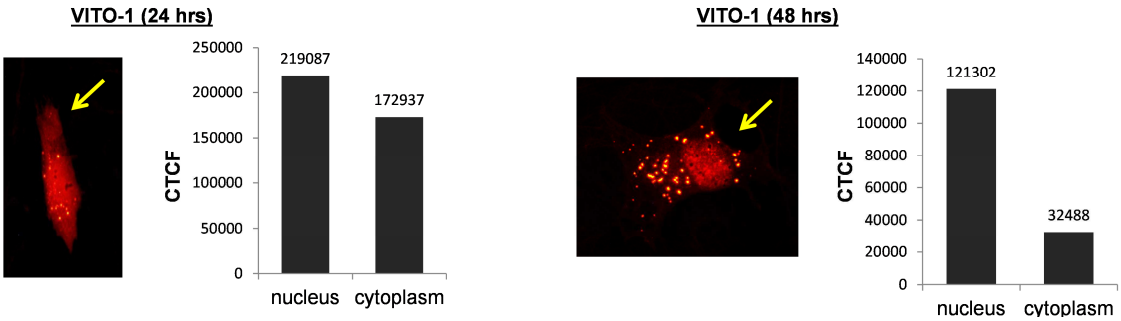


Figure 6. Distribution of VITO-1-DsRed overexpressed in C3H-10T1/2 fibroblast cell line. *Expression of VITO-1 dsRed at different time points after transfection. (A) After 24 hrs VITO-1 is distributed uniformly all over the cells. (E) But after 48 hrs, VITO-1 localizes primarily to the nucleus (histograms represent quantitation using Image J software) although some punctated cytoplasmic distribution could be seen. (B, F) Microtubules were stained for α-tubulin as a control for normal cytoskeletal organization in cells expressing VITO-1. (C, G) Nuclei were visualized using Dapi staining.*

After 24 hrs of transfection VITO-1 was localized all over the cells showing diffused expression in the cytoplasm (Fig. 6A). After 48 hrs, VITO-1 translocated into the nucleus and showed more than 70% nuclear intensity (Fig. 6D). The corrected total cell fluorescence (CTCF) was quantitated using Image J software.

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

This translocation into nucleus could possibly be due to the presence of endogenous TEFs or other regulatory molecules, which facilitate nuclear translocation of VITO-1.

3.1.3 VITO-1 Localizes prominently in the nucleus of undifferentiated C2C12 cells

C2C12 cell line is a subclone of the mouse myoblast cell line established by D. Yaffe and O. Saxel. The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. Care should be taken not to allow the cells to become confluent as this will deplete the myoblastic population in the culture.

Diffentiation and myotube formation of C2C12 cells is stimulated when the medium is supplemented with 2% horse serum instead of FCS.

Northern blot analysis using RNA from C2C12 cells had revealed the presence of m-VITO-1 RNA in proliferating C2C12 cell indicating expression of the gene in dividing myoblasts that have not yet undergone terminal differentiation (Mielcarek, Gunther et al. 2002). The expression level in C2C12 myoblasts was lower compared to differentiated C2C12 myotubes.

To investigate the localization of VITO-1 in C2C12, cells were cultured on 6 well fibronectin coated plates and transfected with 0.8 µg of VITO-1 pEGFPc2 using FuGene 6 reagent. After transfection, cells were incubated with serum free medium without antibiotics for 8 hrs after which the medium was replaced with DMEM containing 10% FCS and penicillin/streptomycin). Cells were fixed with methanol-acetone for 10 min and the nucleus was stained with Dapi.

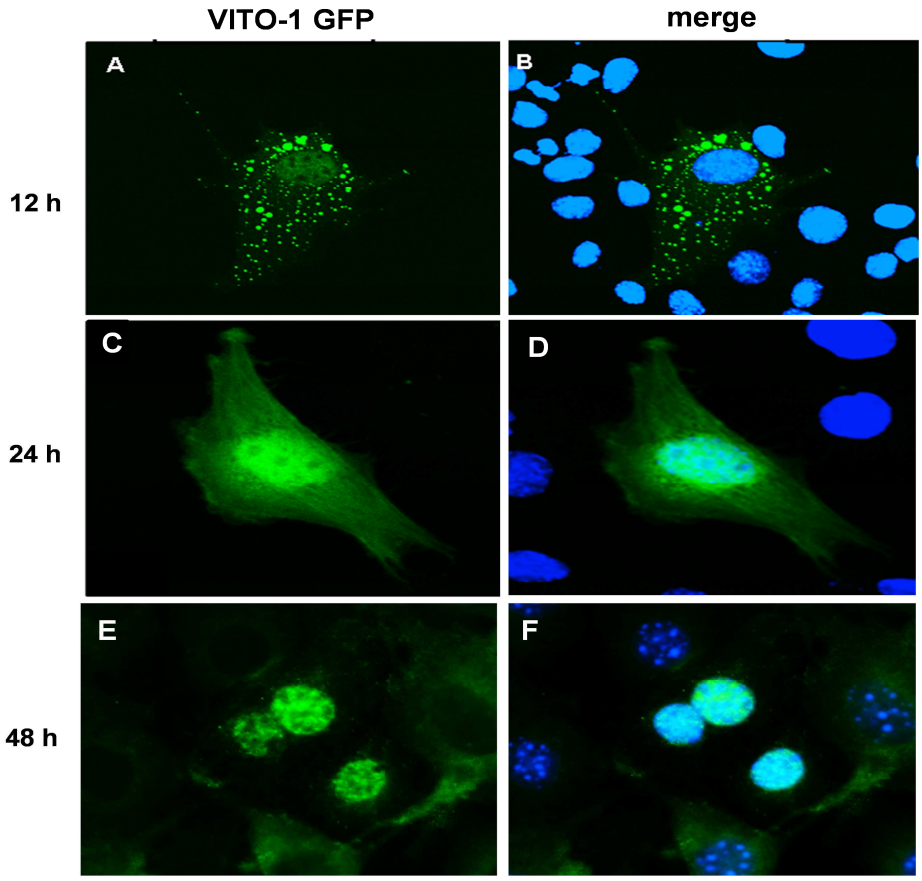


Figure 7. Expression of VITO-1 (green) in undifferentiated C2C12 muscle cell line*. VITO1-GFP was transfected into C2C12 cells using Fugene 6 and the distribution pattern was monitored at different time points. (A) After 12 hrs, VITO1 show a puntacted distribution all over the cytoplasm in most of the cells. (C) After 24 hrs, VITO1 is distributed both in the cytoplasm as well as the nucleus. (E) After 48 hrs, VITO1 is associated prominently in the nucleus which supports its role as a transcriptional co-activator.*

After 12 hrs of transfection, VITO-1-GFP was found predominantly in the cytoplasm in a punctuated pattern in most of the cells although some cells still show a weak nuclear localization (Fig. 7A). In 24 hrs, one can observe the distribution of VITO-1 both in the nucleus as well as in the cytoplasm (Fig. 7C). After 48 hrs, VITO-1 completely translocates into the nucleus (Fig. 7E). VITO-1 shows an endogenous expression in undifferentiated C2C12 myoblasts and the localization of VITO-1 in the nucleus could support its possible role as a co-activator where it might interact with TEFs or other transcriptional factors that are involved in the terminal differentiation of muscle cells and control gene regulation.

3.1.4 Expression of VITO-1 in differentiated C2C12 myotubes

Proliferating C2C12 cells can be triggered to differentiate into myotubes by culturing them in low growth factor medium when they are confluent. To monitor the expression of VITO-1 in differentiated myotubes, a GFP tagged fusion plasmid (VITO-1-pEGFP-c2) was used. C2C12 (mouse myoblast cell line) cells were grown on 6 well plates in DMEM containing 10% FCS and Penicillin/Streptamycin (P/S) antibiotics. When the cells reached 60% confluence, they were transfected with 2 µgs of the plasmid VITO-1-GFP using FuGENE 6 reagent and incubated for 48 hrs. When the cells reached ~ 100% confluence, the medium was replaced with DMEM containing 2% Horse serum. Cells were allowed to differentiate for 4 – 5 days until the formation of multinucleated mature myotubes. Later the cells were fixed with 4% PFA and the nuclei were stained with DAPI. The cover slips containing the fixed cells were visualized using a fluorescence microscope.

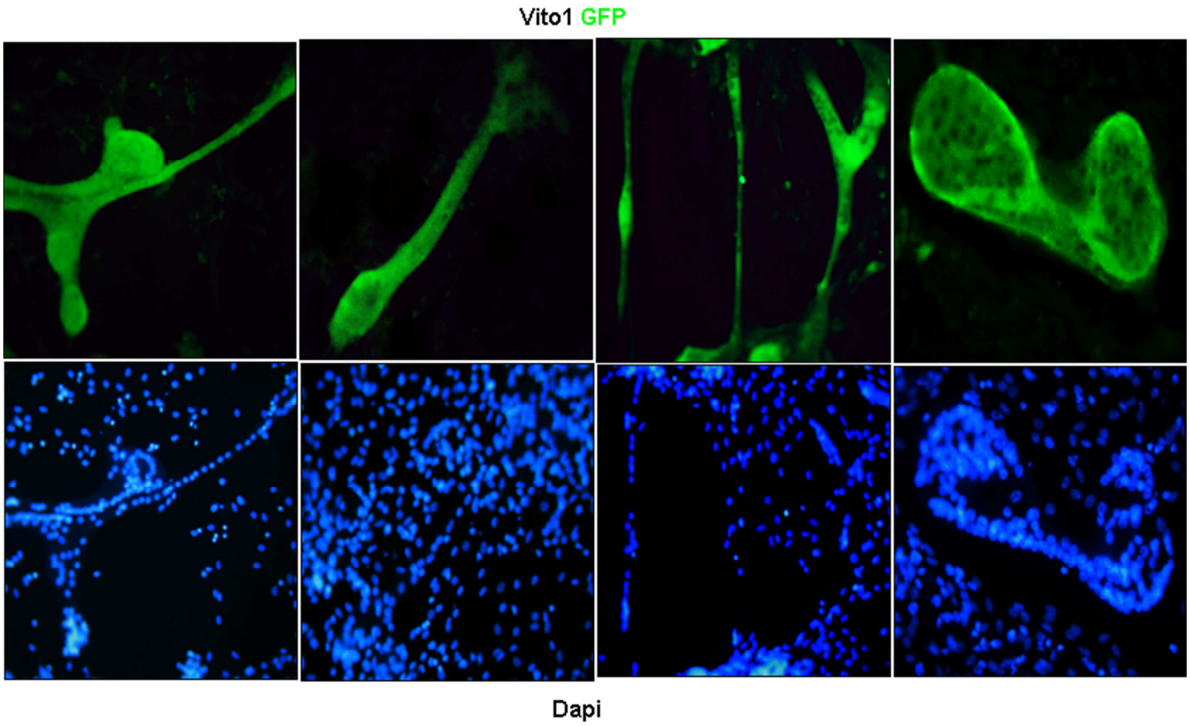


Figure 8. Over expression of VITO-1 in matured myotubes of C2C12 cells*. The top four panels show the expression of VITO-1-GFP in C2C12 cells after differentiation into myotubes. Nuclei were stained using Dapi (bottom panels).*

VITO-1-GFP was predominantly expressed in differentiated myotubes (Fig. 8 top panels) indicating that cells expressing VITO-1 showed an increased property to form myotubes compared to their neighboring untransfected cells which did not form the mature myotubes. These findings show that VITO-1 might play an important role in association with transcription factors to facilitate terminal differentiation of muscle cells.

3.1.5 Expression of mutant VITO-1 constructs in C2C12 cells shows the requirement of SID domain to translocate to the nucleus.

VITO-1 mediates interaction with TEFs and other proteins through its scalloped interaction domain (SID) [(Gunther, Mielcarek et al. 2004)]. We hypothesized that, VITO-1 lacking the SID domain may not interact with TEFs or probably may not associate with other proteins, thus losing its functional properties. To provide further

evidence of the importance of SID domain, a mutant construct lacking the SID domain was fused to IRES-GFP (VITO-1 Δ SID-GFP). This construct was transfected into C2C12 cells plated in 6 well chambers with 1 µg of plasmid DNA using the FuGene 6 reagent and were fixed using 4% PFA. After 48 hrs of transfection, full length VITO-1 was mainly expressed in the nucleus (Fig. 9A). VITO-1 lacking the SID domain did not translocate into the nucleus even after 48 hrs and displayed a prominent cytoplasmic localization (Fig. 9B). The corrected total cell fluorescence (CTCF) was quantitated using Image J software.

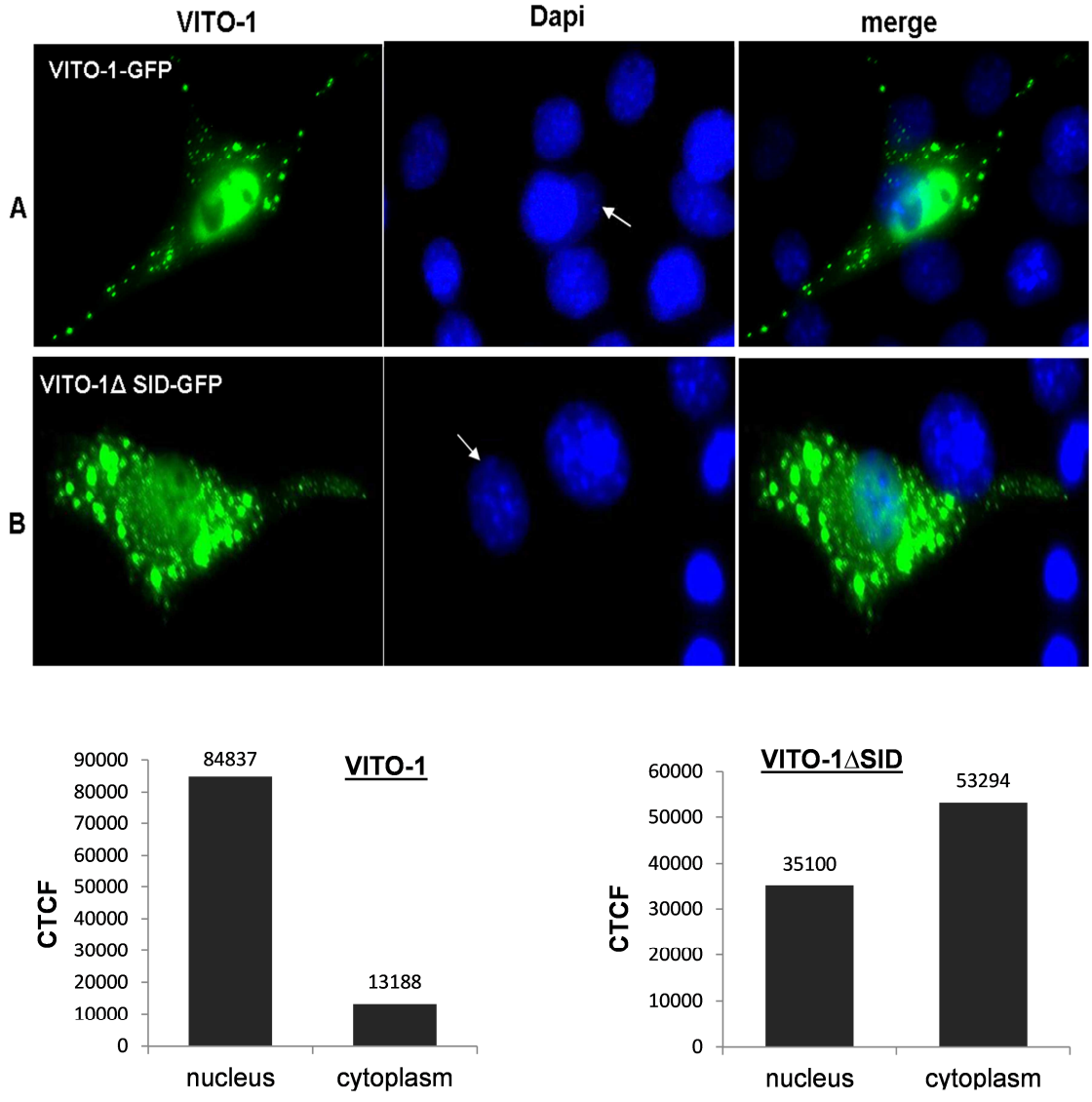


Figure 9. Sub cellular localization of VITO-1 mutant in C2C12 cells.

(A) Full length VITO-1-GFP localized mainly in the nucleus after 48 hrs of transfection. (B) VITO-1 mutant lacking the SID domain (VITO-1Δ SID-GFP) is distributed throughout the cells with prominent punctated pattern. (Histograms represent total cell fluorescence quantitated using Image J software)

The results demonstrate the SID domain of VITO-1 is instrumental for an efficient translocation into nuclei where it may act as a transcriptional co-activator to regulate expression of target genes.

3.2 Over-expression of TEF3 together with VITO-1, results in a complete recruitment of VITO-1 into the nucleus in various cell types

The expression of VITO-1 was analyzed in various cell types like C2C12, HEK293 and 10T1/2 in the presence of TEF3. VITO-1 – GFP was co-transfected with TEF3-pCDNA5 in an equimolar ratio using the FuGene 6 reagent. After 48 hrs of transfection, cells were fixed with 4% PFA and nucleus was stained by DAPI. VITO-1 when transfected alone in these cell lines did not completely localize in the nucleus. After 24 hrs of co-transfection, VITO-1 still remained in the cytoplasm in most of the cells and did not show much difference with cells transfected with VITO-1 alone. But interestingly after 48 hrs, VITO-1 was completed translocated into the nucleus in all cell types including HEK 293 cells (Fig. 10). It should be noted that VITO-1 transfected alone never showed a nuclear expression (Fig. 5). This is probably due to the association of VITO1 with the transcription factor TEF3 during translocation into the nucleus. The endogenous expression of TEF transcription factor seems not sufficient to drag over-expressed VITO-1 into the nucleus.

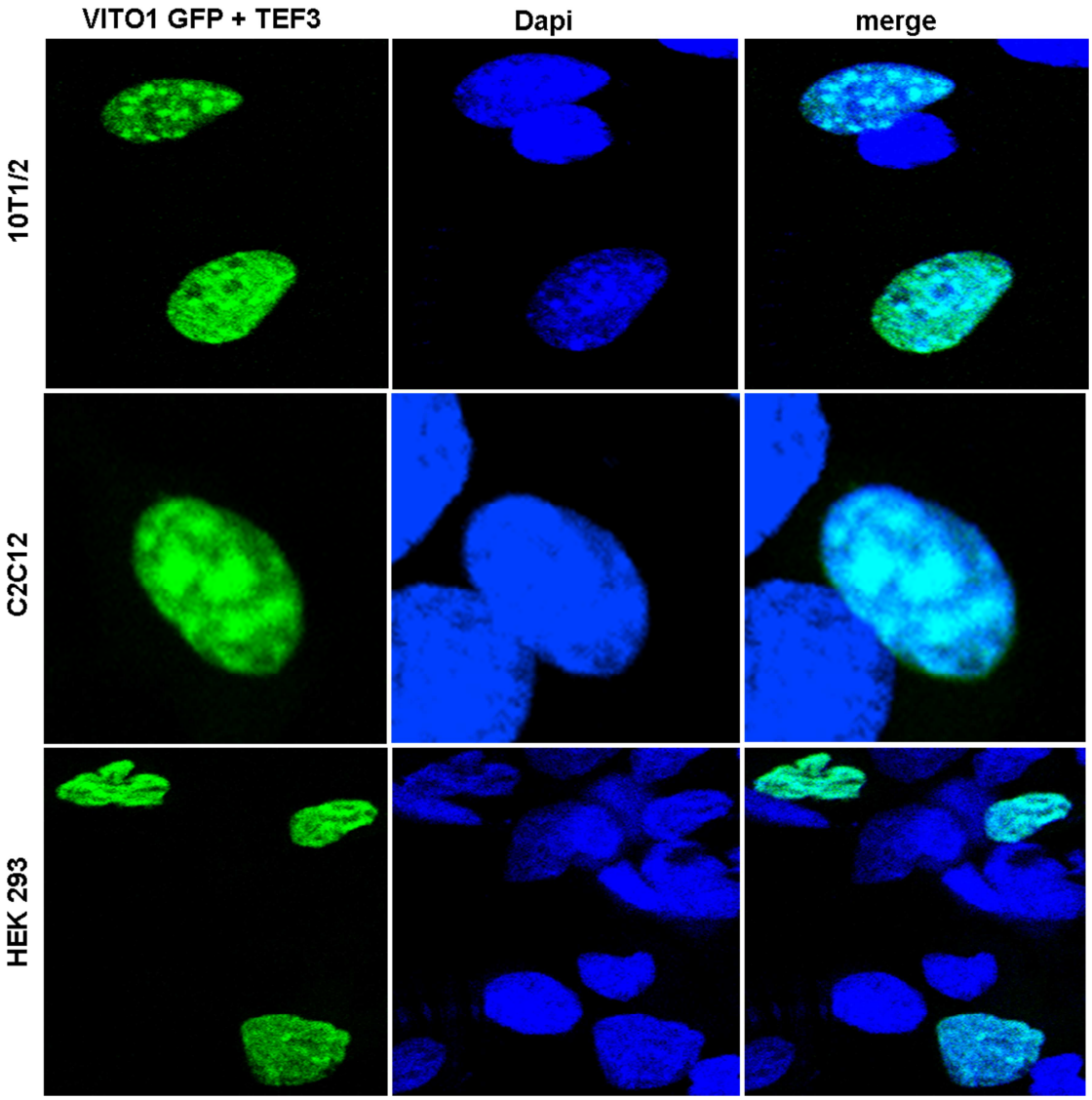


Figure 10. Full length VITO-1 translocates completely into the nucleus in the presence of TEF3 in different cell types*. 10T1/2, C2C12 and HEK 293 cells were trasnfected with VITO-1-GFP and TEF3. In the presence of TEF3, VITO-1 completely translocated to the nucleus in all three cell types. Nuclei were stained with Dapi.*

The next important question was whether VITO-1 lacking the SID domain translocates into the nucleus in the presence of TEF3. To investigate this, VITO-1 Δ SID-GFP was co-transfected in C2C12 cells with TEF3 in an equimolar concentration with a total DNA concentration of 1.5 µg using FuGene 6 into cells plated in 6 well chambers.

