VITO proteins are essential new cofactors of the muscle regulatory network

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1.	**Introduction**	**1**
1.1.	**Regulation of transcription in vertebrates**	**1**
1.2.	**Transcription regulation in skeletal and cardiac muscles**	**3**
1.3.	**Transcription Enhancer Factors (TEF) family**	**4**
1.3.1.	**Evolution and structure of TEFs family**	**4**
1.3.2.	**Expression and regulation of Transcription Enhancer Factor members**	**6**
1.3.2.1.	**TEF-1**	**6**
1.3.2.2.	**TEF-3**	**8**
1.3.2.3.	**TEF-4**	**9**
1.3.2.4.	**TEF-5**	**10**
1.3.3.	**Biological function of TEF gene family**	**11**
1.3.3.1.	**Role of TEFs in skeletal muscles**	**13**
1.3.3.2.	**Regulation of transcription in cardiac muscles by TEFs**	**14**
1.3.4.	**Co-activators of TEFs**	**17**
1.3.4.1.	**YAP65 and TAZ as co-activators of TEFs**	**17**
1.3.4.2.	**p160 family as co-activators of TEFs**	**19**
1.3.4.3.	**TONDU as co-activator of TEFs**	**19**
1.4.	**Aim of the studies**	**20**
2.	**Abbreviations**	**21**
3.	**Materials and Methods**	**24**
3.1.	**Materials**	**24**
3.1.1.	**Basic materials**	**24**
3.1.2.	**Chemicals**	**24**
3.1.3.	**Radiochemicals**	**25**
3.1.4.	**Specific Reagents**	**25**
3.1.5.	**Enzymes**	**26**
3.1.6.	**Kits**	**27**
3.1.7.	**Oligonucleotides**	**27**
3.1.7.1.	**Sequencing primers**	**27**
3.1.7.2.	**Primers for RT-PCRs**	**28**
3.1.7.3.	**Cloning primers**	**29**
3.1.7.4.	**Oligonucleotides for RNAi**	**31**
3.1.8.	**Vectors and Plasmids**	**32**

3.1.8.1.	**Plasmids for riboprobes synthesis**	**34**
3.1.9.	**Bacterial strains**	**34**
3.1.10.	**Cell lines**	**35**
3.1.11.	**Antibodies**	**35**
3.1.12.	**Mice strains**	**35**
3.1.13.	**Buffers and solutions**	**36**
3.2.	**Methods**	**37**
3.2.1.	**Standard molecular biology methods**	**37**
3.2.2.	**Cloning strategies**	**37**
3.2.3.	**Expression and diagnostic plasmids**	**37**
3.2.3.1.	**Expression plasmids**	**37**
3.2.3.2.	**Plasmids for riboprobes synthesis**	**39**
3.2.4.	**Identification of the VITO-1 genomic locus – screening of cosmid libraries**	**40**
3.2.5.	**In situ hybridization**	**42**
3.2.5.1.	**Embryos preparation**	**42**
3.2.5.2.	**Tissue preparation for paraffin embedding**	**42**
3.2.5.3.	**Riboprobes synthesis**	**43**
3.2.5.4.	**Whole mount in situ hybridization**	**43**
3.2.5.5.	**In situ hybridization on paraffin embedded tissue slides**	**45**
3.2.5.6.	**Eosin staining**	**46**
3.2.6.	**Cell culture methods**	**46**
3.2.6.1.	**Basic maintenance**	**46**
3.2.6.2.	**Differentiation of C2C12 cells into myotubes**	**47**
3.2.6.3.	**MyoD dependent conversion of fibroblast cell lines**	**48**
3.2.6.4.	**Transient transfection**	**48**
3.2.6.4.1.	**Calcium phosphate**	**48**
3.2.6.4.2.	**Electroporation**	**49**
3.2.6.5.	**Stable cell line generation**	**49**
3.2.6.6.	**Fluorescence Activated Cell Sorting (FACS)**	**50**
3.2.7.	**Immunocytochemistry**	**50**
3.2.7.1.	**Immunoperoxidase detection**	**50**
3.2.7.2.	**Immunocytochemistry with fluorescent labeled secondary antibody**	**51**
3.2.8.	**Total RNA isolation from tissues and cells**	**51**

3.2.9.	**PCR and RT-PCR**	**51**
3.2.10.	**P^{32} random primed labeling probes preparation**	**52**
3.2.11.	**Southern blot analysis**	**52**
3.2.12.	**Northern blot analysis**	**53**
3.2.13.	**Western blot analysis**	**54**
3.2.14.	**Overexpression of VITO-1 and VITO-2 proteins in *E.coli***	**54**
3.2.15.	**CAT assay**	**57**
3.2.16.	**β-galactosidase activity**	**57**
3.2.17.	**Sumoylation assay**	**58**
3.2.18.	**Statistics**	**58**
4.	**Results**	**59**
4.1.	**VITO family of genes as new homologues of vestigial and TONDU proteins**	**59**
4.2.	**Expression pattern of VITO genes family**	**64**
4.2.1.	**VITO-1 is specifically expressed in skeletal muscles**	**64**
4.2.2.	**VITO-2 is not a muscle specific gene**	**69**
4.3.	**Mouse Vgl-4 is ubiquitously expressed in the adult tissues**	**77**
4.4.	**Expression of TEFs and their co-activators is modulated during the course of C2C12 myoblasts differentiation**	**79**
4.5.	**Intracellular localization of VITO1/2 proteins in the various cell lines**	**81**
4.6.	**VITO-1 but not VITO-2 enhances MyoD-mediated myogenic conversion of fibroblasts**	**84**
4.7.	**Knockdown of VITO-1/2 genes by RNAi**	**87**
4.7.1.	**VITO-1 and VITO-2 can be knocked-down efficiently using RNAi**	**88**
4.7.2.	**Attenuation of VITO-1 but not VITO-2 inhibits MyoD mediated conversion of 10T1/2 and 3T3 cells**	**91**
4.7.3.	**Knockdown of VITO-1/2 genes in terminally differentiated C2C12 cell line by RNAi**	**93**
4.8.	**VITO-2 is not a direct transcriptional activator**	**100**
4.9.	**Expression of VITO-1/2 genes in the Myf-5 and delta1 knockout mice**	**102**
4.10.	**Posttranslational modification of VITO family members**	**106**
5.	**Discussion**	**110**
5.1.	**Identification of vestigial and TONDU related co-activators**	**110**

5.2.	**Tissue distribution of the VITO family of genes**	**112**
5.3.	**Nuclear localization of VITO proteins**	**117**
5.4.	**VITO family of genes lack a transactivation domain**	**117**
5.5.	**MyoD mediated conversion of fibroblasts and role of VITO genes**	**118**
5.6.	**VITO family of genes are required for C2C12 myoblasts differentiation**	**121**
5.7.	**VITO genes are differently regulated by the Notch pathway**	**123**
5.8.	**Lack of VITO-1/2 expression in Myf-5 mutant mice**	**124**
5.9.	**VITO-1 but not VITO-2 is target of SUMO modifier**	**125**
6.	**Summary**	**127**
7.	**Zusammenfassung**	**130**
8.	**Appendix**	**133**
8.1.	**Sequences of mouse and human VITO-1/2 genes and their alignments**	**133**
8.2.	**Curriculum Vitae**	**143**
8.3.	**Publications and scientific activity in congresses during PhD studies**	**146**
8.3.1.	**Publications**	**146**
8.3.2.	**Presentation**	**146**
9.	**Acknowledgements**	**147**
10.	**References**	**149**

1. INTRODUCTION

1.1. Regulation of transcription in vertebrates

Much has been learned about the regulation of the eukaryotic genes transcription over the past three decades. Transcriptional regulation is the framework responsible for a cell specification and development of a complex tissues and organs. Basically, transcription is a polymerisation reaction of single nucleotides leading to mRNA. This reaction is catalysed by polymerase I, II or III depending on DNA in the presence of Mg^{2+} or Mn^{2+} ions. Transcription might be divided in three steps: initiation, elongation and termination.

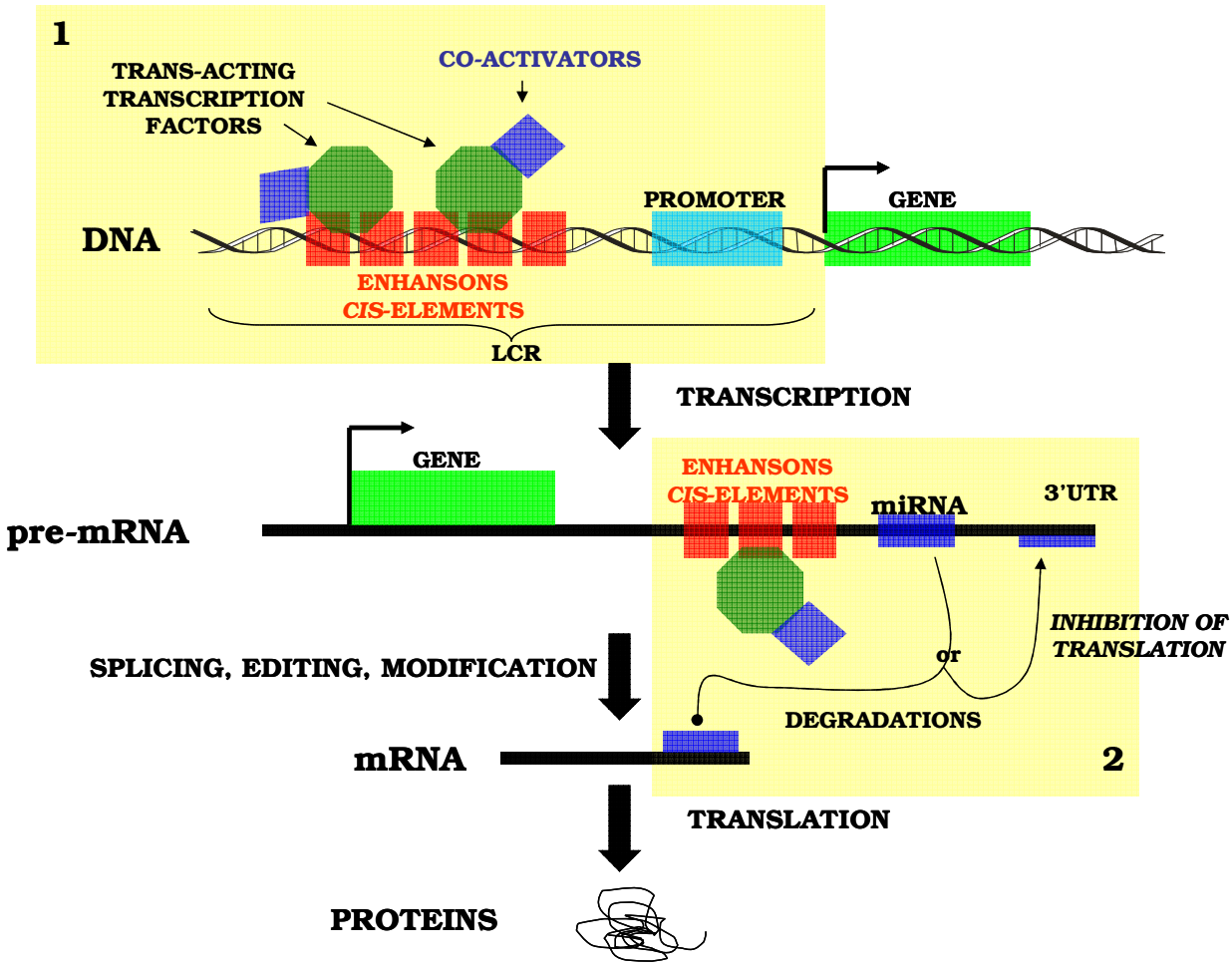

Fig. 1. A schematic drawing of enhancers and their co-activators in the regulation of transcription. Two yellow boxes 1 and 2 show two control steps for protein expression at the transcription level.

From this point of view, transcription seems to be a simple enzymatic reaction. However, the central of transcription requires many specific regulatory proteins, which can interact with DNA via hydrogen and van der Waals bonds in the specific DNA regions, so called promoters. At this point, it is assumed that transcription is regulated by different bounding properties of proteins complexes to specific promoters. Such kind of control of eukaryotic gene expression exists on Locus Control Regions (LCRs) discovered first in the human β-globin locus. LCRs were defined as a *cis*-regulatory elements and their ability to control tissue specific gene expression at physiological levels (reviewed by Li et al. 2002). Extensive studies in various models of organisms revealed a number of factors, which are responsible for the transcription control. Heterogenity of those factors has suggested their specific role as a transcriptional gene expression regulators including enhancers, silencers and has led to hypotheses about detailed mechanism (reviewed by Lemon and Tjian, 2000; Ramji and Foka, 2002). Recently a role of microRNA as *trans*-acting factors that exert their activity by composition of *cis*-regulatory elements has been postulated (Hobert, 2005). It seems that microRNA build a new discovered block (second checkpoint) controlling specific gene expression (Fig. 1.).

Identification of different regulators keeping transcription machinery properly working is a first step to understand how individual genes are turned on or off in cells leading to their specification. It can also give an answer to the question how cells are reprogrammed during differentiation, proliferation and how they can fulfil their specific function in whole organisms. Another topic, which might be solved by a better understanding of the transcriptional regulation, is lineage specification of pluripotent stem cells, which can regenerate adult tissues (Heyworth et al. 2002; Weissman et al. 2001). Recently, transcriptional profiles of embryonic stem cells, neural stem cells and hematopoietic stem cells of the bone morrow origin have been established (Ramalho-Santos et al. 2002), indicating an enormous complexity of regulatory events in different stem cell types.

Tight regulation of the transcription is the major process controlling gene expression networks during embryogenesis in response to physiological and metabolic changes to keep homeostasis. It also governs regenerations processes in adult tissues.

1.2. Transcription regulation in skeletal and cardiac muscles

Transcriptional control in skeletal and cardiac muscles involves a cascade of transcription factors, acting via different *cis*-regulatory elements. Three different major groups of regulators have been described, namely bHLH, MADS and TEF families. All members of those families can bind to the distinct regions of DNA, which are specific and typical for each family. The properties of DNA binding result in the activation of downstream targets genes.

It has been postulated, that skeletal muscles are controlled at the transcriptional level by the Myogenic Regulatory Factors (MRFs) which belong to the bHLH family. MRFs consist of four members: MyoD (Myod1), Myf-5, Myogenin and Myf-6 called also MRF4 or herculin and all of them share a motif of a basic Helix-Loop-Helix domain (bHLH). Myogenic bHLH members bind to the E-box site (CANNTG) within DNA promoter regions to regulate transcription (reviewed by Emerson, 1990; Puri and Sartorelli, 2000; Berkers and Tapscott, 2005). An E-box consensuses have been identified in many muscles specific promoters like: Muscle Creatine Kinase (MCK) (Buskin et al. 1989), cardiac α-actin (Sartorelli et al. 1992), cardiac Troponin T (cTNT) (Iannello et al. 1991), cardiac Myosin Light Chain 2 (cMLC2) (Navankasattusas et al. 1992), β Myosin Heavy Chain (βMyHC) (Thompson et al. 1991; Kariya at al. 1994), α Myosin Heavy Chain (αMyHC) (Gupta et al. 1994), cardiac Troponin C (cTNC) (Parmeck at al. 1992), alpha-tropomyosin (Pasquet et al. 2006) and in skeletal α-actin (MacLellan at al. 1994). It is assumed, that MyoD and Myf-5 play a role in the specification of the muscle cell fate, whereas myogenin and MRF4 regulate the muscle differentiation program (reviewed by Sabourin and Rudnicki, 2000; Buckingham, 2001). In addition two other subfamilies of bHLH have been identified: bHLH lucine zipper, which consist Myc/Max/Mad transcriptional factors (Luscher, 2001) and the bHLH-PAS subfamily (Crews, 1998). MRFs and bHLH leucine zipper subfamilies recognise typical E-box whereas bHLH-PAS factors bind a DNA sequences which are distinct from the prototypical E-box (Luscher, 2001).

MADS box transcription factors (MCM1-a yeast homolog, Agamous, Deficiens,- plants homolog, Serum response factor) is the second major transcription network which govern regulation of skeletal and cardiac muscles (Molketkin et al. 1995; Black and Olson 1998). It is also called myocyte enhancer factor-2 (MEF2). In vertebrates, all known four members MEF2A, MEF2B, MEF2C and MEF2D posses a

highly conserved 56 amino acids motif – so called MADS box. The MADS box is responsible for the dimerisation of proteins containing this motif to create active homodimmers as well as for specific binding to DNA A/T rich elements (Gossett et al. 1989). MEF2A, MEF2B and MEF2D are ubiquitously expressed in adult tissues, while expression of MEF2C is enriched in the spleen and brain as well as in the skeletal and cardiac muscles (Pollock et al. 1991; Yu et al. 1992; Martin et al. 1993; McDermott et al. 1993; Breitbart et al. 1993). The expression pattern of MEF2 members has been also described during mouse embryonic development. MEF2C was detected first at E7.5 in the part of mesoderm, which forms the primitive heart tube. All others MEF2s start to be expressed in the myocardium at E8.5. The earliest expression of MEF2C in the embryonic skeletal muscle was detected at E9.0 in the rostral myotome while MEF2A and MEF2D were found half a day later in the myotome (Edmondson et al. 1994). Another member of MADS box proteins is Serum Response Factor (SRF), which regulates transcription in cardiac, skeletal and smooth muscle cells by binding to the CArG box found in several promoter regions (Norman et al. 1988; Miano, 2003). SRF is the first known trans-acting factor, which regulates also muscle specific microRNA by binding to *cis*-elements in the regulatory region of microRNA. Overexpression of SRF can regulate specific microRNAs, which target the HAND2 transcription factor during mouse development (Zhao et al. 2005).

1.3. Transcription Enhancer Factors (TEF) family

1.3.1. Evolution and structure of TEFs family

Transcription Enhancer Factor (TEF) is the last main family of transcription regulators found in skeletal and cardiac muscles as well as in non muscles cells. All four members of the TEF family (TEF-1, TEF-3, TEF-4, TEF-5) have very high homology in so called TEA/ATTS DNA Binding Domain (DBD), (for nomenclature of TEFs see Tab. 1.)

TEFs family	Synonyms	References
TEF-1	mTEAD-1 N-TEF-1	Xiao et al. 1991 Azakie et al. 1996
TEF-3	FR19 TEFR1 ETFR2 RTEF-1 mTEAD-4	Hsu et al. 1996 Yockey et al. 1996 Yasunami et al. 1996 Stweart et al. 1996
TEF-4	ETF mTEAD-2	Yasunami et al. 1995 Keneko et al.
TEF-5	DTEF-1 ETFR-1 mTEAD-3	Azakie et al. 1996 Yasunami et al. 1996

Tab. 1. Nomenclature of TEFs family members. In the work presented here names listed in the first column will be used.

The name of TEA domain originates from other known homologous, like: yeast TEC1 in *Saccharomyces cerevisiae* involved in the transcriptional activation of the transposon Ty1 element (Laloux et al. 1990) and AbaA in *Aspergillus nidulans,* which can regulate development of the asexual spores (Mirabito et al. 1989). Additionally, maize Golden 2 (g2) gene has been also identified as a homolog of TEFs (Hall et al. 1998). The last known homolog of TEFs family called scalloped was found in *Drosophila melanogaster* (Campbell et al. 1992). The TEA DNA binding domain is the region with the remarkable degree of conservation between yeast and human's (Fig. 2) (Adrianopoulos et al. 1991; Burglin, 1991; Jacquemin et al. 1996)**.** It is located at the N-terminal moiety of TEFs proteins and consists of three α- helices or one α- helix and two β-sheets responsible for their binding to DNA. However, only helix1 and 3 have been recognized as important for interaction with DNA (Hwang et al. 1993). TEFs can bind via this domain to the specific enhansons i.e. M-CAT (5'CATTCCT3'), GT-CII (5'CATTCCA3`) and SpH I and II (5'CATACCT3') motifs to regulate expression of target genes. Comparison of DNA- binding sites suggest that TEFs bind to consensus sequence 5`- (A/T) (A/G) (A/G) (A/T) ATG (C/T) (G/A) - 3` with the core sequence ATG. Biochemical studies have been done to compare the role of flanking regions of different promoters possessing M-CAT motifs. On the other hand, it has been also postulated that TEF-1 has different binding affinity to GT-IIC (GGAATG (67,3%) followed by M-CAT (12%) and SphI (4%) sites (Jiang et al. 2000). Different models were also proposed suggesting that the transcription regulation is mediated via the

binding of others transcription intermediary factors (co-factors) to the flanking sequence of M-CAT elements (Larkin et al. 1996).

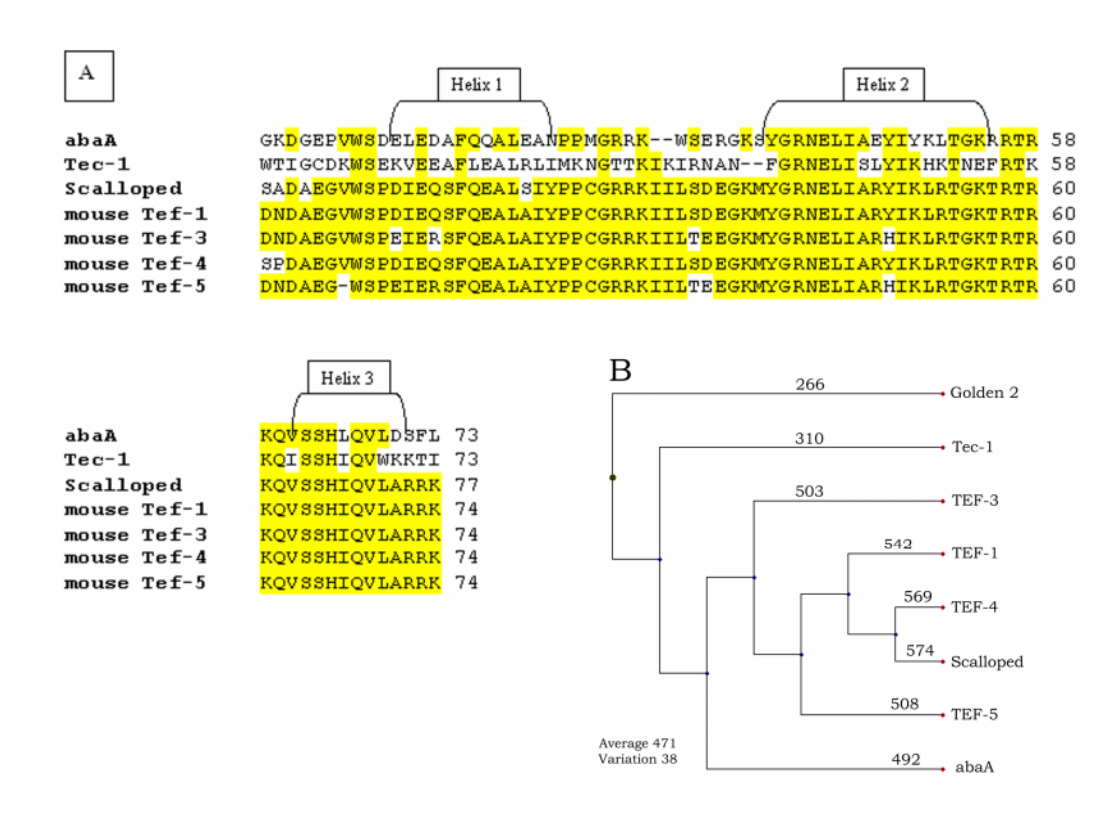

Fig. 2. Alignment of amino acids sequences in the conserved TEA DNA Binding Domain between all homologs of TEFs members from different species (panel A) and phylogenetic relationship among all identified TEFs factors (panel B).

1.3.2. Expression and regulation of Transcription Enhancer Factor members

1.3.2.1. TEF-1

One of the most extensively studied members of TEFs family is TEF-1, which has been identified in HeLa cells by its binding properties to the GT-CII, SpH I and II enhansons of the simian virus 40 (SV40) enhancer (Xiao et al. 1987; Davidson et al. 1988; Xiao et al. 1991). TEF-1 was also isolated independently from various cDNA libraries (Blatt et al. 1993; Melin et al. 1993; Shimizu et al. 1993). It is expressed ubiquitously in different cell lines (Tab. 2) and during murine development. During mouse embryogenesis TEF-1 mRNA was detected in oocytes followed by the absence

of its transcripts up to E8.0 (Kaneko et al. 1998). On the other hand, continues β-galactosidase activity in the TEF-$1^{+/-}$ embryo was observed from the oocytes stage up to later stages of mouse development (Chen et al. 1994). Another study, based on whole mount in situ hybridization revealed TEF-1 transcripts in decidual cells and in the distinct extra-embryonic regions e.g. in the ectoplacental cone at E6.5 (pregastrula or egg cylinder stage) (Jacquemin et al. 1996; Jacquemin et al. 1998). From E8.5, TEF-1 is detected in the entire embryo and between E9.5 and E10.5 it shows restricted expression in specific structures such as: the ventricular layer of the neuroepithelium, the spinal cord in the developing brain and the myocardium at E10.5. At later stages (E13.5 - E18.5) TEF-1 mRNA was found in various facial and axial muscles, in the differentiating myocardium, in the forebrain ventricles as well as in the olfactory and respiratory regions of nasal epithelium. Additionally, TEF-1 was uniformly detected in the metanephros of the developing kidney and adrenal gland. Pronounced expression was observed in the internal layer of the urinary bladder epithelium and in the external layer of the mesenchyme. Furthermore, TEF-1 is localized in the labyrinthine of the chorioallantoic placenta (Jacquemin et al. 1996; Jacquemin et al. 1998). However, there is no clear evidence that TEF-1 is expressed in somites during embryogenesis. In adult tissues TEF-1 is detectable at high level in the: kidney, lungs, skeletal muscles and heart and at low level in the brain and liver (Shimizu et al. 1993). Another report showed a ubiquitous TEF-1 distribution by β-galactosidase staining of TEF-$1^{+/-}$ heterozygous mice carrying LacZ cassette (Chen et al. 1994). As TEF-1 was identified in the developing myocardium during mouse embryogenesis and its transcripts are enriched in adult heart, TEF-1 was used in many reports as a specific marker of adult cardiomyocytes. In contrast, TEF-1 was also found in a subpopulation of the Sca-1^{+} and Sca-1^{-} non-cardiomyocytes isolated from the adult heart. In this respect TEF-1 can not be described as a specific marker of cardiomyocytes (Oh et al. 2003).

Cell line	**TEF-1**	**TEF-3**	**TEF-4**
Cervical carcinoma Hela	+	+	nd
Neuroblastoma IMR-32	+	+	nd
Ovary adenocarcinoma OVCAR-3	+	+	+
Molt4 T cell leukemia	+	+	nd
Embryonic insteine 407	+	+	+

Choriocarcinoma JEG-3	+	+	nd
Colon adenocarcinoma CaCo-2	+	+	nd
Embryonic kidney 293T	+	+	nd
Myoblastoma C2C12	+	+	+
Sol8	+	+	nd
3T3	+	+	+
Carcinoma F9, F19	nd	+	+
Hep-G2	-	+	nd

Tab. 2. Expression of TEF-1/3/4 in different cell lines (Xiao et al. 1987; Jacquemin et al. 1996; Shimizu et al. 1993; Yasunami et al. 1996; Hsu et al. 1996), nd – data not found.