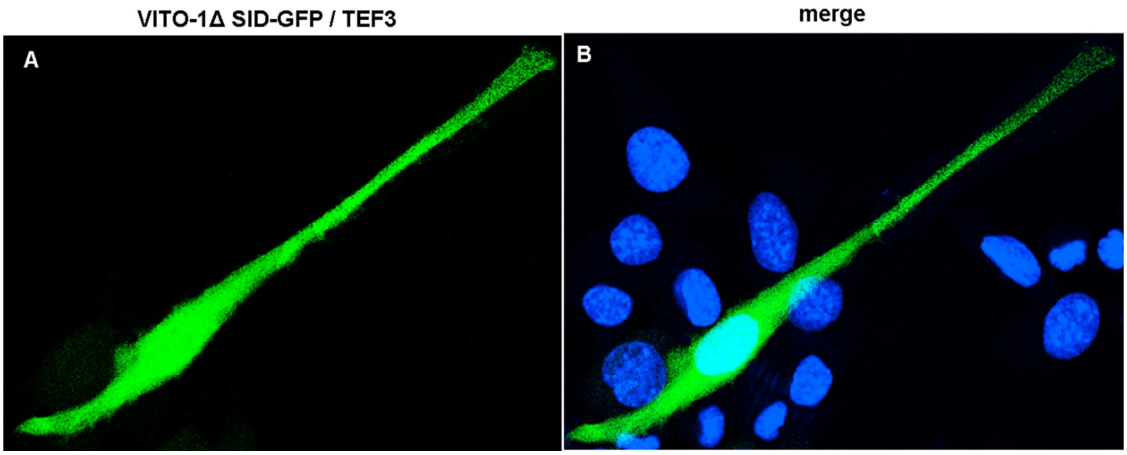


Figure 11. Subcellular localization of VITO-1ΔSID-GFP in the presence of TEF3. *VITO-1ΔSID-GFP is distributed all through the cell and not restricted to nuclear compartment alone.*

Cells were fixed and stained as stated previously and visualized in the microscope. It should be noted that after 48 hrs of transfection, cells expressing VITO-1 Δ SID-GFP did not completely translocate into the nucleus even in the presence of TEF3 (Fig. 11). This shows the requirement of the SID domain to activate TEF3 and its downstream target genes. These findings underline the importance of the SID domain in mediating interaction of VITO-1 with other proteins such as TEF3.

3.3 Screening for novel interacting partners of VITO-1 using the Yeast Two Hybrid (Y2H) system

The yeast two-hybrid system is a versatile method to identify new interaction partners of individual proteins. To identify additional binding partners of VITO-1 which are involved in skeletal muscle regulation and other important biological processes, the yeast two hybrid method was chosen. The yeast two-hybrid system is an invaluable tool for the successful identification of novel protein–protein interactions, both in standard laboratory screens and in high-throughput automated formats.

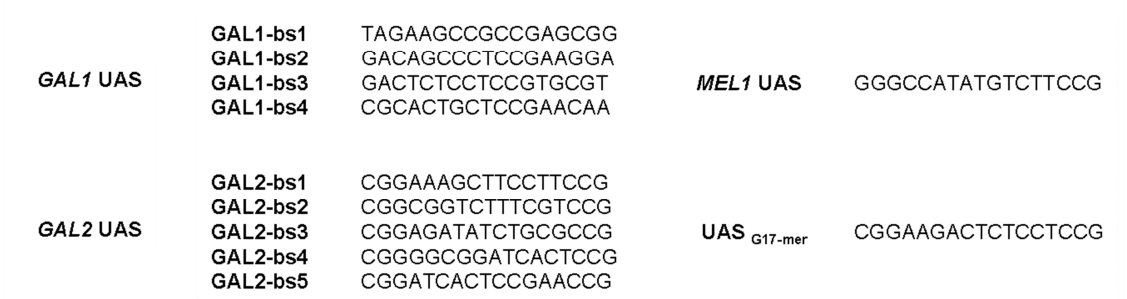
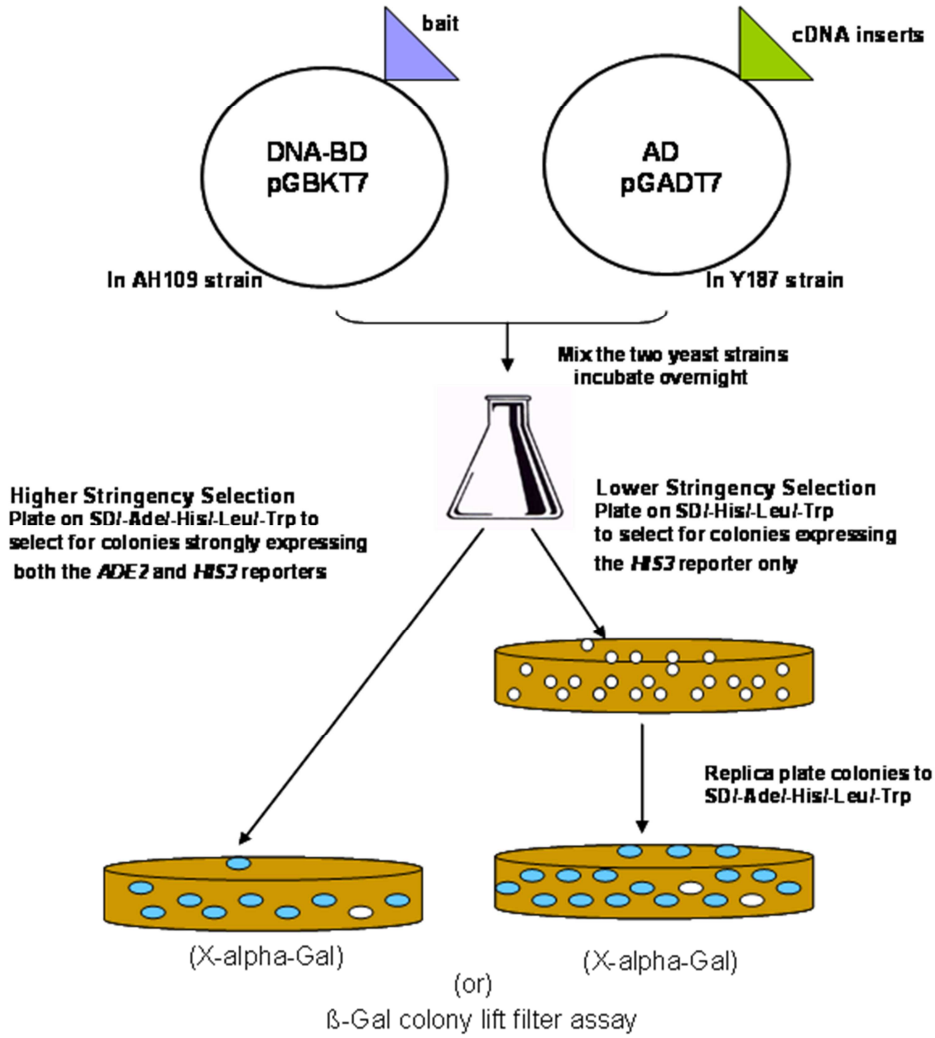


Figure 12. Sequence of the GAL4 DNA-BD recognition sites in the GAL1, GAL2, and MEL1 UASs and the UASG 17-mer consensus sequence (Giniger and Ptashne 1988)

The screening is based on the GAL4 UASs system in which either an intact *GAL1*, *GAL2* or *MEL1* UAS or an artificially constructed UAS consisting of three copies of the 17-mer consensus binding sequence, is used to confer regulated gene expression on the reporter genes.

The yeast strains *Saccharomyces cerevisiae* AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, MEL1) and Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, URA3::$GAL1_{UAS}$-$GAL1_{TATA}$-lacZ, MEL1) were employed as hosts in the two-hybrid assay. AH109 contains 2 nutritional reporter genes for adenine and histidine. Both AH109 and Y187 harbor the LacZ reporter gene. The HIS3 reporter of the AH109 and the LacZ reporter of Y187 are all tightly regulated by the intact GAL1 Promoter (including the GAL1 UAS and *GAL1* minimal promoter). The ADE2 reporter of AH109 is tightly regulated by the intact *GAL2* promoter, whose induction properties are similar to those of the *GAL1* promoter. In AH109, LacZ is under the control of the MEL1 UAS and minimal promoter.

VITO-1 (1 – 291 amino acids) was cloned into the pGBKT7 vector containing the cMyc epitope tag (Bait) and the plasmid was expressed in the yeast strain AH109. A human skeletal muscle library was used as the Prey as VITO-1 expression is confined to skeletal muscle tissue. The library was already cloned into the protein-encoding sequence of pGADT7 containing a HA epitope tag and pre-transformed into the Y187 strain.



- Matchmaker™ Library Construction & Screening kits user manual (modified)

Figure 13. Overview of the Y2H screen for identifying potential VITO-1 interacting target genes. *Vito1 was cloned into the DNA binding vector pGBKT7 and transformed into AH109 strain. The human skeletal muscle cDNA library was already cloned into the AD vector pGADT7 and pretransformed into the Y187 strain. These pretransformed plasmids were subjected to overnight mating and were plated the following day onto plates containing nutritional selection markers which allows the growth of colonies (clones) expressing the reporter genes. These clones were confirmed positive after doing an X-α-Gal assay or a ß-Gal colony lift filter assay.*

3.3.1 Detecting expression of VITO-1 in yeast cells

After generating the bait plasmid (VITO-1-pGBKT7), expression levels in yeasts were tested by western blot. For this, VITO-1 pGBKT7 was transformed into the AH109 strain and plated on agar plates lacking -Trp. Liquid cultures were prepared by inoculating a single colony and grown at 30°C with shaking (250 rpm) until the OD600 reaches 0.4-0.6. Cells were centrifuged and the pellet was either stored at -80°C or proceeded with protein extraction.

Proteins from yeast cannot be extracted simply by boiling or sonication. Protein was extracted by the Urea/SDS method as described in materials and methods. The extracted sample was loaded on a SDS PAGE and electro blotted onto nitrocellulose membrane. The protein was detected using c-myc monoclonal antibody against the c-myc epitope tag of pGBKT7 vector.

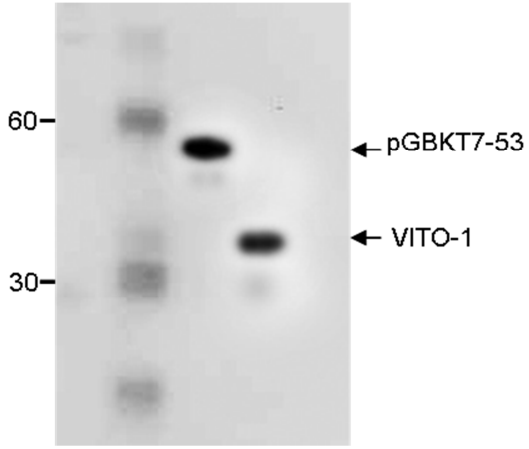


Figure 14. Expression of VITO-1-pGBKT7 transformed into AH109*. Proteins trsnaformed into yeast were extracted and a western blot was performed to test its expression level. pGBKT7-53 was used as a positive control*

VITO-1 could be detected as a 33 kD protein (Fig.14) which confirmed its expression. pGBKT7-53 that expresses a 57 kD protein was used as a positive control (Fig. 14). Before proceeding for screening, the bait was tested for toxicity and auto-activation of the reporter gene.

3.3.2 Testing bait for Toxicity

It is important to demonstrate that the bait protein is not toxic when expressed in yeast. If the bait is toxic to the yeast cells, both solid and liquid cultures will grow more slowly.
To test if VITO-1 is toxic to yeast cells, 0.1 µg of empty pGBKT7 and VITO-1 pGBKT7 were transformed into the AH109 strain and 100 µl was plated on SD/ -Trp agar plates with 1:10 and 1:100 dilutions. Plates were grown at 30°C for 3-5 days.
If VITO-1 (Fig. 15B) is toxic, then one should notice the colonies significantly smaller compared to the control empty pGBKT7 vector (Fig. 15A), which was not the case.

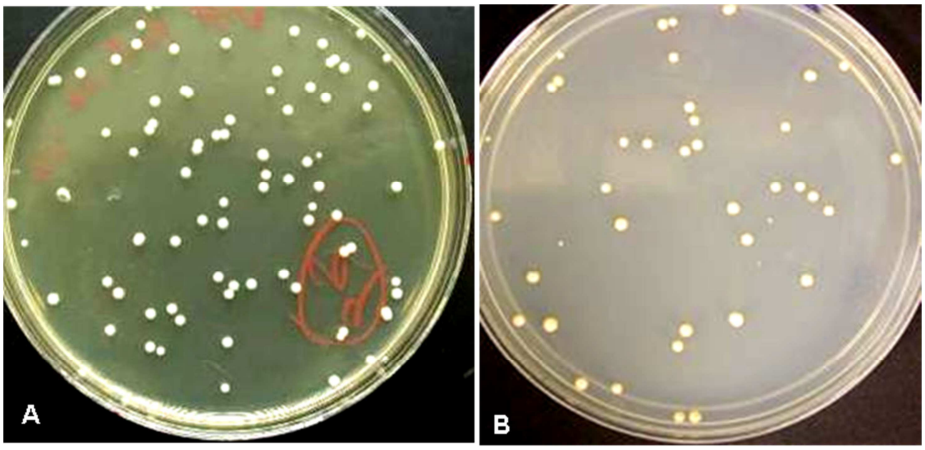


Figure 15. VITO-1 is not toxic when expressed in yeast*. VITO-1(B) and empty pGBKT7 vector (A) was transformed in yeast and plated at dilutions of 1:100. The colony size of VITO-1 is similar to the control which indicates its non-toxicity to grow in yeast cells.*

3.3.3 Testing VITO-1 for Auto-activation

To maximize sensitivity of the *HIS*3 reporter gene, the AH109 strain already expresses a basal level of *HIS*3. Additionally, bait proteins often contain a certain level of transcriptional activity. This is enough to initiate some transcription at the most sensitive

reporter in the system, *HIS*3. Moreover VITO-1 being a transcriptional co-activator, it is very important to check for auto-activation of the reporter gene and to delete this property before initiation of the screen.

To test for auto-activation of VITO-1, 0.1 µg of plasmid DNA was transformed into AH109 and mated with empty AD vector pGADT7 in Y187 strain without the prey and were plated on SD/-Leu-Trp and SD/-Leu-Trp-His agar plates and incubated for 3 days at 30°C. Slight growth of the yeast colony was noticed in SD/-Leu-Trp-His agar plates which showed auto-activation by VITO-1 as confirmed by β-galactosidase assay.

*HIS*3 encodes an enzyme involved in histidine biosynthesis, which can be specifically inhibited in a dose-dependent manner by 3-Amino-1,2,4-Triazole (3AT). The concentration of 3-AT was determined empirically and included in plates lacking histidine, such that even slight increases in HIS3 reporter gene expression are detected. This will enhance the probability of detecting weak protein-protein interactions. To suppress the auto-activation of VITO-1, colonies from SD/-Leu-Trp were replica plated onto SC-Leu-Trp-His plates containing 3AT at concentrations of 0 mM, 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM (Fig. 16) and the plates were incubated for 3-5 days at 30°C.

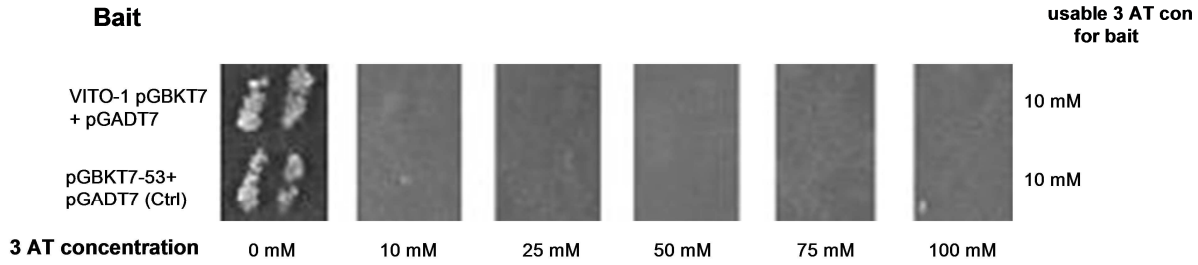


Figure 16. Reporter gene auto-activation test for VITO-1.
10mM concentration of 3AT was sufficient to suppress the auto-activity of the reporter gene by VITO-1. This concentration of 3-AT was used forVITO-1 in all further yeast experiments.

The lowest concentration of 3AT that inhibits the growth of the cells from transformation (containing bait and pGADT7) is the basal amount of 3AT added to all plates lacking histidine. It was found that 10 mM of 3 AT was sufficient to inhibit the auto-activation of the reporter genes by VITO-1 and subsequently this concentration was used for all future experiments during screening.

3.3.4 Two-Hybrid Library Screening Using Yeast Mating

Screening was performed as described in materials and methods. A concentrated overnight culture of the bait strain VITO-1 pGBKT7 was prepared by inoculating a fresh large colony (2-3 mm) in SD/-Trp and incubate shaking (250–270 rpm) at 30°C until the OD600 reached 0.8 (16–20 hr). Cells were harvested and resuspended at a density of > $1x10^8$ cells per ml in SD/–Trp (5 ml). The 1 ml skeletal muscle cDNA library strain pre-transformed in Y187 was thawed and combined with the bait strain together with 45 ml of 2xYPDA liquid medium (with 50 μg/ml kanamycin) under aerobic conditions and incubated at 30°C for 20–24 hr with slowly shaking (40 rpm).

VITO-1 pGBKT7 in *MAT* α AH109 was mated with human cDNA skeletal muscle library-pGADT7 in *MAT* α Y187. Co-culturing the two strains overnight produces an array of diploid yeast clones, each co-expressing the bait with a different library prey protein.

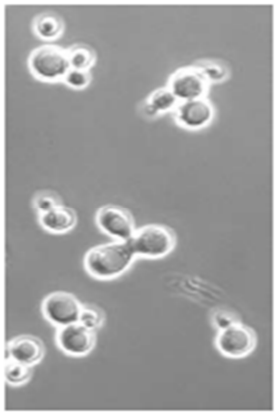


Figure 17. Formation of diploid yeast clones during mating as seen under the light microscope.

The mated culture (100 µl) was spread with dilutions of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions on SD/–Trp, SD/–Leu, SD/–Leu/–Trp (DDO) and SD/-Trp/-Leu/-His and incubated for 5 days at 30°C. The number of screened clones (diploids) was calculated by counting the colonies from the DDO plates after 5 days.

• Number of Screened Clones = cfu/ml of diploids x resuspension volume (ml)

In the case of VITO-1, 23 colonies grew on the 1/1,000 dilution on DDO plates.

Number of clones screened for VITO-1 = 23 x 11.5 x 10 x 1,000 = 2.65 million

The mating efficiency (percentage of diploids) was calculated as follows

No. of cfu/ml of diploids/No. of cfu/ml of limiting partner x 100 = % Diploids

• No. of cfu/ml on SD/–Leu = viability of the Prey Library (1.4×10^7)
(140 colonies grew on the 1/10,000 dilution on SD/–Leu)

• No. of cfu/ml on SD/–Trp = viability of Bait (1.8×10^8)
(1800 colonies grew on the 1/10,000 on SD/–Trp)

• No. of cfu/ml on SD/–Leu/–Trp = viability of diploid (3.4×10^5)
(34 colonies grew on the 1/1,000 dilution on DDO plates)

Since the Prey Library was the limiting partner in this screen,

Mating efficiency is ($3.4 \times 10^5 / 1.4 \times 10^7$) x 100 = 2.42 %

3.3.5 High stringency selection of VITO-1 clones

The VITO-1 clones obtained from SD/-Trp/-Leu/-His are the ones that express the appropriate reporter genes and markers, indicating the presence of interacting hybrid protein pairs. However these clones might also contain false positives which can further be eliminated by selecting them using high stringency conditions. This was achieved by replica plating the clones onto SD/-Trp/-Leu/-His/-Ade (QDO) which provides a tight control to select positive clones that activate both *HIS3* and *ADE2*.

These VITO-1 clones were further confirmed positive by subjecting them to either X-α-Galactosidase assay or a ß-Galactosidase colony lift filter assay.

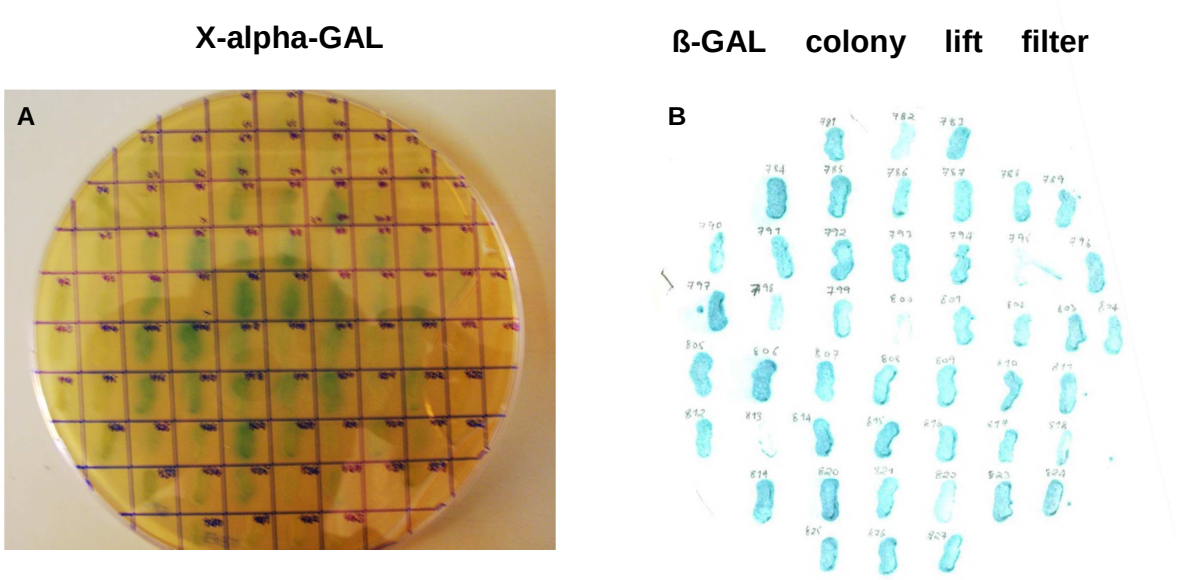


Figure 18. Interaction of Vito1-pGBKT7 with the skeletal muscle cDNA library clones by (A) X-alpha-Gal and (B) ß-galactisodase colony lift filter assay.

(A) - Yeast strains producing alpha-galactosidase the chromogenic substrate X-alpha-Gal can be used to assay GAL4-based two-hybrid interactions directly on the nutritional selection plates which gives a quick report about the protein-protein interactions

(B) - ß-galactisodase colony lift filter assay to confirm the interaction of VITO-1 with its partner clones as identified by the activation of HIS reporter gene. The ß-galactosidase-producing colonies were identified by aligning the filter and the agar plate using the orientation marks. ß-galactocidase assay is 10 times more sensitive than X-α-gal assay and hence can be used to identify even weak interactions between the bait and its partners.

In yeast, alpha-D-galactoside galactohydrolase, an enzyme encoded by the *MEL1* gene is regulated by several *GAL* genes. Secretion of this enzyme in response to *GAL4* activation leads to hydrolysis of X-α-Gal in the medium causing yeast colonies (positives) to develop a blue colour. A big advantage of this reporter system is that the result can be

seen immediately on the selection plates (Fig. 18A). The ß-gal colony lift filter assay is much more sensitive than X-α-Gal assay and is used to measure ß-galactosidase activity to screen large number of cotransformants that survive the HIS3 growth selection. This assay is particularly useful when the cells to be assayed contain one or only a few copies of the *lacZ* reporter gene. (Fig. 18B) shows the positive clones of VITO-1 after ß-gal colony lift filter assay.

3.3.6 Novel interaction partners of VITO-1

From a total of 79 clones obtained after screening for VITO-1, 48 clones were identified truly positive as identified by X-α-Gal and ß-gal colony lift filter assay. The clones are a mixture of both Gal4 DBD and Gal4 AD plasmids. DNA was extracted from these yeast clones and was transformed into E.coli and plated on ampicillin plates to select for Gal4 AD (pGADT7) inserts alone. The DNA of the interacting clones was extracted from E.coli using the Qiagen mini prep kit. The plasmids were cut using the restriction enzyme Alu1 which is a frequent cutter to eliminate repetitive or identical clones. Unique putative clones were sequenced. The sequence of around 25 clones which is a mixture of both overlapping as well as identical clones showed similarities with known proteins (Table. 11).

Since VITO-1 is a transcription co-activator of TEFs, we expected members of the TEF family or other transcription factors or repressors to surface in our yeast two hybrid screen. Surprisingly none of the newly identified binding partners directly had an annotated function in the regulation of transcription. We focused our interests on Telethonin (T-cap) and Myozenin1 (MYOZ1) because they were isolated as independent overlapping clones. All other clones were either single clones like PKIA, PKM2, ARDBK1, DUSP13, MT2A and UNC 119 or identical clones such as ADAMTS8, ATP2A2 and FTL.

Genes Identified	Expression profile	No. of clones
Telethonin **(T-Cap)**	**Muscle**, heart larynx peripheral nervous system, prostate, pancreas, tongue, bone, liver, lung, placenta, skin, eye	4
Myozenin-1 **(MYOZ1)**	**muscle,** heart, soft tissue, prostate, larynx, tongue, kidney, eye, lung	2
Protein kinase inhibitor alpha **(PKIA)**	**heart, muscle**, brain, PNS, liver, thymus, prostate	1
Pyruvate kinase muscle **(PKM2)**	**all tissues**	1
Adrenergic beta receptor kinase 1 **(ADRBK1)**	**thymus, lymph node, blood, PNS,** muscle and all other tissues except heart and larynx	1
Dual specificity Phosphatase 13 **(DUSP 13)**	**muscle, heart, bone marrow, PNS**, pancreas, prostate, testis, eye, liver, brain	1
Metallothionein 2A **(MT2A)**	**muscle, liver, vascular, prostate** and almost all other tissues	1
Adam Metallo peptidase with thrombospodin type 1 motif 8 **(ADAMST8)**	**lung, prostate**, blood, brain, colon, placenta, ovary, pancreas, testis	2
ATPase Ca transporting, cardiac muscle slow twitch 2 **(ATP2A2)**	**muscle, heart, larynx, PNS,** and everywhere except in thymus	2
UNC 119 homolog (*C.elegans*)	**eye**, cervix, ovary, PNS, thymus, uterus, muscle, heart…	1
Ferritin light polypeptide **(FTL)**	**all tissues**	7
Galactosyl transferase polypeptide **(B4GALT5)**	**blood, ovary, PNS**, muscle, heart and all other tissues	1
Proteosome subunit beta type 4 **(PSMB4)**	**skin, cervix** in all other tissues except thymus	1
Chaperonin containing TCP1 subunit 7 **(CCT7)**	**all tissues**	1

Table 11. *Clones identified for VITO1 as potential interaction partners by Yeast Two hybrid screening using a human skeletal muscle library.*

3.4 Interaction of VITO-1 with Telethonin and Myozenin1

3.4.1 VITO-1 interacts with both Telethonin and Myozenin1 in the yeast two hybrid system

To confirm the interaction of VITO-1 with T-cap and MYOZ1, VITO1-pGBKT7 was co-transformed with the overlapping clones of T-cap-pGADT7 and MYOZ1-pGADT7 in individual experiments and were plated on SD/-Trp/-Leu/-His (TDO) plates and incubated at 30°C for 4-5 days.

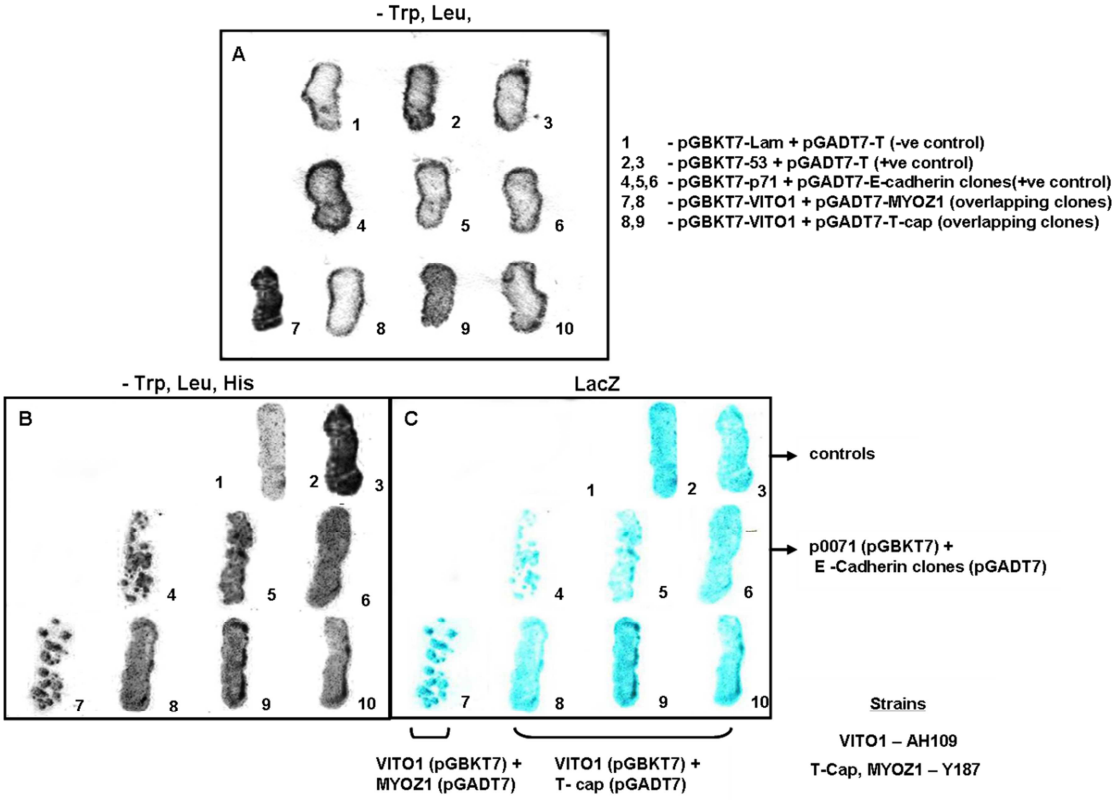


Figure 19. Yeast two hybrid (Y2H)- Interaction of VITO-1 with Telethonin and Myozenin*. Yeast clones obtained after screening were cloned into pGADT7 and retested for interaction by lacZ test. pGBKT7-Lam + pGADT7-T were used as a negative control (A, B, C # 1) and pGBKT7-53 + pGADT7-T as a positive control (A, B, C # 2, 3). The already known interaction between p0071 and different clones of E-cadherin were used as additional positive control (A, B, C # 4, 5, 6). Interaction of Vito1 with its screening partners, MYOZ1 (A, B, C # 7, 8) and different T-cap clones (A, B, C # 9 and 10) were*

tested. Clones were streaked onto plates lacking -trp and -leu as a control to confirm the growth (A- clones 1-10). The same clones were streaked onto – trp, -leu, -his to test for reporter gene activation (B- clones 1-10). These colonies (B- clones 1-10) were subjected to a ß-gal colony lift LacZ filter assay to confirm the interaction between the bait and prey (C- clones 1-10). Vito1 was found to interact with both MYOZ1 (C- clones 7,8) and the different overlapping clones of T-cap (C- clones 9, 10).

The colonies were replica plated again on TDO plates as streaks and were allowed to grow until a thick patch of yeast growth was observed. These patches of yeast colonies were analyzed for positive interaction by ß-gal colony lift filter assay. As a negative control, pGBKT7-Lam was co-transformed with pGADT7-T while pGBKT7-53 co-transformed with pGADT7-T served as the positive control. Moreover already known interaction between p0071 and E-cadherin clones were included as additional positive control (Hatzfeld, Green et al. 2003). As expected VITO1 interacted with both T-cap as well as MYOZ1 clones as indicated by activation LacZ reporter gene (Fig 19).

3.4.2 VITO-1 binds TEFs with greater affinity than T-Cap and MYOZ1

To determine the binding affinity of VITO-1 with the sarcomeric proteins T-cap and MYOZ1 in comparison to the transcription factor TEF3, a ß-galactosidase reporter gene liquid culture assay using Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate was carried out. This assay provides a quantitative measurement of the reporter gene activity depending on the strength of interaction between two proteins fused to the GAL4 system. For this experiment, VITO-1 pGBKT7 was co-transformed with either TEFs or T-cap or MYOZ1 pGADT7 and the liquid cultures were grown on medium with SD/-Trp/-Leu/-His + appropriate 3 AT concentration. The cells were allowed to grow until mid-log phase and the OD_{600} was measured. The cell pellet dissolved in Z-buffer was subjected to freeze (-80°C) thaw cycle followed by the addition of ONPG and ß-mercaptoethanol. If ß-galactosidase is present, the colourless ONPG is split into galactose and o-nitrophenol, a yellow compound. Samples were incubated at 30°C and the time required for the development of yellow color to the sample solutions was recorded. The reaction was stopped by the addition of 1M sodium carbonate. The OD_{420} of the samples

were measured and the reporter gene activity was measured by calculating the ß-galactosidase units.

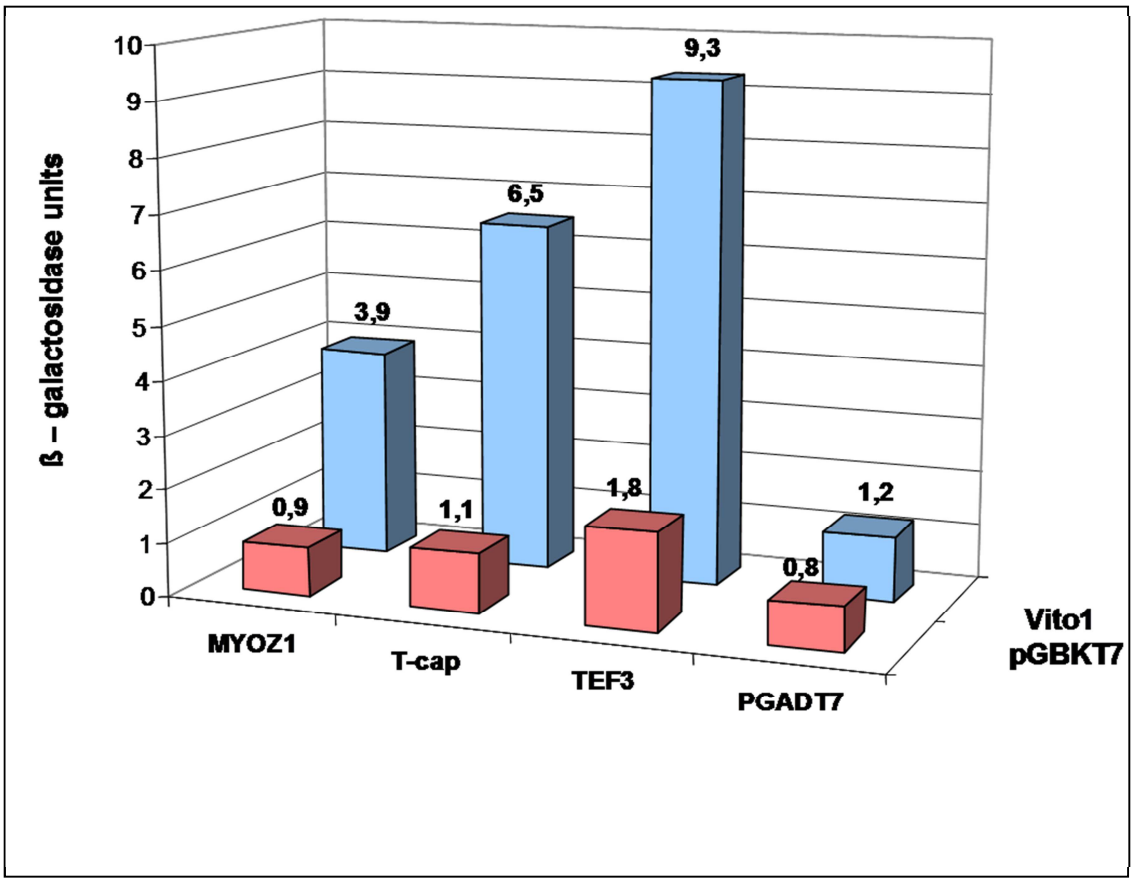


Figure 20. Quantitative measurement of ß-galactosidase activity using ONPG liquid culture assay*. Together with Vito1, MYOZ1 shows > 3 fold activation, T-cap > 6 fold and TEF3 > 9 fold activation of the LacZ reporter gene.´*

The binding affinity of Vito1 with its interacting partners was characterized using ONPG assay. MYOZ1/T-cap/TEF3 (in fusion with GAL4AD) and Vito1 can efficiently activate lacZ reporter gene expression. Enzyme activities are normalized against control containing both pGADT7 and pGBKT7.

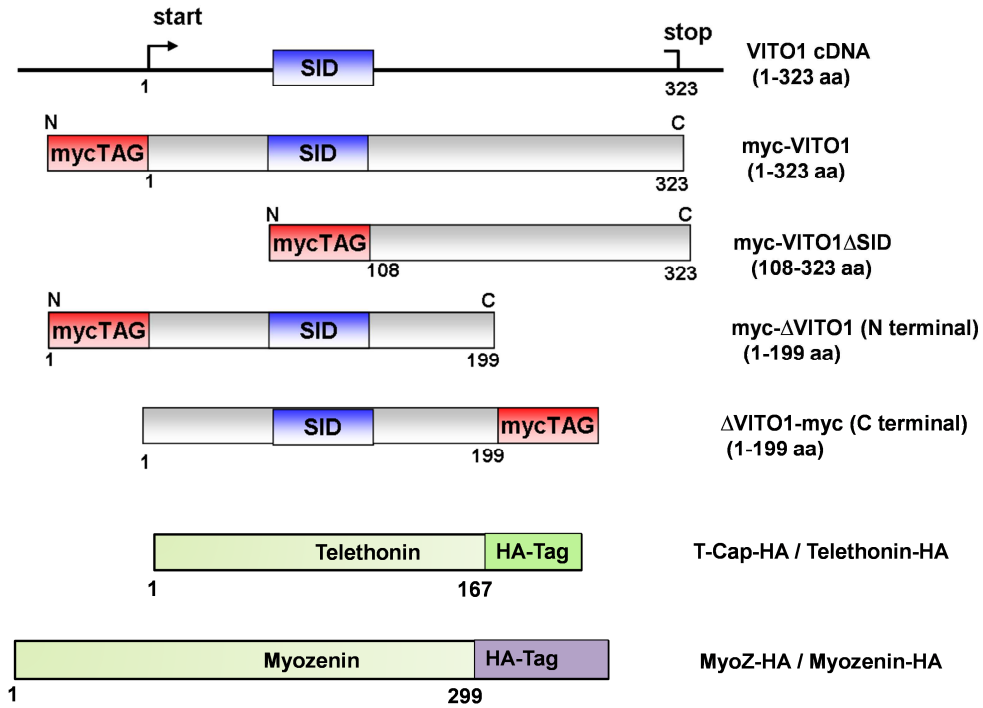
Enzyme activities were normalized against control containing both pGADT7 and pGBKT7. All experiments were performed in triplicates. It was found that VITO-1 was able to synergistically activate the LacZ reporter together with its binding partners namely TEf3, T-cap and MYOZ1. However VITO-1 showed a stronger interaction with

TEFs (> 9 fold) in comparison with T-cap (> 6 fold) and MYOZ1 (> 3 fold) with respect to the controls (Fig 20).

3.4.3 VITO-1 might require other binding partners of eukaryotic cellular environment to stabilize its interaction with Z-disc proteins

After confirming the interaction of VITO1 with both T-cap and MYOZ1 in Y2H, Co-IP from in vitro transcripted/translated protein from rabbit reticulocyte lysate system was used to confirm protein interactions *in vitro*. Full length myc-VITO1 (1-323 aa) and its mutants, a) N-terminal myc-ΔVITO1 (1-199 aa), b) C-terminal ΔVITO1-myc (1-199 aa) which contains the SID domain and c) myc- VITO1ΔSID (108-323 aa) without the SID domain were used to investigate their interaction with T-cap in subsequent co-immunopecipitation experiments. Proteins were fused either with a c-myc tag or with a hemagglutinin (HA) tag. Myf-5 and E2-2 known to form stoichiometric complexes with each other were used as positive controls.

VITO-1 constructs, Telethonin and Myozenin used for subsequent co-immunoprecipitation experiments:



Proteins were translated in vitro by using a TNT T7 polymerase-coupled reticulocyte lysate system (Promega). 2 µl of the protein samples were loaded on SDS PAGE gels and were detected for proper and specific translation of each protein (Fig. 21 – Lysate). Myf-5 and E2-2 known to form stoichiometric complexes with each other were used as positive controls. 10 µl of the translated VITO1 and Telethonin were incubated with 6 µl of c-myc antibody and were immunoprecipitated using Protein A sepharose beads. For Co-Immunoprecipitation, 10 µl of the translated proteins (to be analyzed for interaction) were mixed together, precipitated using *5 µg* c-myc antibody (for VITO1 - T-cap interaction) and were detected by autoradiography. VITO-1 did not co-immunoprecipitate with T-cap (Fig. 21). Myf5 and E2-2 complex was precipitated using 2 *µg* E2-2 antibody as a positive control (Fig.21).

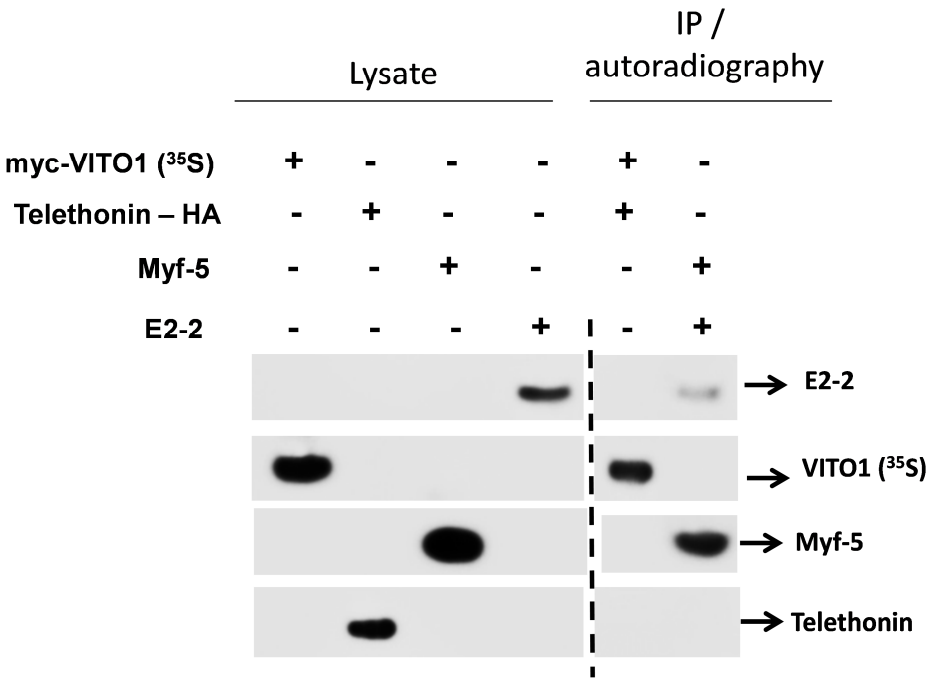


Figure 21. VITO-1 does not co-immunoprecipitate with T-Cap in the rabbit reticulocyte model.

Myc-VITO-1 was radiolabelled with ^{35}S *and mixed with T-cap HA. The known interacting partners Myf-5 and E2-2 were used as positive controls. The protein complexes were immunoprecipitated with c-myc antibody and subjected to SDS PAGE and autoradiography. Protein lysates before immunoprecipitation were loaded as input*

controls. Telethonin did not co-precipitate with VITO-1. The positive control complex partners, Myf5 and E2-2 were co-precipitated.

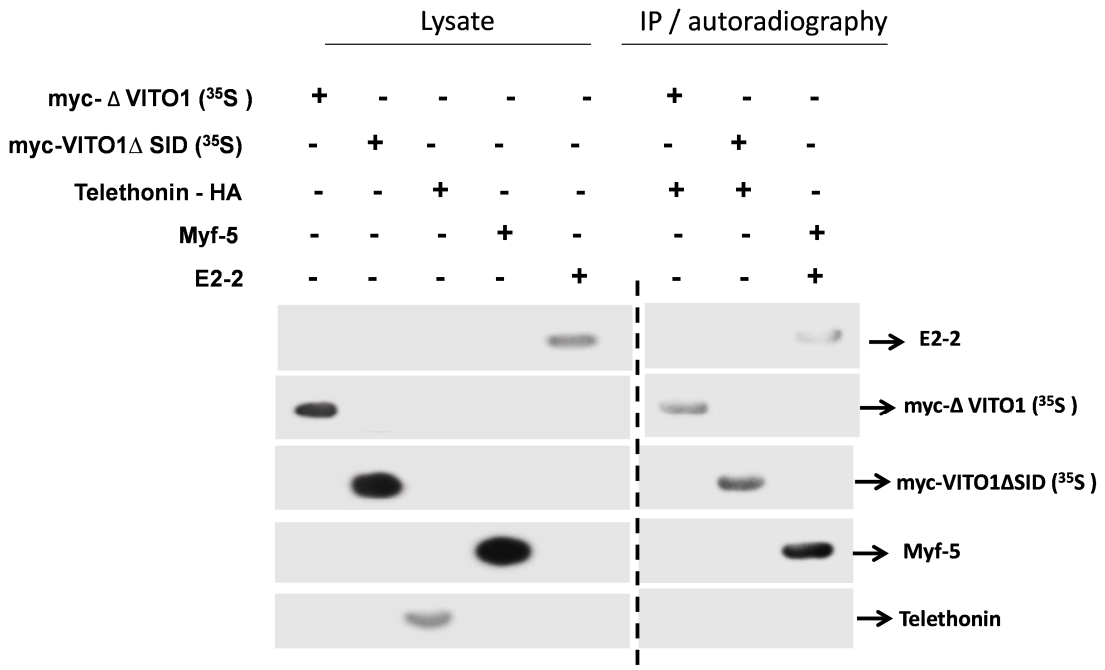


Figure 22. Co-Immunoprecipitaion of VITO-1 deletion constructs with T-cap.
VITO1 deletion constructs myc-ΔVITO1 and myc-VITO1ΔSID were radiolabelled with ^{35}S *and mixed with T-cap HA. The known interacting partners Myf-5 and E2-2 were used as positive controls. The protein complexes were immunoprecipitated with c-myc antibody and subjected to SDS PAGE and autoradiography. Protein lysates before immunoprecipitation were loaded as input controls. Telethonin did not Co-precipitate with VITO-1. The positive control complex partners, Myf5 and E2-2 were co-precipitated.*

Two VITO1 deletion constructs, N-terminal myc-ΔVITO1 (1-199 aa), which contains the SID domain and myc-VITO1ΔSID (108-323 aa) without the SID were tested for interaction with T-cap from proteins that were invitro transcripted/translated. Translated proteins from different plasmids were loaded on SDS PAGE and detected by autoradiograpgy to check for proper translation as input controls (Fig. 22). Myf-5 and E2-

2 known to form stoichiometric complexes with each other were used as positive controls. 10 µl of the translated VITO-1 deletion constructs and T-cap were incubated with 6 µl of c-myc antibody and immunoprecipitated using Protein A sepharose beads to avoid any unspecific cross reactivity between the protein and antibody. For Co-Immunoprecipitation, 10 µl of the translated proteins (to be analyzed for interaction) were mixed together, precipitated using *5 µg* c-myc antibody and were detected by autoradiography. The positive control Myf5/E2-2 complex was co-immunoprecipitated with 2 *µg* E2-2 antibody (Fig. 22).