1.3.2.2. TEF-3

The next member of TEFs family - TEF-3 has been identified parallel by different groups and therefore has different names: FR19 (Hsu et al. 1996), TEFR1 (Yockey et al. 1996) and ETFR2 (Yasunami et al. 1996). Extensive studies generated comprehensive information concerning the expression profile of this gene. The presence of TEF-3 transcript was noted in several cell lines (Tab. 2). During embryonic mouse development TEF-3 starts to be expressed at E6.5 in the maternal deciduas and in the extra-embryonic layers, transcripts were also detectable at high level in the deciduas at E8.5. In contrast to TEF-1, TEF-3 was slightly detected in conceptus. At later stages, TEF-3 signals were found in the myotome and in the cranio-caudal progression from cervical levels to trunk and caudal levels (from E9.5 to E10.5). Later on (E11.5 – E12.5), TEF-3 labeled skeletal muscles precursors including head, body wall and limb muscles. During late developmental stages (E13.5 –E18.5), TEF-3 was localized in the neck, shoulder, and hind limb muscles as well as in the liver, lung, salivary gland, nasal gland epithelia and presumably in the duodenal region. This gene has been never detected in the developing myocardium (Jacquemin et al. 1996; Jacquemin et al. 1998). In adult tissues TEF-3 expression is restricted only to the lungs, heart and skeletal muscles (Yockey et al. 1996). However, Yasunami et al. showed also an expression of TEF-3 in the brain and kidney (Yasunami et al. 1996).

It has been also noted, that TEF-3 is induced by fibroblast growth factor (FGF-1) in NIH 3T3 fibroblast cell line. Transcripts were detected 4 hours after FGF-1 addition, which was dependent on *de novo* RNA and proteins synthesis. Furthermore FGF-2, TGF-β1 (Transforming Growth Factor), EGF (Epidermal Growth Factor), PMA (Phorbol 12-myristate 13-acetate), PDGF-BB (Platelet-derived growth factor) up-regulate TEF-3 expression levels in this cell line. It also appeared that TEF-3 can be expressed as two isoforms in 3T3 cell line treated with FGF-1 (Hsu et al. 1996).

1.3.2.3. TEF-4

Another member of TEFs family, TEF-4 was first described as a neural transcriptional factor in neuronal precursor cells cDNA library and named ETF (Yasunami et al. 1995). TEF-4 transcripts were found in various cell lines (Tab. 1.2.). During mouse development TEF-4 is the first expressed member of TEFs family genes. TEF-4 transcripts were detected during early stages of the zygote development, from the cleavage stage of preimplantation embryos up to day E6.5 (Keneko et al. 1997; Keneko et al. 1998). Expression of TEF-4 at E6.5 was observed in the entire conceptus up to E8.5. In the mid-gestational stages TEF-4 expression was similar to the TEF-1, labeling the ventricular layer of the neuroepithelium (brain and spinal cord). At later stages both TEF-1 and TEF-4 are expressed in the same regions (ventricular zone of CNS and lungs). TEF-4 mRNA was found in the olfactory epithelium, the cortical region corresponding to the nephrogenic zone as well as in the mesynchyme of the intestinal loops, entire bladder mesynchyme and epithelium (Kaneko et al. 1997; Jacquemin et al. 1996; Jacquemin et al. 1998). At stage E10.5, TEF-4 transcripts were visualized in the hindbrain (the highest expression) as well as at low level in the distal portions of the forelimb and the hind limb buds (Yasunami et al. 1995). In adult tissues TEF-4 transcripts are ubiquitously distributed (Kaneko et al. 1997).

Interestingly, the genomic loci of TEF-4 and soggy (Sgy) are closely linked. The TEF-4 gene is connected to Sgy via CpG islands in a bidirectional manner. The Sgy locus (5 exons within a 4,6 kb region) is located 3.8 kb upstream of the TEF-4 start site (12 exons within a 17,9 kb region) (Suzuki et al. 1996; Keneko et al. 2000). As consequence of bidirectional loci of TEF-4 and Sgy, their differential expression in various tested cell lines was observed. For example cell lines such as: lung carcinomas

LL/2 and LLC, colon carcinomas CA51 and MC38, melanoma B16, which expressed TEF-4 were negative for the Sgy mRNA. Vice versa, in spermacytocytes, mastoma P815, MPC-11, TEF-4 was not detectable whereas expression of Sgy was observed (Keneko et al. 2004). Another example of a mutual expression pattern of TEF-4 and Sgy was detected during differentiation of ES cells into embryonic bodies. Two days after differentiation, Sgy expression was repressed while TEF-4 transcripts were up-regulated. DNA methylations, which govern differential gene expression at bidirectional loci, have been postulated as a presumptive mechanism responsible for regulation of TEF-4 and Sgy (Keneko et al. 2004). On the other hand, Tanoue and colleagues in 2001 identified a cell specific 117-bp enhancer in the first intron of the TEF-4 locus and proposed that the GC boxes included in this enhancer direct TEF-4 expression (Tanoue et al. 2001). Recently it was also postulated that TEF-4 can be regulated by MyoD binding to the two E-box enhansons within the first intron of TEF-4 (Zhao et al. 2006)

1.3.2.4. TEF-5

TEF-5 is the last member of TEF multigene family and has been discovered parallel by different groups as DTEF-1 (Azakie et al. 1996), ETFR1 (Yasunami et al. 1996) and TEF-5 (Jacquemin et al. 1997). In the literature, there are no data describing an expression of TEF-5 in cell lines. During mouse embryogenesis TEF-5 mRNA was detected at the oocyte stage (Kaneko et al. 1998). TEF-5 transcripts were also found in the extraembryonic tissues such as the extraembryonic ectoderm of the chorion, the ectoplacental cone and the primary giant cells at stages E6.5 – E7.5. From mid to late gestation TEF-5 expression appears in the ventricular layers of the central nervous system (E9.5 – E10.5) and in the epithelia of the mouth cavity, the pharynx, and the nasal cavities (E13.5 – E17.5). At late gestation TEF-5 was also noted in the epithelial component of the tooth buds, in all compartments of the ear, in the epithelial and mesenchymal components of the oesophagus and wall of the urinary bladder, the stomach and intestine as well as in the bronchial epithelium. TEF-5 was also found in the skin epidermis and the hair follicle epithelia (Jacquemin et al. 1998). Quite controversial is the presence of TEF-5 in the developing heart. Jacquemin showed rather weak expression with restriction to the wall of aorta and intestine (Jacquemin et al. 1998). Another group was able to detect TEF-5 in the atria and ventricles. In addition

they also noted TEF-5 mRNA in the ectoderm of forelimb and hindlimb, the hyoid arch and mandibular and frontonasal structures at stages E9.5 – E10.5. However, somites were not labeled by TEF-5 transcipts (Brunskill et al. 2001). Moreover, in chicken embryos TEF-5 expression was shown in the sinus venosus and in the trabeculated ventricular myocardium and ventricular outflow tract (Azakie et al. 2005). Taking together, it seems that TEF-5 (in addition to TEF-1) is involved in the early heart development and might play a crucial role during cardiogenesis. In adult tissues, it has been indicated, that TEF-5 is also ubiquitously expressed with the highest expression levels in skeletal muscles, heart and lungs (Azakie et al. 1996; Yasunami et al. 1996).

TEF-5 locus is composed of 13 exons with division of TEA domain in three different exons (III, IVA and IVB exons) and is located close to the Fkbp5 locus on chromosome 17 (Jacquemin et al. 1999).

1.3.3. Biological function of TEF gene family

Despite numerous reports, which describe the expression of the TEF multigene family, the biological function of TEFs remains unclear. Most of TEFs seem to be ubiquitously expressed with exception of TEF-3. Additionally, enhansons to, which they can bind, are distributed in promoters of different genes in many tissues. TEF members can play important functions during embryogenesis and also in adult tissues in response to many different stimuli. Specific enhansons for TEFs as well as MEF2 and bHLH families have been detect in the promoters of the same genes, what might suggest potential cooperation between members of those families in a control of transcription.

So far, the most studied member of TEFs was TEF-1 and many groups used this gene as an example for the whole family. As it has been already mentioned, TEF-1 was identified by its binding properties to the GT-IIC and Sph enhansons of the SV40 enhancer (Widelman et al. 1986; Xiao et al. 1987; Davidson et al. 1988) and TEF-1 can control its activity at the early promoter (Nomiyama et al. 1987). It also has a function role in the transactivation of the SV40 late promoter by interaction with the large tumor antigen T (TAg is responsible for transcription regulation of the viral genes). (Cesaz et al. 1991; Berger et al. 1996). Another set of experiments showed that overexpression of TEF-1 in HeLa cells does not activate transcription based on TEF binding sites above the background level, but rather repressed this activity (Xiao et al. 1991; Hwang et al.

1993). However, it has been demonstrated, that TEF-1 stimulates transcription efficiently in HeLa cells than in lymphoid BJA-B cells (Chaudhary et al. 1994). This finding was explained by the presence of the negative enhancer factor-1 (NEF-1) and NEF-2 in the BJA-B and HeLa cell lines respectively (Chaudhary et al. 1995). The repression of TEF-1 activity was explained by competition for co-activators or so called transcription intermediate factors (TIF), a phenomena known as a squelching (Ptashne, 1988). Additionally it was shown that single-stranded DNA binding proteins like Purα, Purβ and MSY1 might mask M-CATs elements to prevent its binding to TEF-1 and eliminate the transcriptional activity (Carlini et al. 2002).

TEF-1 binds to multiple sites of the human papillomavirus type 16 E6/E7 and it is selectively active in keratinocytes (Ishiji et al. 1992). MCBF (MCAT Binding Factors) family members have also been shown to regulate the mouse mammary tumor virus (MMTV) (Henrard et al. 1988). The potential TEFs binding sites were found in the long terminal repeat (LTR) of MMTV (Maeda et al. 2002). TEF-1 was able to squelch its basal activity and abrogate its response to the glucocorticoid dexamethasone while TEF-3 and TEF-5 had no effect. These results suggested a role of TEFs in mammary tumorgenesis (Maeda et al. 2002). In addition, it has been found, that overexpression of TEF-1 in BeWo cells inhibited the basal activity of the human chorionic somatomammotropin promoter (Jiang et al. 1995). It is believed, that this repression is mediated through direct interaction of TEF-1 with TATA-binding Protein (TBP) (Jiang et al. 1996). So far, only two reports revealed potential role of TEF-5: as an activator of the human chorionic somatomammotropin-B at the so called DF-3 element (Jacquemin et al. 1997) and activation of the human 3β-hydroksysteroid dehydrogenase gene (HSD3B1) (Peng et al. 2004).

TEF-binding sites were found in many muscles and non muscles genes promoter regions. Genes such as: cardiac Torponin cTNT (Mar et al. 1988a; Mar et al. 1988b; Mahr and Ordahl, 1990), βMyHC (Flink et al. 1992; Thompson et al.1991), αMyHC (Gupta et al. 1994; Molkentin et. al. 1994), MLC2 (Qasba et al. 1992), s/cTNC (Parmacek and Leiden, 1988), vascular smooth muscles α-actin (Karns et al. 1995), AChrβ (Berberich et al. 1993), c-mos (Lenormand et al. 1995), Foxa2 (Sawada et al. 2005), Fgf4 (Zhao et al. 2006) all contain binding sites for TEFs. In addition, enhansons to which TEFs can bind were also noted in the keratinocyte specific human papilloma virus-16 E6/E7 (Davidson et al. 1988; Ishiji et al. 1992) and in human chorionic

somatomammotropin gene also called placental lactogen–B gene (Jacquemin, et al. 1994a; Jacquemin, et al. 1994b).

Since *cis*-acting elements that bind TEFs were found in many different promoters, various studies tried to establish a link between TEFs, MEF2 and bHLH expression and postulated a cooperative binding of these proteins. Attempts were made to identify potential interaction partners, jointly used by these proteins. Finally, several groups screened for co-activators involved in gene specific regulation especially in skeletal and cardiac muscles.

1.3.3.1. Role of TEFs in skeletal muscles

Since β-MyHC is expressed in adult slow skeletal muscles and in the ventricular myocardium (Lompre et al. 1984), a large numbers of studies tried to identify elements, which drive its differential expression. Another MyHC isoform, α-MyHC is expressed in the atria and weakly in ventricular fibers (Bouvagnet et al. 1984; Gorza et al. 1984). A detailed molecular analysis of the β-MyHC proximal promoter located several *cis*-acting elements - distal and proximal M-CATs, A/T rich region and E-box site (Knotts et al. 1994; Thomson et al. 1991). The ability of TEF-1 as well as other members of TEFs family to bind these elements was proved (Flink et al. 1992; Shimizu et al. 1993; Farrance et al. 1996). It has also been shown that β-MyHC can be induced in fast type fibers by mechanical overload (MOV). Distal M-CAT elements contributed to the basal expression of β-MyHC in the slow fiber by binding of a multiproteins complex consisting of TEF-1, poly(ADP-ribose) polymerase (PARP) and Max bHLH proteins. Furthermore, it was noted, that only TEF-1 can bind to the distal M-CAT element and can physically interact with PARP and Max to form a functional complex. However, such kind of regulation is not absolutely required for MOV responsiveness (Wiedenman et al. 1996; Vyas et al. 1999; Vyas et al. 2001). The β-MyHC promoter region contains also an additional enhanson A/T rich element that is necessary for its expression in slow type muscles, which enhances MOV responsiveness. Surprisingly, it has been described, that this element (which otherwise bind exclusively GATA and MEF2 transcription factors) binds also TEF-1 under the basal as well hypertophic conditions. That means that TEFs family might contribute to the regulation of transcription in cardiac and skeletal muscles by binding to *cis*-regulatory elements that differ from the MCAT motif (Tsika et al. 2002; Karasseva et al. 2003). This finding also contradict data

that TEF-1 and MEF2 physically interact to regulate specific gene expression (Maeda et al. 2002). Another study published by Giger showed, that TEF-1 and Myogenin proteins were significantly attenuated in the unloaded Soleus muscle in comparison to control, which correspond to the transition of β-MyHC gene (Giger et al. 2004).

1.3.3.2. Regulation of transcription in cardiac muscles by TEFs

Since TEF-binding *cis*-elements are present in the enhansons of various structural genes, they might also control transcription regulation in cardiac muscle cells. Several groups described TEF-dependent gene regulation of cardiac myocytes, and it seems that the cardiac transcription machinery is based on the direct interaction or competition between MEF2, TEFs families and their co-activators. One of many examples is the identification of *cis*-elements in the promoter region of αMyHC. Many regulatory elements have been found within the promoter sequence such as: an element specific for the erg-1 cellular oncogene, a thyroid regulatory element (TRE) by which αMyHC can be regulated (Markham et al. 1990; Tsika et al. 1990) and an E-Box-M-CAT (EM) hybrid motif (Gupta et al. 1991). The EM regulatory element is responsible for the basal as well as cyclic AMP-inducible expression of the αMyHC gene. It has been reported that TEF-1 and Max protein, which belongs to the basic helix-loop-helix leucine zipper (bHLH LZ) subfamily, can trans-activate the αMyHC gene through their binding to the EM motif. This effect seems to occur due to the synergistic cooperation between both factors (Molkentin et al. 1994; Gupta et al. 1997). Moreover, TEF-1 can interact with SRF (MADS box family) to activate the skeletal α-actin promoter in COS-1 cells. The strong interaction between both proteins is mediated through the C-terminal subdomain of the MADS box of SRF (204-224 amino acids) and the second and the third α-helix of TEAD DBD domain of TEF-1. However, no data are available whether other members of TEFs family can interact with SRF by the same structural elements (Gupta et al. 2001). It was postulated, that the skeletal α-actin promoter can be regulated by TEFs family members and SRF in response to stimuli like pressure overload, α_1-adrenergic agonists, FGF and TGF-β or interleukin-1β (MacLellan et al. 1994; Karns et al. 1995; Patten et al. 1996), underscoring a potential role of TEFs in the regulation of tissue homeostasis.

M-CAT elements together with the TATA box, GC box in the proximal promoter region as well as with A/T-rich MEF2 and GATA elements do also control the cardiac troponin T activity (cTNT) (Mar et al. 1988; Mar et al. 1990; Iannello et al. 1991). The cTNT gene is expressed in cardiac and skeletal muscles during development but at late embryonic stages is repressed in developing skeletal muscles with strong up-regulation in cardiac tissue (Long et al. 1988). It has been noted that in cultured myocytes, TEF-3 is not able to activate the cTNT promoter (Stewart et al. 1994). In contrast to this observation TEF-5 can trans-activate cTNT promoter in a tissue specific fashion, independently on A/T-rich, MEF2 or GATA elements. In contrast TEF-5 did not activate the cTNT gene in transiently transfected embryonic skeletal muscles and fibroblasts (Azakie et al. 2005).

Another gene that can be possibly regulated by TEFs is the muscle creatine kinase (MCK). MCK is expressed in skeletal and cardiac muscles. Its promoter has different enhansons and following elements were identified CArg, AP2, A/T-rich, right and left E-boxes, MEF-2 sites as well as transcriptional regulatory element x (TREX) - similar to the M-CAT (7/8 bases) (Buskin et al. 1989; Amacher et al. 1993; Fabre-Suver et al. 1996). Due to MCK expression in both skeletal and cardiac muscles, the obvious question was which elements were responsible for the heart and skeletal muscle expression. Using sites specific mutation analysis it was shown that the right E-box and TREX are necessary elements to regulate MCK expression in skeletal muscles, while CArG and A/T-rich elements were responsible for the expression in the heart. Other elements such as the AP2, MEF-2 sites and the left E-box are necessary in both skeletal and cardiac muscles. Moreover, it was shown that the TrexBF factor, which binds to the TREX element, is specific for skeletal muscle but distinct from TEF-1 (Amacher et al. 1993; Fabre-Suver et al. 1996).

In the β-myosin heavy chain promoter four M-CATs (additional two in comparison to skeletal muscle) and GATA as well as NFAT binding elements were identified (Simpson et al. 1991; McLean et al. 2003). Site direct mutagenesis showed that one M-CAT is responsible for the basal activity of the promoter. Mutation of a second M-CAT motif revealed decreasing activity of this promoter but only in response to the α_1-adrenergic agonist while mutation in both M-CATs generally decreased basal activity of the promoter in response to pressure overload and leukaemia inhibitory factor (Morimoto et al. 1999; Hasegawa et al. 1997). Moreover, a GATA binding element within the βMyHC promoter seems to play a role in the transcriptional

activation of this gene in response to the aortic constriction (Hasegawa et al. 1997). An additional region between -71/+34 containing no consensus element and can also mediate a pressure overload response (Wright et al. 2001). It was postulated that all four M-CAT elements (by binding TEF factors) maintain the basal activity of βMyHC in slow type muscles and the heart. NFAT sites within the βMyHC promoter can bind NFAT factors *in vitro* but are not required for its basal activity *in vivo* (McLean et al. 2003).

Another studies showed that the α1A/C –adrenergic receptor promoter has multiple M-CATs with binding properties for TEF-1, but only one is required for its activity in cardiomyocytes and in response to β-adrenergic agonists' (O'Connell et al. 2001). Another study presented that one from two M-CATs is responsible for activity of vascular smooth muscle α-actin promoter (Swartz et al. 1998). The cardiac ankkyrin repeat protein is another example of a gene under control of TEF transcription factors, since MCATs were found within its promoter (Aihara et al. 2000). Biochemical data supported the notion that MCATs binding sites (TEFs) are controlled by p38 and Rac1 –components of the stress activated MAPK pathway (Aihara et al. 2000; Ambrosino et al. 2006). Additional, data revealed that both TEF-3 (activates skeletal muscle α-actin and βMyHC) and TEF-5 (activates skeletal muscle α-actin) are targets for the α_1-adrenergic signalling pathway (Stewart et al. 1998; Ueyama et al. 2000; Maeda et al. 2002). TEF-3 has been also recognized as a positive regulator of the VEGF promoter region (Vascular Endothelial Growth Factor) (Shie et al. 2004).

Despite of numerous studies, which tried to uncover functions of TEFs *in vitro* in cell culture, only a little attempt was made to address the function of TEFs *in vivo*. TEF-1 was disrupted by a retroviral gene trap insertion. TEF-1 mutant embryos die between E10.5 and E11.5. The earliest phenotype appeared at E10.5, indicated by a pale yolk sac and a dilated fourth ventricle in the brain. Later on, TEF-1 deficient embryos showed a dilated heart, a slow heart beating rate as well as tissue edema. The thin ventricular wall was accompanied by a reduced trabeculation. Hence, TEF-1 seems to play an important role in the maturation of the embryonic heart but not during initiation of the heart development. Surprisingly, no changes were reported in other tissues or organs. According to the biochemical data, which suggest a stimulatory role of TEF-1 in the expression of several sarcomeric genes, no significant changes were noted in cTNT, cTNI and myosin expression (Chen et al. 1994). Similarly to TEF-1 knock out mice, SRF null mice also did not show any changes in the expression of βMyHC, cardiac

actin and slight changes in desmin, αMyHC and ANF expression was observed. Surprisingly, mutant embryos lacking SRF showed a lower expression of transcription factors like TEF-1, Nkx2.5, GATA4 as well as myocardin – the co-activator of SRF (Parlakian et al. 2004). In contrast, another group showed that mice deficient in SRF are characterized by a dramatic reduction in the expression of ANF, skeletal, cardiac and smooth muscle α-actins as well as SM22α transcripts (Niu et al. 2005). In addition, transgenic mice overexpressing TEF-3 develop progressive atria arrhythmias with slower conduction velocities across the atria and the ventricular myocardium. The conduction defect was concluded due to the dephosphorylation of connexins 40 and 43 with parallel up-regulation of the protein phosphatase 1β (Chen et al. 2004).

1.3.4. Co-activators of TEFs

Despite of many published data, the question remained open, how TEFs specifically regulate transcription. So far, the main explanation of the mode of action was based on the potential role of co-activators, which might modulate TEFs function, acting as their positive or negative regulators. Such co-factors might bind directly to TEFs family or to other proteins that might interact with TEFs (Majumder et al. 1997; Roeder, 2005; McKenna et al. 2002).

1.3.4.1. YAP65 and TAZ as co-activators of TEFs

A potential and rather unexpected co-activator of TEFs is YAP65 (Yes Associated Protein 65 kDa). YAP65 has been described previously as an interaction partner of the Src/Yes protein kinase family (Sudol et al. 1994) and of the PDZ domain protein EBP50 (Mohler et al. 1999). YAP65 has been identified as a potent trans-activator comparable to herpes simplex virus VP16 (Yagi et al. 1999). It was observed, that this co-activator can interact specifically with the carboxyl region of all members of TEFs family by a newly identified TEAD protein binding domain in YAP65 (aa 32-139) (Vassilev et al. 2001). The TEAD protein binding domain of YAP65 protein is localized at the N-terminal end before 14-3-3 binding domain. The protein does also contain two WW domains (called also RSp5) followed by proline rich motif (SH3-

binding motif), and activation domain (Yagi et al. 1999; Kanai et al. 2000; Vassilev et al. 2001). Interestingly, an overexpression of YAP65 strongly enhanced activity of TEFs family in the lyphocytic MPC11 cell line, in which TEFs normally are not active. Moreover, TEFs overexpression squelched YAP activity. YAP65 appeared to be accumulated in the cytoplasm as a complex with cytoplasmatic localised protein called 14-3-3 whereas all TEFs members are located in the nucleus. Hence, it was postulated that YAP65 might control TEFs specific activation of target genes in response to the unknown stimuli (Vassilev et al. 2001). YAP65 seems to be a specific co-activator of TEF-4. Both bind to enhansons in the Pax-3 promoter region, so called NCE2 resembling GT-IIC element and this complex is able to activate Pax-3 expression in neural crest cells (Milewski et al. 2003).