Telethonin did not co-immunoprecipitated with either myc-ΔVITO1 or myc-VITO1ΔSID (Fig. 22).

A similar experiment was carried out with the same conditions and procedure to find if VITO-1 interacts with Myozenin-1 (Fig 23). VITO-1 did not co-immunoprecipitate with Myozenin-1.

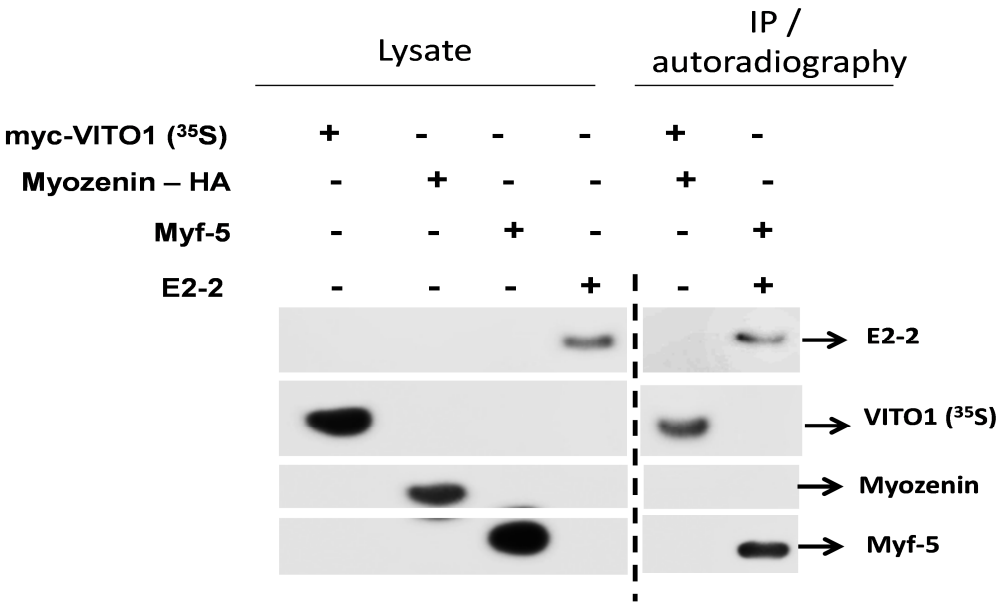


Figure 23. VITO-1 does not co-immunoprecipate with Myozenin1 in the rabbit reticulocyte system. *Myc-VITO-1 was radiolabelled with* ^{35}S *and mixed with Myozenin-HA tagged fusion protein. The known interacting partners Myf-5 and E2-2 were used as positive controls. The protein complexes were immunoprecipitated with c-myc antibody and subjected to SDS PAGE and autoradiography. Protein lysates before immunoprecipitation were loaded as input controls. Myozenin1 did not Co-precipitate*

with VITO-1. The positive control complex partners, Myf5 and E2-2 were co-precipitated.

The rabbit reticulocyte system system failed to provide evidence of physical interactions between VITO-1 / T-Cap and VITO-1 / Myozenin1. These results did not support or confirm the physical interaction of VITO-1 with T-cap and MYOZ1 previously observed in the Yeast 2 Hybrid system.

The failure of this system to detect VITO-1/T-cap and VITO-1/MYOZ1 interactions in the coupled *in vitro* transcription / translation system could be due to various reasons. Analysis of protein-protein interactions in the transcription / translation system is artificial since additional components that might facilitate or stabilize interactions are missing. In addition, newly synthesized proteins in the reticulocyte system are not properly modified, which might also compromise molecular interactions. It seems possible that VITO-1 requires other proteins to form a complex to allow a stable interaction, which is missing in this *in vitro* system or requires post-translational modification. Although yeast two hybrid system has its own disadvantages, it embodies an *in vivo* technique using the yeast host cell as a live test tube reflecting processes in higher eukaryotic cells better than most *in vitro* approaches. Thus VITO-1 might require other binding partners of eukaryotic cellular environment to mediate its interaction with T-cap and MYOZ1. To verify the Y2H interaction of VITO-1 with T-cap and MYOZ1, we hence switched to mammalian cell lines where the proteins are expressed in their native environment. Due to high transfection efficiency and easy handling, HEK 293 and C2C12 cell lines were chosen for the Co-IP assay.

3.4.4.1 VITO-1 does not bind with T-cap in both HEK 293 cells and undifferentiated C2C12 cells

HEK293 cells

HEK 293 cells were grown until 50% confluency and were transfected using FuGene 6 reagent. VITO-1 full length myc-VITO1 (1-323 aa) and mutant constructs, N-terminal myc-ΔVITO1 (1-199 aa), C-terminal ΔVITO1-myc (1-199 aa) which contains the SID

domain and myc- VITO1ΔSID (108-323 aa) without the SID domain were co-transfected with Telethonin-HA (T-cap-HA). Cells were harvested after 48 hrs of transfection and Co-IP was performed as described in experimental procedures. Proteins in total cell lysate were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls. Proteins were immunoprecipitated with $5\ \mu g$ c-myc and the bound proteins were detected by western blot analysis using mouse anti-HA (1:200 dilution) and mouse anti-c-Myc (1:100 dilution). HRP conjugated goat α mouse (1:5000) was used for chemiluminescent detection.

VITO-1 full length and their mutant constructs did not co-immunoprecipitate with T-cap in HEK293 cells (Fig. 24).

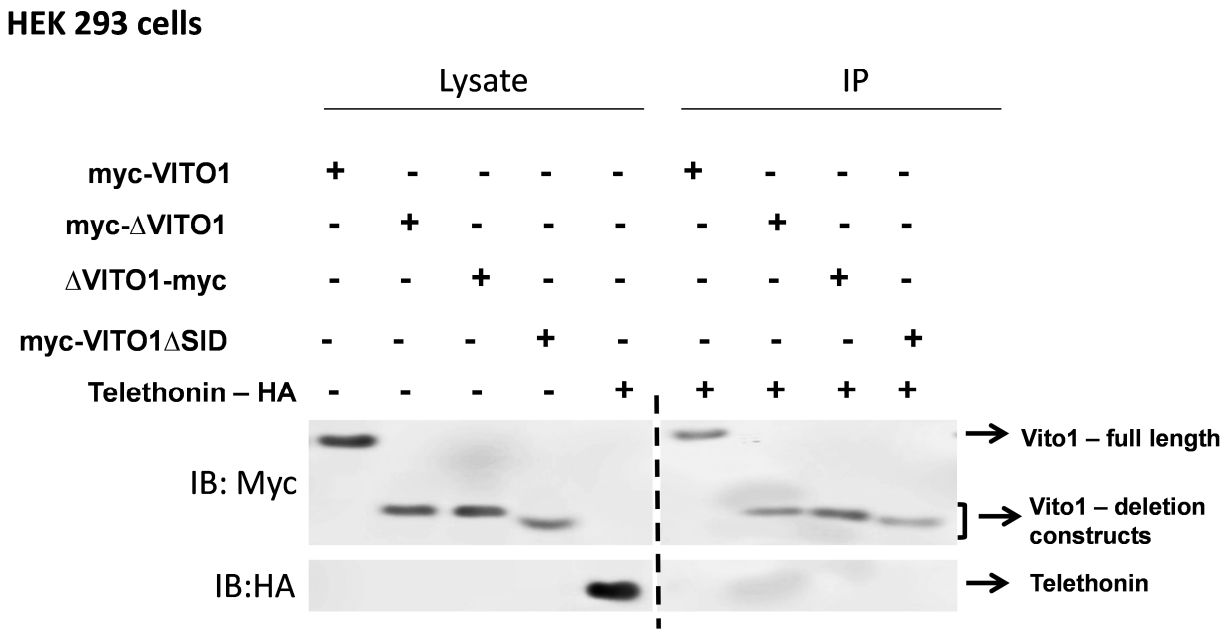


Figure 24. Co-immnunoprecipitation of Vito1 constructs with T-cap from transfected HEK 293 cells.

HEK 293 cells were cotransfected using Fugene 6 with either myc-VITO1, myc- Δ VITO1, ΔVITO1-myc, myc-VITO1-ΔSID together with telethonin-HA. Protein lysates saved before immunoprecipitation and 10% of total protein lysate used for IP was loaded as input controls. Telethonin was not co-precipitaed with Full length VITO-1(myc-VITO1) and its deletion constructs (myc- Δ VITO1, ΔVITO1-myc and myc-VITO1-ΔSID).

C2C12 cells

Since VITO-1 is a muscle specific protein, C2C12 cells which is a muscle cell line was chosen to address the interaction of VITO-1 with telethonin. VITO-1 full length myc-VITO1 (1-323 aa) and mutant constructs myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID were co-transfected with Telethonin-HA (T-cap-HA). Cells were harvested after 48 hrs when the C2C12 cells were still in undifferentiated state (myoblasts). Proteins in total cell lysate were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls (Fig. 25). Proteins were immunoprecipitated with 10 μg c-myc and the bound proteins were detected by western blot analysis using mouse anti-HA (1:200 dilution) and mouse anti-c-Myc (1:100 dilution). HRP conjugated goat α mouse (1:5000) was used for chemiluminescent detection.

VITO-1 full length and their mutant constructs did not co-immunoprecipitate with T-cap in undifferentiated C2C12 cells (Fig. 25).

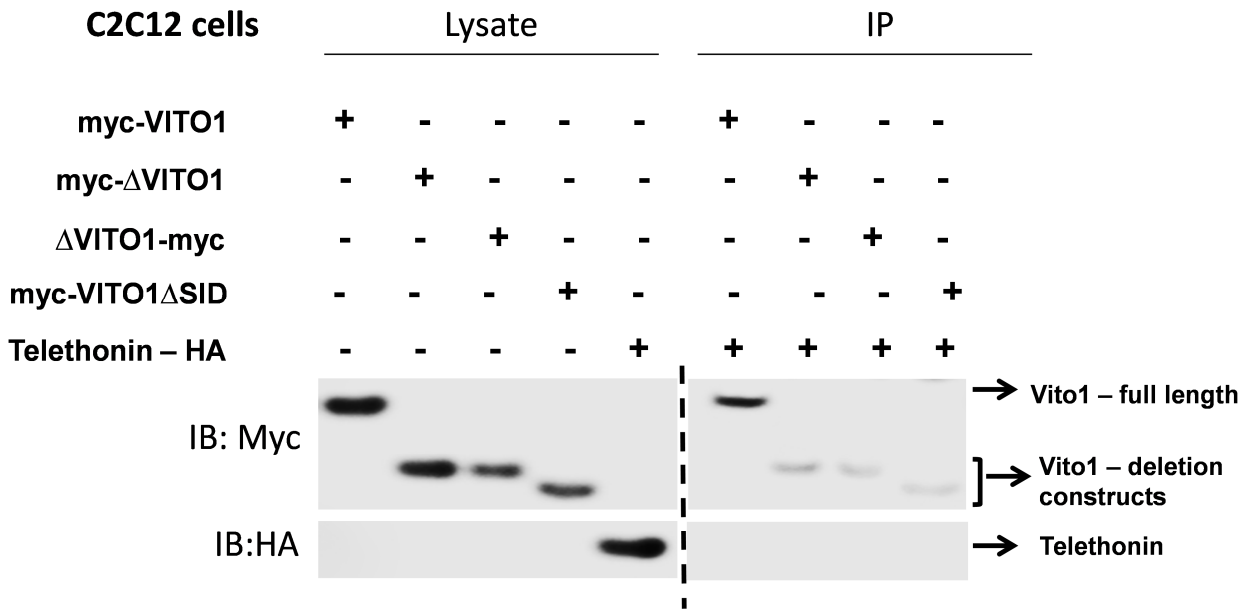


Figure 25. VITO-1 does not co-immunoprecipitate with T-cap in C2C12 myoblasts *C2C12 cells were cotransfected using Fugene 6 with either myc-VITO1, myc- Δ VITO1, ΔVITO1-myc, myc-VITO1-ΔSID together with telethonin-HA. Protein lysates saved before immunoprecipitation were loaded as input controls. Full length VITO-1(myc-VITO1) and its deletion constructs (myc- Δ VITO1, ΔVITO1-myc & myc-VITO1-ΔSID) did not co-immunoprecipitate with telethonin-HA.*

3.4.4.2 VITO-1 did not interact with Myozenin 1 in both HEK293 cells and undifferentiated C2C12 cells

With similar experimental conditions as the previous experiment, VITO-1 full length myc-VITO1 (1-323 aa) and mutant constructs myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID were co-transfected with Myozenin1-HA (MyoZ-HA) in HEK293 cells and undifferentiated C2C12 cells.

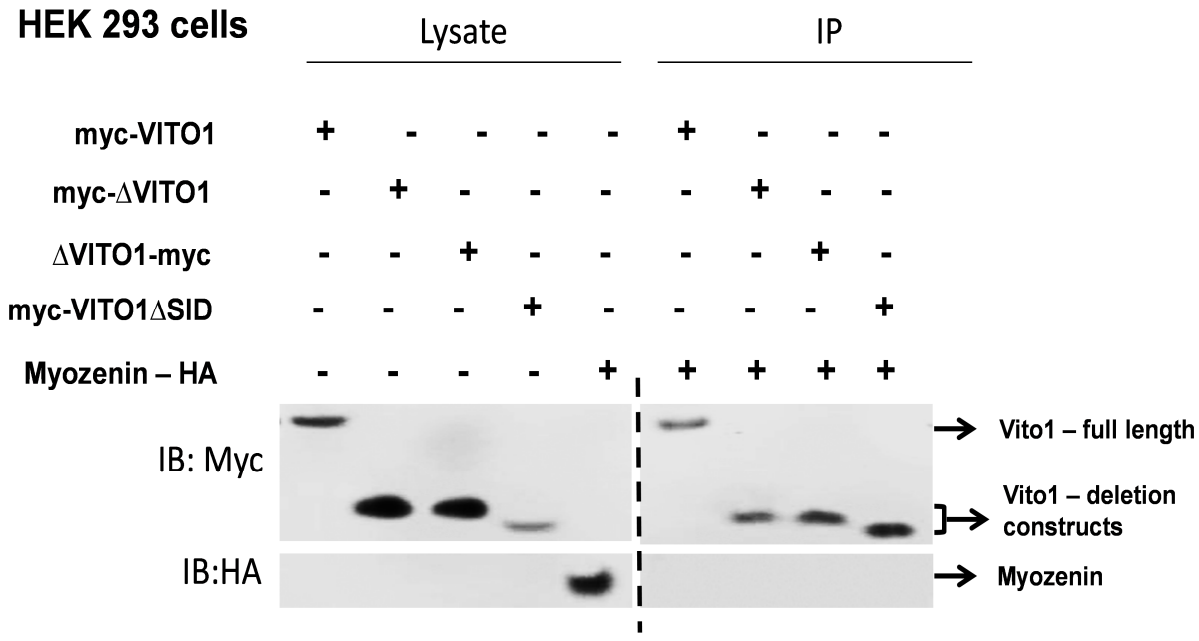


Figure 26. VITO-1 does not co-immunoprecipitate with Myozenin-1 in HEK 293 cells.. *HEK 293 cells were cotransfected using Fugene 6 with myc-VITO1, myc- Δ*

VITO1, ΔVITO1-myc, myc-VITO1-ΔSID together with Myozenin-HA. Protein lysates saved before immunoprecipitation and 10% of total protein lysate used for IP was loaded as input controls. Full length VITO-1 (myc-VITO1) and its deletion constructs (myc- Δ VITO1, ΔVITO1-myc & myc-VITO1-ΔSID) did not co-immunoprecipitate with Myozenin-HA.

VITO-1 full length and their mutant constructs did not co-immunoprecipitate with Myozenin 1 in both HEK293 cells (Fig. 26) and undifferentiated C2C12 cells (Fig. 27).

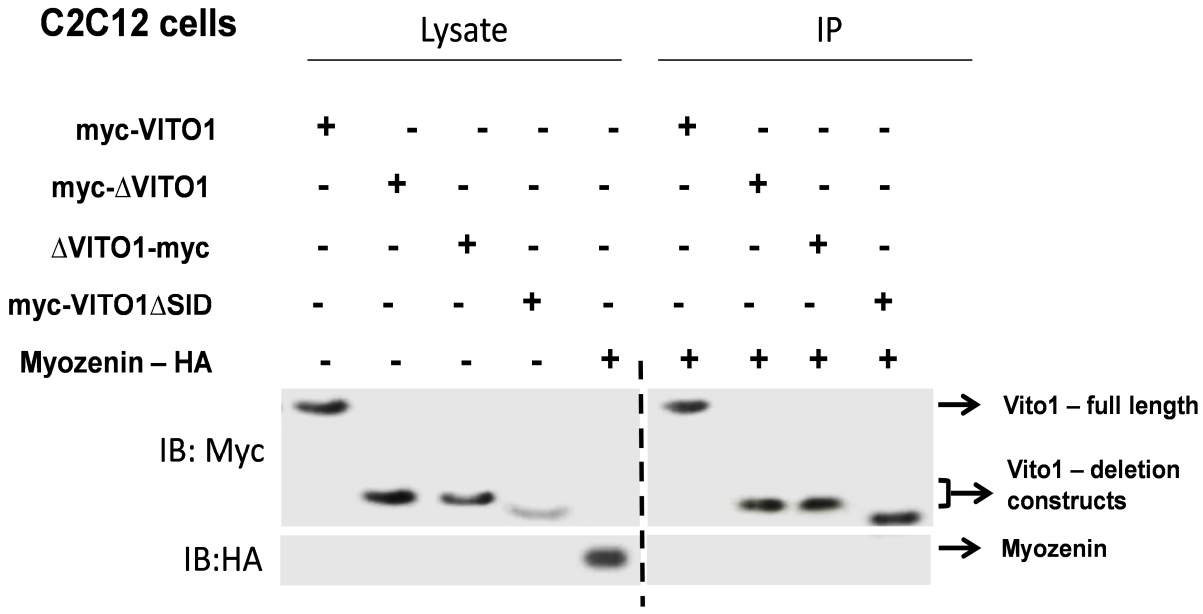


Figure 27. VITO-1 does not co-immunoprecipitate with Myozenin 1 in C2C12 myoblasts *Undifferentiated C2C12 cells were cotransfected using Fugene 6 with myc-VITO1, myc- Δ VITO1, ΔVITO1-myc, myc-VITO1-ΔSID together with Myozenin-HA. 10% of total protein lysate used for IP was loaded as input controls before immunoprecipitation. Full length VITO-1 (myc-VITO1) and its deletion constructs (myc-Δ VITO1, ΔVITO1-myc & myc-VITO1-ΔSID) did not co-immunoprecipitate with Myozenin-HA in C2C12 cells.*

3.4.5 VITO1 interacts with the Z-disc proteins T- cap and MYOZ1 in differentiated C2C12 cells and Chicken primary Myocytes via its Scalloped Interaction Domain (SID)

Based on Northern blot analysis, it was known that VITO-1 is specifically expressed in skeletal muscles and upregulated in differentiated C2C12 myotubes. Since we failed to detect stable protein-protein interaction with VITO-1 and the sarcomeric proteins T-cap and myozenin1 by Co-IP in undifferentiated C2c12 cells, we wondered whether interaction of VITO-1 and the Z-disc proteins requires additional components not present in undifferentiated cells. It seemed possible that other proteins expressed during differentiation of muscle cells or during formation of sarcomeric proteins serve as a bridging complex facilitating the interaction of VITO-1 with telethonin and myozenin1. To verify this hypothesis, differentiated myotubes from C2C12 cells and primary chicken myocytes which form mature sarcomeres were chosen for the Co-IP experiments.

3.4.5.1 Interaction of VITO-1 with T-cap in differentiated C2C12 myotubes

C2C12 cells were grown to 50% confluence in DMEM with 10% FCS and were co-transfected using Lipofectamine 2000 reagent with Telethonin-HA (T-cap-HA) and VITO1 constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID). After 48 hrs, when the cells were almost 100% confluent, the medium was replaced with differentiating medium (DMEM with 2% Horse Serum) and the cells were allowed to differentiate for 72-96 hrs until the formation of mature myotubes Removal of growth factors from proliferating C2C12 cells causes them to reversibly withdraw from the cell cycle and to rapidly begin expressing a variety of muscle-specific proteins.

Medium was changed every day until the cells were well differentiated showing formation of large mature myotubes. Cells were harvested and Co-IP was performed as described in experimental procedures. Proteins in total cell lysate were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls (Fig. 28).

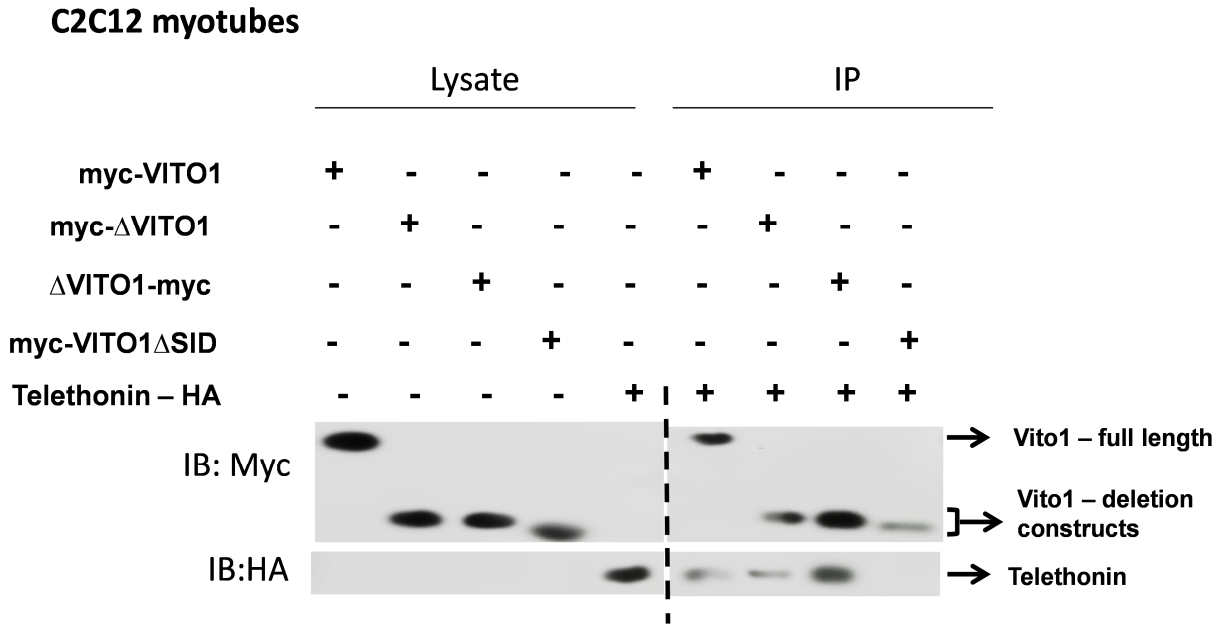


Figure 28. Vito1 co-immunoprecipitates with T-cap in differentiated C2C12 myotubes*. C2C12 cells were transfected using Lipofectamine 2000 with VITO-1 constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID) and T-cap and were allowed to differentiate to form mature myotubes before harvestion for the immunoprecipitation (IP) experiment. Protein lysates from the diffentiated myotubes were loaded as input controls (10% of total lysate used for IP). Telethonin Co-precipitated with all the three VITO-1 constructs (myc-VITO1, myc-ΔVITO1 and ΔVITO1-myc which contained the SID domain. VITO-1 mutant lacking the SID domain (myc-VITO1ΔSID) did not Co-Immunoprecipitate with T-cap.*

Proteins were immunoprecipitated with 10 µg of c-myc antibody and the bound proteins were detected by western blot analysis using mouse anti-HA (1:200) and mouse anti-c-myc (1:100). T-cap co-immunoprecipitated with Full length myc-VITO1 (1-323 aa) and its mutants, N-terminal myc-ΔVITO1 (1-199 aa) and C-terminal ΔVITO1-myc (1-199 aa), all containing the SID domain (Fig. 28). It should be noted that myc- VITO1ΔSID (108-323 aa) without the SID domain did not interact with T-cap. These results suggest the requirement of the Vito1 SID domain to mediate interaction with its binding partners like T-cap or TEFs.

3.4.5.2 VITO-1 Co-immunoprecipitates with T-cap when over-expressed in Primary Chicken Myocytes

Primary myocytes from chicken embryos are a good model to study the interaction of various Z-disc proteins as they form well defined sarcomeres, easy to handle and mainly due to its high transfection efficiency when studying ectopically expressed proteins such as VITO-1.

Primary myocytes were isolated from 11 day old chicken embryos as described in materials and methods and plated at 60% confluency in DMEM containing 10% FCS. The following day, medium was changed and the cells were transfected using the Calcium phosphate method with with Telethonin-HA (T-cap-HA) and VITO1 expression constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID). After 8 hrs of transfection, the medium was changed to DMEM with 10% Horse serum. Cells were allowed to differentiate for 72 hrs until they formed multinucleated myotubes and matured into functional myofibers. At this stage the cells were harvested, lysed and subjected to immunoprecipitation using 10 µgs of c-myc antibody. Precipitated proteins were detected using anti-HA (1:200) and anti-c-myc (1.100) by immunoblotting. HRP conjugated goat α mouse (1:5000) secondary antibody was used for chemiluminescent detection. Proteins in total cell lysate were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls.

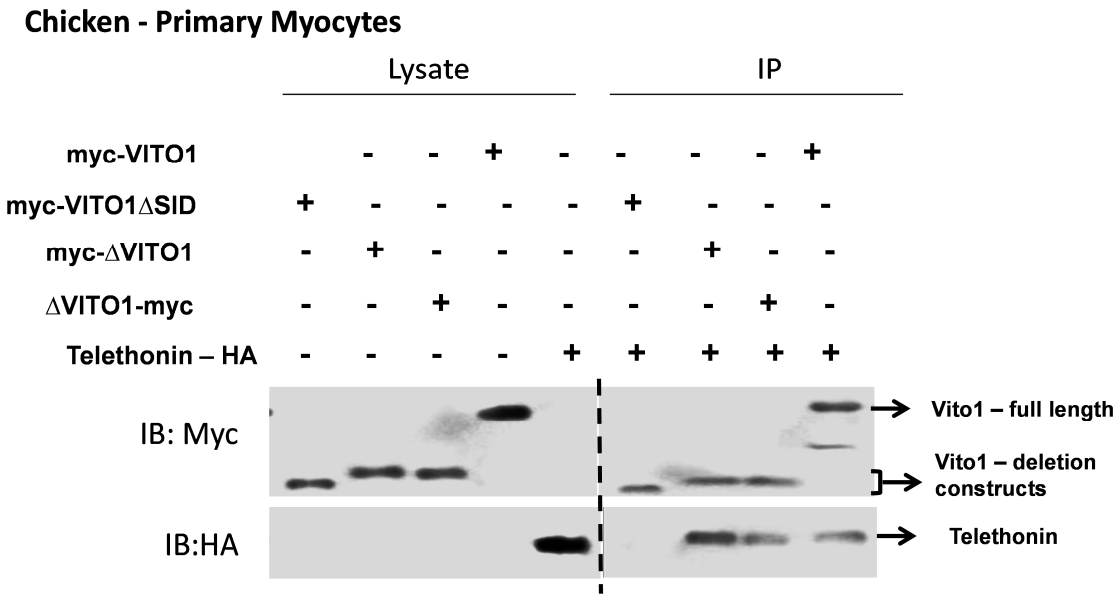


Figure 29. Vito1 interacts with T-cap in vitro via its SID domain.

Primary myocytes were isolated from 11 day old chicken embryo transfected using Calcium Phosphate method with Vito1 contructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID) and T-cap. 10% 0f total protein lysates from differentiated myocytes were loaded as input controls.

Telethonin Co-precipitated with all the three VITO-1 constructs (myc-VITO1, myc-ΔVITO1 and ΔVITO1-myc which contained the SID domain. VITO-1 mutant lacking the SID domain (myc-VITO1ΔSID) did not co-immunoprecipitate with T-cap.

T-cap co-immunoprecipitated with full length myc-VITO1 (1-323 aa) and its mutants, N-terminal myc-ΔVITO1 (1-199 aa) and C-terminal ΔVITO1-myc (1-199 aa), all containing the SID domain (Fig. 29). VITO1 mutant without the SID domain myc-VITO1ΔSID (108-323 aa) did not physically associate interact with T-cap (Fig.29).

3.4.5.3 VITO-1 associates with MYOZ1 in both differentiated C2C12 myotubes and primary chicken myocytes.

Since VITO-1 associated with T-cap in C2C12 myotubes and primary chicken myocytes, we used the same model and conditions to test the physical interaction of VITO-1 with Myozenin1 (MYOZ1).

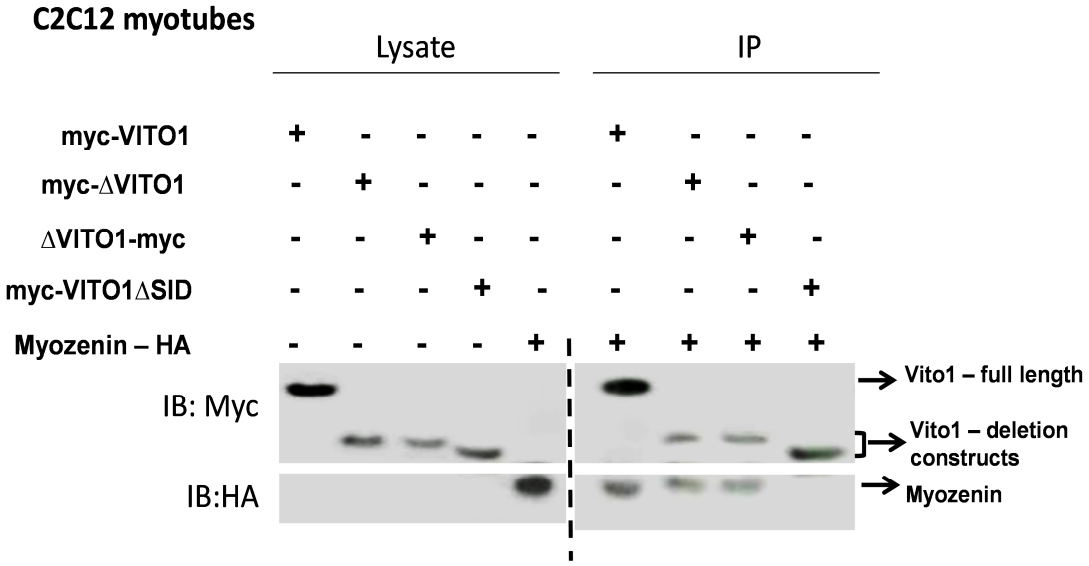


Figure 30. VITO1 Co-immunoprecipitates with MYOZ1 in differentiated C2C12 myotubes*. C2C12 cells were transfected using Lipofectamine 2000 with VITO-1 constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID) and Myozenin1 were allowed to differentiate to form mature myotubes before harvestion for the immunoprecipitation (IP) experiment. 10% of total protein lysates from the diffentiated myotubes were loaded as input controls. Myozenin1 Co-precipitated with all the three VITO-1 constructs (myc-VITO1, myc-ΔVITO1 and ΔVITO1-myc which contained the SID domain. VITO-1 mutant lacking the SID domain (myc-VITO1ΔSID) did not Co-immunoprecipitate with Myozenin1.*

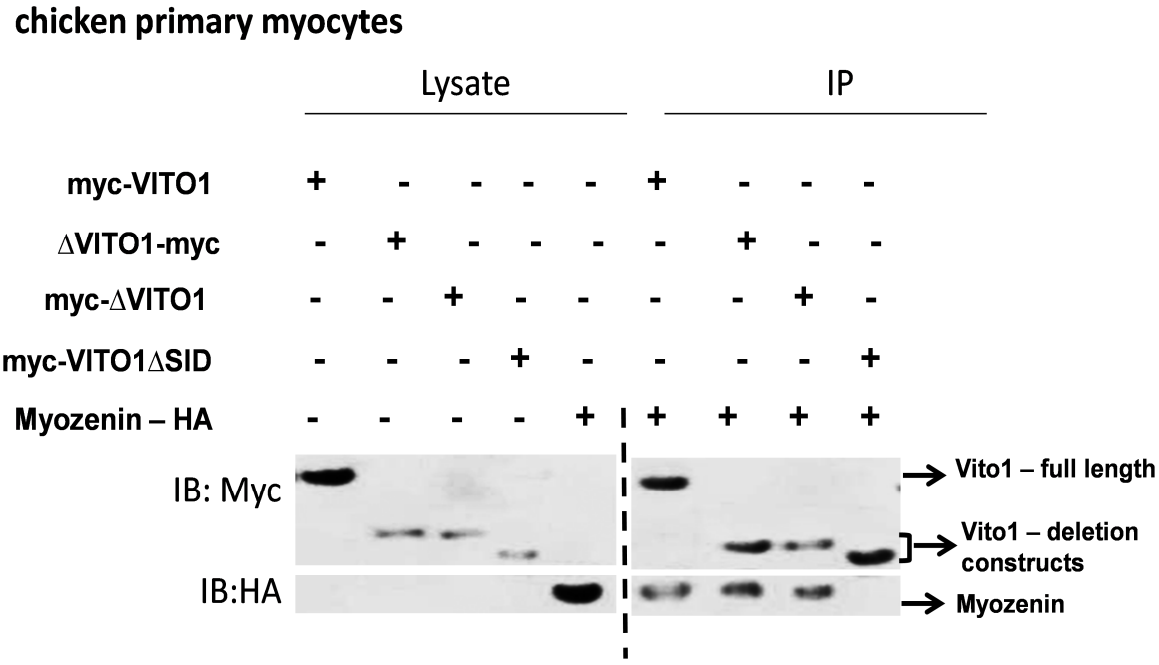


Figure 31. Interaction of VITO-1 with MYOZ1 in chicken primary myocytes required the SID domain*. Primary myocytes were isolated from 11 day old chicken embryo transfected using Calcium Phosphate method with VITO1 contructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID) and HA-tagged Myozenin1. 10% of total protein lysates (used for IP) from differentiated myocytes were loaded as input controls.*

Myozenin1 Co-precipitated with all the three VITO-1 constructs (myc-VITO1, myc-ΔVITO1 and ΔVITO1-myc which contained the SID domain. VITO-1 mutant lacking the SID domain (myc-VITO1ΔSID) did not Co-Immunoprecipitate with Myozenin1.

The experimental procedures for Co-IP were the same as previously described for telethonin. C2C12 cells were co-transfected using Lipofectamine 2000 reagent with Myozenin-HA and VITO1 constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID) and allowed to form mature myotubes before harvesting for IP experiment. Proteins in total cell lysate were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls (Fig. 30).
For primary chicken myotubes, myocytes were isolated from 11 day old chicken embryos and transfected using calcium phosphate method. Cells were allowed to differentiate for 72 hrs until they form multinucleated myotubes and harvested for IP. Proteins in total cell lysate were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls (Fig. 31).
Proteins were immunoprecipitated with 10 µg of c-myc antibody and the bound proteins were detected by western blot analysis using anti-HA and anti - c-myc. Myozenin1 co-immunoprecipitated with full length myc-VITO1 (1-323 aa) and its mutants, N-terminal myc-ΔVITO1 (1-199 aa) and C-terminal ΔVITO1-myc (1-199 aa), in both differentiated C2C12 myotubes (Fig. 30) and primary chicken myocytes (Fig. 31). All these VITO1 constructs contained the SID domain. VITO1 mutant without the SID domain myc-VITO1ΔSID (108-323 aa) did not interact with Myozenin1 in both the cell types (Fig. 30 and Fig. 31)

3.4.5.4 VITO-1 forms a complex with Z-disc proteins by physically interacting with T-cap and MYOZ1 in differentiated C2C12 myotubes and Chicken primary myocytes

To verify if VITO-1 forms a complex with both T-cap and MYOZ1, VITO-1 constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID), Telethonin-HA tagged and Myozenin1-HA tagged were over-expressed transiently in equimolar

concentrations in both differentiated C2C12 myotubes (using Lipofectamine 2000) and Chicken primary myocytes (calcium phosphate method) as described earlier.

Protein complex were immunoprecipitated with 10 µg of c-myc antibody and the bound proteins were eluted after washing the beads to remove unspecifically bound proteins. Eluted proteins were detected by western blot analysis using mouse anti-HA (1:200) and mouse anti-c-Myc (1:100). Proteins in total cell lysate (C2C12 myotubes) were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls (Fig. 32). Both the Z-disc associated proteins Telethonin and Myozenin1 co-immunoprecipitaed with full length myc-VITO1 (1-323 aa) and its mutants, N-terminal myc-ΔVITO1 (1-199 aa) and C-terminal ΔVITO1-myc (1-199 aa), in C2C12 myotubes (Fig. 32).

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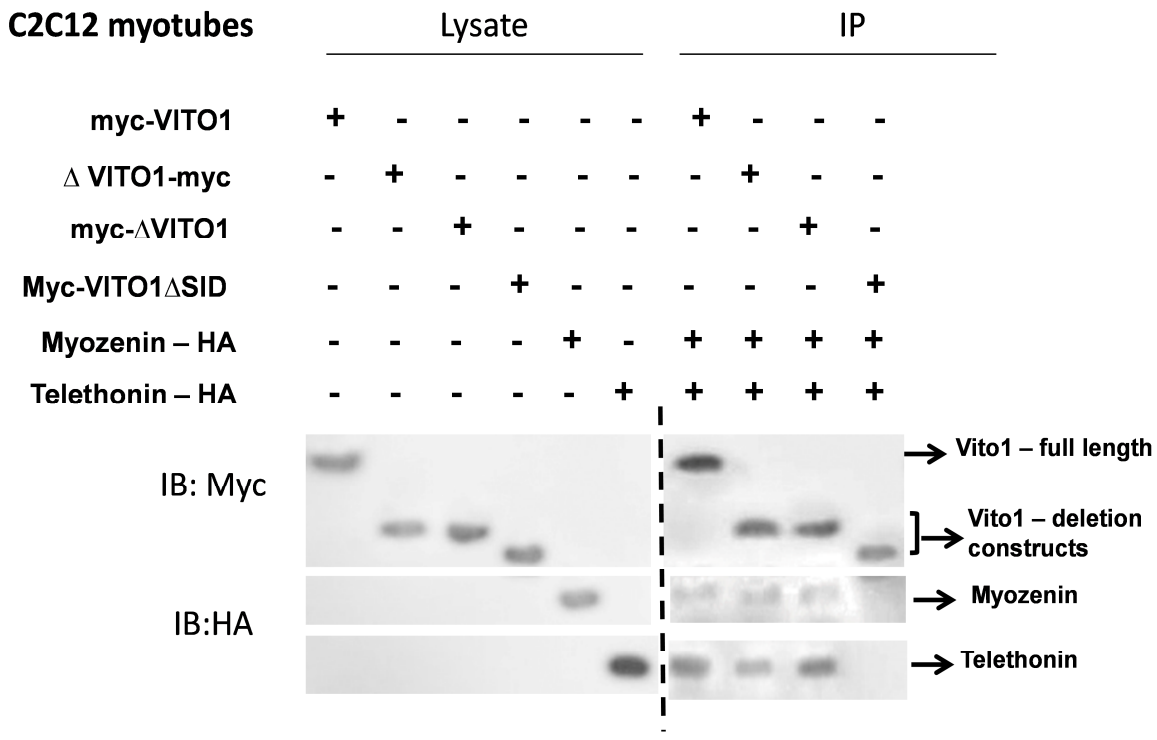


Figure 32. VITO-1 Co-immunoprecipitates with T- Cap and Myozenin in C2C12 myotubes forming a complex*. C2C12 cells were transfected using Lipofectamine 2000 with VITO-1 constructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID), Telethonin and Myozenin. 10% of total protein lysate used for IP was loaded as input controls. All the three VITO-1 constructs (myc-VITO1, myc-ΔVITO1 and ΔVITO1-myc*

which contained the SID domain co-immunoprecipitated with telethonin and myozenin1 thus forming a complex. VITO1 mutant without the SID domain myc-VITO1ΔSID did not show interactions with Telethonin and/or Myozenin1.

For primary chicken myotubes, myocytes were isolated from 11 day old chicken embryos and transfected using calcium phosphate method. Cells were allowed to differentiate for 72 hrs until they form multinucleated myotubes and harvested for IP. Proteins in total cell lysate (primary chicken myotubes) were quantified using the standard Bradford assay and 10% of total protein lysate used for IP was loaded as input controls (Fig. 33). Both the Z-disc associated proteins Telethonin and Myozenin1 co-immunoprecipitaed with full length myc-VITO1 (1-323 aa) and its mutants, N-terminal myc-ΔVITO1 (1-199 aa) and C-terminal ΔVITO1-myc (1-199 aa), in primary chicken myotubes (Fig. 33).
Thus VITO-1 forms a complex with Telethonin and Myozenin1 in both differentiated C2C12 myotubes and primary chicken myocytes. Again, VITO1 mutant without the SID domain myc-VITO1ΔSID did not show interactions with Telethonin and/or Myozenin1 in both the cell models (Fig. 32 and Fig. 33).

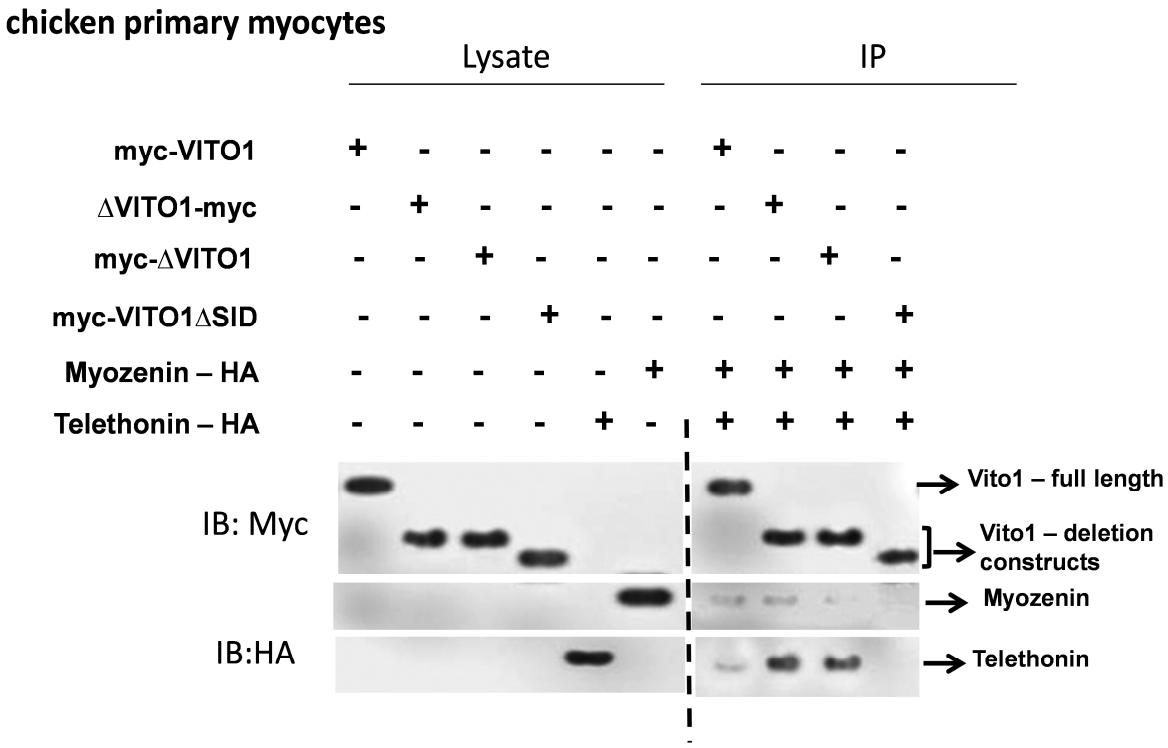


Figure 33. VITO-1 Co-immunopecipitates with Telethonin and MYOZ1 in chicken primary myocyte forming a complex*. Primary myocytes were isolated from 11 day old chicken and were transfected using Calcium Phosphate method with VITO-1 contructs (myc-VITO1, myc-ΔVITO1, ΔVITO1-myc and myc-VITO1ΔSID), Telethonin and Myozenin. 10% of total protein cell lysate used for IP was loaded as input controls. All the three VITO-1 constructs (myc-VITO1, myc-ΔVITO1 and ΔVITO1-myc which contained the SID domain co-immunoprecipitated with telethonin and myozenin1 thus forming a complex. As anticipated, VITO1 mutant without the SID domain myc-VITO1ΔSID did not show and interaction with Telethonin and/or Myozenin.*

Our results confirm that VITO-1 interacts with Telethonin and Myozenin1 in differentiated C2C12 myotubes and primary chicken myocytes and forms a complex with both these Z-disc proteins. Since more T-cap proteins co-immunoprecipitated with VITO-1 compared to MYOZ1, it seems likely that VITO-1 interacts more efficiently with T-cap compared to MYOZ1. This is consistent with the findings from ONPG reporter gene assay (Fig.20). From the above Co-IP experiments it is clear that the functional SID domain of VITO-1 is required to mediate its interaction with the Z-disc proteins. VITO-1 also associates with TEFs via its SID domain (Gunther, Mielcarek et al. 2004). It would be interesting to see if TEFs and the Z-disc proteins (T-cap and MYOZ1) both compete for the same binding sites in the SID domain or have different binding sites.

3.5 VITO-1 localizes at the sarcomeres of both cardiomyocytes and primary myocytes and translocates between the nucleus and cytoplasm

VITO-1 being a transcriptional co-activator is supposedly expressed in the nuclei of different muscle cell types where it interacts with TEFs. But its interaction with the sarcomeric proteins prompted us to investigate if VITO-1 shuttles between the nucleus and cytoplasm. Since the interaction between VITO-1 and the Z-discs proteins T-cap and MYOZ1 occurs in differentiated and striated muscle cells, differentiated C2C12 myotubes, primary chicken myocytes and mouse cardiomyocytes were used as a model system.