TAZ (Transcription co-activator with PDZ motif) shares homology with the YAP65 protein and can bind to 14-3-3 proteins, similarly to YAP65 (Kanai et al. 2000) (Fig. 3). TAZ has been shown to act as a potential co-activator of the core-binding factor 1 (CBFA1) and is involved in the regulation of osteoblast differentiation (Cui et al. 2003). Recently, a pivotal role of the TAZ protein as a co-activator of the Runx-2 dependent gene transcription has been demonstrated.

```
mouse TAZ          MNPSSVPHPLPPP----------------------GQQVIHVTQDLDTDLEALFNSVMNP 38
mouseYAP65         MEPAQQPPPQPAPQGPAPPSVSPAGTPAAPPAPPAGHQVVHVRGDSETDLEALFNAVMNP 60

mouse TAZ          KPS------SWRKKILPESFFKEPDSGSHSRQSSTDSSGGHPGPRLAGGAQHVRSHSSPA 92
mouseYAP65         KTANVPQTVPMRLRKLPDSFFKPPEPKSHSRQASTD--AGTAG---ALTPQHVRAHSSPA 115

mouse TAZ          SLQLG-------TGAGAAGGPAQQHA--HLRQQSYDVTDELPLPPGWEMTFTATGQRYFL 143
mouseYAP65         SLQLGAVSPGTLTASGVVSGPAAAPAAQHLRQSSFEIPDDVPLPAGWEMAKTSSGQRYFL 175

mouse TAZ          NHIEKITTWQDPRKVMNQPLNHVN-LHPSITSTSVPQRS--------MAVSQPNLAMNHQ 194
mouseYAP65         NHNDQTTTWQDPRKAMLSQLNVPAPASPAVPQTLMNSASGPLPDGWEQAMTQDGEVYYIN 235

mouse TAZ          HQQVVATSLSPQNHP--TQNQ---PTGLMSVPNALTTQQ----------QQQQKLRLQR 238
mouseYAP65         HKNKTTSWLDPRLDPRFAMNQRITQSAPVKQPPPLAPQSPQGGVLGGGSSNQQQQIQLQQ 295

mouse TAZ          IQMERERIRMRQEELMRQEAALCRQLP-METETMAP--VNTPAMSTDMRSVTNSSSDPFL 295
mouseYAP65         LQMEKERLRLKQQELFRQELALRSQLPTLEQDGGTPNAVSSPGMSQELRTMTTNSSDPFL 355

mouse TAZ          NGGPYHSREQSTDSGLGLGCYSVPTTPEDFLSNMDEMDTGENSGQTPMTVNPQQTRFPDF 355
mouseYAP65         NSGTYHSRDESTDSGLSMSSYSIPRTPDDFLNSVDEMDTGDTISQS--TLPSQQSRFPDY 413

mouse TAZ          LDCLPGTNVDLGTLE-------SEDLIPLFN--------DVESVLNKS----EPFLTWL  395
mouseYAP65         LEALPGTNVDLGTLEGDAMNIEGEELMPSLQEALSSEILDVESVLAATKLDKESFLTWL  472
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Fig. 3. Alignment of amino acids of mouse YAP65 and TAZ shows a high homology among those two co-activators of TEFs family.

It is believed that TAZ might act as a master switch gene which promotes differentiation of mesynchymal stem cells into osteoblasts, it was also described as a target of BMP-2 (Hong et al. 2005). On the other hand, the TAZ protein is a potential

co-activator of TEF-1 and/or TEF-3. In contrast, TEF-4 and TEF-5 are only weakly activated by TAZ. Authors postulated that TAZ is a new co-activator of TEFs. TAZ can interact via its N-terminal domain. Interaction with TAZ occurs by C-terminal domain of TEFs (Mahoney et al. 2005).

1.3.4.2. p160 family as co-activators of TEFs

p160 is a member of the bHLH-PAS gene family, because of their highly conserved N-terminal domain (basic helix-loop-helix/Per-Arnt-Sim) (reviewed by Mckenna et al. 2005). This conserved region is typical for three members: SRC1, TIF2 and RAC3. The PAS subdomain is responsible for dimerisation to form active homodimmers (Kewley et al. 2004). The strongest interaction was demonstrated between SRC-1 and TEF-4, however interactions with others TEFs members are also possible. SRC1 binds to TEFs via the bHLH-PAS domain and it was shown, that this domain is necessary to enhance the transcriptional activation from TEF response elements in transiently transfected cells. Moreover TIF2 and RAC3 are also potent co-activators of TEFs. Furthermore, GRIP-1 a mouse homolog of TIF2 is an interaction partner of MEF-2C as well as myogenin and binds them via the bHLH-PAS domain (Chen et al. 2000; Belandia et al. 2000). In contrast, GRIP-1 acts as a co-repressor of MyoD whereas SRC-1 and RAC3 are potent co-activators (Wu et al. 2005).

1.3.4.3. TONDU as co-activator of TEFs

TONDU is a human homolog of vestigial gene, which was identified in Drosophila (Vaudin et al. 1999). Vestigial interacts with scalloped (a homolog of TEFs family in fruits flay) and both proteins control target gene expression and promote the wing formation. In Drosophila wing cells, vestigial interacts with scalloped via the scalloped interaction domain (SID) (Simmonides et al. 1998, Paumard-Rigal et al. 1998; Halder et al. 1998). It was postulated that binding of vestigial can modulate the conformation of scalloped. Moreover, scalloped alone shows a different affinity to enhansons than in a heterodimeric complex with vestigial (Vaudin et al. 1999; Halder et al. 2001). Additionally, it was noted, that ectopic expression of vestigial induces a wing

tissue overgrowth and activates wing specific genes (Kim et al. 1996; Paumard-Rigal et al. 1998). On the other hand, the vestigial expression is controlled by different pathways like decapentaplegic (dpp) a delta ligand of Notch, escargot and snail genes (Kim et al. 1997; Guss et al. 2001; Neumann et al. 1996; Celis, 1999). Interestingly, human TONDU can rescue loss of vestigial function in Drosophila by forming transcriptional active complex with scalloped (Vaudin et al. 1999). In summary, TONDU represents a novel potential co-activator of TEFs family with the highest homology to vestigial in Drosophila.

1.4. Aim of the studies

Transcription is a decisive process for proper function of all organisms. It requires many transcription factors, enhancers and co-activators. Cooperation and synergism between them play an important role in regulation of the transcription. However, to fully understand mechanism controlling transcription it is necessary to identified all components of this machinery including co-activators, which might modulate the role of transcription factors. The primary aim of presented study was the identification and functional characterization of vestigial and TONDU homologues in mammals. Further aims of the current work were:

- to determine of the expression pattern of those homologous during mouse development as well as in adult tissues.
- To investigate the role of vestigial/TONDU homologues in myogenesis
- To determinate their potency as transactivators.

2. ABBREVIATIONS

A	Adenine
ADP	adenosino di phosphate
AMP	adenosino mono phosphate
ANF	Atrial natriuetic factor
ATCC	American type culture collection
bHLH	Basic helix-loop-helix
bHLH-PAS	Basic helix-loop-helix-per-arnt-sim
BLAST	Basic Local Alignment Search Tool
BMP-2	Bone morphogenetic protein 2
bp	Base pairs
BSA	Bovine Serum Albumine
C	Cytosine
CAT	Chloramphenicol Acetyl Transferase
cDNA	DNA complementary to mRNA
cMLC2	Cardiac myosin light chain 2
cTNC	Cardiac troponin C
cTNT	Cardiac troponin T
Dig	Digoxigenin
DM	Differentiation medium
dpc	Days post coitum
E	Embryonic day
EM	E-box-M-CAT hybrid motif
ES	Embryonic stem cells
EST	Expressed sequence tag
EtOH	ethanol
Ex	Exon
FACS	Fluorescent activated cell sorter
FGF	Fibroblast growth factor
G	Guanine
GFP	Green Fluorescence Protein
GM	Growth medium

H	Hour
HS	Horse serum
Ig	Immunoglobulin
k.o.	Knockout
kb	kilo base pairs
kDa	kilo Daltons
LCR	Locus Control Regions
LSC	Liquid Scintillation Counting
LTR	Long Terminal Repeat
M	mol
mA	mili amper
MADS	MCM1-a, Agamous, Deficiens, Serum response factor
MAPK	Mitogen activated protein kinase
MCBF	MCAT Binding Factors
MEF2	Myocyte enhancer factor 2
MeOH	Methanol
min.	minute
ml	milliliter
mM	mili Mol
MMTV	mammary tumor virus
MOV	mechanical overload
MRF	Myogenic regulatory factor
mRNA	messenger RNA
MW	molecular weight
MyHC	Myosin heavy chain
ng	nanogram
nm	nanometer
o.n.	over night
PARP	poly(ADP-ribose) polymerase
Pax-3	Paired box-3 protein
PBS	Phosphate Buffered Saline
PBT	PBS+Tween-20
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde

RNAi	interference RNA
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Sca-1	Stem cell antigen-1
Sgy	soggy
SID	Scalloped interaction domain
siRNA	small interfering RNA
MCK	Muscle Creatine Kinase
SRF	Serum response factor
SUMO	Small Ubiquitous Modifier
SV40	Simian Virus 40
T	Thymine
TAZ	transcription co-activator with PDZ motif
TBP	TATA Binding Protein
TE	Tris-EDTA buffer
TEF	Transcription Enhancer Factor
TGF-ß	Transforming Growth Factor ß
TK	Thymidine cassette
TLC	Thin layer chromatography
TRE	Thyroid Regulatory Element
U	Uracil
V	volt
VEGF	Vascular Endothelial Growth Factor
Vgl-4	Vestigial related factor 4
VITO	vestigial and TONDU related protein
VITO-1/2	VITO-1 and VITO-2
wt	wild type
YAP65	Yes Associated protein 65 kDa
αMyHC	α Myosin Heavy Chain
βMyHC	β Myosin Heavy Chain

3. Materials and Methods

3.1. Materials

3.1.1. Basic materials

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)

3.1.2. Chemicals

All basic chemicals were bought from the following companies: Roth (Hamburg/Karlsruhe, Germany), Boehringer Mannheim (Mannheim), Invitrogen (Karlsruhe), Merck (Darmstadt), Molecular Probes (Goettingen), New England Biolabs (Schwalbach), Pharmacia (Freiburg), Promega (Mannheim), Quiagen (Hilden), Serva Feinbiochemika (Heidelberg), Sigma-Aldrich (Deisenhofen), Stratagene (Heidelberg).

3.1.3. Radiochemicals

Radioactive labelled nucleotides were bought from Amerscham Buchler (Braunschweig) or PerkinElmer (Boston):

- [α-^{32}P] dCTP (6000 Ci/ml)
- [γ-^{32}P] dATP (6000 Ci/ml)
- Chloamphenicol, D-Threo-[dichloroacetyl-1,2-^{14}C] (50μCi/ml)

3.1.4. Specific Reagents

▪ BCIP (5-Bromo-4-chloro-3-indolylphosphat)	Boehringer (Mannheim)
▪ Chloroquine	Sigma-Aldrich(Deisenhofen)
▪ DAB (3,3'-diaminobenzidine)	Sigma-Aldrich(Deisenhofen)
▪ DAPI (4',6-diamidino-2-phenylindole)	Invitrogen (Karlsruhe)
▪ Dimethylsulfoxid	Sigma-Aldrich(Deisenhofen)
▪ Dimethylformamid	Sigma-Aldrich(Deisenhofen)
▪ Digoxygenin-UTP	Boehringer (Mannheim)
▪ Dithyotreithol (DTT)	Promega (Mannheim)
▪ Dubelcco's Modified Eagle Medium (D-MEM) glutamine and sodium pyruvate	1.000 mg/ml D-glucose, L-Invitrogen (Karlsruhe)
▪ Eosin	Division Chroma
▪ Fetal Bovine Serum	Invitrogen (Karlsruhe)
▪ Geneticin G-418	Invitrogen (Karlsruhe)
▪ Heparin	Sigma-Aldrich(Deisenhofen)
▪ Horse serum	Invitrogen (Karlsruhe)
▪ IPTG (Isopropyl-β-D-tiogalactopyranosidase)	Roth (Karlsruhe)
▪ Levamisole	Sigma-Aldrich(Deisenhofen)
▪ NBT (4-Nitro-Blue-Tetrazoliumchlorid)	Boehringer (Mannheim)
▪ n-Butyryl CoA	Promega (Mannheim)
▪ NP-40	Roth (Karlsruhe)
▪ Mowiol	Merck (Darmstadt)
▪ Opti-MEM® with GlutaMAX^{TM}-I	Invitrogen (Karlsruhe)
▪ Penicillin-Streptamycin-Glutamine	Invitrogen (Karlsruhe)

- PFA (paraformaldehyde) — Merck (Darmstadt)
- Prestained Protein Ladder 10-180 kDa — Fermentas (Lithuania)
- Probond™ Resin — Invitrogen (Karlsruhe)
- Protease inhibitor coctail EDTA-free — Roche (Karlsruhe)
- RNasin® — Invitrogen (Karlsruhe)
- Sheep serum — Sigma-Aldrich(Deisenhofen)
- Triton X-100 — Roche (Karlsruhe)
- Trizol® — Invitrogen (Karlsruhe)
- Trypsin 2,5% — Invitrogen (Karlsruhe)
- Tween-20 — Roche (Karlsruhe)
- Vectabond™ — Vector Laboratories
- X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) — Roth (Karlsruhe)
- Yeast tRNA — Boehringer (Mannheim)

3.1.5. Enzymes

Restriction endonucleases were from Jena Bioscience, New England Biolabs and Fermentas.

ENZYME	COMPANY
Taq DNA Polymerase	Eppendorf
RNA Polymerases (T7, T3, SP6)	Promega
DNA Polymerase I Large Klenov	Promega
Expand High Fidelity Polymerase	Roche
T4 Polynucleotide Kinase	Promega
T4 DNA Ligase	Promega
RQ1 RNase Free DNase	Promega
Chloramphenicol Acetyltransferase CAT	Promega
SuperScript™ II Reverse Transcriptase	Invitrogen

3.1.6. Kits

KITS	COMPANY
JetQuick PCR purification kit	Genomed
JetQuick Gel extraction kit	Genomed
JetStar Maxi plasmid purification kit	Genomed
JetStar Mega plasmid/cosmid purification kit	Genomed
Rapid Ligation Kit	Fermentas
TOPO®TA Cloning Kit	Invitrogen
ECL developing system	Amersham
FUGENE 6 transfection reagent	Roche
Comassie Plus™ Protein Assay Kit	Pierce

3.1.7. Oligonucleotides

All oligonucleotides were bought from Roth, Invitek or Invitrogen.

3.1.7.1. Sequencing primers

Primer	Sequence 5'→3'
T7	TAATACGACTCACTATAGGG
T3	ATTAACCCTCACTAAAGGGA
Sp6	ATTAGGTGACACTATAG
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
5`-GFP	CAAGTCCGGCCGGACTCAGATC
3`-GFP	GCTGCAATAAACAAGTTAAC
5`-pDSRED	CCAAAATGTCGTAACAACTC
3`-pDSRED	GAAGGACAGCTTCTTGTAGTC
5`-GST	GACCATCCTCCAAAATCGGATCTG
3`-GST	CGGGAGCTGCATGTGTCAGAG

5`-GAL4	GGTCTCCGCTGACTAGGGCACATC
3`-GAL4	GATGCCGTCACAGATAGATTGG
5`-VITO-1-seq	GAGTAGTAGGCTAGTTTCTGG
5`-VITO-1-SID-seq	CTCCAGATGTGTCCTCTTCAC
5`-E5-VITO-1-5`	GTACCAGGTCTACGGTCCCCC
5`-E5-VITO-1-3`	GTAGACCTGGTACATAACATC
5`-E4-VITO-1-5`	CAGATGTGTCCTCTTCACCTAC
5`-E4-VITO-1-3`	GCTGGGGTTGGGTTGGAAAATG
5`-E3-VITO-1-5`	GCTGGTCAAAATTGTAGCCAC
5`-E3-VITO-1-3`	GTGGCCGTGCAGAGTGGCGGG
5`-E1-VITO-1-5`	CTATTATGTCCAGCGGTTGCC
5`-E1-VITO-1-3`	CATAATAGAACATGCAAATTC
5'-VITO-2-seq1	GGGTCAGTAGTGGATGAACAC
5'-VITO-2-seq2	CACCCCACGGCACCTTTACTAC
5'-VITO-2-seq3	CTAATGCCATCAGTGCGAGCTG
5'-VITO-2-seq4	ATGAGTTGTGCGGAGGTGATG
5'-Vgl-4-seq1	CACAGGCCCGCCTCCCATC
5'-Vgl-4-seq2	GGAGACTGCCGCAGAGAT
5'-Vgl-4-seq3	CCACCTGTGACCCTGTGGTGG
5`- TEF-1-seq1	GATACATCAAACTCAGGACGG
5`- TEF-1-seq2	CGAATCTGTGGACATTCGTCAG
5`-TEF-3-seq1	GTCTCGGCCACAGCCTTCCAC
5`-TEF-3-seq2	GGACATCCGCCAAATCTATGAC
5`-pGBKT7	GCCGCCATCATGGAGGAGCAGAAG

3.1.7.2. Primers for RT-PCRs

No.	Primer	Sequence 5'→3'	Product size
1A	5`-HPRT	GCTGGTGAAAAGGACCTCT	280bp
1B	3`-HPRT	CACAGGACTAGAACACCTGC	
2A	5`-TEF-1-EX9	GGCCTGGGATGATACAGACAGGA CAG	570bp

2B	3`-TEF-1-EX12	CATTGAACCTCGCATACTCCGTCTCTAC	
3A	5`-TEF-3-EX7	GCATAGCTAGCTCCAAGCTCTGG	560bp
3B	3`-TEF-3-EX11	GGTCTCCTGTGTGTCTCGGTTG	
4A	5-TEF5-EX2	GAGGACTGGAAAAACCAGGACAAG	760 bp
4B	3`-TEF5-EX9	GACGCTGATGGTCATGCTGTCGGC	
5A	5`-VITO-2-EX2	GGATGAGGAGGAGGAGGAGAAAG	310 bp
5B	3`-VITO-2-EX3	GCAGTGACTTGGAAGTCAGGATG	
6A	5`-mTAZ	ATGTGAACCTCCACCCGTCCATCA	360 bp
6B	3`-mTAZ	GAAGAGAGGGATCAGATCTTCAGACTC	
7A	5`-mYAP65	CCCTGATGATGTACCACTGCC	380 bp
7B	3`-mYAP65	CCACTGTTAAGAAAGGGATCGG	
8A	5`-MyoD	GCCCGCGCTCCAACTGCTCTGAT	420 bp
8B	3`-MyoD	CCTACGGTGGTGCGCCCTCTGC	
9A	5`-Myogenin	GGGCCCCTGGAAGAAAAG	460 bp
9B	3`-Myogenin	AGGAGGCGCTGTGGGAGTT	
10A	5'-GAPDH	GTGGCAAAGTGGAGATTGTTGCC	360 bp
10B	3'-GAPDH	GATGATGACCCGTTTGGCCC	
11A	5`-p202	CAGAGAGCAAATATTACCGTGTG	380bp
11B	3`-p202	GAGAAGAGTTGGAGTTTATCTCC	
12A	5`-p204	CCCAGAGGTGCTGTTCTCCACTC	490bp
12B	3`-p204	GTGCCATTTTCCACTCCCCACCAC	
13A	5`-p205	GGGAAAGCAGATCAGCCTCCCTG	420bp
13B	3`-p205	CAGTTGGGCACTTCAATCATTTGTTTAG	
14A	5`-Vgl-4	GAACCCAGGATGCAGACCCTCCC	350bp
14B	3`-Vgl-4	GCAAGCTCACGGCGGGGGCTG	
15A	5`-Utrophin	GGGGAAGATGTGAGAGATTT	410 bp
15B	3`-Utrophin	GTGTGGTGAGGAGATACGAT	
16A	5`VITO-1-Ex1	GCCCAGAGAAAGAGCGCCCGC	320 bp
16B	3`VITO-1-Ex2	GCCAGTCGGCCGCGCCCTGGTG	

3.1.7.3. Cloning primers

No.	Primer	Sequence 5'→3'	Product size
17A	5`-VITO-2-pDsRED-EcoRI	GTTAGCGAATTCATGAGTTGTGCGGAGGTGATGTATC	980bp
17B	3`-VITO-2-pDsRED -SacI	GCTGATACGAATTCCAGTACCAAGTTGATTCTTTGCTCTTGTCC	

18A	5`-VITO-2-GAL-KpnI	GTATACGGTACCATGAGTTGTG CGGAGGTGATGTATC	980bp
18B	3`-VITO-2-GAL-SacI	GCTGATACCTCGAGTCAGTACC AAGTTGATTCTTTGCTCTTGTC	
19A	5`-Vgl-4-GFP-EcoRI	GACGAATTCATGCTGTTTATGA AGATGGACC	950bp
19B	3`-Vgl-4-GFP-BamHI	GTGGATCCCCCTTCAGGAGACC ACAGAG	
20A	5`-TEF-3-pGBKT7-EcoRI	GAGGAAGAATTCATGTAGGGT CGGAATGAGCTG	1.3kb