3.5.1 Ectopically expressed VITO-1 is localized predominantly in the nucleus of cardiomyocytes isolated from mouse

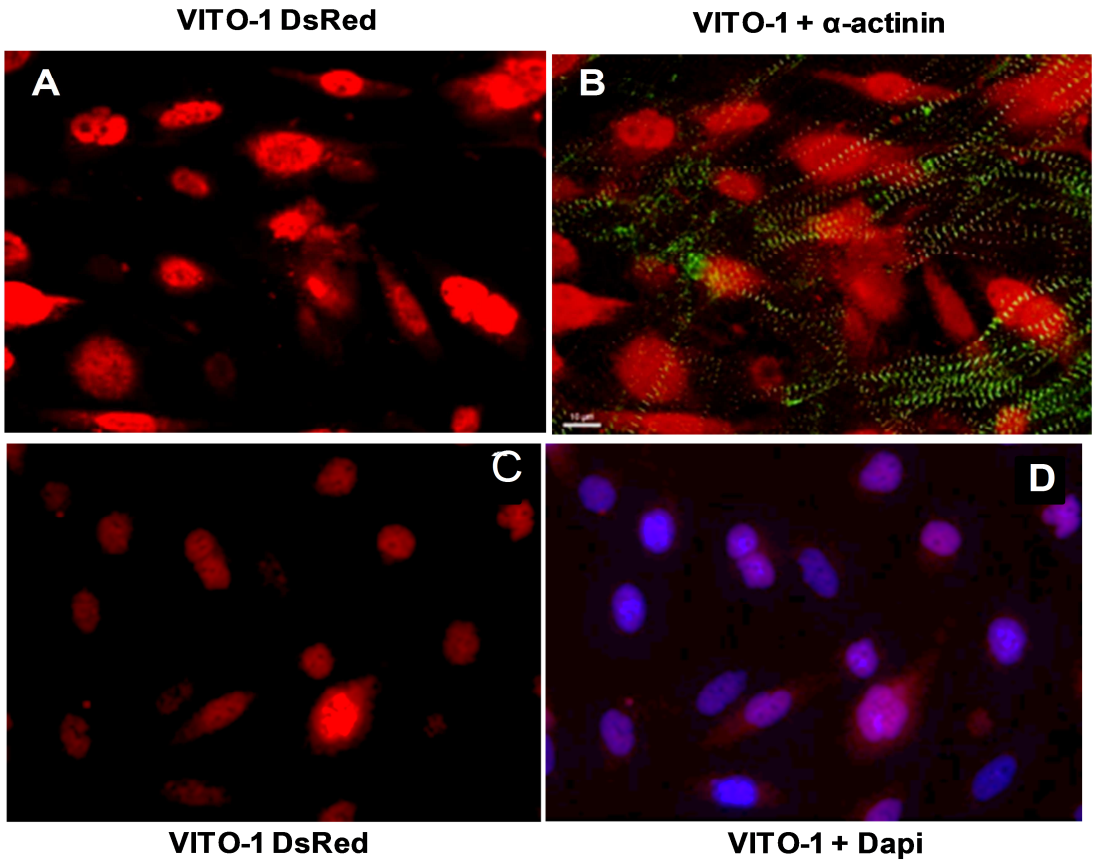


Figure 34. VITO-1 localizes prominently in the nuclei of cardiomyocytes*. Cardiomyocytes were electroporated with VITO-1DsRed using the nucleofector kit and the expression was analyzed after 72 hrs. (A, C) - Majority of the transfected cells expressing VITO-1 were confined to the nucleus. (B) - α-actinin staining as a sarcomeric marker control. (D) – dapi nucler staining.*

Even though VITO-1 is a skeletal muscle specific protein, cardiomyocytes isolated from mice were used to analyze the exogenous expression of VITO-1 as these cells mimic muscle cells in many aspects like striated pattern, stress-sensor proteins, etc. We chose this model to study the localization VITO-1 in Cardiomyocytes.

Neonatal cardiomyocytes were isolated from mouse as described in experimental procedures and these cells were immediately electroporated using the Nucleofector kit to transiently express VITO-1-DsRed. After electroporation, the cells were plated on collagen coated cover slips and were incubated in DMEM with 10% FCS for 2-3 days. Cells were later fixed with acetone and stained using mouse monoclonal sarcomeric alpha-actinin (1:100), a marker for Z-discs and detected with ALEXA 488 (green) goat anti mouse secondary antibody (dilution 1:1000) for visualization. Cells were further prepared for fluorescent microscopy

VITO-1 was localized mainly in the nuclei of cardiomyocytes (Fig. 34 A, C) although one could see some diffused pattern in the cytoplasm. This association in the nucleus could possibly be due to the presence of increased amount of endogenous TEFs or other transcription factors to which VITO-1 might interact with to form a complex.

3.5.2 VITO-1 is localized at the Z-discs of sarcomeres in mouse cardiomyocytes

As described previously, even though VITO-1 was mainly localized in the nucleus of cardiomyocytes, some cells still showed a diffused cytoplasmic localization. Neonatal cardiomyocytes were prepared and electroporated with Vito-1 DsRed as described previously. Interestingly we found cells where VITO-1 was expressed in the Z-discs of cardiomyocytes (Fig. 35 A).

This may be due to high expression of its Z-disc interacting partners in thses cells which might have undergone terminal differentiation.

The question remains open, under what circumstances VITO-1 shuttles between the nucleus and Z-discs. This could be due to some external stimuli or stress conditions or many other external factors which needs a closer and deeper analysis.

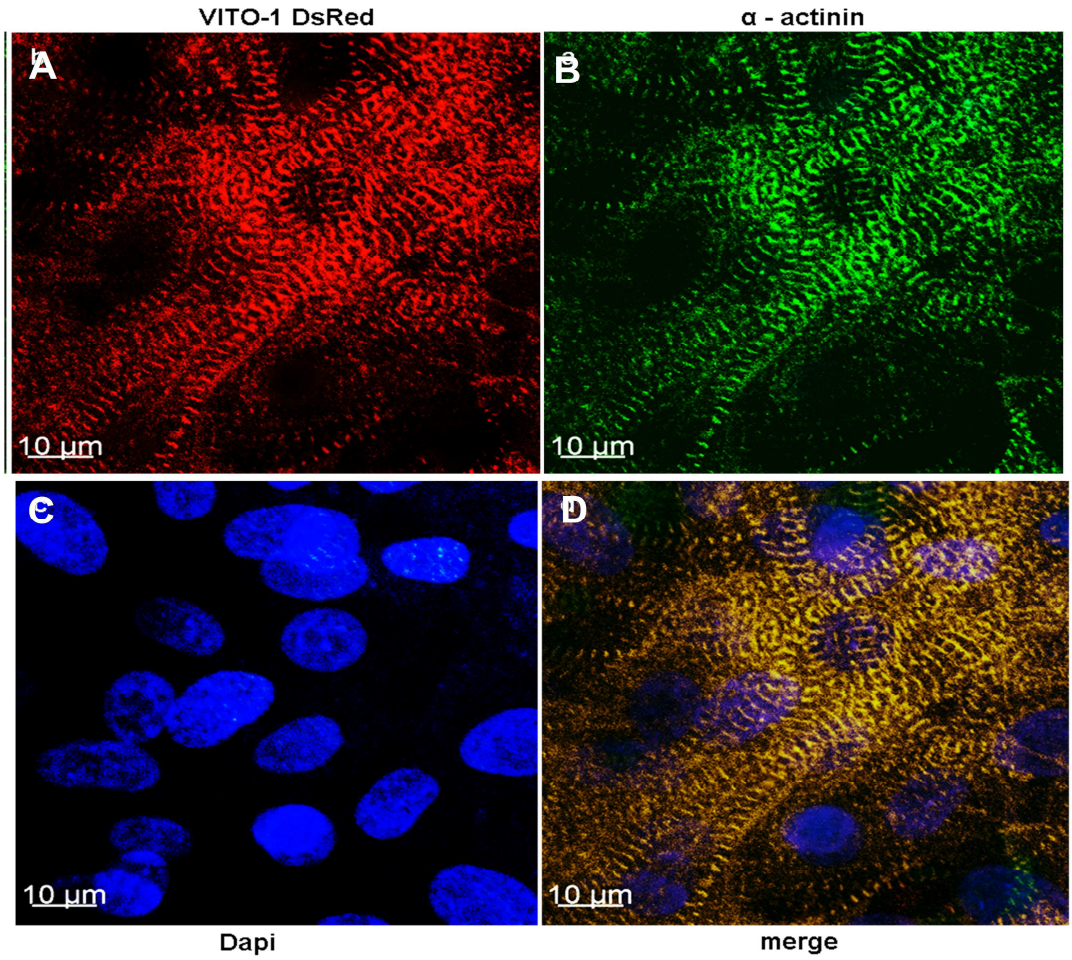


Figure 35. VITO-1 (red) associates at the Z-discs of neonatal mouse cardiomyocytes*. Cardiomyocytes were freshly isolated from neonatal mice and were electroporated with Vito1-DsRed using the Cardiomyocyte-Neonatal Nucleofector kit and the cells were plated on collagen coated dishes. (A) – After 72 hrs, VITO-1-DsRed localized at the Z-discs. (B) - α-actinin staining a marker for Z-discs.*

3.5.3 VITO-1 localizes at the Z-discs of primary chicken myocytes after over-expression of T-cap

VITO-1 physically interacted with T-cap in the Co-IP experiments from isolated myocytes from chicken embryos. Hence we attempted to look for the sarcomeric association of both these proteins. T-cap is a well known Z-disc protein found in striated and cardiac muscle that serves as a scaffold to which myofibrils and other muscle related

proteins are attached. Primary chicken myocytes cultures form well-structured sarcomeres and are comparatively easier to transfect than cardiomyocytes which are quite challenging. Hence this model was chosen to test our hypothesis that VITO-1 might associate with T-cap at the sarcomeres.

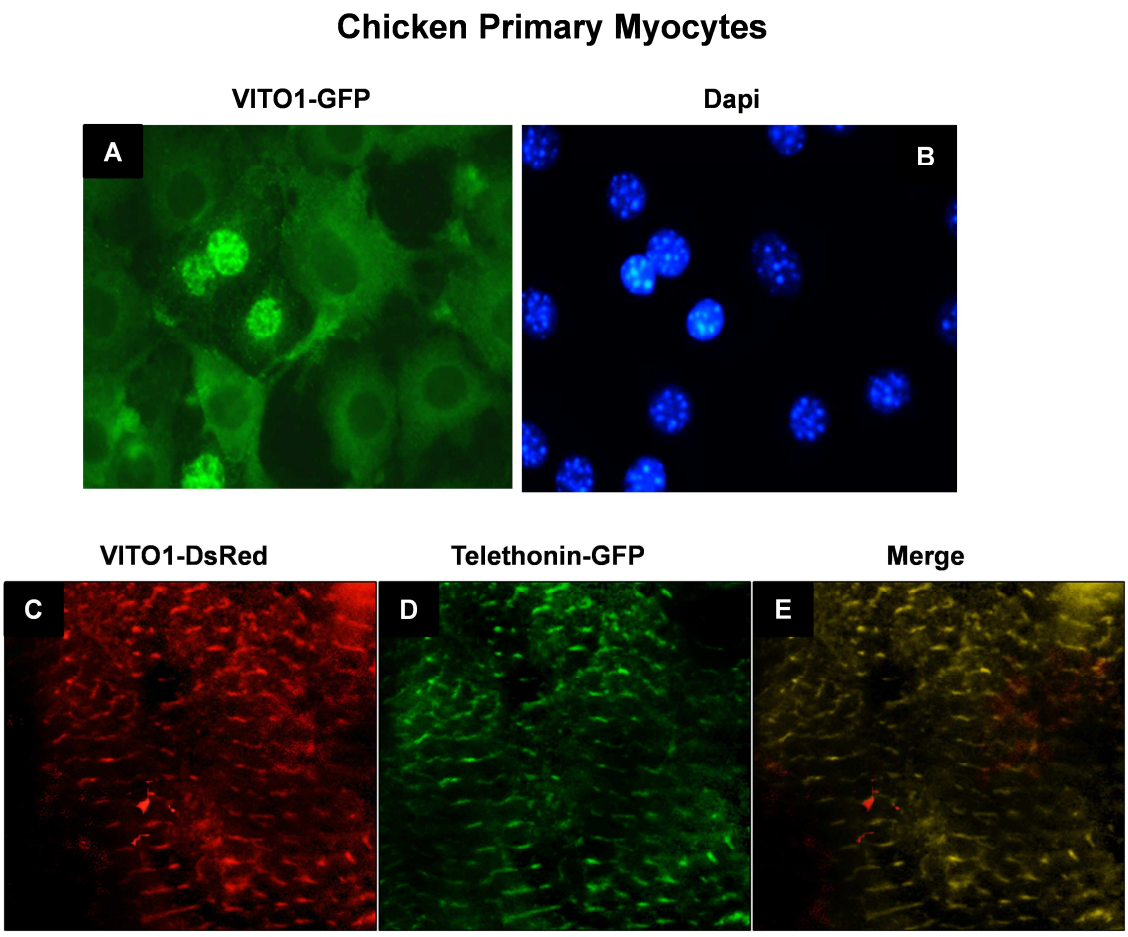


Figure 36. VITO-1 (red) colocalizes with T-cap (green) at the z-bands of sarcomere from primary myocytes.

Primary myocytes were isolated form 11 day old embryo and cotransfected with VITO-1-DsRed and T-cap-GFP and allowed to differentiate. After 72 hrs (C) VITO1-dsRed and (D) Telethonin-GFP associated at the Z-discs and (E) co-localized with each other. A) VITO1-GFP transfected alone localized at the nucleus.

Primary chicken myocytes were isolated from the breast muscle of 11 days old chicken embryos and were plated on collagen coated 6 well culture dishes containing cover slips in DMEM with 10% FCS. The following day, when the cells reached a confluence of 70%, VITO-1-DsRed and T-cap-GFP were co-transfected using calcium phosphate method. After 8 hrs of transfection, the medium was replaced with DMEM containing 10% Horse serum and the cells were allowed to differentiate for 3 to 5 days until the formation of mature myofibers. The cells were later fixed with 4% PFA and the cover slips were mounted on glass slides with moviol.

VITO-1 was expressed strongly at the sarcomeres and localized at the Z-discs where it associated with Telethonin (Fig. 36 C, D & E). VITO1-GFP transfected alone, localized at the myoctes' nuclei (Fig. 36 A). This shows that VITO-1 translocates from the nucleus to cytoplasm when its Z-disc partners are expressed abundantly in striated muscle cells.

3.6 Vito1 might be involved in the initial stage of myofibrillogenesis

Myofibrillogenesis occurs basically in three stages from premyofibrils to nascent myofibrils to mature myofibrils. It is known from literature that a premyofibril model for myofibrillogenesis accounts for sarcomeric formation in skeletal muscle cells. Premyofibrils are the starters of sarcomeres, which are composed of Z-bodies containing mainly the muscle specific isoform of alpha-actinin. When titin and muscle myosin II filaments are expressed, they modify the premyofibrils in such a way that they align to form nascent myofibrils. Several Z-bodies fuse together to form Z-bands in which muscle myosin II filaments are eliminated finally resulting in the formation of mature myofibrils.

Telethonin does not represent a static component of the myofibril, but might be a protein involved in the dynamic control of early events of Z-disc formation during of myofibrillogenesis where it co-localizes with titin and thus plays an important role in myofibril turnover. Since VITO-1 interacts with T-cap in both yeast two hybrid system

and protein binding assays, we asked whether VITO-1 is also associated in the early stages of myofibrillogenesis.

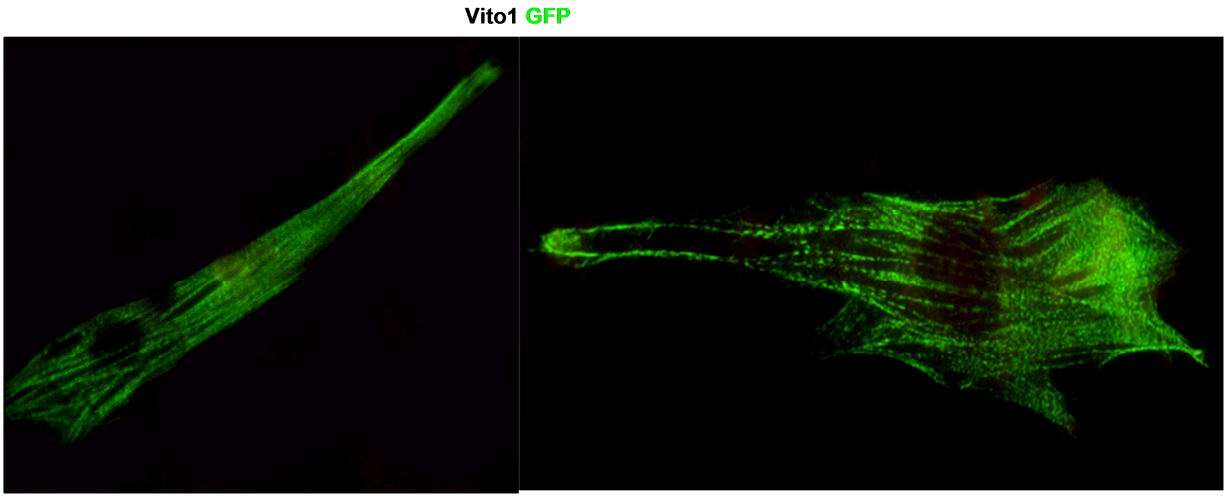


Figure 37. Vito1 might be involved during myofibrillogenesis of skeletal muscle cells. *Skeletal muscle cells were isolated from 11 day old chicken embryo and cotransfected with Vito1 GFP and T-cap N1. VITO1 could be seen involved in myofibrillogenesis where it localized to the Z-bodies and nascent myofibrills and thus might play an important role in skeletal muscle myogenesis*

Primary myocytes cultures from 11 day old chicken embryos were isolated as described in materials and methods. Cells were transfected with VITO-1 GFP and T-cap-N1 using calcium phosphate method and the spreading edge of myocyte expressing VITO-1-GFP was monitored over a 15h time period. Cells were fixed at two different time points expressing VITO-1-GFP (4 hrs and 8hrs) and visualized under a fluorescent microscope. VITO-1 is expressed in punctuated patterns which are supposedly premyofibrils or nascent myofibrils containing the z-bodies which later mature to form Z-bands containing mature myofibrils (Fig. 37). These findings need to be confirmed by using early myofibrillogenesis markers like muscle alpha-actinin, muscle myosin II, titin, etc.

4. Discussion

Translocation of proteins from nucleus to cytoplasm plays a very important role in regulating gene expression. In the past it was assumed that regulatory factors are confined to either the nucleus or the cytoplasm, until an appropriate signal triggers transport of these factors to the other side of the nuclear envelope. Recently several pieces of evidence suggest that most of these proteins are moving in and out of the nucleus thus transmitting various signals necessary for gene regulation. Depending on the equilibrium between import and export rates, such proteins localize either in the nucleus or in the cytoplasm. Compared to the model of retention versus transport, continuous shuttling may allow for a tighter regulation of protein activity. Firstly, interaction of transport receptors with shuttling proteins most often generates inactive protein complexes, thus preventing action in the lack of appropriate signals. Secondly, the existence of a permanent pool of the protein in both compartments favors a more rapid association to target molecules upon signal induction, without the delay associated with translocation across the nuclear envelope. Interfering with nucleocytoplasmic shuttling opens a novel and exciting window to manipulate cellular processes.

4.1 Other partners of VITO-1 obtained from the Y2H Screen

The Y2H screen of VITO-1 also displayed some other interesting candidates which have to be still investigated. A single clone of the dual specificity phosphotase (DUSP13) was detected as a possible partner of VITO-1. Members of the protein-tyrosine phosphatase superfamily cooperate with protein kinases to regulate cell proliferation and differentiation. This superfamily is separated into two families based on the substrate that is dephosphorylated. One family, the dual specificity phosphatases (DUSPs) acts on both phosphotyrosine and phosphoserine/threonine residues. This gene encodes different but related DUSP proteins through the use of non-overlapping open reading frames, alternate splicing, and presumed different transcription promoters (Lang, Hammer et al. 2006). Expression of the distinct proteins from this gene has been found to be tissue specific and

the proteins may be involved in postnatal development of specific tissues. A protein encoded by the upstream ORF was found in skeletal muscle MDSP (Muscle-restricted dual specific phosphotase), whereas the encoded protein from the downstream ORF was found only in testis TMDP (Testis and Muscle specific dual specificity phosphatase). In the mouse, a similar pattern of expression was found. Multiple alternatively spliced transcript variants were described, but the full-length sequence of only some were determined. Both MDSP and TPDP, has been reported as a nuclear protein in cultured cells (Tanner, Trievel et al. 1999; Todd, Tanner et al. 1999) and to be diffusely distributed in the cytosol of resting T-cells (Alonso, Rahmouni et al. 2003). Hsu-Hsin Chen, Ralf Luche et al (Chen, Luche et al. 2004) overexpressed untagged MDSP in differentiated C2C12 myotubes and performed a direct immunofluorescence. MDSP displayed a diffuse staining in the cytosol and was apparently excluded from all nuclei. The same results were obtained COS-1, Hela cells and also when C2C12 myoblasts were transfected with MDSP prior to induction of differentiation and hence concluded that MDSP is primarily cytosolic. VITO-1 is also present in a diffuse pattern in the cyotoplasm when over-expressed alone. In the presence of T-cap, VITO-1 is strongly expressed in the cytosol and translocate into the nucleus in the presence of TEF. It is quite possible that MDSP dephosphorylates VITO-1 and this post-translational modification might be involved in the shuttling effect of VITO-1 in regulating TEF dependent genes as well as the Z-disc proteins. However the true interaction of VITO-1 with MDSP needs to be established before coming to any final conclusions.

4.2 Nucleocytoplasmic translocation of VITO-1 might play an important role in skeletal muscle myogenesis.

Initially VITO-1 was identified as a co-activator of TEFs in regulating the transcription of muscle specific genes. Reporter gene assays indicate that VITO-1 by itself was not able to trans activate the reporter gene but was able to do so in the presence of TEFs (Gunther, Mielcarek et al. 2004). This indicates that VITO-1 plays a supportive but significant role in activating the TEFs from its dormant state to regulate its target genes.

MyoD, a member of the myogenic regulatory factors (MRFs) family, is a key protein in regulating muscle differentiation. This gene encodes a nuclear protein that regulates muscle cell differentiation by inducing cell cycle arrest, a prerequisite for myogenic initiation and also plays a role in muscle regeneration. Being one of the earliest markers of myogenic commitment, it activates its own transcription which may stabilize commitment to myogenesis. VITO-1 seems to be an important component of this program that appears to be necessary to activate TEF-1 transcription factors. The knock down of VITO-1 using siRNA inhibits expression of myogenin and results in the attenuation of C2C12 myotube formation. The supportive role of VITO-1 in MyoD mediated myogenic conversion of 10T1/2 fibroblasts into muscle cells further established the role of VITO-1 as an important co-factor of the muscle regulatory program. Studies from this thesis work reveal a novel interaction between VITO-1 and the Z-disc proteins T-cap and MYOZ1. When T-cap is over-expressed, VITO-1 shuttles from nucleus to the cytoplasm where it localizes at the Z-discs of sarcomeres. Quantitative ONPG reporter gene assay show that VITO-1 binds to TEFs with stronger affinity than with T-cap or Myozenin1 suggesting that relative amounts of interaction partners as well as their binding activity will dictate the eventual localization of VITO-1.

Several other muscle proteins are known to shuttle between the cytoplasm and nucleus. The muscle LIM protein (MLP) is a muscle-specific LIM-only factor that exhibits a dual subcellular localization (Kong, Flick et al. 1997), being present in both the nucleus and in the cytoplasm. Overexpression of MLP in C2C12 myoblasts enhances skeletal myogenesis, whereas inhibition of MLP activity blocks terminal differentiation as observed for VITO-1. MLP functions through a physical interaction with the muscle basic helix-loop-helix (bHLH) transcription factors MyoD, MRF4, and myogenin but does not associate with the myocyte enhancer factor-2 (MEF2) protein, which acts cooperatively with the myogenic bHLH proteins to promote myogenesis. Similar to VITO-1, MLP also lacks a functional transcription activation domain and serves as a cofactor for the myogenic bHLH proteins by increasing their interaction with specific DNA regulatory elements.

Cardiac ankyrin repeat protein (CARP, also known as Ankrd1) has been independently identified by several groups as a cytokine-inducible transcriptional regulator, a protein interacting with transcription factor YB-1, and a cardiac doxorubicin-responsive protein. In skeletal muscles, CARP expression is up-regulated under particular conditions including hypertrophy, eccentric contraction, denervation, and muscular dystrophy (Carson, Nettleton et al. 2002; Tsukamoto, Senda et al. 2002; Nakada, Tsukamoto et al. 2003; Barash, Mathew et al. 2004; Witt, Labeit et al. 2005; Hentzen, Lahey et al. 2006). Although CARP is known to be involved in the regulation of gene expression in the heart, Bang et al. 2001demonstrated that CARP located to both the sarcoplasm and nucleus, suggesting a shuttling of CARP in cellular components. CARP also localizes in cardiac sarcomere although the roles of "sarcomeric CARP" are not fully elucidated. Several reports have demonstrated that CARP binds titin/connectin, myopalladin, and desmin at the Z/I-region of sarcomere. Myopalladin can also bind nebulin and alpha-actinin, (Bang, Mudry et al. 2001) and these proteins are able to bind other members of the Z-disc, thus constituting a network of interactions. Unlike VITO-1 and MLP, CARP expression is up-regulated not only by myogenin and MyoD, but also induced by myocyte enhancer factor 2 (MEF2), which acts as a transcriptional regulator of skeletal muscle remodeling and is expressed from embryogenesis through to adulthood.

Ankrd2 (also known as Arpp) is a sarcomeric protein found in the I band, (Kemp, Sadusky et al. 2000; Moriyama, Tsukamoto et al. 2001; Pallavicini, Kojic et al. 2001) that is involved in skeletal muscle hypertrophy. Ankrd2 is able to interact with the Z-disc protein telethonin and with three other transcription factors PML, p53 and the ubiquitous transcription factor YB-1 similar to CARP (Kojic, Medeot et al. 2004). The Ankrd2 gene is significantly up-regulated in response to prolonged mechanical induced stretch1 as well as denervation4 and the protein is up-regulated upon myoblast differentiation (Pallavicini, Kojic et al. 2001). It should be noted that VITO-1 is also upregulated in differentiated C2C12 myotubes although some expression was found in proliferating myoblasts. Ankrd2 has putative binding motifs for the muscle specific transcriptional factor MyoD (Pallavicini, Kojic et al. 2001). Camilla Bean et al (Bean, Salamon et al. 2005)used MyoD – silenced C2C12 cells to demonstrate that Ankrd2 is a MyoD

downstream target gene. The promoter activity of Ankrd2 was investigated under conditions where C2C12 cells express altered levels of MyoD and found that a minimal region of 290 bp contains putative E-boxes and is the critical regulator of Ankrd2. Ankrd2 expression is regulated by MyoD during earlier stages of myogenic differentiation (1d time-point) and later (3d and 6d time-points) seems to be replaced by unknown alternative mechanism in regulating Ankrd2 expression.

It was previously shown that NFAT proteins translocate from the cytoplasm to the nucleus in response to sustained Ca^{2+} mobilization and return to the cytoplasm when the Ca^{2+} signal ends. NFATc remained completely cytoplasmic when both the Rel-similarity domain (RSD NLS) and the NFAT-homology region (NHR) nuclear localization signal (NLS) were mutated (Beals, Clipstone et al. 1997). After having translocated to the nucleus and bound to DNA, NFAT proteins can enhance transcription by recruiting or facilitating the binding of AP-1 transcription factors to NFAT–AP-1-binding sites (Luo, Burgeon et al. 1996). The components of the AP-1 transcription factor (Fos and Jun polypeptides) then mediate the induction of transcription by recruiting co-activators such as CBP (CREB-binding protein), p300 and JAB1 (Jun activation domain binding protein), through their transcriptional activation domains (Karin, Liu et al. 1997). These co-activators augment transcription activity by recruiting the basal transcription machinery through direct protein–protein interactions and by acetylating histones, which increases accessibility of nucleosomal DNA to transcription factors (Hertel, Lynch et al. 1997). Co-transfection of ribosomal S6 kinase (RSK2) and NFAT3 markedly enhanced multinucleated myotube differentiation of C2C12 myoblasts (Cho, Yao et al. 2007). In addition, RSK2 mutation or knockdown dramatically decreased NFAT3 activity and promoter activity of NFAT3 target genes as well as myotube differentiation. These results suggest that NFAT3 is critical for myotube differentiation. Moreover, RSK2 is shown to be a key protein kinase that phosphorylates NFAT3, which is critical in the process of differentiation.

The functional complex of VITO-1 with TEFs and the Z-disc proteins reveals a novel mechanism for both initiating and maintaining the myogenic program and suggests a global strategy by which VITO-1 proteins may control a variety of developmental

pathways**.** The mechanism by which VITO-1 promotes skeletal muscle differentiation remains to be investigated. Further functional analyses are required to understand the molecular basis of the relationship of VITO-1 and other genes mentioned above with MyoD and to clarify their direct role in muscle development or other pathological situations.

4.3 The subcellular localization of VITO-1 is modulated by TEFs and the Z-disc proteins

VITO-1 functionally and physically interacts with TEF-1 factors (Gunther, Mielcarek et al. 2004) as reported also for Vgl-2 (Maeda, Chapman et al. 2002) which is a homologue of VITO-1. The contribution of the interaction of VITO-1 with TEFs in muscle-specific gene regulation needs to be further studied in detail. Chen et al (Chen, Mullett et al. 2004), showed that the amount of endogenous TEF-1 that binds to the MCAT DNA sequence decreases as skeletal muscle differentiation progresses. This might suggest that TEF-1 expression declines with muscle differentiation. Alternatively, an up-regulation of VITO-1 expression during muscle differentiation might modulate TEFs-DNA interactions resulting in a reduction of the occupancy of MCAT binding sites by TEFs.

Differentiation of muscle cells into myotubes and finally into mature myofibers requires the interplay of many proteins that are involved in sarcomere assembly. In striated muscle cells, the Z-disc constitutes the border of individual sarcomeres, where anti-parallel actin filaments spanning the sarcomeres are cross-linked. The Z-disc plays a key role in the supramolecular assembly of the sarcomeric unit during myogenesis, in the transmission of the tension generated by various sarcomeres along myofibrils and in the regulation of contractile activity. Mutations in Z-disc components have been found to be responsible for various forms of muscle disorders, proving the importance of these proteins in maintaining the integrity of striated skeletal muscle cells (Dalkilic and Kunkel 2003). Some of the Z-disc proteins including α-actinin 2 (Luther 2000), telethonin (Mues, van der Ven et al. 1998), ZASP-1 (Faulkner, Pallavicini et al. 1999), FATZ-1 (filamin, α-actinin and telethonin binding protein of the Z-disc; also termed myozenin-1 or calsarcin

2) (Faulkner, Pallavicini et al. 2000; Frey, Richardson et al. 2000; Takada, Vander Woude et al. 2001), myotilin (Salmikangas, Mykkanen et al. 1999) and myopalladin (Beggs, Byers et al. 1992; Bang, Mudry et al. 2001) are primarily or exclusively found at that location. According to this study, VITO-1 is a novel Z-disc associated protein which interacts with both telethonin, myozenin1 as well as the transcription factor TEFs. Over-expression of VITO-1 together with T-cap results in the translocation of VITO-1 from Nucleus to the Z-discs in mouse cardiomyocytes as well as in chicken primary myocytes. From the ONPG reporter gene assay it is clear that VITO-1 binds to TEFs with a higher efficiency compared to either T-cap or Myozenin1.

When VITO-1 was over-expressed in the presence of TEF in differentiated C2C12 myotubes, VITO-1 translocates into the nucleus (Fig. 38 D, I, N) as observed in other cells types like HEK293 cells and 10T1/2 cells. On the other hand, when VITO-1 is over-expressed together with T-cap VITO-1 does not show any specific localization in the nucleus but is completely distributed all over the differentiated cell (Fig. 38 C ,H, M).

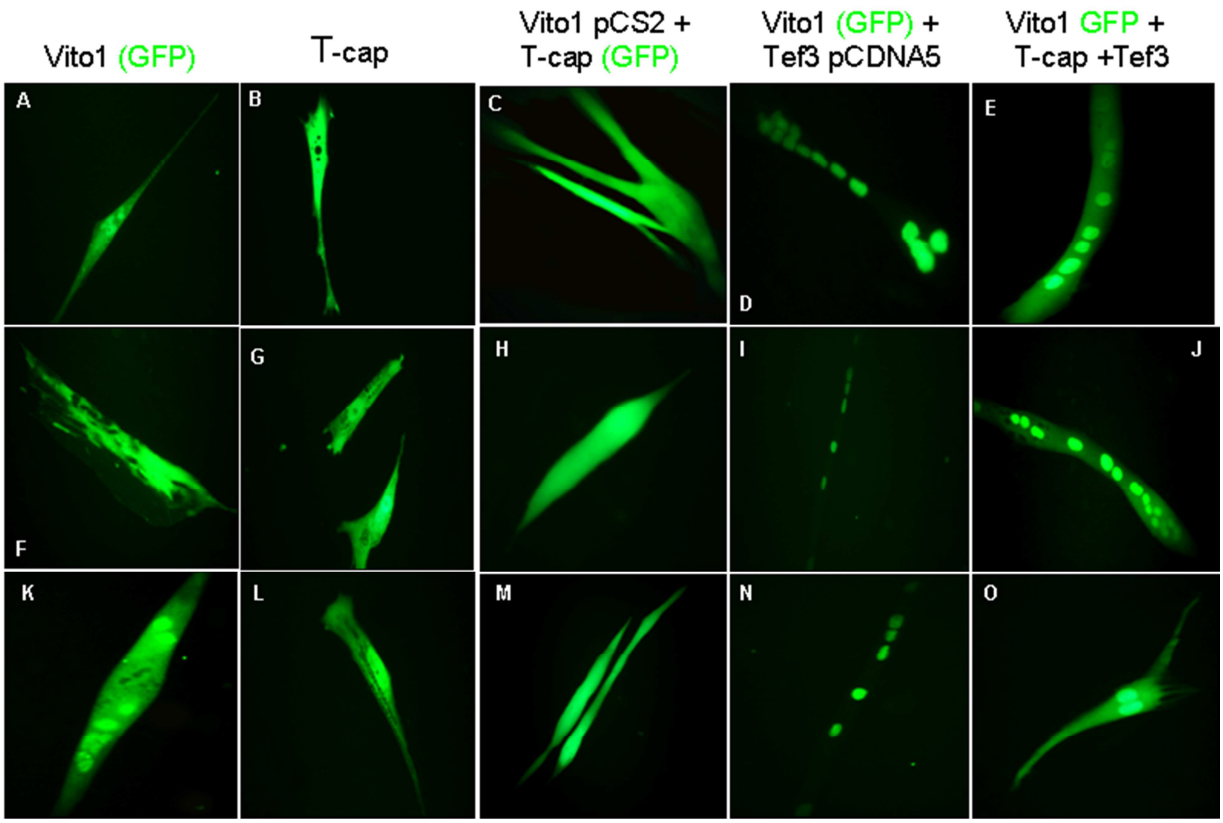


Figure 38. VITO-1 translocates between cytoplasm and nucleus in differentiated C2C12 myotubes. *(D, I, N) - VITO-1 GFP is completely transported to the nucleus when co-transfected with TEF3. (C, H, M) – When VITO1 is co-transfected with T-cap, VITO-1 is distributed throughout the cells. (E ,J, O) - Co-transfection of all three plasmids, VITO-1, T-cap and TEF3 directs VITO-1 to the nucleus as well as cytoplasm. However VITO-1 was more intensely expressed in the nucleus.*

Interestingly, when VITO-1 was co-expressed with TEF-3 and T-cap, VITO-1 is distributed both in nucleus as well as cytoplasm (Fig. 42 E, J, and O). However it should be mentioned that VITO-1 is localized at the nucleus with more intensity where it supposedly interacts with TEF-3 than in the cytoplasm where it might associate with T-cap. This is consistent with the ONPG assay in which VITO-1 activated the reporter gene with TEF-3 two to three folds more than with T-cap or MYOZ1 (Fig. 20). This nucleocytoplasmic property of VITO-1 needs to be analyzed further to attribute the significance of VITO-1's role in different cellular components.

4.4 Post-translational modification might be involved in trafficking VITO-1

Posttranslational modification is the chemical modification of a protein after its translation. It is one of the later steps in protein biosynthesis for many proteins. During protein synthesis, 20 different amino acids can be incorporated in proteins. After translation, the posttranslational modification of amino acids extends the range of functions of the protein by attaching to it other biochemical functional groups such as acetate, phosphate, various lipids and carbohydrates, by changing the chemical nature of an amino acid or my making structural changes, like the formation of disulphide bonds. Transcriptional factors and co-factors are modified quite often by phosphorylation, SUMOylation, ubiquitination, glycosylation, acetylation, methylation and many others that change the regulation of genes thus inducing structural changes and addition of other proteins or peptides. The computational biology software NetPhos 2.0 Server predicted several phosphorylation sites and two potential sumoylation sites for VITO-1 (Fig. 43w)

VITO-1 sequence

MSCLDVMYQVYGPPQPYFAAAYTPYHQKLAYYSKMQEAQECASPGSSASGSSSFSNPTPASVKEE
EGSPEKERPPEAEYINSRCVLFTYFQGDISSVVDEHFSRALSHPSSYTPSCTSSKAHRSSGPWRAEGT
FPMSQRSFPASFWNSAYQAPVPAPLGSPLAAAHSELPFATDPYSPATLHGHLHQGAADWHHAHPH
HAHPHHPYALGGALGAQASAYPRPAVHEVYAPHFDPRYGPLLMPAATGRPGRLAPASAPAPGSPP
CELAAKGEPAGSAWAAPGGPFVSPTGDVAQSLGLSVDSGKRRRECSLPSAPPALYPTLG

Potential Phosphorylation sites predicted: Serine (S): 19
Threonine (T): 2; Tyrosine (Y): 3

Sumoylation sites predicted: 2 – VKEE, AKGE

The amino acids underlined and highlighted in yellow color is the SID domain through which VITO-1 mediates its interaction with other partners.

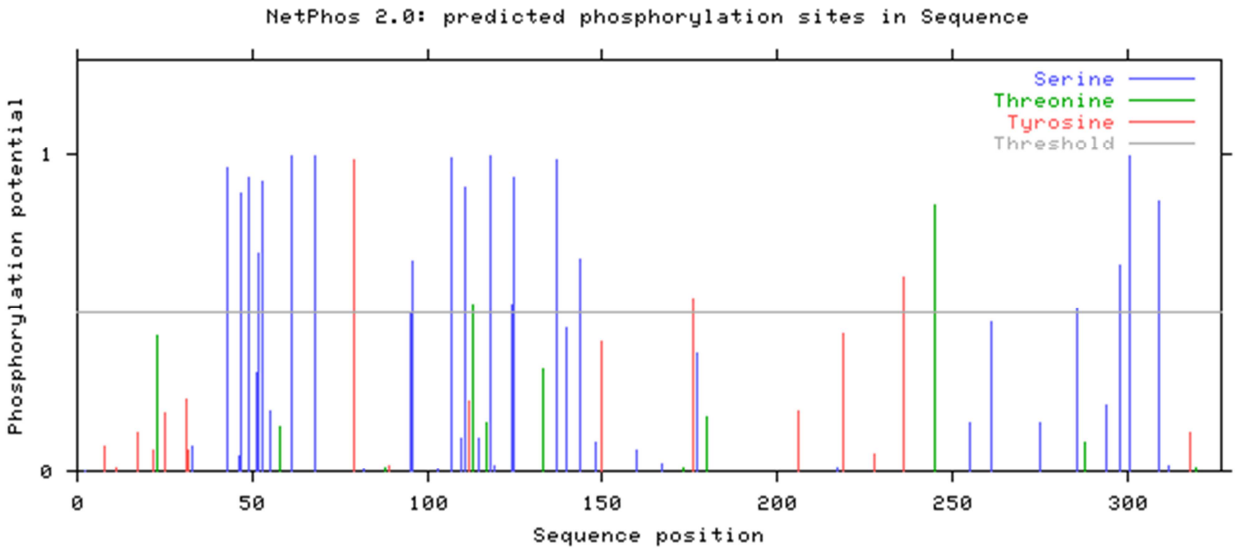


Figure 39. *Phosphorylation sites of VITO-1 as predicted from Netphos 2.0 software*

As predicted from Netphos software, VITO-1 has 19 Serine (green), 2 Threonine (gray) and 2 tyrosine potential phosphorylation sites. Importantly considered should be the phosphorylation sites in the SID domain of VITO-1 (60 – 156 amino acids) as VITO-1

requires the SID domain to interact and activate the TEF-1 and TEF-3 genes (Gunther, Mielcarek et al. 2004) and control the regulation of their dependent genes. VITO-1 also interacts with the sarcomeric Z-disc proteins T-cap and MYOZ1 (FATZ) through its SID domain as seen from the yeast two hybrid, Co-IP and immunocytochemistry experiments. The SID domain of VITO-1 contains 10 serine, 1 threonine and 1 tyrosine phosphorylation sites. Although one might be tempted to look at the consensus sequence containing the phosphorylation sites, even phosphorylation at non-consensus sites might be important to modify the function of VITO-1 to transclocate from nucleus to the sarcomeres in striated muscle cells. Point mutagenesis at different serine residues in the SID domain of VITO-1 will help to evaluate the potential impact of phosphorylation events at these sites. The nucleoplasmic shuttling of many proteins is modulated by different kinases and phosphotase responsible for the posttranslational modifications thus exporting the protein from nucleus to cytoplasm and vice versa in response to various stimuli and extracellular growth factors. Vestigial, a homologue of VITO-1 interacts with Scalloped (sd) through a 56-amino-acid domain, as Sd does not bind to a deleted Vg protein missing only these amino acids, and a construct encoding only this portion of the protein will still bind to Sd. Significantly, a duplicate panel of Vg deletion proteins probed with TEF-1 shows that TEF-1 interacts with Vg via the same protein domain. Affinity columns containing this protein fragment of Vg bind Sd and TEF-1 protein as well as full-length Vg (Halder and Carroll 2001). This Sd/TEF-1-binding domain of Vg is serine rich and includes putative phosphorylation sites (Williams, Bell et al. 1991). Phosphorylation of Vg at these sites may potentially modify the Vg–Sd interaction similar to that of VITO-1 and TEF.

Muscle LIM protein (MLP) and myopodin are two sarcomeric proteins of the Z-band that bind α-actinin and move between the sarcomere and the nucleus: muscle LIM protein (MLP) and myopodin. Faul et al (Faul, Dhume et al. 2007) showed how phosphorylation and dephosphorylation regulate myopodin's localization to the Z bands or nucleus. Phosphorylation of myopodin by either protein kinase A (PKA) or calcium-calmodulin–dependent protein kinase II (CaMKII) induces myopodin's release from α-actinin, its binding partner in the Z band, and its entry into the nucleus. Dephosphorylation of

myopodin by the calcium-activated phosphatase calcineurin permits myopodin to remain or relocalize to the Z band. If the phosphatase activity of calcineurin is inhibited, myopodin moves into the nuclei of cardiac myocytes. Moreover, pharmacological inhibition of the phosphorylation action of PKA reduced or abolished the import of myopodin into the nucleus. Activation of PKA by pharmacological agents leads to diffuse Z-band localization and nuclear localization of myopodin in adult cardiomyocytes. The mechanism of MLP movement from the Z band into the nucleus has not been determined. Protein kinase inhibitor α (PKIA) was also identified as a single clone from the VITO-1 Y2H screen. VITO-1 might also be phosphorylated by PKA or CaMKI kinase which leads to the translocation of VITO-1 from the sarcomeres into the nucleus. De-phosphorylation of VITO-1 by DUSP-13 (Dual specificity phosphatase) which is another potential interaction partner might control the relocation of VITO-1 back to the Z-discs.

VITO-1 interacts with telethonin and is localized at the Z-discs in its presence. It is known that telethonin can be phosphorylated by the kinase region of titin. This domain of titin is, surprisingly, located in the M-band region of the A band, about as far apart as two partners might be in a sarcomere. These disparate locations led to speculation that titin might be released from the middle of the thick filaments to phosphorylate the telethonin in the Z band.. Studies also reveal the presence of T-cap in the nucleus however its role in the nucleus is not yet investigated. VITO-1 as a transcriptional co-factor is located in the nucleus even in the absence of T-cap. It seems that unbound T-cap translocates to the nucleus where it associates with VITO-1 and induces posttranslational modifications that lead to a release from the nucleus into the sarcomere. Such hypotheses have to be analyzed carefully. The predicted phosphorylation sites of VITO-1 should be confirmed by eastern blotting or mass spectrometry. Unpublished previous work from Mielcarek et al claim that VITO-1 protein was most probably phosphorylated in 293T, 3T3 and C2C12 cell lines since an additional band with a molecular weight shift of 1-2 kD was observed. Nevertheless this needs further confirmation.