20B	3`-TEF-3-pGBKT7-SalI	GCTCTCCAGTCGACTCATTCTT TCATAAGTC	
21A	5`-VITO-1-pRset-BamHI	CCGCGGGGATCCGCCATGAGCT GTCTG GATGTTATG	500bp
21B	3'-VITO-1-pRset-HinDIII	GGGCACATCAAGCTTGGGGCTC ACGAAGGGTCCCC	
22A	5`-VITO-1-pGEX-EcoRI	CCGCGGGAATTCGCCATGAGCT GTCTGG	870bp
22B	3`-VITO-1-pGEX-SalI	GATGGCAGTCGACCTCTCAGCC ACATGG	
23A	5`-VITO-1-pDsRED-EcoRI	CCGCGGGAATTCGCCATGAGCT GTCTGG	870bp
23B	3`-VITO-1-pDsRED-SalI	GATGGCATGCAATGTCGACGCC ACATGG	
24A	5`-Vgl-4-full length	GCCGGACAGATAAATGCTGTTT ATGAAT	950bp
24B	3`-Vgl-4-full length	GTTCCCCCTTCAGGAGACCACA GAG	
25A	5`-TEF-3-GFP-XhoI	GCAAGGCCGCTCGAGTTACCTC CAACGAGTG	1.3kb
25B	3`-TEF-3-GFP-SalI	GCTCTCCAGTCGACTCATTCTT TCACAAGTCG	
26A	5`-VITO-2-pRset-SacI	GCTTTGGAGCTCGCCAACACCT TGCATCCC	520 bp
26B	3`-VITO-2-pRset-EcoRI	GCTGAATTCATGCTTGTACCAA GTTGATTCTTTG	
27A	5`-PROBE-5`-ko-VITO-1	CATGCTCCAGGATCCTCATAGC AGC	400bp
27B	3`-PROBE-5`-ko-VITO-1	GGCATATAAAGTTTGCGGATCC AATGG	
28A	5`-PROBE-3`-ko-VITO-1	CTTCGACCCGCGGGATCCGCCG CTGCTCATGC	1.3kb
28B	3`-PROBE-3`-ko-VITO-1	GGTTTTCCCAACAGGTGGGGGG ATCCGAAGTCTC	
29A	5`-3`ARM-ko-VITO-1	GGCACAAAGCTTAGACAGGGA TCCCAGTTAC	2.2kb
29B	3`-3`ARM-ko-	GTGCTCTTCTAAGGATCCTTCT	

	VITO-1	GCGGATGAC	
30A	5`-TEF-1-SalI	GCGAGAGCCCTGTCGACAACA TGGAAAGG	1.3kb
30B	3`-TEF-1-SalI	CTATATAAATAAGTCGACTCAG TCCTTCAC	
31A	5'-VITO-1-pGBKT7-EcoRI	CCGCGGGAATTCGCCATGAGCT GTCTGGATGTTATG	870 bp
31B	3'-VITO-1-pGBKT7-Pst I	GGGCCACATCCTGCAGGGGGC TCACGAAGGGTCCC	
32A	5'-VITO-2-full length	ATGAGTTGTGCGGAGGTGATG	980bp
32B	3'-VITO-2- full length	GTACCAAGTTGATTCTTTGCTC	

3.1.7.4. Oligonucleotides for RNAi

No	Primer	Sequence 5'→3'
33A	5'-VITO1-A	GATCCCCGACATCAGCTCTGTGGTGGT TCAAGAGACCACCACAGAGCTGATGTC TTTTTGGAAA
33B	3'-VITO1-A	AGCTTTTCCAAAAAGACATCAGCTCTG TGGTGGTCTCTTGAACCACCACAGAGC TGATGTCGGG
34A	5'-VITO-1-B	GATCCCCGCGAGCTTGCGGCCAAGGGT TCAAGAGACCCTTGGCCGCAAGCTCGC TTTTTGGAAA
34B	3'-VITO-1-B	AGCTTTTCCAAAAAGCGAGCTTGCGGC CAAGGGTCTCTTGAACCCTTGGCCGCA AGCTCGCGGG
35A	5'-Myogenin-A	GATCCCCGTGAATGAGGCCTTCGAGGT TCAAGAGACCTCGAAGGCCTCATTCAC TTTTTGGAAA
35B	3'-Myogenin-A	AGCTTTTCCAAAAAGTGAATGAGGCCT TCGAGGTCTCTTGAACCTCGAAGGCCT CATTCACGGG
36A	5`-VITO-2-A	GATCCCCGACCAGCCTGCCGAGATGGT TCAAGAGACCATCTCGGCAGGCTGGTC TTTTTGGAAA
36B	3`-VITO-2-A	AGCTTTTCCAAAAAGACCAGCCTGCCG AGATGGTCTCTTGAACCATCTCGGCAG GCTGGTCGGG
37A	5`-VITO-2-B	GATCCCCGCCAACACCTTGCATCCCGT TCAAGAGACGGGATGCAAGGTGTTGGC TTTTTGGAAA

37B	3`-VITO-2-A	AGCTTTTCCAAAAAGCCAACACCTTGC ATCCCGTCTCTTGAACGGGATGCAAGG TGTTGGCGGG
38A	5`-VITO-2-C	GATCCCCGCCATCAGTGCGAGCTGCCT TCAAGAGAGGCAGCTCGCACTGATGGC TTTTTGGAAA
38B	3`-VITO-2-C	AGCTTTTCCAAAAAGCCATCAGTGCGA GCTGCCTCTCTTGAAGGCAGCTCGCAC TGATGGCGGG

3.1.8. Vectors and Plasmids

Vector/ Plasmid	Source	Application
pGEM T-Vector	Promega	Contains TA ends, for cloning of a PCR products.
pCR TOPO 2.1	Invitrogen	Contains TA ends, for cloning of a PCR products.
pCR TOPO II	Invitrogen	Contains TA ends, for cloning of a PCR products.
pSuper	R. Agami	Contains the H1 promoter, for expression of RNAi in eukaryotic cells (Agami et al. 2002).
pDSRed-N1-5.1	Clontech	Contains the CMV promoter and the RED coding sequence at the N-terminal end; constructed for expression of the fused protein in eukaryotic cells.
pGBKT7	Clontech	Contains the truncated ADH1 promoter and the GAL4-DBD from *S. cerevisiae*; for Two Hybrid Screen.
pKJ-1	Generous gift from Prof. T. Braun	Contains the neomycin cassette under the mouse pGK-1 promoter.
pEGFP-C2	Clontech	Contains the CMV promoter and the GFP coding sequence at the C-terminal end; constructed for expression of the fused protein in eukaryotic cells.
pGEX-5X-1	Clontech	Contains the C-terminal glutatione-S-transferase; for overexpression of the fused GST protein in *E.coli*.
pRSET A	Invitrogen	Contains 6 copies of the Histidine tag, which is recognized by a monoclonal antibody; constructed for production of the epitope-tagged protein, which can be overexpressed in *E.coli*.
pPNT	Generous gift from Prof. T. Braun	Contains the PGK-neo cassette and the TK cassette; constructed for the conventional inactivation of target gene in mice.

pXJ40-TEF-1	Generous gift from Prof. T. Braun	Contains the full length of TEF-1 cDNA; for expression in eukaryotic cells.
pXJ40-TEF-3	Generous gift from Prof. T. Braun	Contains the full length of TEF-3 cDNA; for expression in eukaryotic cells.
pBluscriptSK-TEF5	Generous gift from Prof. T. Braun	The basic vector contains the coding region of TEF-5.
2MEF2mt-TK	Generous gift from Prof. T. Braun	The reporter construct carrying mutated MEF-2 binding sites (Moltkentin et al. 1995).
2MEF2-TK	Generous gift from Prof. T. Braun	The reporter construct carrying functional MEF-2 binding sites (Moltkentin et al. 1995).
pEMSV-MyoD	Generous gift from Prof. T. Braun	Contains the full length of MyoD cDNA; for expression in eukaryotic cells.
GAL4-VP16	Generous gift from Prof. T. Braun	Contains the activation domain of the viral activator VP16 fused to the DNA binding domain of the yeast GAL4.
GAL4-TAD	Generous gift from Prof. T. Braun	Wild-type GAL4 containing GAL4-transctivation domain (TAD) (Braun et al. 1990).
pG5E1bCAT	Generous gift from Prof. T. Braun	The GAL4-dependent reporter plasmid (Moltkentin et al. 1995).
pCDNA-MEF2C	Generous gift from Prof. T. Braun	Contains the full length of MEF2C cDNA; for expression in eukaryotic cells.
pBluscript–VITO-1	Generous gift from Prof. T. Braun	The basic vector contains the coding region of VITO-1.
pHook-LacZ	Invitrogen	Contains the CMV promoter and LacZ ORF
pGM4poly II	Generous gift from Prof. T. Braun	Contains the SV40 early promoter, the β-globin intron II and GAL4-DBD.
RSV β-gal	Generous gift from Prof. T. Braun	The expressing vector of β-galactosidase in eukaryotic cells (Braun et al. 1990).
pCS2-VITO-1	Generous gift from Prof. T. Braun	Contains the full length of VITO-1 cDNA; for expression in eukaryotic cells.
pCS2-VITO-2	Generous gift from Prof. T. Braun	Contains the full length of VITO-2 cDNA; for expression in eukaryotic cells.
pMT-VITO-1 (N-myc tag)	Generous gift from Prof. T. Braun	The expressing vector of VITO-1 linked to the N-terminal myc tag, for expression of the fused protein in eukaryotic cells.
pMT-VITO-1	Generous gift	The expressing vector of VITO-1 linked to the

(C-myc tag)	from Prof. T. Braun	C-terminal myc tag, for expression of the fused protein in eukaryotic cells.
pMT-VITO-2 (N-myc tag)	Generous gift from Prof. T. Braun	The expressing vector of VITO-2 linked to the N-terminal myc tag, for expression of the fused protein in eukaryotic cells.
pSG9M-Gam1	Kindly provided by Prof. Suske	The expressing vector of Gam-1 for expression of the protein in eukaryotic cells.
pSG5LINK-EGFP-SUMO-1	Kindly provided by Prof. Suske	The expressing vector of SUMO-1 fused with the EGFP tag, for expression of the fused protein in eukaryotic cells.

3.1.8.1. Plasmids for riboprobes synthesis

GENE	INSERT	SIZE(kb)	VECTOR	Antisense probe	
				Enzyme	Pol.
Myf-5	Myf-5 full length	1,8kb	pBluscript	NcoI	T3
MyoD	MyoD full length	1,2kb	pV2C	MluI	T3

3.1.9. Bacterial strains

Strain	Source	Description
TOP10F` *E.coli*	(Invitrogen)	*[F`[lacI^q,Tn10(Tet^R)]], mercA,* Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, deoR, recA1, araD139 Δ(ara-leu)7697, galK, rpsL(StrR), endA1, nupG,
XL1-Blue *E. coli*	(Stratagene)	*rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17(r_K^-,m_K^+), *sup*E44, *rel*A1, l^-, *lac*$^-$, [F' *pro*AB, *lac*I^{q}ZD *M15*, Tn10(Tet^r)]
Stbl2 *E. coli*	(Invitrogen)	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *rec*A1 *end*A1 *lon gyr*A96 *thi*-1 *sup*E44 *rel*A1 λ$^-$ Δ(*lac-pro*AB)
BL21(DE3)pLysE *E. Coli*	(Stratagene)	*B,F, dcm, ompT, hsdS_B($r_B^-m_B^-$)gal(DE3) [pLysS Cam^r, T7 lysozyme, T7 RNA polymerase inhibitor*

3.1.10. Cell lines

C3H10T1/2	ATCC No: CCL-226; murine fibroblasts
C2C12	ATCC No: CRL-1722; murine myoblastoma cell line
293T	ATCC No: CRL-11268; human kidney cell line, contains Adeno and SV-40 viral DNA sequence
NIH/3T3	ATCC No: CCL-1658; murine fibroblasts

3.1.11. Antibodies

- TEF-1: Mouse monoclonal antibody anti human transcription enhancer factor-1 (TEF-1); (Transduction Laboratories)
- MF-20: Mouse monoclonal antibody anti myosin heavy chain, (Schafer et al. 1999)
- Mouse monoclonal Anti HisG antibody (Invitrogen)
- Myogenin: mouse monoclonal antibody anti-myogenin (laboratory collection)
- Digoxygenin FAB Fragment (Roche)
- Alexa Fluor 488 labelled chicken anti-rabbit IgG, (Molecular Probes)
- Alexa Fluor 594 labelled chicken anti-rabbit IgG, (Molecular Probes)
- Alexa Fluor 488 labelled goat anti-mouse IgG, (Molecular Probes)
- Alexa Fluor 594 labelled chicken anti-mouse IgG, (Molecular Probes)
- C-Myc (9E10): sc-40 mouse monoclonal antibody anti c-Myc (Santa Cruz)
- Sheep Anti- mouse IgG horse radish peroxidase linked (Amersham)
- Donkey Anti- rabbit IgG horse radish peroxidase linked (Amersham)
- Polyclonal rabbit anti-GFP (Invitrogen)

3.1.12. Mouse strains

- ICR
- BL6C/57
- k.o. Myf-5 (Generous gift from Prof. Braun) as described by Braun (Braun et al. 1992)

- k.o. delta (dll1) (kindly provided by Prof. Gossler) as described by de Angelis (de Angelis et al. 1997)

3.1.13. Buffers and solutions

All buffers and solutions were prepared in the accordance with the standard protocols described in "Molecular Cloning" (Sambrook et al., 1989) or "Current Protocols in Molecular Biology" (Ausubel et al., 1992). All solutions were made in double-distilled water or demineralized MilliQ water. Solutions were either autoclaved or filter-sterilized.

3.2. Methods

3.2.1. Standard molecular biology methods

All molecular biology procedures were done according to the standard protocols of "Molecular Cloning" (Sambrook at al. 1989) and "Current Protocols in Molecular Biology" (Ausubel et al. 1992). Procedures, buffers and solutions used during this study are presented below only if they differ from published instructions.

3.2.2. Cloning strategies

All plasmids were prepared as follow. 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 sequenced from both sides and if necessary also with internal primers (3.1.7.1). A sequencing reaction contained around 100 ng of the plasmid DNA, sequencing primer (3.1.7.1) at concentration 3.2 pmol and buffers included in DNA Cycle Sequencing Kit (Abi, Weiterstadt). 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 (3.1.8). All sequence and alignments were prepared using data bases at www.ncbi.nih.gov. BLAST or www.ensembl.org and DNA-Star, Lasergene 99 software.

3.2.3. Expression and diagnostic plasmids

3.2.3.1. Expression plasmids

All plasmids were generated as described in 3.2.2. Table (3.1.7.3) lists the primers pairs used for PCR amplification together with restriction sides employed for subcloning.

Name of plasmid	No. of primers	Cloning sites	Applications
pCRII-TOPO-VITO-2	32A 32B	-	Contains the full length of VITO-2 cDNA
pDsRED-5.1-VITO-2	17A 17B	EcoRI, SacI	Contains the full length of VITO-2 fused to the N-terminal DsRED tag, for expression in eukaryotic cell lines.
Gal4-VITO-2	18A 18B	KpnI, SacI	Contains the VITO-2 full length fused to the N-terminal Gal4-DBD, for expression in eukaryotic cell lines; CAT assay.
pEGFP-C2-Vgl-4	19A 19B	EcoRI, BamHI	Contains the Vgl-4 full length fused to the N-terminal GFP tag, for expression in eukaryotic cell lines.
pGBKT-TEF-3	20A 20B	EcoRI, SalI	Two Hybrid screen.
pGBKT-VITO-1	31A 31B	EcoRI, PstI	Two Hybrid screen.
pCS-DBD-VITO-1	-	HinDIII	Generated from pGBKT-VITO-1 plasmid, for expression in eukaryotic cells.
pRSET-A-ΔVITO-1	21A 21B	BamHI, HinDIII	Contains the ΔVITO-1 protein fused to the N-terminal His-tag, for overexpression in *E.coli.*
pGex-5.1-VITO-1	22A 22B	EcoRI, SalI	Contains the VITO-1 protein fused to the N-terminal GST-tag, for overexpression in *E.coli.*
pDsRED-5.1-VITO-1	23A 23B	EcoRI, SalI	Contains the VITO-1 full length fused to the N-terminal DsRED tag, or expression in eukaryotic cell lines.
pEGFP-C2-TEF-3	25A 25B	XhoI, SalI	Contains the TEF-3 full length fused to the C-terminal GFP tag, for expression in eukaryotic cell lines.
pRSET-A-ΔVITO-2	26A 26B	SacI, EcoRI	Contains the ΔVITO-2 protein fused to the N-terminal His-tag, for overexpression in *E.coli.*
pGEM-T-vector-5`-probe-k.o.	27A 27B	BamHI	Contains fragment of genomic VITO-1 DNA; probe for ES genotyping.

pGEM-T-vector-3`-probe-k.o.	28A 28B	BamHI	Contains fragment of genomic VITO-1 DNA; probe for ES genotyping.
T-vector-5`-3`-Arm	29A 29B	BamHI	Contains 3` flanking region of the VITO-1 genomic locus.
pSuper-VITO-1A	33A 33B	EcoRI, HinDIII	Contains RNAi specific for VITO-1 (variant A), for expression in eukaryotic cell lines.
pSuper-VITO-1B	34A 34B	EcoRI, HinDIII	Contains RNAi specific for VITO-1 (variant B), for expression in eukaryotic cell lines.
pSuper-Myogenin	35A 35B	EcoRI, HinDIII	Contains RNAi specific for myogenin, for expression in eukaryotic cell lines.
pSuper-VITO-2A	36A 36B	EcoRI, HinDIII	Contains RNAi specific for VITO-2 (variant A), for expression in eukaryotic cell lines.
pSuper-VITO-2B	37A 37B	EcoRI, HinDIII	Contains RNAi specific for VITO-2 (variant B), for expression in eukaryotic cell lines.
pSuper-VITO-2C	38A 38B	EcoRI, HinDIII	Contains RNAi specific for VITO-2 (variant C), for expression in eukaryotic cell lines.

3.2.3.2. Plasmids for riboprobes synthesis

All plasmids used for riboprobes synthesis were generated on the basis of pCR TOPO II (Invitrogen) or pGEM-T-Vector (Promega). After PCR amplification using primers (3.1.7.2), fragments were cloned into TA vectors and verified by restriction digestion and sequencing (3.2.3).

GENE	No. of primers	INSERT	SIZE	VECTOR	ANTISENSE PROBE	
					Enzyme	Pol.
VITO-1	-	VITO-1	1,4kb	pKS-VITO-1	XhoI	T3
VITO-2	-	VITO-2	2,2kb	pSPORT6-	SalI	T7

				VITO-2		
VITO-2	-	VITO-2	0,6 kb	pSPORT6-VITO-2	SalI	T7
VITO-2	5A 5B	VITO-2 (RT-PCR)	310 bp	pCR TOPO II	NotI	SP6
TEF-1	2A 2B	TEF-1 (RT-PCR)	570 bp	pCR TOPO II	NotI	SP6
TEF-3	3A 3B	TEF-3 (RT-PCR)	560 bp	pCR TOPO II	BamHI	T7
TEF-5	4A 4B	TEF-5 (RT-PCR)	760 bp	pCR TOPO II	BamHI	T7
Vgl-4	24A 24B	Vgl-4 full length	950bp	pCR TOPO II	BamHI	T7
Vgl-4	14A 14B	Vgl-4 (RT-PCR)	350bp	pCR TOPO II	NotI	SP6

3.2.4. Identification of the VITO-1 genomic locus – screening of cosmid libraries