VITO-1 might also undergo sumoylation by SUMO proteins as VITO-1 sequence contains 2 sumoylation sites (marked in pink in the VITO-1 sequence) and especially the

one at the beginning of the SID domain (VKEE) adjacent to the serine residue. SUMO is a member of multiple ubiquitin family proteins that becomes covalently attached to other proteins via an amide linkage between the C-terminal carboxyl group of the ubiquitin family protein and lysine side chains in the target proteins (Melchior, Schergaut et al. 2003; Seeler and Dejean 2003; Verger, Perdomo et al. 2003; Johnson 2004; Hay 2005). Conjugation of ubiquitin, the best characterized member of the family, to other proteins usually marks these proteins for destruction by the 26S proteasome; the ubiquitin/proteasome pathway is the major pathway for regulated protein degradation in eukaryotic cells. In contrast, conjugation of SUMO to proteins does not mark them for destruction, but modifies their properties in other ways. Previous genetic analyses have demonstrated a role for the sumoylation machinery in embryonic patterning. For example, in C. elegans embryos, loss of SUMO, Ubc9, or the SUMO activating enzyme results in homeotic transformations apparently due to a role for sumoylation in the function of the Polycomb group protein SOP-2 (Zhang, Christoforou et al. 2004). In Drosophila embryos, loss of Ubc9 results in the deletion of variable numbers of thoracic and anterior abdominal segments, but in this case the relevant sumoylation target is not known (Epps and Tanda 1998). Previous genetic analysis also suggests a role for sumoylation in immune system function as mutations in *sumo* or *ubc9* compromise the Drosophila innate immune response by attenuating the LPS-induced expression of genes encoding anti-microbial peptides such as Cecropin A1 (Bhaskar, Smith et al. 2002). Sumoylation significantly stimulates the function of the Drosophila rel family protein Dorsal since rel family proteins play critical roles in both vertebrate and invertebrate innate immunity. Finally, a recent yeast two-hybrid screen indicates that Dof, a cytoplasmic component of the FGF signaling pathway, interacts with multiple components of the SUMO conjugation pathway. This suggests possible roles for SUMO conjugation in the morphogenetic processes controlled by FGF receptors such as mesodermal and tracheal morphogenesis (Battersby, Csiszar et al. 2003). Preliminary results obtained from sumoylation assay by unpublished data from the dissertation work of Michael Mielcarek (2007) suggested that VITO-1 is a potential candidate to bind SUMO protein covalently, since two additional bands, which caused an increase of the MW of 10-15 kDa were found. Addition of GAM1 protein leads to inhibition of the

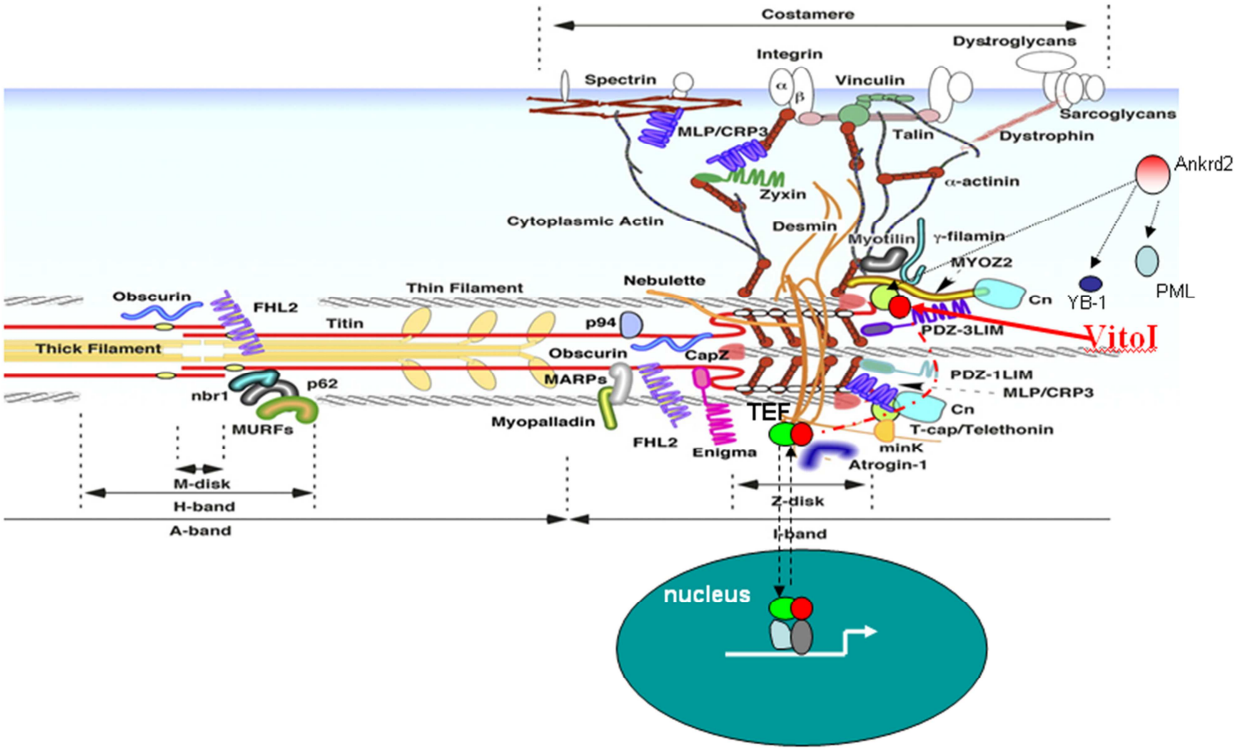
global sumoylation machinery. Co-transfection of VITO-1 with GAM1 efficiently inhibited formation of several VITO-1 bands. Takanaka et al (Takanaka and Courey 2005) show that the SUMO machinery acts to augment Vestigial (Vg) function. SUMO loss-of-function mutations act as genetic enhancers of *vg* loss-of-function mutations. Flies doubly heterozygous for recessive hypomorphic *vg* alleles and recessive *sumo* or *ubc9* alleles exhibit wing notching that is as severe as that exhibited by flies homozygous for the *vg* mutant alleles. In addition, co-overexpression of SUMO and Vg in the wing or eye significantly exacerbates the phenotype due to overexpression of Vg alone.

Conjugation of SUMO with VITO-1 might direct VITO-1 to specific subcellular locales including the nuclear PML oncogenic domains (PODs) as shown by (Ishov, Sotnikov et al. 1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. Sumoylation can also stabilize proteins by antagonizing ubiquitylation (Desterro, Rodriguez et al. 1999; Lin, Liang et al. 2003) and can also modulate the function of VITO-1 which eventually might have an effect on TEFs transcriptional activity. There are two lysine residues (Lys 67 and Lys 269) that are located in a motif resembling the consensus sumoylation site. Lys 67 is located in the sequence VKEE and Lys 269 in the sequence AKGE, while the sumoylation consensus is ψKxE (with ψ signifying a hydrophobic residue). Mutagenesis of lysine to arginine should significantly reduce the ability of VITO-1 to serve as a target for sumoylation. It may be well possible that sumoylation of VITO-1 occurs at non-consensus sites. There are multiple precedents for such non-consensus sites in other sumoylation targets (Johnson and Blobel 1999; Rodriguez, Dargemont et al. 2001). The prediction of a likely role for sumoylation in VITO-1 adds to a growing body of evidence suggesting pleiotropic roles for sumoylation in the development and function of multicellular organisms.

4.5 VITO-1 a novel Z-disc protein?

Z-discs are not simple mechanical joints but are a compartment built by a complex set of proteins which contribute to well defined structures that act as stress sensors in transmitting signal from the extracellular membrane into the nucleus and regulating the

fate of many cardiac and skeletal muscle specific genes. These include a subset of adaptor molecules that have putative binding motifs. The accumulation of signaling-related proteins at z-discs and their physiological significance were extensively reviewed by Pyle and Solaro (Pyle and Solaro 2004).



- *Hoshijima, M. 2006, Am J Physio Heart Circ Physiol (modified)*

Figure 40. Z-disc associated cytosketal structure proteins*. Many of these proteins have been linked to intrinisic mechanical sensor-signal modulator functions. VITO-1(red) seems to be a novel member of this complex interacting with T-cap and Myozenin in addition to oits role as a co-factor of TEFs (green). VITO-1, T-cap, MYOZ1, PDZ-3LIM, one-PDZ and 3-LIM domain protein, MLP/CRP3, Titin, Ankrd2, MARPS, α-actinin, actin, nebulin, FHL2, MURFS and other proteins of the a and I band of the Z-discs is shown above.*

In this study, Telethonin and Myozenin1 were identified as interaction partners of VITO-1 which is a co-factor of TEF dependent skeletal muscle gene regulation. Yeast two

hybrid analysis, Co-IPs and immunocytochemistry experiments clearly suggest that VITO-1 physically interacts with T-cap and MYOZ1. However the physical interaction of VITO-1 with these Z-disc proteins could only be established in differentiated muscle types and not in non-muscle or undifferentiated muscle cells. VITO-1 requires the striated muscle environment where it needs the assistance of other cofactors or chaperones to mediate interaction with T-cap and MYOZ1.

Studies suggest that a hypo-phosphorylated rather than a hyper-phosphorylated form of MyoD may be more subject to acetylation as cells undergo differentiation (Mal et al., 2001). MyoD is highly phosphorylated in proliferating myoblasts, but undergoes substantial de-phosphorylation once these cells are induced to differentiate (Kitzmann et al., 1999). Thus acetylation of MyoD occurs only in differentiated cells.

Similarly VITO-1 might undergo certain post-translational modifications in differentiated cells and/or undergo a conformational change which facilitates interaction with telethonin and myozenin. Although yeasts do not have sarcomeres, VITO-1 still interacts with telethonin and myozenin in the Y2H system. A similar modification of amino acid residues might possibly occur in VITO-1 during or after translation thus exposing their protein binding sites to interact with telethonin and myozenin. Some proteins might specifically interact when they are co-expressed in the yeast, although in reality they are never present in the same cell at the same time. However, in most cases it cannot be ruled out that such proteins are indeed expressed in certain cells or under certain circumstances.

Co-IPs also revealed T-cap and MYOZ1 both precipitating together with VITO-1 thus forming a complex. MYOZ1 (FATZ-1), together with FATZ-2 and FATZ-3, belongs to a family of proteins, named calsarcins, and they interact with sarcomeric calcineurin, a calcium/calmodulin-dependent phosphatase involved in the regulation of genes affecting muscle differentiation and the formation of different fiber types (Delling, Tureckova et al. 2000; Frey, Richardson et al. 2000; Frey, Barrientos et al. 2004). MYOZ1 interacts with actinin-2, Zasp, T-cap, filamin and myotilin which is mainly expressed in striated muscle. Interaction between these two proteins was established by yeast two-hybrid studies, biochemical and cell transfection assays as in the case of VITO-1. Pathogenic mutations

in the myotilin gene cause a subset of myofibrillar myopathies and limb girdle muscular dystrophy (LGMD) type 1A that are characterized by streaming of Z-discs and degeneration of myofibers (Hauser, Horrigan et al. 2000; Hauser, Conde et al. 2002; Selcen and Engel 2004). Mutations in T-cap also cause LGMD. Telethonin interacts with ZASP, Titin, MINK, myostatin, MURFs, MYOZ1, PKD and Ankrd2. Ankrd2 is also an interesting protein with bi-functional action in both nucleus and Z-discs like VITO-1. Ankrd2 can interact with p53, Pre-myelocytic leukemia protein (PML) and YB-1, in vitro (Kojic, Medeot et al. 2004) and can translocate from the I-band into the nucleus after muscle injury (Tsukamoto, Hijiya et al. 2008). Recent studies show that Ankrd2 is expressed mainly in slow muscle type and is not involved in hypertrophy. Unpublished data also reveal that VITO-1 is a slow muscle type protein whose knock out results in a loss of slow fibers, which are markedly increased in atrophic conditions. CARP, a transcription factor, is known to be involved in the regulation of gene expression in the heart. Bang et al (Bang, Mudry et al. 2001) demonstrated that CARP localized to both the sarcoplasm and nucleus, suggesting a shuttling of CARP in cellular components. CARP, ankrd-2/Arpp, and DARP, are three members of the conserved gene family of muscle ankyrin repeat proteins whose expression is induced upon injury and hypertrophy (CARP), stretch or denervation (ankrd2/Arpp), and during recovery following starvation (DARP), suggesting that they are involved in muscle stress response pathways. Stretch of fetal rat cardiac myocytes for 90 minutes resulted in a striking increase of CARP staining in both the nucleus and on myofibrils, in most, if not all, myocytes, relative to unstretched control cells (Miller, Bang et al. 2003). Stretch did not appear to affect ankrd2/Arpp staining, titin N2A or α-actinin, showing that stretch did not cause myofibrillar damage. It would be interesting to study the expression of VITO-1 under such stretch conditions to see if VITO-1 remains in nucleus or at the Z-discs or results in an increased expression in both the nucleus and Z-discs as in the case of CARP.

To conclude, we can say that Z-disc proteins have not only a single or two partners, but are generally associated with a bundle of proteins depending on the extracellular stimuli and pathological conditions involved and change their functions thereby acting as a component of the mechanical sensor that signals to the transcription machinery (Fig. 40).

VITO-1 is a novel member of this family of proteins with a dual role and it can expected that VITO-1 probably associates with other partners amidst T-cap, MYOZ1 and the transcriptional factor TEFs, playing an active role in the signaling cascade between the nucleus and sarcomeres to regulate skeletal muscle-specific genes and muscle stress response pathways.

5. Summary

Transcriptional regulation in heart and skeletal muscle is controlled by the combined action of 3 major families of transcription factors namely the the bHLH, MADs box transcription factors and the Transcriptional enhancer factor (TEFs) which play important roles for the development of muscle tissues and regulated expression of muscle specific genes. MCAT element (5'-CATTCCT-3') has been found in a number of cardiac, smooth, and skeletal muscle-specific genes, including cardiac troponin T, β-myosin heavy chain (β-MHC), smooth and skeletal muscle α-actin. The proteins that bind to the MCAT element belong to the TEF family of transcription factors. VITO-1 was identified as an essential co-factor of TEF1 and activates TEF1 through its SID domain, which results in the transcription of genes involved in muscle cell differentiation. In this thesis, I have analyzed various molecular characteristics of VITO-1 and studied its role in different cell types.

To analyze and compare the subcellular localization in different cell types, VITO-1 was over-expressed in HEK 293, C2C12 and CH10T1/2 cell line. VITO-1 displayed a predominant localization in the nucleus of C2C12 and CH10T1/2 cells whereas a cytoplasmic distribution in HEK 293 cells which might be attributed to the absence of endogenous TEFs in this cell line. Co-expression of TEFs with VITO-1 in HEK 293, C2C12 CH10T1/2 cell lines resulted in a complete translocation of VITO-1 into the nucleus. Expression of VITO-1 deletion mutant constructs in C2C12 cells proved the requirement of SID domain to translocate into the nucleus. In the presence of VITO-1, differentiated C2C12 cells formed large myotubes which supports its role in muscle cell formation. To identify additional binding partners of VITO-1, a yeast two hybrid screen was performed with a skeletal muscle cDNA library using the GAL4 system. From a total of 48 positive clones we focused on Telethonin (T-cap) and Myozenin1 (MYOZ1, FATZ) as they were isolated as independent overlapping clones. Telethonin (T-cap) is one of the most abundant transcripts expressed in striated muscle at the Z-discs. MYOZ1 is also a sarcomeric protein that is known to interact with T-cap. The interactions of T-cap and MYOZ1 clones with VITO-1 were reconfirmed using the Y2H assay. An ONPG

β-galactosidase reporter gene assay revealed that VITO-1 binds TEFs with greater efficiency than T-cap and MYOZ1. A failure of Co-IP using the *in vitro* transcripted / translated proteins to substantiate the interaction between these proteins might be due to the absence of native cellular environment. Since VITO-1 is known to be up regulated in differentiated C2C12 cells and T-cap and MYOZ1 are Z-disc proteins, we used differentiated C2C12 myotubes and chicken primary myocytes for the Co-IP experiments. Indeed VITO-1 containing the SID domain co-immunoprecipitated with T-cap and Myozenin1 in differentiated C2C12 myotubes and primary chicken myocytes thus demonstrating efficient physical interaction. This interaction is mediated through the SID domain as VITO-1 lacking the SID domain did not interact with T-cap and MYOZ1. Immunohistochemistry analysis showed that VITO-1 co-localizes with T-cap in HEK 293 and cos-1 cells. Ectopically expressed VITO-1 was localized both in the nucleus and the Z-discs of neonatal cardiomyocytes. When VITO-1 was co-expressed with T-cap in chicken myocytes, it was localized predominantly at the Z-discs of sarcomeres. A co-expression of VITO-1 with TEF and T-cap in differentiated C2C12 myotubes showed that VITO-1 is distributed both in nucleus as well as cytoplasm. VITO-1 is localized at the nucleus with more intensity where it supposedly interacts with TEF-3 than in the cytoplasm where it might associate with T-cap. This nucleo-cytoplasmic property of VITO-1 needs to be further analyzed. To summarize, the transcriptional co-activator VITO-1 plays a novel role by interacting with the transcriptional machinery (TEFs) as well as the Z-disc (T-cap and MYOZ1) to regulate muscle specific genes.

6. Zusammenfassung

Die Genexpression in der Skeletalmuskulur unterliegt hauptsächlich der gemeinsamen Kontrolle von drei Transkriptionsfaktor-Familien (bHLH, MADs and TEFs. Diese spielen eine wichtige Rolle in der Muskelentwicklung und bei der Expression von muskelspezifischen Genen. Das MCAT-Element (5´-CATTCCT-3´) konnte in herzspezifischen Genen, wie Troponin T und in der schweren kette bes β-Myosins (β-MHC), sowie in Skelett- und Glattmuskelzellen α - Aktin nachgewiesen werden. MCAT bindende Protein gehören zur Familie der TEF-transkriptionsfaktoren. Ein essentieller Ko-faktor von TEF1 ist VITO-1. Die SID-Domäne des VITO-1 interagiert mit TEF1 und ist involviert in die Aktivierung von TEF Target-Genen, die verantwortlich für die muskelspezifische Genregulation sind. In dieser Arbeit sollte VITO-1 charakterisiert werden und die funktionelle Rolle von VITO-1 für das myogene Programm untersucht werden.

Die Beteiligung von VITO-1 an Prozessen der Muskelzellbildung konnte durch die Ausbildung von großen Myotuben aus differenzierten C2C12-Zellen in Anwesenheit von VITO-1 bestätigt werden. Zur Analyse und zum Vergleich der subzellulären Localisation wurde VITO-1 in verschiedenen Zellenlinien überexprimiert. In C2C12- und CH10T1/2-Zellen konnte VITO-1 hauptsächlich im Zellkern nachgewiesen werden. Bei Überexpression in HEK 293-Zellen war VITO-1 jedoch im Zytoplasma localisiert, wahrscheinlich aufgrund der niedrigen Konzentration von endogenen TEFs. Die Co-Expression von TEF3 and VITO-1 in HEK-Zellen, sowie in der Zelllinien C2C12 and CH10T1/2 zeigte eine Translocalisation von VITO-1 im Nucleus. Durch ein in C2C12-Zellen eingebrachtes verkürztes VITO-1 Konstrukt konnte nachgewiesen werden, dass die SID-Domäne fur diese Translocalisation notwendig ist.

Zur Identifizierung weiterer VITO-1 Bindungspartner wurde ein Yeast-two-Hybrid Screen durchgeführt. Insgesamt konnten 48 positive Klone isoliert werden, von denen Telethonin (T-cap) und Myozenin1 (MYOZ1, FATZ) weiter untersucht werden. Die Interaktion der beiden sarkomerischen Proteine Telethonin und MYOZ1 konnte durch die Verwendung eines Y2H-assays bestätigt werden. Durch eine ONPG β – Galactosidase-

Analyse wurde gezeigt, dass VITO-1 effizienter an TEF3 bindent, als an T-cap oder MYOZ1. Die Interaktionen zwischen VITO-1 und T-Cap, sowie T-Cap und MYOZ1 wurden *in vitro* nachgewiesen. Hierfur wurden differenzierte C2C12 Myotuben und primäre Myozyten aus dem Huhn verwendet. Es konnte gezeigt werden, das VITO-1 mit T-cap und Myozenin-1 interagiert. Diese Interaktionen sind abhängig von der SID-Domaene von VITO-1. Immunohistochemische Analysen und konfokale Mikroskopie zeigten eine Co-Localization von VITO-1 mit T-cap in HEK 293- und Cos-1 Zellen. Ektopische experimentes VITO-1 war sowohl im Zellkern, als auch in den Z-Banden von murinen neonatalen Kardiomyozyten lokalisiert. Die Koexpression von VITO-1 und T-cap in Myozyten aus dem Huhn war hauptsächlich in den Z-Banden der Sarkomere zu finden, wobei in differenzierten C2C12 Myotuben VITO-1 sowohl im Zytoplasma, als auch im Nukleus nachgewiesen werden konnte. Zur Aufklarung der genauen Funktion von VITO-1 im Nukleus und im Zytoplasma bedarf es weiterer Studien. Zusammenfassend lasst sich sagen, dass VITO-1 durch die Bindung mit Transkriptionsfaktoren (TEFs) und Z-Scheiben-specifischen Proteinen (T-cap und MYOZ1) eine Rolle bei der Regulation versciedener muskelspezifischer Gene spielt.

7. Abbreviations

3-AT	3-amio 1, 2, 3-triazole
A	Adenine
ADP	adenosine di phosphate
AMP	adenosine mono phosphate
Amp	Ampicillin
ANF	Atrial natriuetic factor
ATCC	American type culture collection
ATP	Adenosine Tri-phosphate
bHLH	Basic helix-loop-helix
BHLH-PAS	Basic helix-loop-per-arnt-sim
BLAST	Basic Local Alignment search Tool
BMP-2	Bone morphogenetic protein 2
Bp	Base pairs
BSA	Bovine Serum Albumin
C	Cytosine
CaCl2	Calcium Chloride
CaM	Calmodulin-like
CAT	Chloramphenicol Acetyl Transferase
cDNA	DNA complementary to mRNA
cMLC2	Cardiac myosin light chain
C-myc	Myc protein implicated in cancer cells
cTNC	Cardiac troponin C

cTNT	Cardiac troponin T
DCM	Dilated cardiomyopathy
DM	Diffentiation medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EM	Electron Microcropy
EM	E-box-M-CAT hybrid motif
ES	Embryonic stem cells
EST	Expressed Sequence Tag
EtOH	Ethanol
FACS	Fluorescence activated cell sorter
FATZ	Filamin, actinin, telethonin binding protein of the Z-discs
FeCl3	Ferric chloride
FGF	Fibroblast growth factor
G	Guanine
GAL4 AD	Gal4 transcription activation domain
GAL4 BD	Gal4 DNA binding domain
GFP	Green Fluorescence Protein
GM	Growth Medium
HA	Hem agglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ig	Immunoglobin

kD	Kilo Dalton
KH2PO4	Potassium dihydrogen phosphate
Lac Z	β – galactosidase reporter gene
LB	Luria Broth
LCR	Locus control region
LGMD	Limb-girdle muscular dystrophy
MAPK	Mitogen Activated Protein Kinase
MEF2	Myocyte Enhancer Factor
MRF	Myogenic Reulatory Factor
mRNA	Messenger RNA
MYOZ1	Myozenin 1
Na2HPO4	Disodium hydrogen phosphate
NaCl	Sodium Chloride
NaVO4	Sodium Orthovanadate
NES	Nuclear Export Signal
NH4Cl	Ammonium chloride
NLS	Nuclear Localization Signal
OD	Optical Density
ORFs	Open reading frame
PMSF	phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RNAP I	RNA polymerase I
rRNA	Ribosomal RNA

SC-H	Synthetic medium without histidine
SD	Snythetic Dropout
SDS	Sodium dodecyl sulphate (SDS)
siRNAs	small interfering RNAs
SMC	Smooth muscle cells
SOC medium	Super Optimal Broth
SUMO	Small Ubiquitous Modifier
T-cap	Titin cap – Telethonin
TE	Tris EDTA
TEF	Transcription Enhancer Factor
TRABS	transcription balancing sequences
tRNA	Transfer RNA
VEGF	Vascular Endothelial Growth Factor
VITO	Vestigial and TONDU related protein
X-GAL	bromo-chloro-indolyl-galactopyranoside
Y2H	Yeast two hybrid
YAP65	Yes Associated Protein 65 kD
β-ME	β - Mercaptoethanol
β-MHC	β – myosin heavy chain

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