Mouse strain 129 ola genomic library was provided by RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH on 11 filters with spotted DNA cosmids. Filters were hybridized with P^{32}- labeled random primed murine 1kb XhoI/SacI cDNA fragment of VITO-1 gene. Hybridization signals were visualized on the X-ray films. More than 30 positives signals were detected. After calculation of the coordinates, the strongest spots were selected according to the manufacture's protocol (RZPD). Around 18 cosmid clones in Lavrist 7 vector were obtained. All of the cosmids were analyzed by restriction digestion using diagnostic enzymes according to the sequence of the VITO-1 genomic locus obtained from the www.ensembl.org database (Fig. 3.1). Next, cosmids giving appropriate fragments size after restriction analysis were sequenced with T7 and T3 sequencing primers. Two cosmid clones were also sequenced with the internal primers (3.1.8.1). Only one from them has shown identical sequence as a genomic locus of VITO-1. To verify fragments obtained after restriction analysis an oligohybridization was performed. Cosmid fragments after restriction analysis were blotted onto the Bio-Traces® nitrocellulose membrane. Oligonucleotides specific for VITO-1 exons (3.1.8.1) were labeled with [γ-^{32}P] dATP and hybridized at 42^{O}C to the membrane. Results were visualized on the X-ray films (data not shown). Schematic overview of restriction sites localized in the genomic locus of VITO-1 is shown in Fig. 3.2 A. A 4.6 kb fragment, flanked by Kpn I sites (5' arm) was cloned into

the modified pGex-5.1/KpnI vector. Next, pGex-5.1-5'-arm plasmid was digested with XhoI to obtain the 4.6 kb 5' arm flanked by XhoI site.

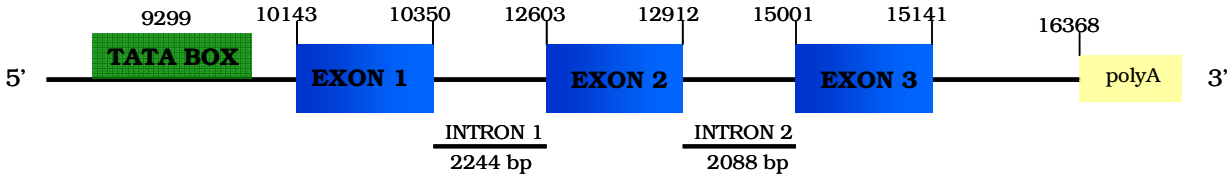


Fig. 3.1. The mouse VITO-1 genomic locus is placed on chromosome 10. The coding region of VITO-1 is divided in three exons. Scalloped interaction domain (SID) is located in the second exon.

This fragment was subcloned into the XhoI site of the pPNT vector (Tybulewicz et al. 1991). The 3' arm was amplified by PCR using specific primers containing BamHI sites. A 2.2 kb 3' flanking sequence was cloned into the pGem-T-vector (Promega).

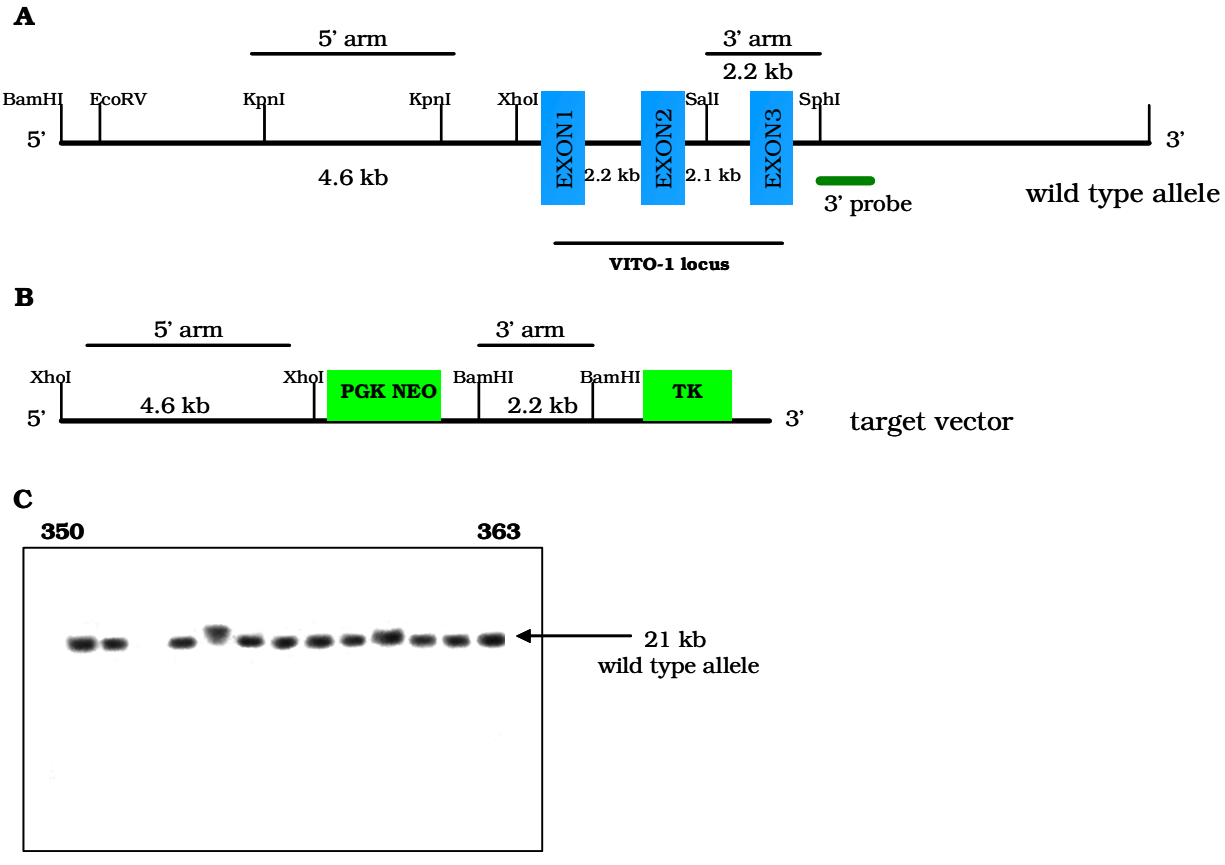


Fig. 3.2. The organization of the VITO-1 gene locus including the major restriction sites (panel A). The structure of the targeting vector is shown in panel B. Genotyping of ES cells clones. Genomic DNA was digested with BamHI and analyzed by Southern blot analysis. Hybridization with the 3' probe showed only

the expected 21 kb fragment representing wild type allele, but no targeted allele represents by a 6.5 kb fragment was observed (panel C).

PCR amplified sequence of the 3' arm was verified by sequencing. This fragment was subloned into the BamHI site of the pPNT/5'arm plasmid. The targeting vector contains a neomycin resistance gene (neo) under the control of the phosphoglycerol kinase (PGK) promoter and a thymidine kinase cassette (Fig. 3.2.B). The pPNT-5'-3'arms plasmid was linearized with NotI for ES cells transfection. ES cells growth, electroporation with generated contruct as well as selection in G418 and gancyclovir was done by technical assistant K. Zabel. Genomic DNA was isolated from more than 1000 clones and southern blot analysis was done using 3' probe (Fig. 3.2 C.). None of the screened clones was positive for mutated allele.

3.2.5. In situ hybridization

3.2.5.1. Embryos preparation

After isolation, embryos were washed with ice-cold PBS and fixed in 4%PFA over night, followed by step dehydratation in methanol 25%, 50%, and 75% in PBT and 2 times in 100% methanol -10 minutes each at room temperature.

3.2.5.2. Tissue preparation for paraffin embedding

After dissection in ice-cold PBS, tissues were fixed over night in 4% PFA followed by step dehydratation in ethanol (25%, 50%, 75%, 96% for 60 minutes each and in 99.8% ethanol 2x10 minutes followed by 99,8% EtOH at -20^{o}C over night). Tissues samples were treated twice with 99.8% ethanol/xylol (1:1) 1 hour, twice per 1 hour with xylol at room temperature and incubated for 2 hours at 60^{o}C with mixture of Xylol/ Paraffin (1:1) followed by two changes of paraffin at 60^{o}C. In the last step, tissues were embedded in paraffin. Sections were cut (7-13 μm) using microtome (Leica) and were attached to glass slides coated with Vectabond^{TM} solution. Briefly,

slides were washed with acetone followed by incubation with 7 ml of Vectabond™ solution in 343 ml of acetone. Slides were washed with distilled water and dried.

3.2.5.3. Riboprobe synthesis

For cRNA synthesis RNA polymerase (T7, T3, SP6) were used. The reaction mixture contains:

Linearized plasmid	1 µg
5 transcription buffer	4 µl
0.1M DTT	2 µl
Dig-Mix	2 µl
RNA polymerase	1 µl
Rnasin	2.5 µl
H_2O	ad 20 µl

The reaction mixture was incubated for 2 hours at 37°C. A DNA template was digested with DNase I for 30 min. at 37°C followed by DNase heat inactivation at 65°C for 15 min. Synthesized riboprobe was ethanol precipitated and redissolved in water with RNase inhibitor. Quality of cRNA was checked by electrophoresis on the 1% agarose gel.

3.2.5.4. Whole mount in situ hybridization

The procedure used is modified protocol of D. Wilkinson and J. Wittbrodt.

Solution/Buffer	Time	Temperature
100% Methanol	5 min	RT
75% Methanol	5 min	RT
50% Methanol	5min	RT

25% Methanol		5min	RT
PBT		5min	RT
PBT		5min	RT
6% H_2O_2	e9.5 dpc	30 min	RT
	e10.5 dpc	45 min	
	e11.5 dpc	60 min	
3x PBT		5 min	RT
Proteinase K (10µg/ml)/PBT	e9.5 dpc	9 min 30 sec	RT
	e10.5 dpc	11 min	RT
	e11.5 dpc	14 min	RT
Glycine (2mg/ml)/PBT		5 min	RT
0.2% glutaraldehyde/4% PFA		20 min	RT
Prehybridization Buffer		60 min	65°C
Hybridization		o.n.	65°C
Posthybridization wash			
2 x Solution 1		30 min	70°C
Solution 1:Solution 2 (1:1)		10 min	70°C
3x Solution 2		5 min	RT
Rnase A (1 mg/ml)/ Solution 2		30 min	37°C
Solution 2		5 min	RT
Solution 3		5 min	RT
2 x Solution 3		30 min	65°C
3 x TBST		5 min	RT
10% Inactivated Sheep serum/TBST		60 min	RT
Anti-digoxygenin antibody 1:2000/TBST		o.n	4°C
3 x TBST		5 min	RT
5 x TBST		60 min	RT
3 x NTMT		10 min	RT
Alkaline Phosphatase Staining			
NBT/BCIP/NTMT			RT

After staining, embryos were washed twice with PBT buffer at room temperature and fixed over night in 4% PFA at 4°C. Embryos were documented; embedded in albumin/gelatin followed by preparation of vibratoma sections.

3.2.5.5. In situ hybridization in paraffin embedded tissue slides

Buffer/Solution	Time	Temperature
2 x 99.8% Ethanol	2 min	RT
96% Ethanol	1 min	RT
70% Ethanol	1 min	RT
50% Ethanol	1 min	RT
30% Ethanol	1 min	RT
PBS	5 min	RT
4% PFA	15 min	RT
PBS	5 min	RT
Proteinase K (10µg/ml)	5 min	RT
Glycine (2mg/ml)	10 min	RT
PBS	5 min	RT
4% PFA	15 min	RT
Acetic Anhydrate/0.25% TEA/H_2O	10 min	RT
2 x PBS	5 min	RT
Hybridization Buffer	15 min	65°C
Hybridization	o.n.	65°C
Posthybridization wash		
3 x Solution I	15 min	65°C
3 x Solution III	15 min	65°C
3 x TBST	10 min	RT
Blocking Solution	30 min	RT
Anti-digoxygenin antibody 1:2000/TBST	o.n	4°C
3 x TBST	15 min	RT
3 x NTMT	10 min	RT
Alkaline Phosphatase Staining		

NBT/BCIP/NTMT		RT

Solutions and buffers used for in situ hybridization

No.	Solutions/Buffers	Components
1.	Solution 1	50% Formamide 5 x SSC pH 4.5 1% SDS
2.	Solution 2	0.5 M NaCl 10 mM Tris-HCl pH 7.5 0.1% Tween-20
3.	Solution 3	50 % Formamide 2 x SSC pH 4.5
4.	Blocking solution	10% inactivated sheep serum in 1 x TBST
5.	NTMT	100 mM Tris-HCl pH 9.5 100 mM NaCl 0.05 M $MgCl_2$ 0.1% Tween-20 levamisole

3.2.5.6. Eosin staining

Usually hydrated sections were stained with 1% eosin in 70% ethanol solution for 5 minutes and washed several times with water. Next, sections were dehydrated in growing step gradient of ethanol and finally in xylol followed by embedding in Enthelan.

3.2.6. Cell culture methods

3.2.6.1. Basic maintenance

C3H10T1/2, C2C12, NIH3T3 and 293T cell lines were grown in D-MEM (1.000 mg/ml glucose) medium containing 10% FCS, 100 U/ml of penicillin, 100 µg/ml of

streptomycin and 0.292 mg/ml L-glutamine (Growth Medium - GM). Cells were grown for 2-3 days to reach 60-80% confluence in a humidified atmosphere containing 10% CO_2 at 37° C. Cells were detached from dishes with trypsin solution (0.05% trypsin, 0.02% EDTA in 1×PBS) and split in 1 to 6 ratio. Frozen stocks were made in the freezing medium: 20% FCS and 10% DMSO in D-MEM.

3.2.6.2. Differentiation of C2C12 cells into myotubes

To differentiate C2C12 cell line to myotubes, medium was changed from growth medium to Differentiate Medium (DM), which contains 2% Horse Serum (HS), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.292 mg/ml L-glutamine. Cells were cultured in D-MEM medium supplemented with 2% HS up to 96 hours after removal of mitotic stimuli, until myotubes were formed (Fig. 3.3). Every day medium was replaced and fresh medium was applied.

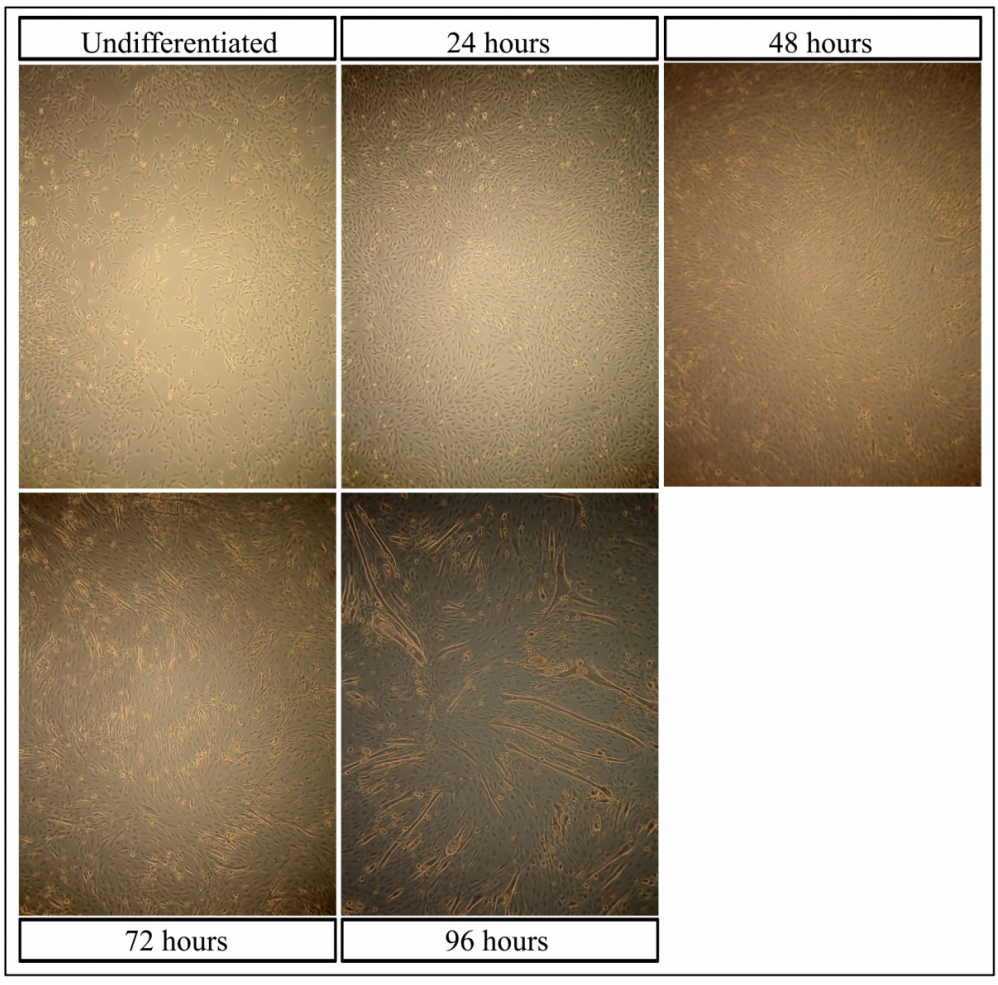


Fig. 3.3. Differentiation of C2C12 myoblasts into myotubes grown in D-MEM medium supplemented with 2% HS.

3.2.6.3. MyoD dependent conversion of fibroblast cell lines

C3H10T1/2 and NIH3T3 fibroblasts cell lines are also able to form myotubes in so called conversion assay. To converse fibroblasts into myotubes, cells were transiently transfected with plasmid containing the full length of MyoD and 24 hours after transfection medium was replaced to differentiate medium (DM) supplemented with 5% Horse Serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.292 mg/ml L-glutamine. Every day medium was replaced and fresh medium was applied. After 4-5 days, cells were PFA fixed followed by β-gal staining or GFP visualization.

3.2.6.4. Transient transfection

3.2.6.4.1. Calcium phosphate

One day before transfection cells were trypsinized and plated at 3×10^4 cells per cm^2. Typically, 6cm plates were used. In some experiments cells were seeded on sterile glass cover slides. Next day, when cells reach 60-70% confluence, 10 minutes before transfection medium was aspirated and changed to fresh growth medium supplemented with 50μM chloroquine (Sigma). Next, 1-30μg of DNA was mixed with 500μl 2 x HBS (100mM NaCl, 6.5mM KCl, 0.8M $Na_2HPO_4{\cdot}2H_2O$, 210mM HEPES pH 7.05) and 62μl of 2M $CaCl_2$. Transfection cocktail was immediately mixed by pipeting and transferred to a single plate in dropwise fashion. After 3-4 hours medium was changed for standard growth medium. Cells were photographed or collected by trypsinization after 24-72 hours.

3.2.6.4.2. Electroporation

On the day of transfection, cells were 70-80% confluent. Cells were detached with trypsin, centrifuged 1000 rpm 5 min., counted and 2×10^6 of cells were resuspended in 400µl of electroporation buffer: Opti-MEM (Gibco) and cytosalts (1M KCl, 10mM $CaCl_2$, 0.5M K_2HPO_4 pH 7.6, 100mM $MgCl_2$) in 25 to 75 ratio respectively. Depending on application around 30 to 50µg of plasmid DNA was added to this electroporation cocktail and the whole volume was transferred into sterile 4mm gap electroporation cuvettes (PeqLab). Electroporation was performed using BTX ECM 600 electroporator (BTX) with the following settings:

Low Voltage Mode
Voltage: 475 V
Pulse Length: 1 milisecond
Number of Pulses: 4

Cells were plated in 6cm or 10cm plates. After 12 hours medium was changed for standard growth or differentiation medium. Cells were photographed or collected by trypsinization after 24-72 hours.

3.2.6.5. Stable cell line generation

4 x 10^5 C2C12 cells were transfected with 10µg of pKJ-1 vector, 40µg of pSuper-VITO1A or pSuper-VITO1B and 10µg of pEGFP-C2 (Clontech) using the standard electroporation protocol (3.2.6.4.2). Usually, 10^4 cells were seeded per 10cm plate. The next day, medium was replaced with selection medium (standard growth medium supplemented with G-418 at 1.2 mg/ml concentration). Medium was changed every second day. Cells were cultivated for 9 days until colonies became apparent. Single colonies were isolated by trypsinization in cloning cylinders. Each trypsinized colony was transferred into the single well of a 96-well plate and expanded in standard growth medium containing 0.6 mg/ml G-418 or without selection. However after 9-10 days after selection all clones died when cells were co-transfected with VITO-1 siRNA.

Parallel, using the same procedure after 14 days, C2C12 cells stably expressing GFP were obtained.

3.2.6.6. Fluorescence Activated Cell Sorting (FACS)

Cells were cultured in triplicates on 6-cm plates. After transfection cells were detached with trypsin and washed with PBS. Next, cells were resuspended in 1 ml of prewarmed ($37^{O}C$) PBS. Cells were sorted in FACS Flow cytometer (Becton Dickinson) and data were analyzed in CellQuest™ software. Instrument calibrations were performed on non transfected cells. Fluorescence was analyzed in the continuous presence of fluorescence DsRED proteins. DsRED was analyzed in FL-1 channel.

3.2.7. Immunocytochemistry

Depending on the aim of experiment and/or primary antibody, different staining protocols and detection systems were used.

3.2.7.1. Immunoperoxidase detection

After culture, cells were washed with PBS, fixed in 4% PFA for 20 minutes followed by washing with PBS with 0.1% Triton-X100. To inactivate endogenous peroxidase cells were treated with 3% H_2O_2 in methanol at room temperature for 10 minutes. Next, as a blocking reagent 5% Horse Serum in PBS with 0.1% Triton-X100 was used, followed by incubation with primary antibody at $4^{o}C$ over night. Next day, cells were washed with PBS/0.1% Triton-X100 solution and incubated with biotinylated secondary antibody for 45 minutes, followed by washing with PBS and incubation with Vectabond® ABC Reagent in PBS for 30 minutes. For signal detection cells were treated with staining solution (1 tablet of DAB (Sigma) was dissolved in 10 ml of 0.1M Tris-HCl pH 7.2 and after filtration 5µl of 30% H_2O_2 was added).

3.2.7.2. Immunocytochemistry with fluorescent labeled secondary antibody

After culture, cells were washed with PBS, fixed in 4% PFA for 10-20 minutes followed by incubation with PBS/0.1% Triton-X100 solution and blocked as above (3.2.7.1). Cells were incubated with the primary antibody at $4^{O}C$ over night or for 1 hour at room temperature, washed with PBS and incubated with the secondary fluorochrom-conjugated antibody for 45 minutes. After three washes with PBS, cells were embedded in Mowiol (6g of glycerol was mixed with 2.4g of Mowiol and 6ml H_2O, followed by incubation for 4 hours at room temperature, mixed with 12ml of 0.2M Tris-HCl pH 8.5, heated for 10 minutes at $50^{O}C$ and aliquoted).

3.2.8. Total RNA isolation from tissues and cells

Total RNA was isolated using the Trizol^{TM} reagent according to manufacture's instruction (Invitrogene). For isolation all instruments were sterilize by treating in 0.5M NaOH for 30 minutes at room temperature followed by washing with DEPC treated water. All solutions used during RNA isolation procedure were prepared with RNAse free water. Usually 1 ml of the Trizol^{TM} reagent was used for 100mg of tissue or in the case of cells per 1×10^6 cells. Tissues samples were homogenized using an Ultra Turrax homogenizer (IKA Works, Wilmington, USA).

3.2.9. PCR and RT-PCR

All PCR conditions were optimized experimentally for each primers pair according to the manufacturer's instruction (Eppendorf). Usually around 1-5ng of a template DNA was used, when necessary $MgCl_2$ was added. The typical reaction mixture contains also 10 x buffer, 5 x Enhancer buffer, 0.2 pmol of each primer and 5U of Taq polymerase (Eppendorf) or Expand High Fidelity Polymerase (Roche). Thermal cycling conditions were also optimized empiricaly.

For RT-PCR, the total RNA was extracted with the Trizol^{TM} reagent according to manufacturer's protocol (3.2.8). Concentration of RNA was determined with Eppendorf BioPhotometer by measuring absorbance at 260nm. In the first step, RNA

extract was purified from DNA contamination by treating with RQ1 RNase-free DNase (1U of DNase per 1µg of RNA) followed by DNase heat inactivation (65^{O}C, 10 minutes). DNA free samples were used as templates for first stranded cDNA synthesis. All reverse transcription reactions were prepared using the reverse transcriptase SuperscriptII (Invitrogen) according to manufacture's protocol (Invitrogen) and oligod$T_{(15)}$ (Promega). Reaction was done at 42^{O}C for 60 minutes followed by heat inactivation of Superscript II at 70^{O}C for 10 minutes. Usually 2-4µl of cDNA was used for PCR reaction, depending on the level of gene expression in different tissues. cDNA obtained using this methods were directly used for PCR reaction or stored at -20^{O}C.

Semiquantitative RT-PCR analysis was performed by decreasing the number of cycles below the plateau phase.

3.2.10. P^{32} random primed labeling probes preparation

Probes were digested from respective plasmids depending on the experiment. Proper bends were cut out from agarose gel and purified with JetQuick Gel extraction kit (Genomed). Usually 25-50ng of DNA and random hexanucleotides as primers were denatured at 95^{O}C for 5 minutes, cooled down on ice and mixed with the following components of reaction mixture: dNTPs, BSA, Klenow enzyme, 5µl of [α-^{32}P] dCTP (6000 Ci/ml) and incubated at 37^{O}C for 2 hours. Before hybridization probe was purified from not incorporated nucleotides using NAP-5^{TM} columns (Sephadex® G-25) according to protocol (Pharmacia Biotech).

3.2.11. Southern blot analysis

Procedure for Southern blotting was performed essentially as described in Sambrook et al. 1989. The genomic DNA was obtained from mouse tails or ES cells clones. Tail cuts were incubated over night in 0.5 ml of buffer containing 100mM Tris-HCl pH 8.5; 5mM EDTA; 0.2% SDS; 200mM NaCl and 200 µg/ml of proteinase K at 55^{O}C. Next, DNA was precipated with 0.7 volume of isopropanol, air dried and dissolved by over night shaking at 55^{O}C in 100µl of TE (Tris-HCl/EDTA) buffer. The genomic DNA was digested with different restriction enzymes or with BamHI for

testing probes for targeted allele and separated on 1% agarose gel. For DNA depurination, the gel was incubated in 0.25% HCl for 15-20 minutes at room temperature. The final transfer was done in alkaline conditions using 0.4M NaOH over night. After more then 12 hours membrane was washed in 2xSSC for 10 minutes and dried. Membrane with bound DNA was used for hybridization with radioactively labeled DNA probes. Membrane was pre-hybridized at $65^{O}C$ for 2-3 hours in Church and Gilbert hybridization buffer (0.5M Na_2HPO_4, 1mM EDTA, 7% SDS) containing 0.2 mg/ml denaturated herring sperm DNA. Hybridization was carried out in Church and Gilbert hybridization buffer containing 0.1 mg/ml denaturated herring sperm DNA and $3x10^6$ cpm/ml of labeled probe at $65^{O}C$ with shaking over night. After hybridization the membrane was washed 3 times with solution containing 1xSSC pH 7.0, 1% SDS at $65^{O}C$. Radioactively labeled DNA was visualized using X-ray films (Kodak).

3.2.12. Northern blot analysis

Northern blot analysis was performed according to the protocol described in Sambrook et al. (1989). Briefly, 20 or 50μg of the total RNA isolated from different tissues and cell lines was denatured in glyoxal mixture (6ml of DMSO, 2ml of deionised glyoxal, 1.2ml of 10xBPTE buffer, 0.6ml of 80% glycerol in H_2O, 0.2ml of 10 mg/ml ethidium bromide) at 65° C for 10 minutes, chilled on ice, mixed with RNA gel loading buffer and separated in 1.2% agarose gel in 1×BPTE buffer (300mM Bis-Tris, 100mM Pipes, 10mM EDTA). Electrophoresis was run at $5V/cm^2$ of the gel. Capillary transfer of RNA onto the nylon membrane was performed at alkaline conditions using 0.01M NaOH with 3M NaCl buffer at room temperature over night. The membrane was washed once with 6xSSC pH 4.5 for 10 minutes and once in 0.2xSSC/1% SDS for 5 minutes and dried. RNA blots were hybridized with radioactively labeled specific probes for different genes as described in (3.2.10). Pre-hybridization, hybridization and post-hybridization washes steps were prepared as described in the paragraph (3.2.11). Probe removal for re-hybridization was done by boiling membrane in 0.1% SDS/ 1×SSC for 10-30 minutes.

3.2.13. Western blot analysis

Proteins were separated on standard denaturing SDS-PAGE gels. Stacking gel was 5%, separating gel 6% to 15%. Upon completion of electrophoresis proteins were transferred onto the nitrocellulose membrane by semi-dry electroblotting (Fastblot B 43, Biometra). Four pieces of Whatman 3MM paper and one piece of the BioTrace® nitrocellulose membrane were cut to the size of the gel and were soaked together with the gel for few minutes in electroblotting buffer. Two layers of 3MM paper were placed on the anode. On the top of this assembly a nylon membrane was placed. Gel was laid on the membrane and covered with 2 layers of 3MM paper. The whole stack was covered with the cathode and pressed gently. Electroblotting was run at a current of 5 mA per cm^2 of the blot for 45 minutes. As alternative, the wet system of proteins transfer was used according to manufactures' instruction (Invitrogen). Membrane was stained with PonceauS for few minutes to visualize transferred proteins bands and blocked in 1% non-fat dry milk powder (Roth) in TBST solution for 30 minutes at room temperature or over night at 4^OC. Next, the membrane was incubated at 4^OC over night or for 1 hour at room temperature with the primary antibody in the blocking buffer. Three 5 minutes washings in TBST preceded 45 minutes incubation (room temperature) with the secondary antibody in TBST with 1% non-fat dry milk. Depending on the type of the experiment different detection systems were used. In case, when highly overexpressed proteins were detected, system based on DAB staining was used. Briefly, after 3 washings (5 minutes each) in TBST, membrane was incubated with ABC solution (2 drops of 'label A' and 2 drops of 'label B' in 5 ml TBST prepared 30 minutes in advance, Vector Laboratories) for 30 minutes. After that the membrane was washed three times with TBST buffer. Detection was done with DAB (2.5 mg/ml) in 10ml of 0.1M Tris-HCl pH 7.2 with 5µl 30% H_2O_2. Staining was stopped by rinsing in water. When proteins were not abundant or isolated from different cell lines ECL detection system was used according to the manual (Amersham).

3.2.14. Overexpression of VITO-1 and VITO-2 proteins in *E.coli*

BL21(DE3)pLysE strain of *E.coli* was transformed with pRSET-A-ΔVITO-1 or pRSET-A-ΔVITO-2 plasmids. VITO-1 and VITO-2 genes were amplified by PCR

using specific primers, which were designed outside of the SID domain in the case of both genes. Colonies were picked and grown in suspension culture o.n. at 37^{O}C. After over night culture at 37^{O}C, bacterial cells were seed to fresh culture with dilution 1:50. Culture was grown up to 0.6-0.8 $OD_{600.}$ The protein overexpression was induced with 1mM IPTG. After 4 hours of culture cells were centrifuged at +4^{O}C 3500 rpm. Crude extracts of cells were loaded into SDS-PAGE gel and the level of overexpressed proteins was monitored by Coomasie staining (Fig. 3.4 A for ΔVITO-1 and Fig. 3.5 A - ΔVITO-2). In addition, western blot analysis was performed to verify the identity of the protein band. The membrane was incubated with the anti-HisG monoclonal antibody. The anti-HisG antibody was detected by biotinylated secondary antibody, followed by DAB staining (Fig. 3.4 B for ΔVITO-1 and Fig. 3.5 A ΔVITO-2). The solubility of proteins in phosphate buffer was checked. ΔVITO-1 protein was efficiently soluble in phosphate buffer (Fig. 3.4 B left panel). In contrast, ΔVITO-2 was not soluble in indicated buffer (Fig. 3.5 B).

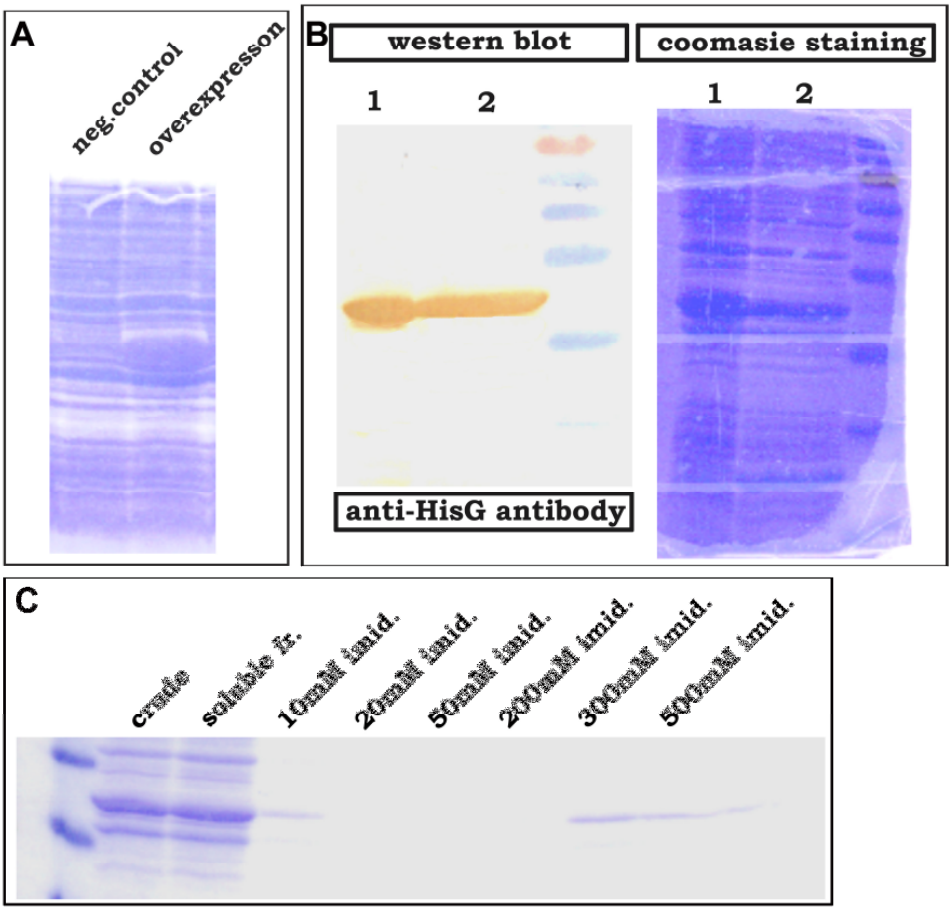


Fig. 3.4. Overexpression and purification of the ΔVITO-1 protein. Overexpression in *E.coli* was checked 4 hours after induction with 1mM IPTG (panel A). Overexpression of the ΔVITO-1 protein was verified by detection

with the anti-HisG antibody (panel B left). The ΔVITO-1 protein was soluble in phosphate buffer (panel B, lane1, right). Purification of the ΔVITO-1 protein on Ni-chelating resins with increased imidazole concentration is shown in panel C.

Purification of the ΔVITO-1 protein was based on the affinity of the fused tag (Histidine repetition) linked to the ΔVITO-1 protein using Ni-chelating resins. The ΔVITO-1 protein was soluble in phosphate buffer. Next, the ΔVITO-1 protein was bound to Ni-chelating resins for 2 hours with rotation at 4^{O}C. Column was washed twice with binding phosphate buffer followed by washes with increased imidasole concentration from 10mM up to 500mM. The purest fractions were obtained in the range of imidasole concentration between 300-500mM (Fig. 3.4 C). Purification of the ΔVITO-2 protein was based on the similar system like in case of the ΔVITO-1 protein. The ΔVITO-2 protein was bound to Ni-chelating resins for 2 hours with rotation at room temperature. Column was washed twice with 8M pH 8.0 urea followed by step gradient of the pH washes. The purest fractions were obtained in range of the pH 5.4-3.3 (Fig. 3.5 C).

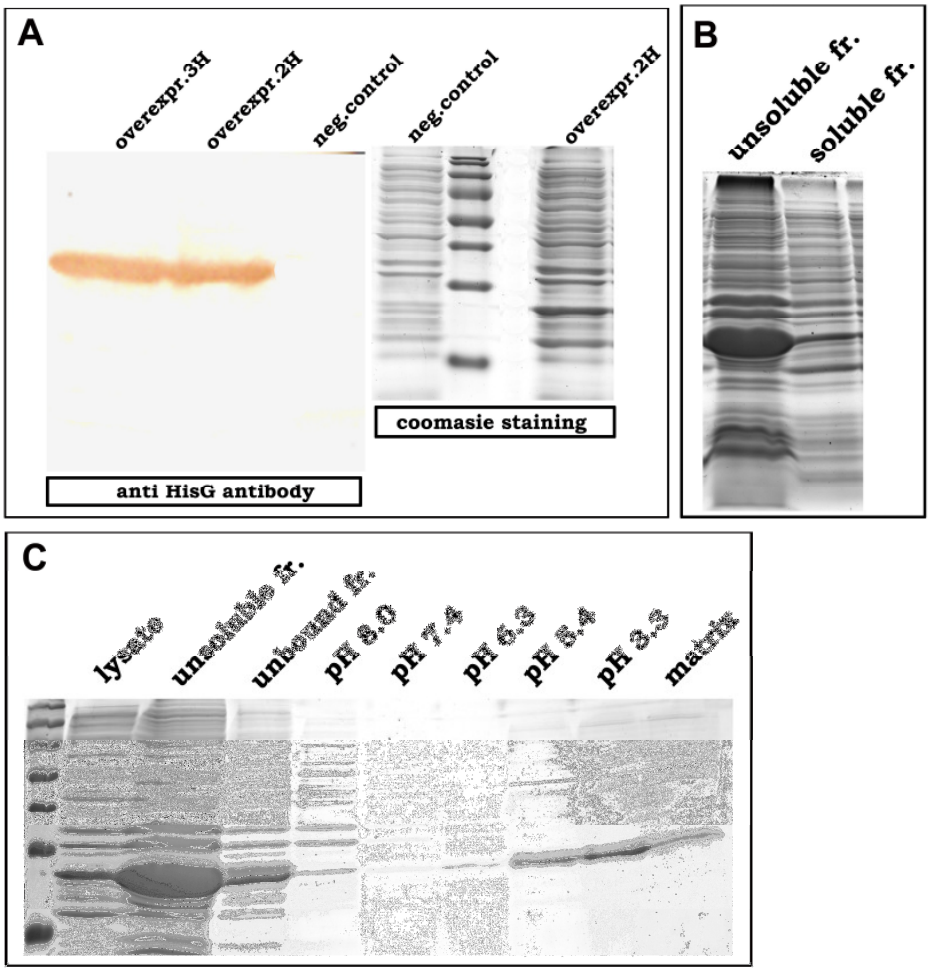


Fig. 3.5. Overexpression and purification of the ΔVITO-2 protein. Overexpression in *E.coli* was checked 4 hours after induction with 1mM IPTG (panel A right). The overexpressed protein was verified by detection with the anti-HisG antibody (panel A, left). The ΔVITO-2 protein was not soluble in phosphate buffer (panel B). Purification of the ΔVITO-2 protein on Ni-chelating resins with step gradient of the pH (panel C).

3.2.15. CAT assay

293T and C2C12 cell lines were grown as described in (3.2.6.1). Cells were transiently transfected with different combination of expression vectors including RSV-β-gal as a standard. Transient transfections of 293T and C2C12 cell lines were done using the calcium phosphate method (3.2.6.4.1) or by electroporation (3.2.6.4.2), respectively. The efficiency of tranfected cells was normalized against β-galactosidase activity (3.2.16). Depends on the transfection efficiency, 10-70μl of proteins extract was used for the reaction containing [^{14}C] chloamphenicol and n-Butyryl CoenzymeA, 0.25M Tris-HCl pH 8.0 to final volume of 125μl. The reaction mixture was incubated at 37^{O}C for 2 hours. As positive control chloamphenicol acetyltransferase (CAT) was added instead of the cell extract. Reaction products – chloamphenicol derivates were analyzed by thin layer chromatography (TLC) or by liquid scintillation counting assay (LSC). In case of TLC, samples were extracted with ethyl acetate and air dried. Samples were resuspended in 20μl of ethyl acetate and spotted on the TLC plate. Plates were developed in chloroform/methanol (97:3) driver, air dried and exposed to X-ray films. After autoradiography, products were scraped from the plate and counted in the scintillation counter. LSC assay was based on xylenes extraction of chloramphenicol derivatives followed by loading to scintillation vial together with scintillation fluid.

3.2.16. β-galactosidase activity

Cells were washed with PBS and fixed in 4%PFA for 5 minutes at room temperature, followed by three times PBS washes. Cells were treated with the staining solution containing X-Gal for 5-30 minutes. Reaction was stopped by washing with PBS.

β-gal assay was used to control the efficiency of transfection in CAT or conversion assay. Briefly, cells were detached by adding lysis buffer (1% NP-40 – 2ml; 1MTris-HCl pH 7.66 – 10ml; 4M NaCl – 7.5ml; 0.5M EDTA pH 8.0 – 0.8ml and H_2O to final volume 200ml). Next, cells were subjected three times to a freeze and thaw cycle, centrifuged at 2000 rpm, at $+4^{O}C$ for 2 min. Next 20µl of lysate, 70µl of Z buffer (0.06M Na_2HPO_4, 0.04M NaH_2PO_4, 0.1M KCl and 1mM β-mercaptoethanol) and 10µl 50mM CPRG was mixed and incubated for 30 minutes at $37^{O}C$. Reaction was stopped by addition of 500µl of 1M Na_2CO_3. Then reaction mixture was measured with the Eppendorf BioPhotometer by absorbance measurement at 600 nm wave length.

3.2.17. Sumoylation assay

293T, C2C12 and fibroblast 3T3 cell lines were grown under the standard conditions (3.2.6.1). Cells were transiently transfected with different combination of expression vectors. Transient transfections of HEK 293, C2C12 and 3T3 cell lines were done using the FUGENE 6 transfection reagent according to manufacture's instruction (Roche). Forty-eight hours after transfection, medium was removed and standard lysis buffer was applied. Cells were scraped from plates followed by boiling for 10 minutes. The protein extracts were centrifuged and supernatants were transferred to fresh tubes. Protein concentration was quantified using Coomasie Plus^{TM} protein assay kit according to the manual (Pierce). Usually, around 4-6µg of total lysate protein was separated on standard 8% separating SDS-PAGE gel. Proteins transfer was done using the wet system according to the manual (Invitrogen). For this assay ECL plus detection system was used (Amersham) to detect sumoylated proteins.

3.2.18. Statistics

Unless otherwise stated all data are means ± SD. Statistical analysis was performed using Exel software (Microsoft). Student's t tests were used. Data were considered significant when $p<0,05$.

4. Results

4.1. VITO family of genes as new homologues of vestigial and TONDU proteins

In order to identify novel genes expressed in skeletal muscles, a subtractive hybridization approach for genes expressed in human skeletal muscles was performed. Subtractive hybridization was performed by incubating driver (fetal human heart cDNA) and tester DNA (human skeletal muscle) in a 100:1 ratio. After two rounds of subtraction using fetal liver cDNA as driver three additional subtraction rounds were performed with fetal heart cDNA as driver (100:1). The PCR fragments from the substraction procedure were cloned into the pGEM-T vector and used to screen a human fetal muscle cDNA library in λgt11 (generous gift from Prof. Braun). The positive clone was sequenced and due to its similarity to the SID (Scalloped Interaction Domain) in Drosophila vestigial and Human TONDU proteins, was named VITO-1 (vestigial and TONDU related) (sequence of mouse and human VITO-1 are presented in Fig. 8.1.1 and Fig. 8.1.2, respectively). Primers used for sequence verification are collected in the Material and Methods (3.1.7.1). Mouse VITO-1 shows 82% similarity to its human homologue at amino acid level. Alignment of mouse and human VITO genes is shown in Appendix (Fig. 8.1.5).

VITO-1 gene shows 16% of similarity to the Drosophila Vestigial sequence and 54% of homology within SID domain. Mouse and human sequence of VITO-1 with accession numbers CAE17332 and CAE17331 respectively, was used to screen different EST databases at www.ncbi.nih.gov./BLAST/ and www.ensemble.org for a potential new family members. Sequence of the SID domain was taken as major criteria to select positives EST clones. On the basis of virtual screening, several candidates were selected (Tab. 3). Clones collected in the Tab. 3 were ordered from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH followed by their sequence verification. Unfortunately only sequences of two clones were correct accordingly to EST database at www.ncbi.nih.gov. Three mouse EST clones with different length were identified which represented the same gene. Sequence analysis revealed a high homology to the Drosophila Vestigial, human TONDU as well as mouse and human VITO-1, especially in the SID domain (35%). Due to its similarity to VITO-1 this new

gene was named VITO-2 (Appendix, Fig. 8.1.3). A human homolog of mouse VITO-2 was also identified in the NCBI database. The sequence is presented in Appendix (Fig. 8.1.4). Alignment of both homologues at DNA and amino acid level is presented in Appendix (Fig. 8.1.6). The human homolog shows 89% of the identity to the mouse VITO-2 gene. Alignment between mouse and human VITO-1/2 genes at the amino acid level is shown in Appendix (Fig. 8.1.7).

The architecture of genomic locus of mouse and human homologues of VITO-1 gene is identical. The coding region of both proteins is divided into the three exons on the basis to www.ensemble.org data base. In contrast to the VITO-1, genomic locus of VITO-2 poses 4 exons (www.ensemble.org).

No.	Accesion no.	Organism	Homology	Length
1	BE285904	Mouse	46%	629
2	BC042696	Mouse	46%	2200
3	XM_283372	Mouse	46%	3200
4	AA571483	Mouse	52%	595
5	BB567795	Mouse	64%	306
6	AA474871	Mouse	48%	371
7	BB649129	Mouse	86%	626
8	BU515761	Mouse	34%	760
8	BF993713	Human	50%	395
9	BG013637	Human	48%	336
10	BG013641	Human	53%	387
11	AW673587	Human	46%	576
12	AL549944	Human	32%	791
13	BI052989	Human	46%	446
14	BG620384	Human	38%	1023
15	AL542319	Human	75%	1006
16	AL542198	Human	37%	980
17	NP_057290	Human	46%	2500

Tab. 3. Potential homologues of the mouse and human VITO-1 gene selected on the basis of a homology to the SID domain. The last column shows a length of EST clones.

To summarize, a new family of gene, named VITO, which poses two members was identified (mouse and human VITO-1/2). Also already known human TONDU and its mouse homolog vestigial related factor 1 (mvr-1) shows a high homology to the Drosophila Vestigial (Fig. 4). It should be emphasized that the SID domain in the Drosophila vestigial protein has 85 amino acids (grey shaded box, Fig. 4). However based on the similarity within the SID domain, only 26 amino acids are highly conservatives among all known VITO family members (Fig. 5).

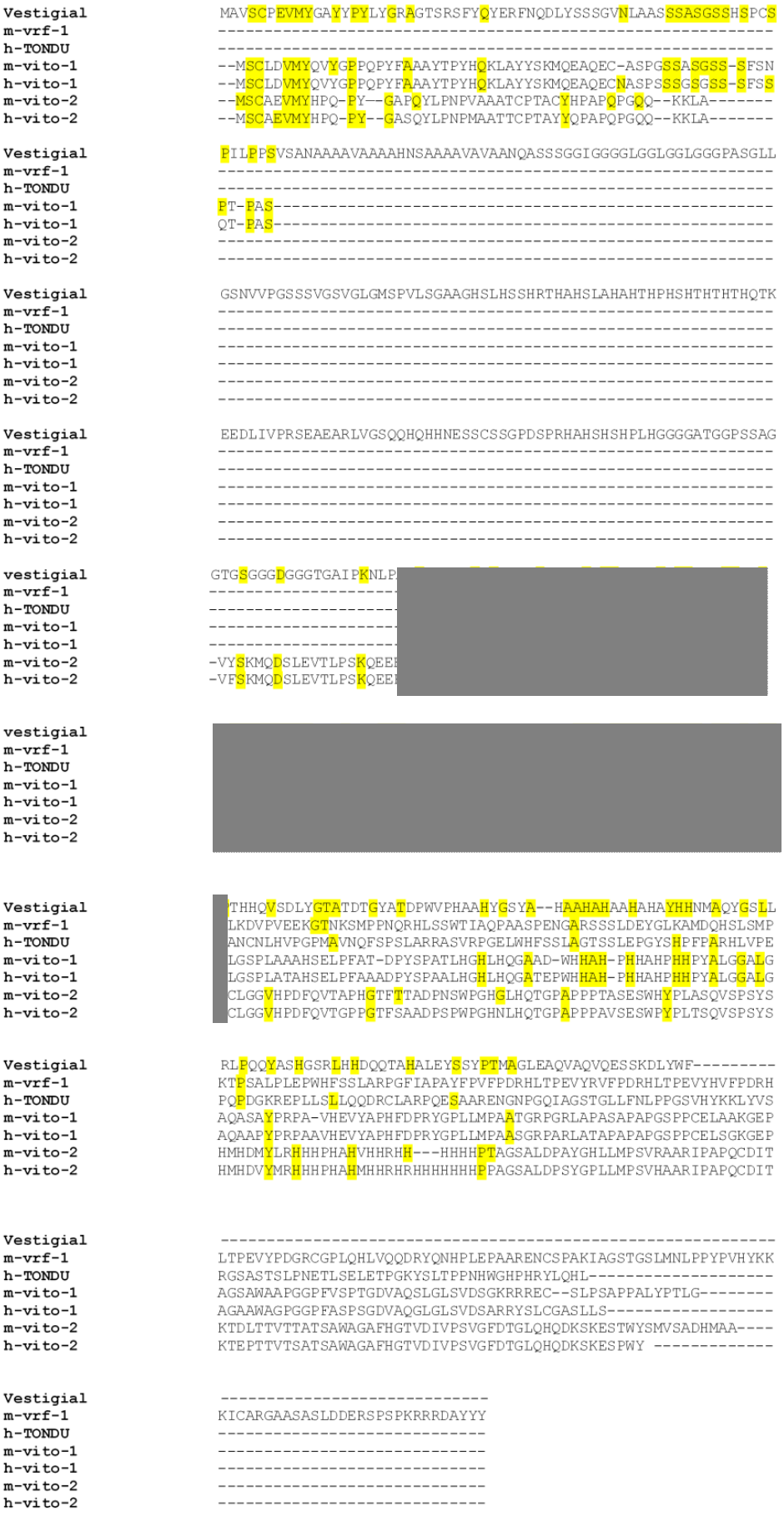

Vestigial MAVSCPEVMYGAYYPYLYGRAGTSRSFYQYERFNQDLYSSSGVNLAASSSASGSSHSPCS
m-vrf-1
h-TONDU
m-vito-1 --MSCLDVMYQVYGPPQPYFAAAYTPYHQKLAYYSKMQEAQEC-ASPGSSASGSS-SFSN
h-vito-1 --MSCLDVMYQVYGPPQPYFAAAYTPYHQKLAYYSKMQEAQECNASPSSSGSGSS-SFSS
m-vito-2 --MSCAEVMYHPQ-PY--GAPQYLPNPVAAATCPTACYHPAPQPGQQ--KKLA-------
h-vito-2 --MSCAEVMYHPQ-PY--GASQYLPNPMAATTCPTAYYQPAPQPGQQ--KKLA-------
Vestigial PILPPSVSANAAAAVAAAAHNSAAAAVAVAANQASSSGGIGGGGLGGLGGLGGGPASGLL
m-vrf-1
h-TONDU
m-vito-1 PT-PAS----------
h-vito-1 QT-PAS----------
m-vito-2
h-vito-2
Vestigial GSNVVPGSSSVGSVGLGMSPVLSGAAGHSLHSSHRTHAHSLAHAHTHPHSHTHTHTHQTK
m-vrf-1
h-TONDU
m-vito-1
h-vito-1
m-vito-2
h-vito-2
Vestigial EEDLIVPRSEAEARLVGSQQHQHHNESSCSSGPDSPRHAHSHSHPLHGGGGATGGPSSAG
m-vrf-1
h-TONDU
m-vito-1
h-vito-1
m-vito-2
h-vito-2
vestigial GTGSGGGDGGGTGAIPKNLP
m-vrf-1
h-TONDU
m-vito-1
h-vito-1
m-vito-2 -VYSKMQDSLEVTLPSKQEE
h-vito-2 -VFSKMQDSLEVTLPSKQEE
vestigial
m-vrf-1
h-TONDU
m-vito-1
h-vito-1
m-vito-2
h-vito-2
Vestigial THHQVSDLYGTATDTGYATDPWVPHAAHYGSYA--HAAHAHAAHAHAYHHNMAQYGSLL
m-vrf-1 LKDVPVEEKGTNKSMPPNQRHLSSWTIAQPAASPENGARSSSLDEYGLKAMDQHSLSMP
h-TONDU ANCNLHVPGPMAVNQFSPSLARRASVRPGELWHFSSLAGTSSLEPGYSHPFPARHLVPE
m-vito-1 LGSPLAAAHSELPFAT-DPYSPATLHGHLHQGAAD-WHHAH-PHHAHPHHPYALGGALG
h-vito-1 LGSPLATAHSELPFAAADPYSPAALHGHLHQGATEPWHHAH-PHHAHPHHPYALGGALG
m-vito-2 CLGGVHPDFQVTAPHGTFTTADPNSWPGHGLHQTGPAPPPTASESWHYPLASQVSPSYS
h-vito-2 CLGGVHPDFQVTGPPGTFSAADPSPWPGHNLHQTGPAPPPAVSESWPYPLTSQVSPSYS
Vestigial RLPQQYASHGSRLHHDQQTAHALEYSSYPTMAGLEAQVAQVQESSKDLYWF---------
m-vrf-1 KTPSALPLEPWHFSSLARPGFIAPAYFPVFPDRHLTPEVYRVFPDRHLTPEVYHVFPDRH
h-TONDU PQPDGKREPLLSLLQQDRCLARPQESAARENGNPGQIAGSTGLLFNLPPGSVHYKKLYVS
m-vito-1 AQASAYPRPA-VHEVYAPHFDPRYGPLLMPAATGRPGRLAPASAPAPGSPPCELAAKGEP
h-vito-1 AQAAPYPRPAAVHEVYAPHFDPRYGPLLMPAASGRPARLATAPAPAPGSPPCELSGKGEP
m-vito-2 HMHDMYLRHHHPHAHVHHRHH---HHHHPTAGSALDPAYGHLLMPSVRAARIPAPQCDIT
h-vito-2 HMHDVYMRHHHPHAHMHHRHRHHHHHHHPPAGSALDPSYGPLLMPSVHAARIPAPQCDIT
Vestigial ----------
m-vrf-1 LTPEVYPDGRCGPLQHLVQQDRYQNHPLEPAARENCSPAKIAGSTGSLMNLPPYPVHYKK
h-TONDU RGSASTSLPNETLSELETPGKYSLTPPNHWGHPHRYLQHL----------
m-vito-1 AGSAWAAPGGPFVSPTGDVAQSLGLSVDSGKRRREC--SLPSAPPALYPTLG--------
h-vito-1 AGAAWAGPGGPFASPSGDVAQGLGLSVDSARRYSLCGASLLS----------
m-vito-2 KTDLTTVTTATSAWAGAFHGTVDIVPSVGFDTGLQHQDKSKESTWYSMVSADHMAA----
h-vito-2 KTEPTTVTSATSAWAGAFHGTVDIVPSVGFDTGLQHQDKSKESPWY ----------
Vestigial ----------
m-vrf-1 KICARGAASASLDDERSPSPKRRRDAYYY
h-TONDU ----------
m-vito-1 ----------
h-vito-1 ----------
m-vito-2 ----------
h-vito-2 ----------

Fig. 4. Amino acids sequence alignments of Drosophila Vestigial, mouse vestigial related factor 1 (m-vr1), human TONDU, mouse and human VITO-1 and VITO-2. Residues matching the Drosophila vestigial sequence are indicated in yellow. The position of the scalloped interaction domain is indicated by grey shaded box.

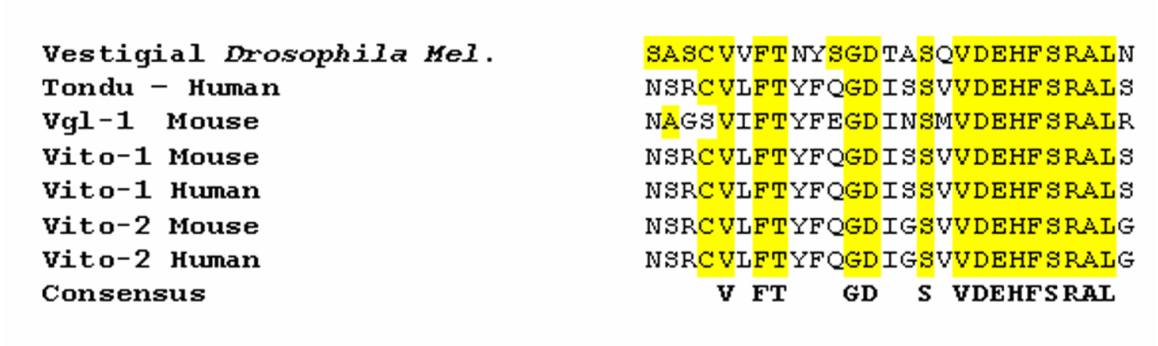
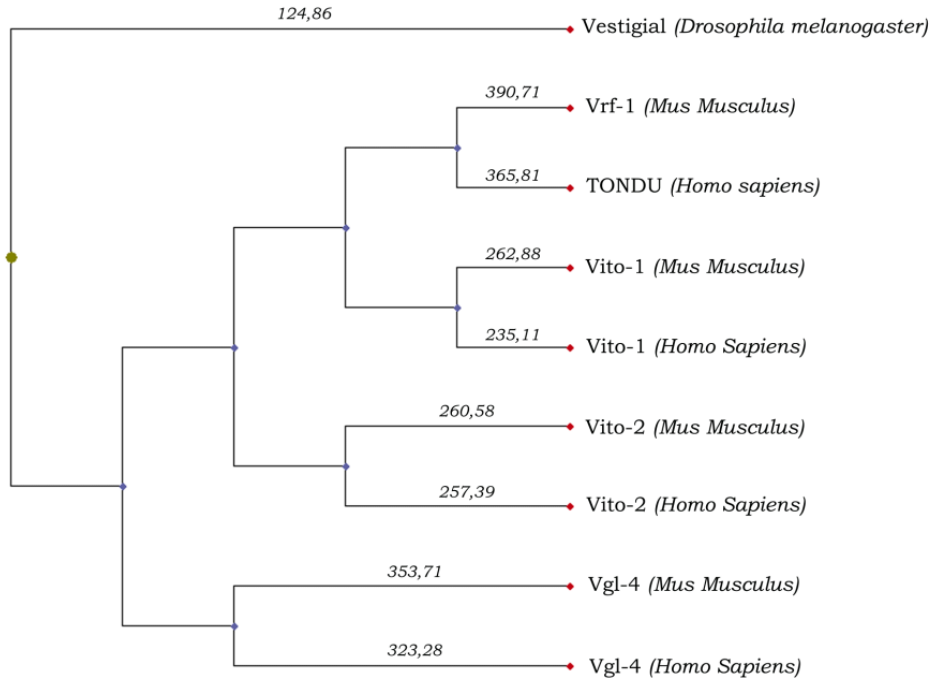


Fig. 5. Amino acids sequence alignments of Drosophila vestigial, human TONDU, mouse vestigial related factor 1 (m-vr or Vgl-1), mouse and human VITO-1/2 within the scalloped interaction domain (SID). Residues matching the vestigial sequence are indicated in yellow. Particularly this part of the SID domain of vestigial shows a high consensus between all VITO family genes members.

Recently a new human homolog of vestigial as member of VITO family genes has been discovered (Chen et al. 2004) and named Vgl-4 (vestigial related factor 4). The authors postulated, that h-Vgl4 (accession number NM_0146667) contains two SID domains. However, the h-Vgl-4 gene shows only a low homology to human TONDU or human VITO-1/2 genes at the DNA as well as amino acids level. The two potential SID domains within h-Vgl-4 have only a similarity of 40% to the consensus core of the SID region (Fig. 5). To investigate the phylogenetic distance between all known members of VITO family genes, including h-Vgl-4 and its mouse homologue as well as Drosophila vestigial a phylogenetic tree was build. The phylogenetic tree was prepared using aligned amino acids sequences by ClustalW software at www.ebi.ac.uk/CLUSTALW/ followed by phylogenetic analysis using PhyloDraw Ver.0.8 software at www.pearl.cs.pusan.ac.kr. (Fig. 6). Results of phylogenetic analysis clearly indicates that VITO-1/2 genes as well as human TONDU, mouse Vrf-1 are the closest homologues of vestigial. A long phylogenetic distance of the mouse and human Vgl-4 genes to the Drosophila vestigial protein, discriminates them as real homologues.

A



B

	Vestigial	Vrf-1	TONDU	m-Vito-1	h-Vito-1	m-Vito-2	h-Vito-2	m-Vgl-4	h-Vgl-4
Vestigial									
Vrf-1	515,57								
TONDU	490,67	74,51							
m-Vito-1	387,74	236,40	211,51						
h-Vito-1	359,97	208,64	183,74	27,76					
m-Vito-2	385,43	280,14	255,24	152,31	124,54				
h-Vito-2	382,25	276,95	252,05	149,12	121,35	10,21			
m-Vgl-4	478,57	494,70	469,81	366,87	339,11	364,57	361,38		
h-Vgl-4	448,14	464,27	439,38	336,44	308,68	334,14	330,95	30,42	

Average: 295,93
Variation: 23,56

C

Name of gene	Species	Accession number
Vestigial	*Drosophila Mel.*	NM_078999
Vrf-1	*Mus Musculus*	NM_133251
TONDU	*Homo Sapiens*	NM_016267
m-Vito-1	*Mus Musculus*	CAE 17332
h-Vito-1	*Homo Sapiens*	CAE 17331
m-Vito-2	*Mus Musculus*	XM_283372
h-Vito-2	*Homo Sapiens*	NP_057290
m-Vgl-4	*Mus Musculus*	NM_177683
h-Vgl-4	*Homo Sapiens*	NM_014667

Fig. 6. A phylogenetic relation between all known members of VITO family proteins and the Drosophila Vestigial are shown in panels A and B. The distances among proteins are summarized in the table (panel B).The tree was obtained by alignment of indicated proteins (panel C).

4.2. Expression pattern of VITO genes family

To begin to unveil the role of the VITO family genes for a development, cell differentiation and tissue specific transcriptional activation, the expression of VITO family genes during moue embryogenesis and in adult mouse tissues were analyzed.

4.2.1. VITO-1 is specifically expressed in skeletal muscles

The expression studies of VITO-1 started with Northern blot analysis as described in Material and Methods (3.2.12). Total RNA was isolated (3.2.8) from different developmental stages of mouse embryos, adult tissues and differentiated and undifferentiated C2C12 cell line.

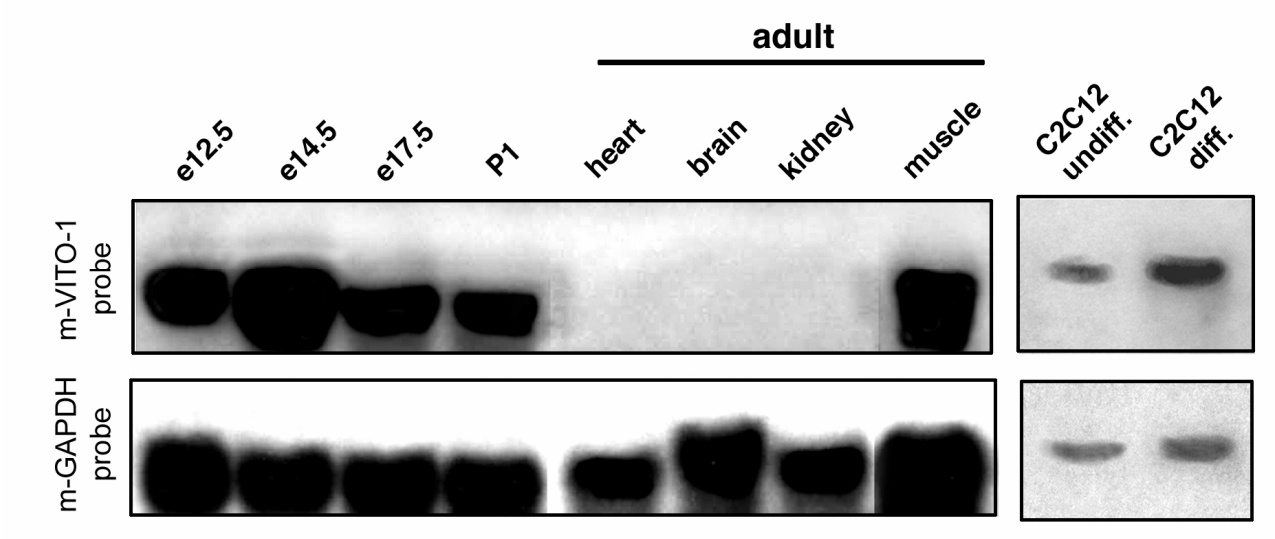


Fig. 7. Northern blot containing 50 µg of total RNA isolated from different adult mouse tissues, cells and embryos was hybridized with mouse VITO-1 probe. An equal amount of RNA was controlled by hybridization with m-GAPDH probe.

Northern blot analysis revealed the presence of a 2.5 kb mRNA product in the developing mouse embryo at E12.5, E.14.5 and E17.5 and in the newborn mice at P1. A strong hybridization signal was detected in adult skeletal muscles but not in others adult tissues. In addition, m-VITO-1 transcripts were found in proliferating C2C12 cells indicating the expression of the gene in dividing myoblasts that have not yet undergone terminal differentiation. The expression level in C2C12 myoblasts was lower compared to differentiated C2C12 myotubes (Fig. 7). To investigate the expression of VITO-1 in the individual skeletal muscle, the semiquantitative RT-PCR analysis was performed.

The total RNA was extracted from various types of skeletal muscles (soleus - slow type muscle), EDL - extensor digitorum longus - example of fast type muscle and Gastrocnemius - example of red and white type of muscle). The reverse transcription was carried as described in 3.2.9. Primers sequences that selectively amplified mouse VITO-1, Utrophin (as marker of slow type muscle) and mouse GAPDH are described in chapter 3.1.7.2.

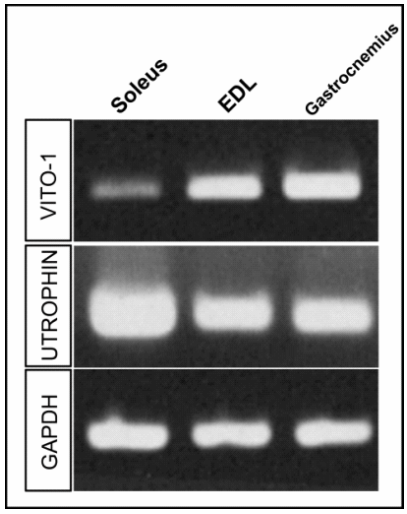


Fig. 8. VITO-1 transcripts are more abundant in the fast than slow muscle types. The semiquantitative RT-PCR analysis of the VITO-1 and utrophin expression in the different types of adult skeletal muscles: soleus, EDL, Gastrocnemius. An equal amount of RNA was controlled by the level of GAPDH of PCR product (22 cycles). In the case of VITO-1 and utrophin the cycle numbers were decreased from 35 to 27.

The semiquantitative RT-PCR analysis showed different level of VITO-1 transcripts in adult skeletal muscles, dependent on the type of fibers (Fig. 8). VITO-1 was found at higher level in fast skeletal muscles than in slow type of skeletal muscles. Utrophin was used as typical marker for the slow type of adult skeletal muscles (Gramolini et al. 2001) and it expression was higher in the soleus as shown above.

Since VITO-1 is highly expressed during mouse development, whole mount in situ hybridization was applied to determine the localization of VITO-1 mRNA.

At E 8.0 a strong expression of VITO-1 was found in the first and second branchial arches structures adjacent to the respective pharyngeal clefts (Figs. 9 A,B). Half a day later at E8.5 the initial expression domains became more restricted and a new area of expression arose in the mesenchyme of the second brancial arch (Figs. 9 C,D). Expression of VITO-1 in the maxillary and mandibular components of the first branchial arch (Fig. 9 G) and in the second branchial arch (Fig. 9 H) was also noted at E9.5 (Fig. 9 G).

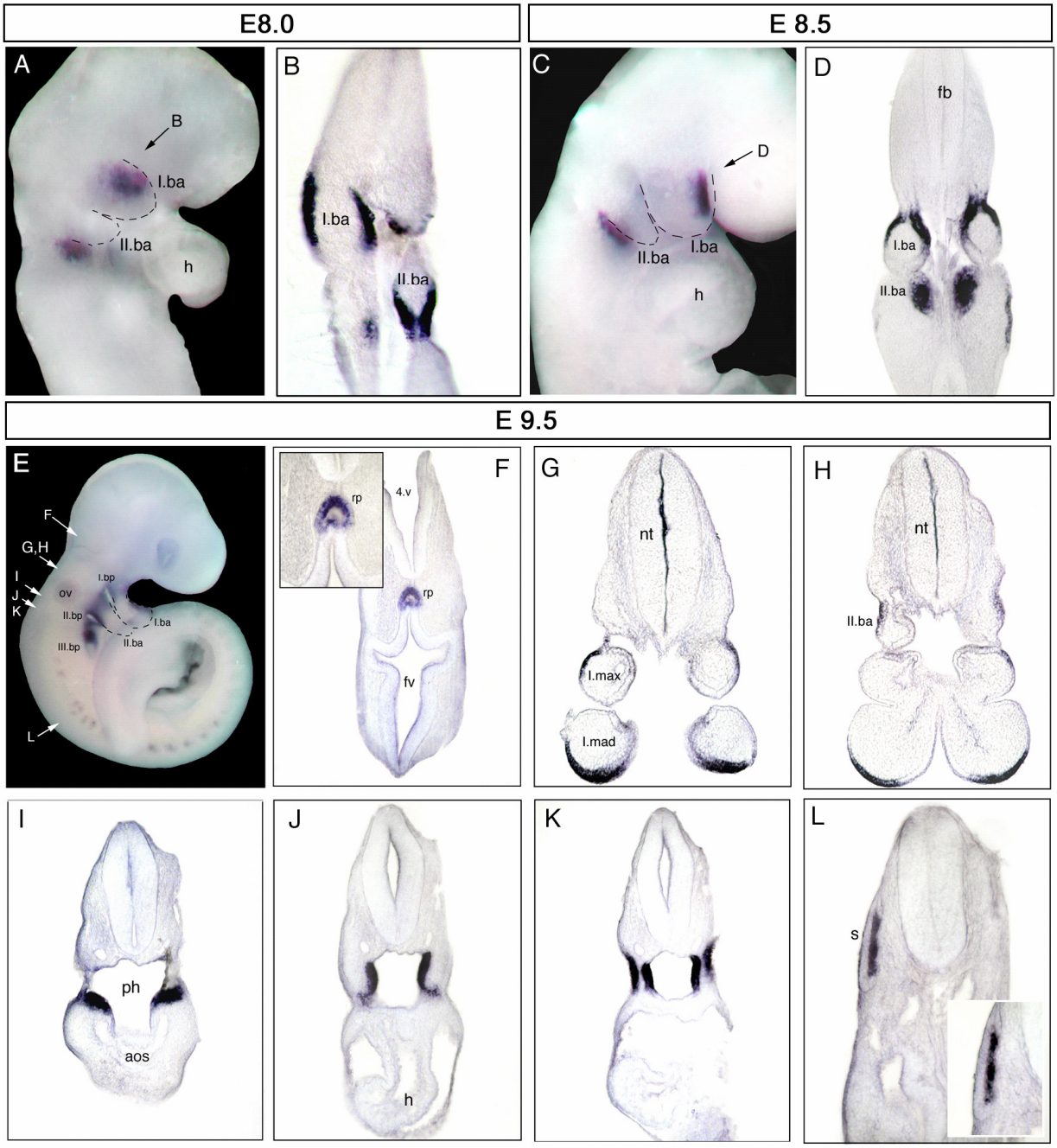


Fig. 9. Whole-mount in situ hybridization of mVITO-1 and Vibratome sections of hybridized mouse embryos at E8.0 to E9.5. Whole-mount preparations (A, C,E) and vibratome sections are shown (B, D, F–L). Sections in B, D, G and H were prepared in the frontal plane as indicated by arrows. Sections in F, I–L were prepared in the transversal plane. The section in B is slightly tilted. Sections in G–K are arranged in a cranial to caudal order. Expression is apparent in the I and II branchial arch at E8.0 and E8.5 (A–D). At E9.5 VITO-1 is expressed in Rathkes pouch (F) and in derivatives of the cranial pharynx (I–K). The expression in somites is restricted to the myotome (L). ba, branchial arch; h, heart; nt, neural tube; max, maxilla part of the I ba; mad, mandibular part of the I ba, ph, pharynx; aos, aortic sac; and s, somite.

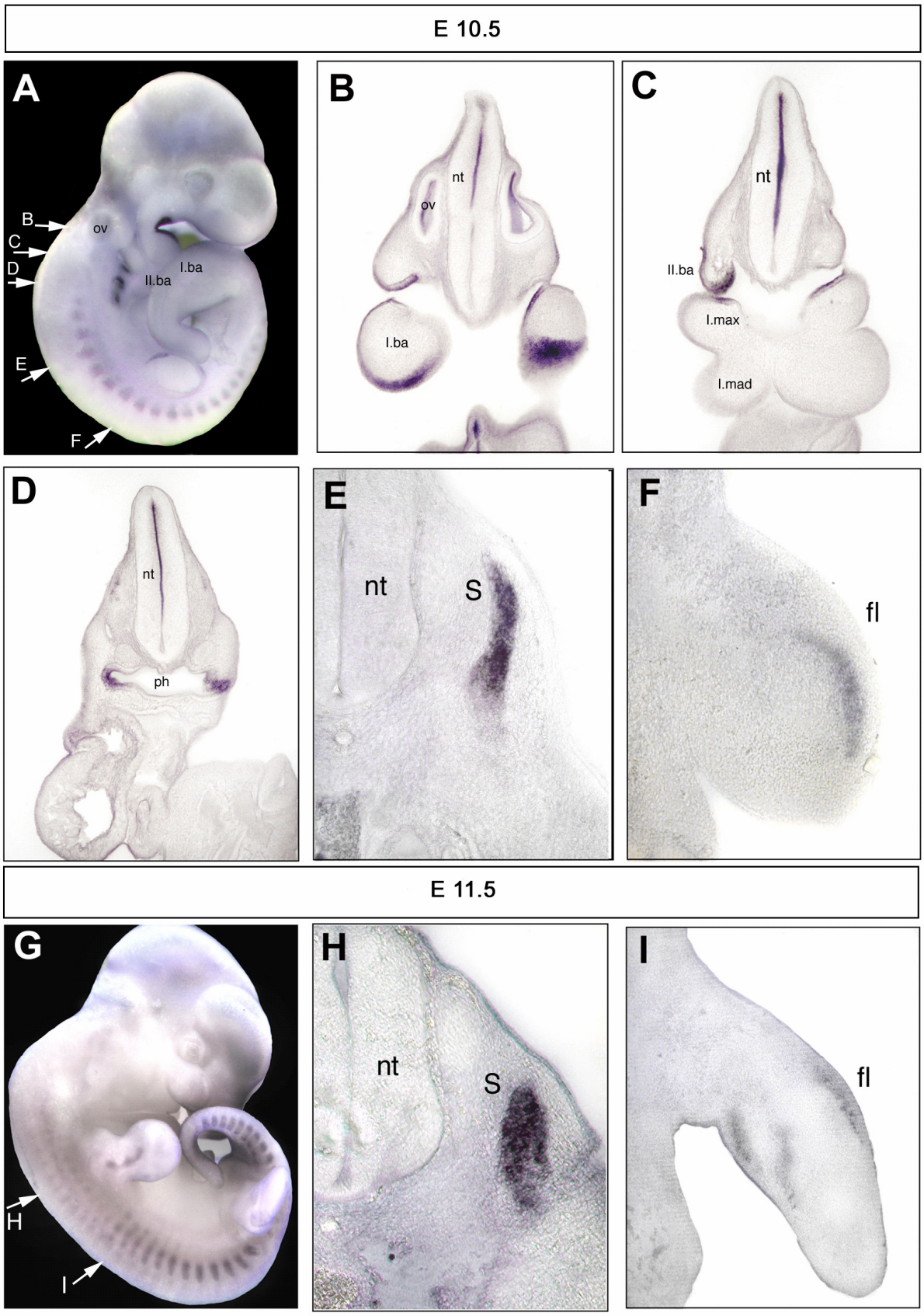


Fig. 10. Whole-mount in situ hybridization of mVITO-1 and Vibratome sections of hybridized mouse embryos at E10.5 and E11.5. Whole-mount preparations (A, G) and vibratome sections are shown (B–FD, H–I). Sections were prepared in the transversal plane as indicated by arrows. Expression is apparent in the I and II

branchial arch (B, C); and in restricted areas of the cranial pharynx (D). The expression in somites at E10.5 (E); and E11.5 (H) is still restricted to the myotome. At E10.5 (F); and E11.5 (I) expression is found in the prospective muscle forming areas of the fore limb. ba, branchial arch; nt, neural tube; max, maxilla part of the I ba; mad, mandibular part of the I ba, ph, pharynx; s, somite; and fl, fore limb.

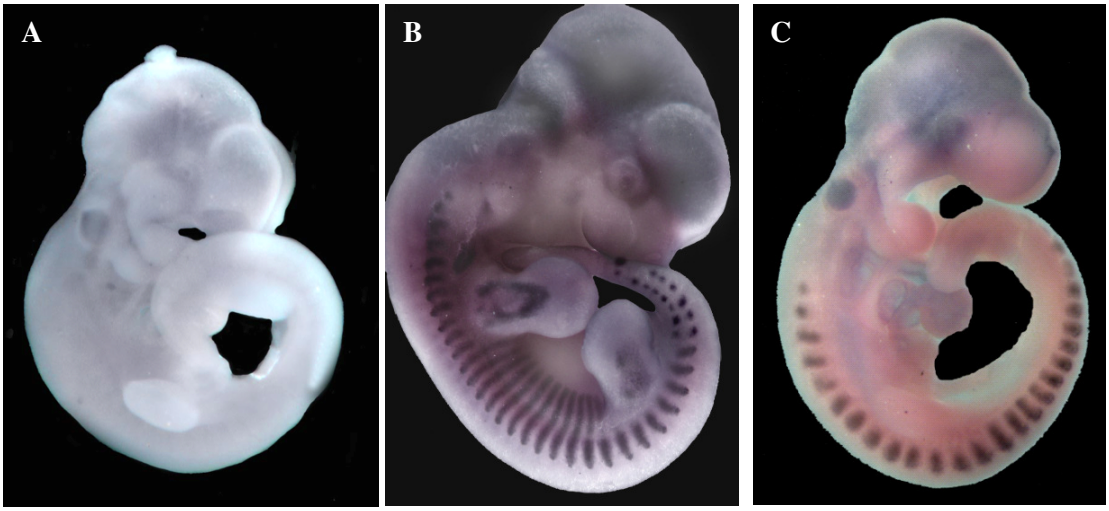


Fig. 11. Control whole-mount in situ hybridization of E10.5 embryos. Panel A – sense m-VITO-1 probe served as negative control. Panel B and C – as positive controls served muscle specific genes – MyoD (E11.5 – panel B) and Myf-5 (E10.5 – panel C).

Sections taken more caudally (indicated by white arrows Fig. 9E) revealed strong expression of VITO-1 in the caudal branchial arches (Figs. 9 I,J), in the caudal branchial cleft, and in the cranial pharynx (Figs. 9 J,K). The earliest expression of VITO-1 in somites was observed at E8.75 with a specific expression restricted to the myotome. No expression was detected in the dermatome or in the dermomyotomal lips. Myotomal expression of VITO-1 started in somite IX or X according to nomenclature of Christ and Ordahl (Christ and Ordahl, 1995). At E9.5 VITO-1 transcripts were localized in the branchial arches and branchial pouches I-III as well as in the Rahkes pouch (Fig. 9 F). At this stage the youngest somites that expressed VITO-1 was somite IX. Expression was still restricted to the myotome (Fig. 9 L). A very similar expression pattern was found at E10.5 with prominent signals in somites and in the second pharyngeal pouch. In addition, VITO-1 expression was observed in the two newly emerging caudal pharyngeal pouches (III and IV) (Figs. 10 A-D). As shown in Fig. 10,

newly forming somites in the caudal part of E10.5 embryos were still negative for VITO-1. Additionally, VITO-1 transcripts were detected in muscle precursor that have already arrived in the fore limb bund (Fig. 10 F). At E11.5 expression of VITO-1 was maintained in matured cranial somites and in the presumptive muscle forming areas of the fore and hind limbs (Figs. 10 G-I). The expression in the pharyngeal pouches had disappeared completely by E11.5. As a negative control sense probe for VITO-1 was hybridized at E10.5 (Fig. 11).

4.2.2. VITO-2 is not a muscle specific gene

To unveil the expression of VITO-2 a similar set of experiments as in the case of VITO-1 was performed. The expression studies of VITO-2 started with Northern blot analysis as described in Material and Methods (3.2.12). Total RNA was isolated from different developmental stages of mouse embryos, adult tissues and differentiated and undifferentiated C2C12 cell line.

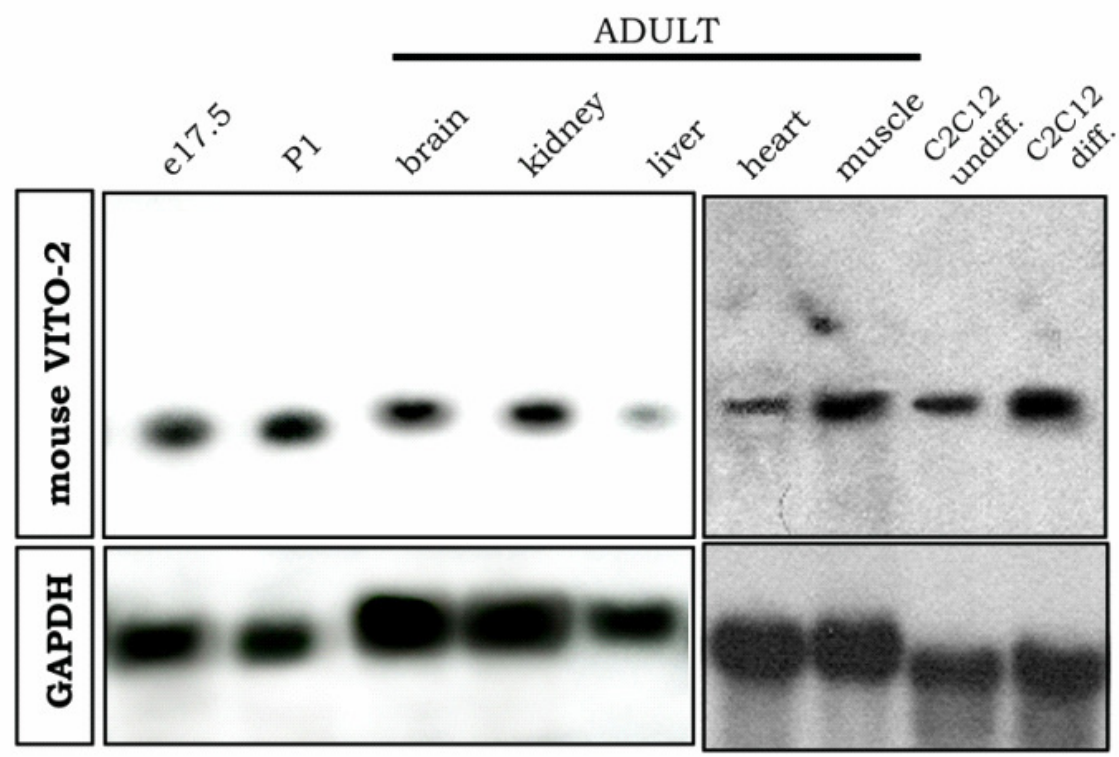


Fig. 12. Northern blot containing 50 µg of total RNA isolated from different adult mouse tissues, cells and embryos was hybridized with mouse VITO-2 probe. An equal amount of RNA was controlled by hybridization with m-GAPDH probe.

Northern blot analysis showed the presence of a 2.5 kb VITO-2 mRNA in the developing mouse embryo at E17.5 and in newborn mice at P1 as presented in Fig. 12. In the adult mouse tissues VITO-2 transcripts were detected in the brain, kidney and in skeletal muscles. A much lower hybridization signal was detected in the liver and the heart. Apparently VITO-2 was found to be predominantly expressed during mouse embryonic development compared to adult tissues. In addition, m-VITO-2 transcripts were found in proliferating C2C12 cells. The expression level in C2C12 myoblasts was lower compared to differentiated C2C12 myotubes (Fig. 12). Expression of VITO-2 was also determined by semiquantitative RT-PCR methods using specific primers (3.1.7.2). Total RNA was extracted from different types of skeletal muscles as in case of VITO-1: soleus, EDL and Gastrocnemius.

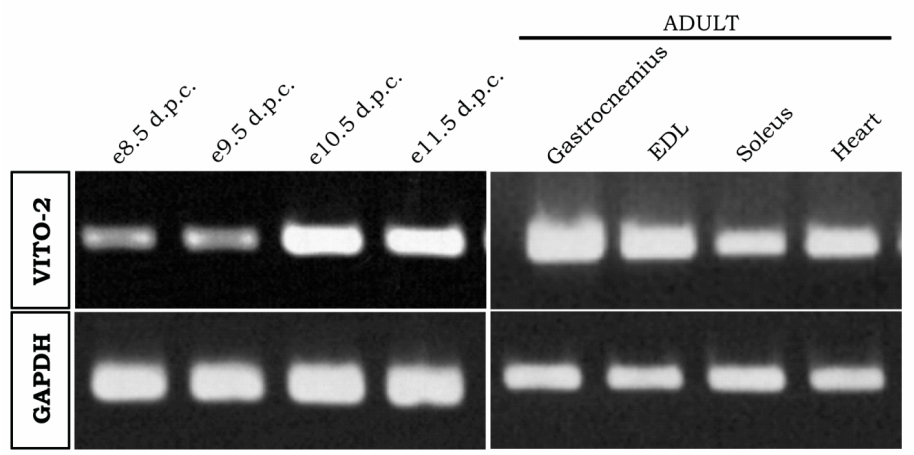


Fig. 13. Semiquantitative RT-PCR analysis of VITO-2 expression in different types of adult skeletal muscles: Gastrocnemius, EDL, soleus (right panel) and during different mouse development from E8.5 to E11.5 (left panel). Equal loading of RNA was controlled by GAPDH expression (22 cycles). In the case of VITO-2 the cycle numbers were decreased from 35 to 25 (left panel) and to 29 cycles (right panel).

Semiquantitative RT-PCR analysis (Fig. 13) revealed different levels of VITO-2 transcripts in adult skeletal muscles. VITO-2 is present at higher levels in fast muscles (Gastrocnemius and EDL) compared to slow muscles (Soleus) (Fig. 13. right panel). During mouse embryonic development, VITO-2 starts to be expressed at E8.5 up to P1 (Figs. 12 and 13, left panel).

Because VITO-2 transcripts are present at high levels during mouse embryonic developmental stages, whole mount in situ hybridization was used to determine the VITO-2 expression pattern.

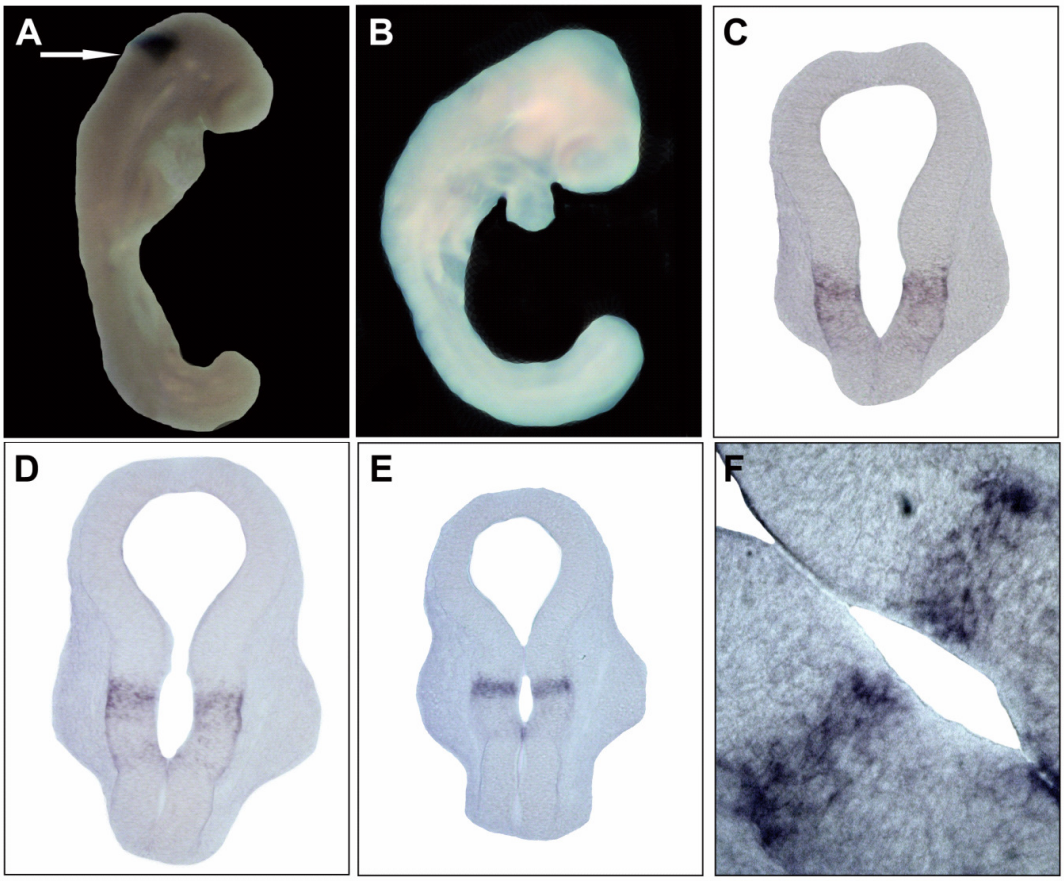


Fig. 14. Whole mount in situ hybridization of mVITO-2 (A,B) and vibratome sections of hybridized mouse embryos at E8.5 (C-F). Embryos were hybridized with antisense (A) and sense probes for VITO-2 (B), respectively. Sections were prepared in the transversal plane as indicated by the arrow and were derived from the cranial end of hybridized embryos. Expression was detected in the midbrain (C,D) and most probably in the rombomers (E,F).

At E8.5 first structures labeled by VITO-2 transcripts were detected in the neuroepithelium of the prospective midbrain (Figs. 14 C-F). As negative control sense probe was hybridized at E8.5 (Fig.14 B). One day later at E9.5 (Figs. 15 A-E) the initial expression in midbrain had completely vanished (Figs. 15 B, 16 B) and the first transcripts of VITO-2 in somites were detected (Figs. 15 A,C-E). The signals were found in the myotome of the somite X or XI (Figs. 15 C,D). No expression of VITO-2 was detected in the dermatome. Similar expression patterns were noted at E10.5 and E11.5 with prominent signal only in the myotome of somites (Figs. 16 A-E, 17 A-D). In contrast to earlier stages, at E11.5 the youngest somites that expressed VITO-2 was somite VI or VII. At this stage there was no expression of VITO-2 in the putative muscles forming areas of the fore and hind limbs.

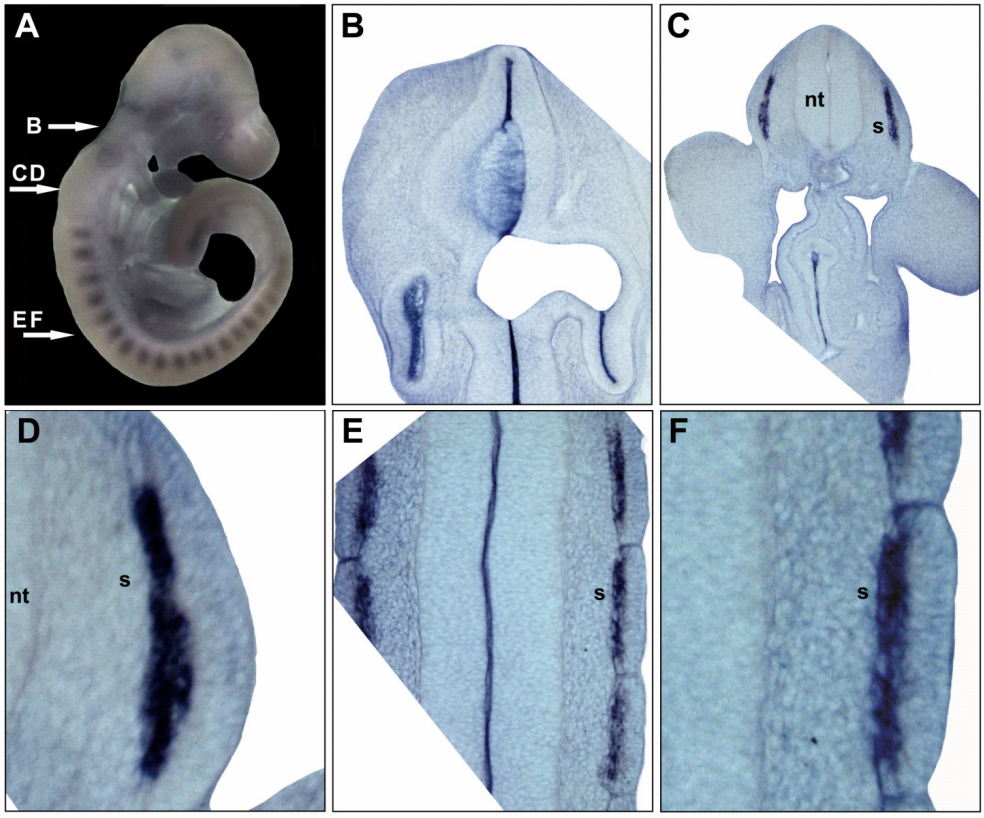


Fig. 15. Whole mount in situ hybridization of m-VITO-2 and vibratome sections of hybridized mouse embryos at E9.5 (A). Sections B-F were prepared in the transversal plane as indicated by arrows. No expression was detected in the prospective region of the midbrain (B). Sections C-F show an apparent expression in somites with signal restricted to the myotome (C,D). s, somite; nt, neural tube.

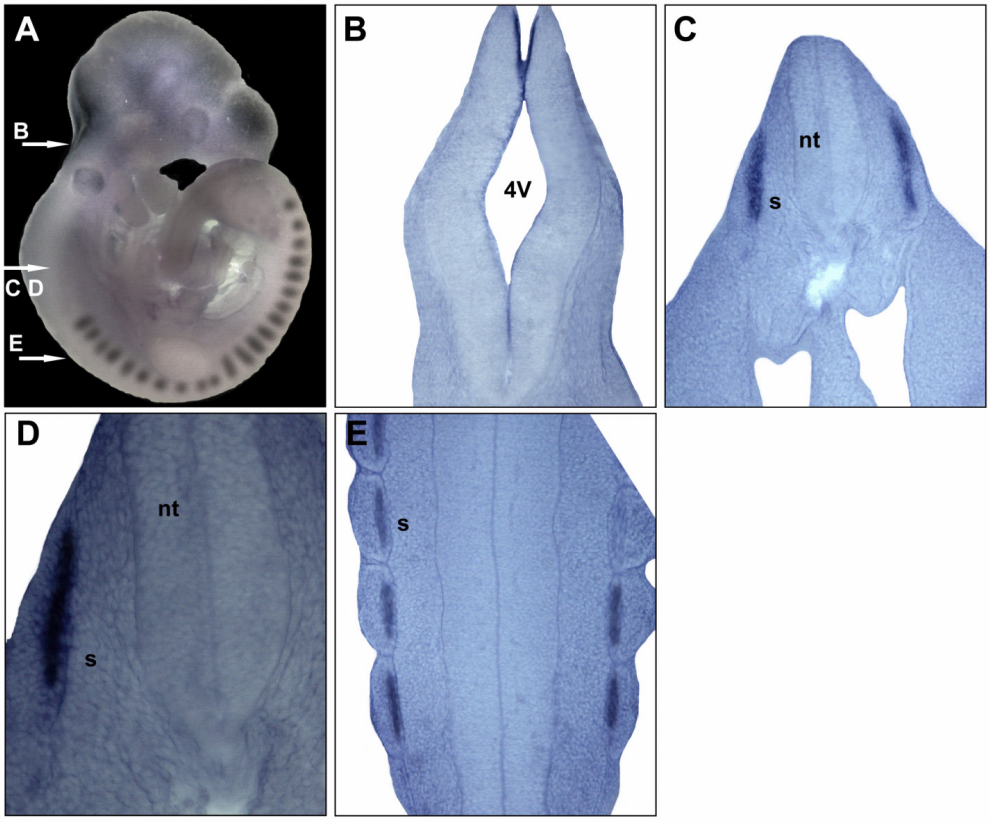


Fig. 16. Whole mount in situ hybridization of m-VITO-2 and vibratome sections of hybridized mouse embryos at E10.5 (A). Sections B-E were prepared in the transversal plane as indicated by arrows. No expression was detected in the prospective region of the midbrain (B). Sections C-E show an apparent expression of VITO-2 in somites with signal restricted to the myotome (C,D). s, somite; nt, neural tube.

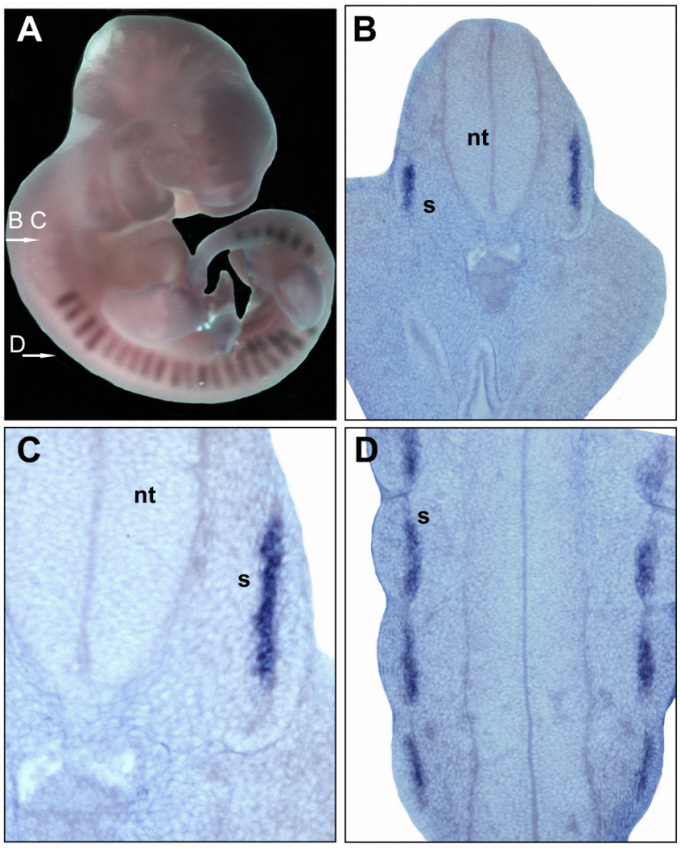


Fig. 17. Whole mount in situ hybridization of m-VITO-2 and vibratome sections of hybridized mouse embryos at E11.5 (A). Sections B-D were prepared in the transversal plane. Sections (B-D) show an apparent expression of VITO-2 in somites with signal restricted to the myotome (B,C). s, somite; nt, neural tube.

Since VITO-2 mRNA was detected in adult heart by RT-PCR (Fig. 12), localization of its transcripts was analyzed by in situ hybridization in adult heart sagital sections (3.2.5.4) to identify the origin of this signal. The same antisence VITO-2 probe as for whole mount in situ hybridization was hybridized to 10µm thick paraffin sections of adult mouse hearts. VITO-2 transcripts were detected in the upper atrial area of the heart (Figs. 18 A-D). Structures positives for VITO-2 seemed to be intracardiac ganglia (Figs. 18 A and a higher magnification B-D). Axons were negative for sense probe of VITO-2 as well as for antisense probe of VITO-1 (data not show). VITO-2 was not found in cardiomyocytes of the adult heart. To confirm its expression RT-PCR analysis was performed. Total RNA was obtained from isolated mouse adult cardiomyocytes after 36 hours in culture (kindly provided by Marion Wiensnet). VITO-2 specific

product was not amplified on RNA from isolated cardiomyocytes, further confirming the lack of VITO-2 expression in cardiac myocytes (Fig. 18 E.). Guided by the expression pattern of VITO-2 as indicated by situ hybridization, additional sets of RT-PCRs were performed. Total RNA was isolated from the atrial part of the heart positive for VITO-2 (Fig. 18 E – 1) and ventricles, which were negative for VITO-2 transcripts as judged by in sit hybridization results (Fig. 18 E -2).

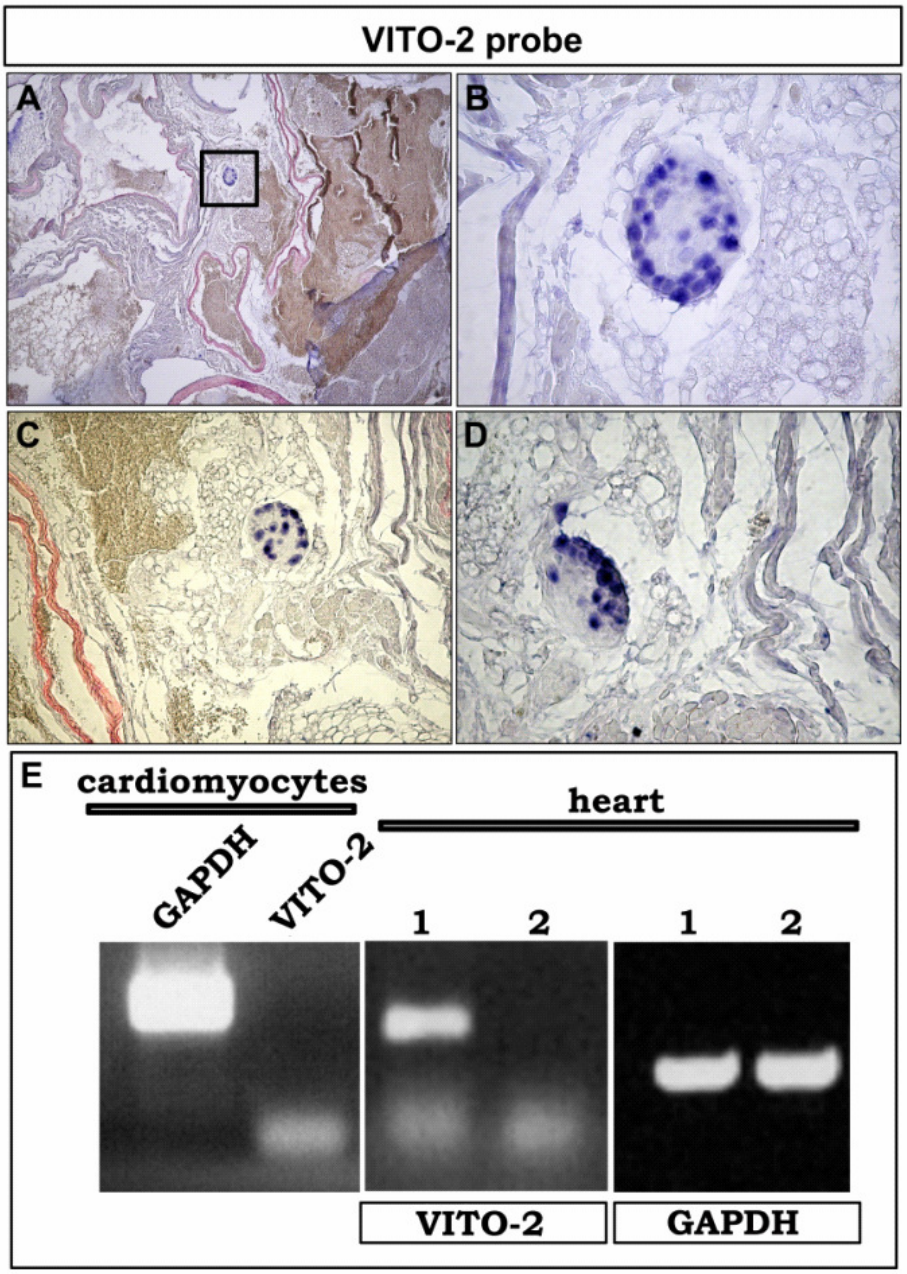


Fig. 18. In situ hybridization of m-VITO-2 in paraffin embedded sagital sections of the mouse adult heart (A-D). VITO-2 is present in the atrial area which contains nerves ganglia. VITO-2 was not found by RT-PCR (E) in adult cultured cardiomyocytes. Product of RT-PCR analysis was detected in the atrial area (E-1) but not in the ventricles of the adult mouse heart (E-2). Quality of RNA was normalized to mouse GAPDH

By the Northern blot analysis VITO-2 transcripts were also found in the adult brain, hence to find out whether its expression is restricted to specific structures of the adult brain, in situ hybridization were performed. Sagital sections of the paraffin embedded brain were hybridized to the mouse antisense VITO-2 probe. Signals of the VITO-2 transcripts were abundant in the cerebellar neurons (Fig. 19 A-D) including the purkinje cells (Fig. 19 C,D). Additional, VITO-2 transcripts were detected in the area of corpus callosum (Fig. 19 E) and in the cortical neurons close to the limbic hippocampus (Fig. 19 F). A strong expression was also noted in the cortex (Fig. 20 A,B,G,H) and a cortical population of neurons was positive for VITO-2 mRNA (Fig. 20 F,G,H). Neurons of subcortical nuclei were also positive for VITO-2, including pyramidal population of neurons (Fig. 20 C,D,E).

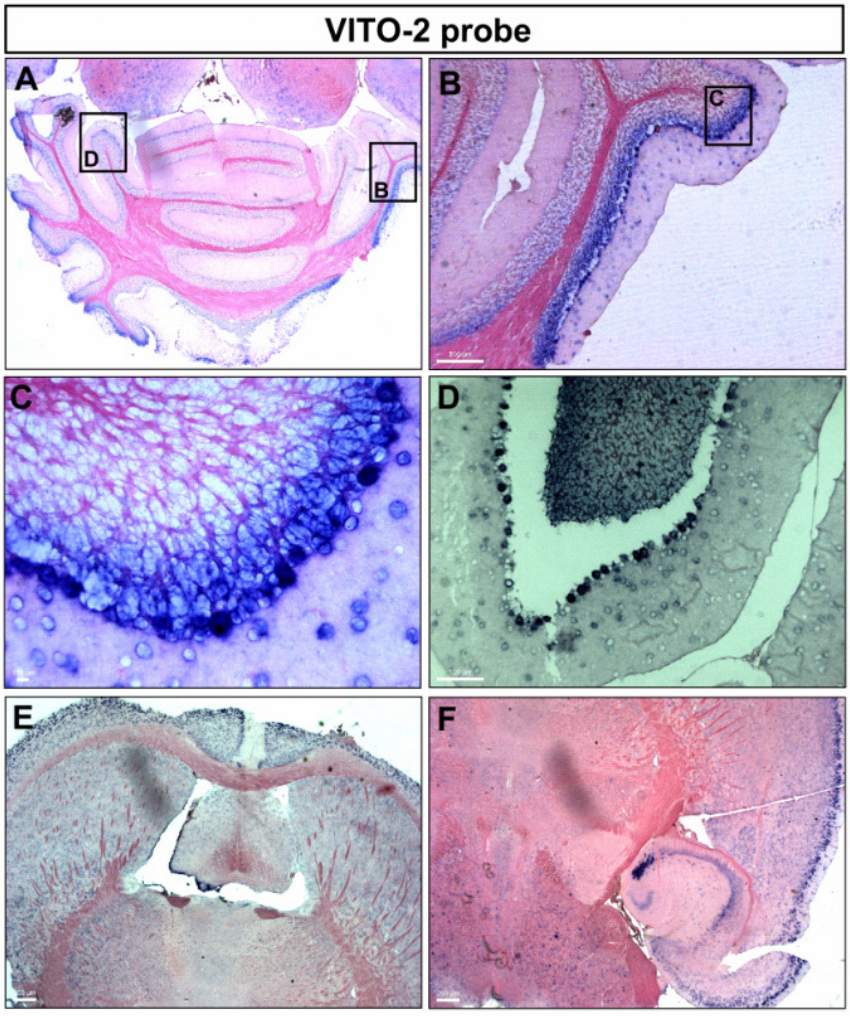


Fig. 19. Localization of VITO-2 mRNA by in situ hybridization in the adult mouse brain. Paraffin embedded sagital sections were hybridized with an antisense VITO-2 probe. VITO-2 transcripts were detected in neurons of cerebellum (A) including the purkinje cells (B,C,D). Signal was also detectable

in the cortical neurons, in the area of corpus callosum (E) and limbic hippocampus (F).

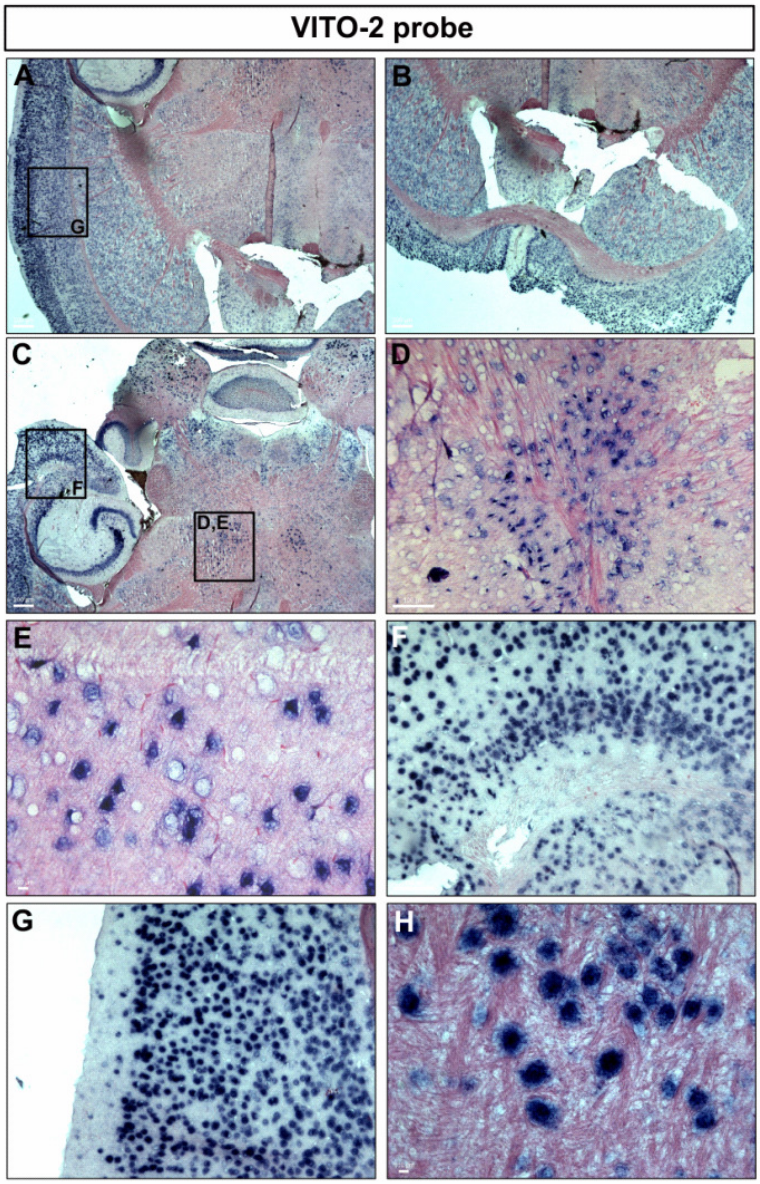


Fig. 20. Localization of VITO-2 mRNA by in situ hybridization in adult brain. Paraffin embedded sagital sections were hybridized with an antisense VITO-2 probe. VITO-2 transcripts were detected in neurons of the cortex (A,B and higher magnification G,H). The cortical population of neurons closed to the hippocampus (C,F) as well as in the subcortical nuclei area (C,D,E) expressed VITO-2. The pyramidal subpopulation of neurons is also positive for VITO-2 (E).

In contrast to VITO-2, no VITO-1 signals were detected in all areas of the brain which were positive for VITO-2 expression (data not show). Since VITO-2 is a potential co-activator of TEFs family its expression in the adult brain was correlated to the signal of mouse TEF-3 (3.2.3.2). A strong expression of TEF-3 was observed in the

similar regions where VITO-2 is normally expressed like cortical neurons (Fig. 21 A) and the purkinje cells of the cerebellum (Figs. 21 B-D).

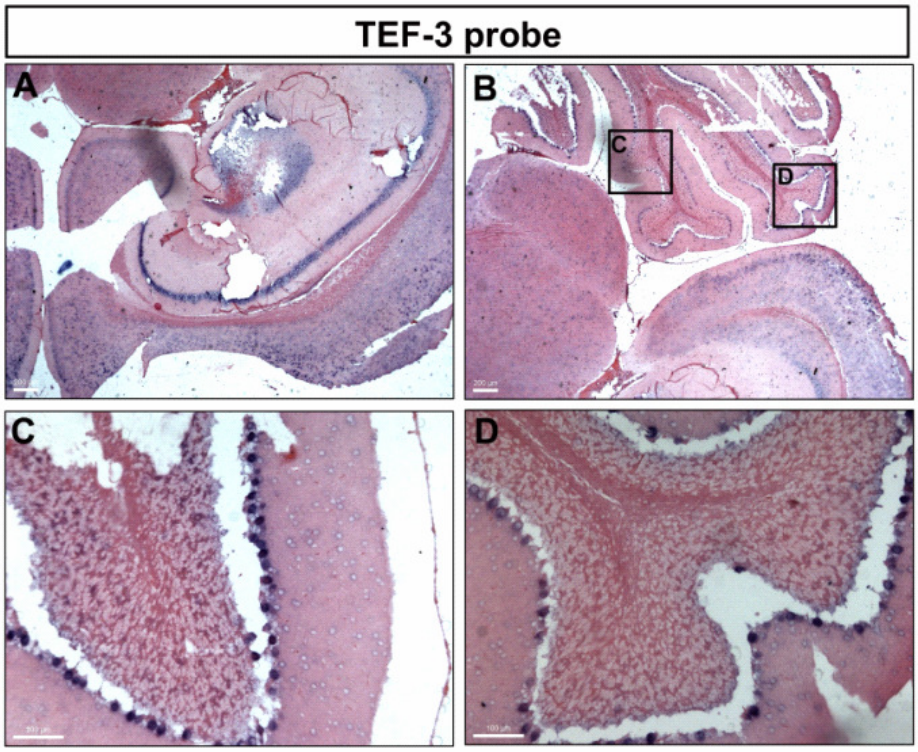


Fig. 21. Localization of TEF-3 transcripts by in situ hybridization in the adult brain. Paraffin embedded sagital sections were hybridized with an antisense TEF-3 probe. TEF-3 mRNAs were detected in the neurons of the cortex (A) as well as in the purkinje cells of the cerebellum (B-D).

4.3. Mouse Vgl-4 is ubiquitously expressed in the adult tissues

Human and mouse homologues of Vgl-4 have been described by Chen et al. (Chen et al. 2004) as potential members of the vestigial Drosophila and human TONDU and VITO family of genes as described in chapter 4.1. A mouse Vgl-4 cDNA was obtained as EST clone (NM_177683) from the RZPD. The clone was verified by sequencing. Northern blot was performed using P^{32} - random primed 950bp EcoRI fragment from the pCR-TOPOII-m-Vgl-4 plasmid. Expression of m-Vgl-4 was analyzed at different stages of mouse embryonic development from E11.5 to E15.5 as well as in adult tissues (Fig. 22). Vgl-4 transcripts (1.8 kb) were detected at all developmental stages tested and in the various adult mouse tissues. The Northern blot

analysis of m-Vgl-4 revealed a broad expression pattern of this gene during embryogenesis and in adult tissues (Fig. 22).

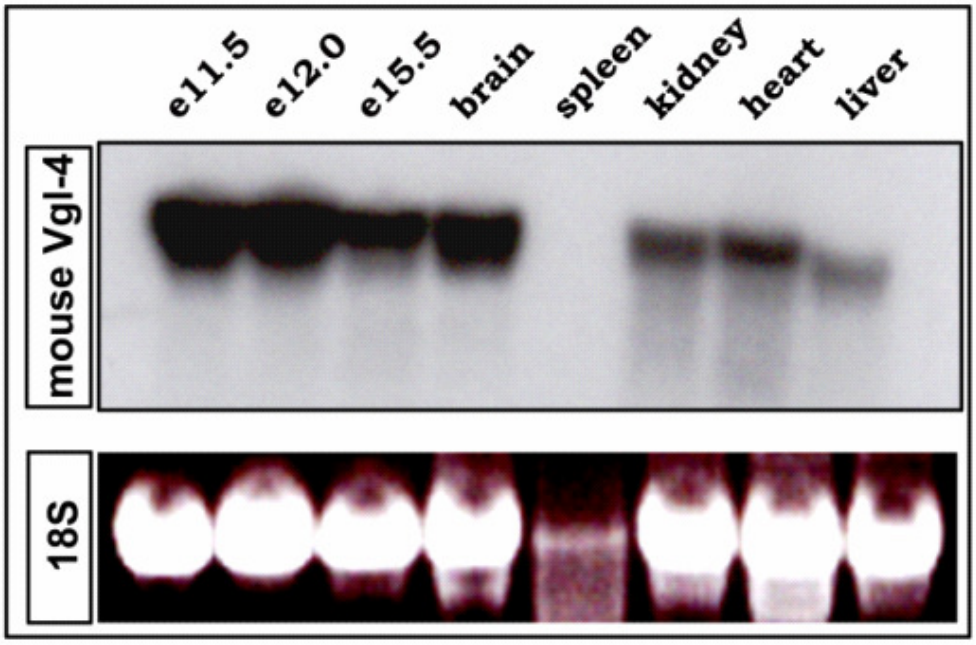


Fig. 22. A mouse-Vgl-4 probe was hybridized to the 50 µg of total RNA isolated from the different stages of mouse development as well as from adult tissues like the brain, kidney, heart and liver. An equal amount of RNA was normalized by visualization of 18S rRNA by Ethidium Bromide.

To localize m-Vgl-4 mRNA expression in situ hybridization method was applied. Vgl-4 expression was found in the intraporitoneal part of midgut (Fig. 23 B), the cranial lobe of the lungs (Fig. 23 C), the submandibular gland (Fig. 23 E) as well as in the right lobe of the thyroid gland (Fig. 23 F). Vgl-4 transcripts were also found in the pectoralis superficialis and the pectoralis profundus muscles (Fig. 23 A). In addition, in the heart a week expression of Vgl-4 was noted in the trabeculated wall of ventricles at this stage (Fig. 23 D). Sense probe did not give any signal (data not show).

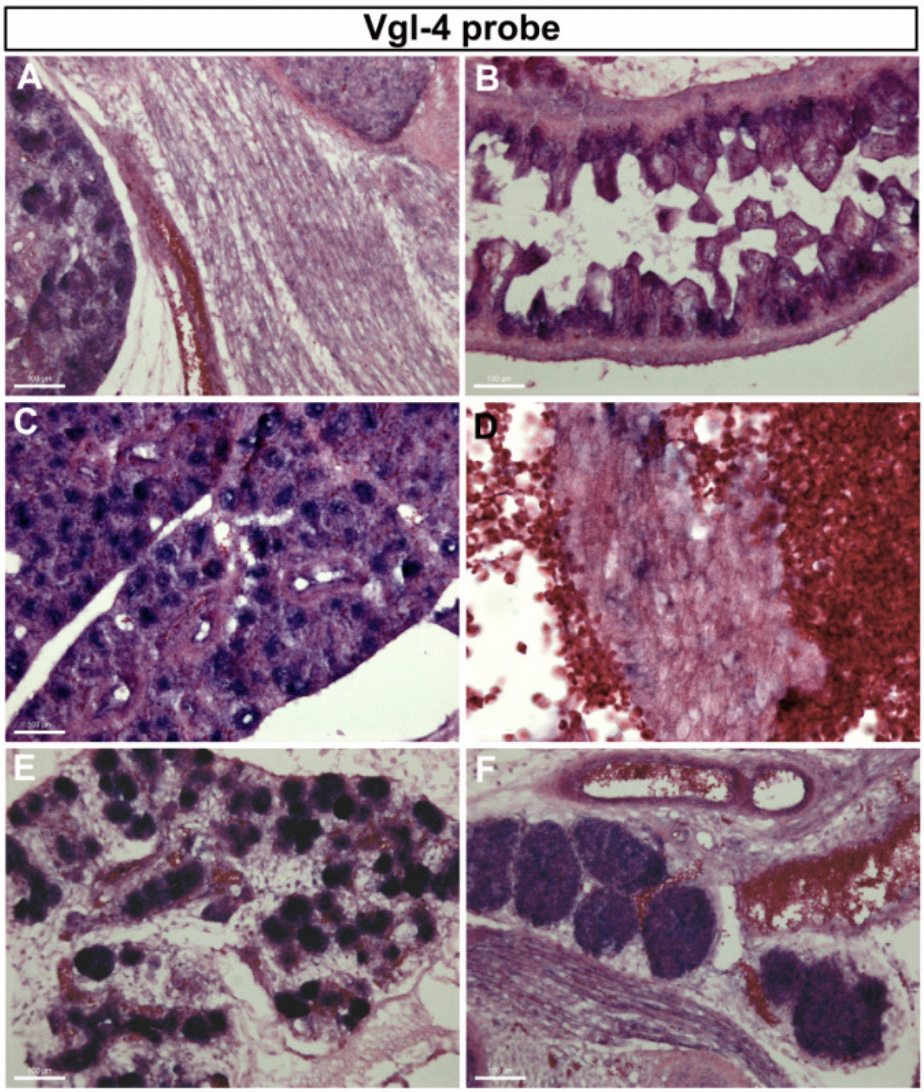


Fig. 23. Localization of mouse Vgl-4 transcripts in embryos at E15.5. An antisense mouse Vgl-4 probe was hybridized in the sagital paraffin embedded sections of embryos at E15.5. Mouse Vgl-4 mRNA was detected in skeletal muscles (A), part of the midgut (B), the cranial lobe of the lungs (C), the heart (D), the submandibular gland (E) and the thyroid gland (F).

4.4. Expression of TEFs and their co-activators is modulated during the course of C2C12 myoblasts differentiation

To assess the expression pattern of TEFs and their known co-activators during myotubes formation, the C2C12 cell line was differentiated into myotubes as describe in Materials & Methods (3.2.6.2). During the course of differentiation cells were harvested every 24 hours and total RNA was isolated from two plates. Semiquantitative RT-PCR analysis was performed using specific primers as listed in 3.1.7.2.

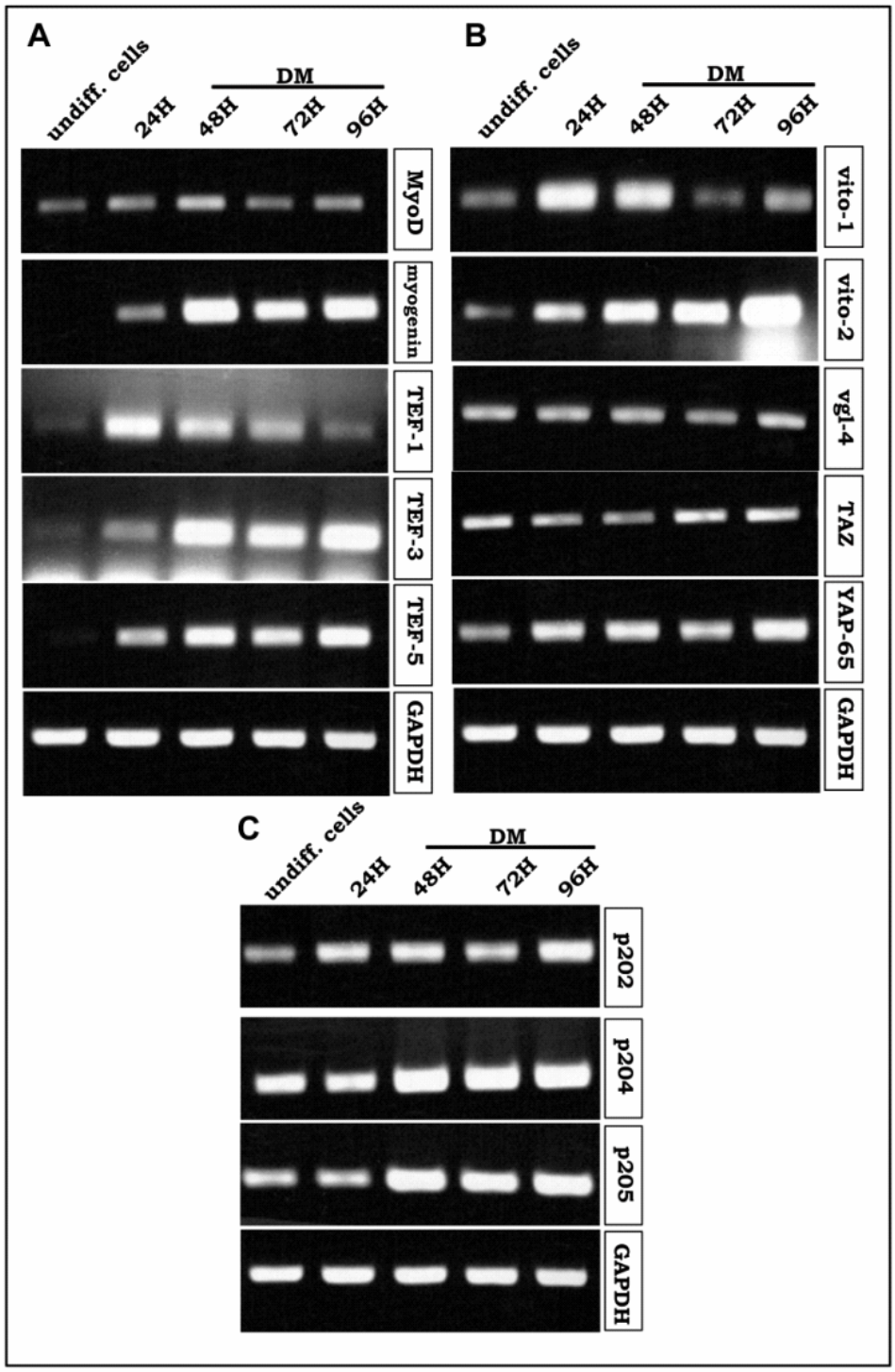


Fig. 24. Expression kinetics of TEFs and their co-activators during differentiation of C2C12 cells into myotubes. Panel A shows expression of MyoD and myogenin (used as positive markers) with 27 cycles as well as TEF1/3/5 with 27/30/30 cycles respectively. Panel B shows expression of known co-activators of TEF members, like Vgl-4 (27cycles), TAZ (27 cycles), YAP-65 (25 cycles) and the VITO family of genes i.e. VITO-1 (27 cycles) and VITO-2 (29 cycles). Panel C shows expression of various members of the p200 family: p202, p204 and p205 (27 cycles). An equal amount of RNA was monitored by the level of GAPDH PCR product (22 cycles).

The first change in expression of monitored genes was observed 24 hours after replacing medium with differentiation medium (DM). At this time point a strong up-regulation was noted for myogenin, TEF-1/5 (Fig. 24 A), VITO-1 (Fig. 24 B) and slight changes for TEF-3 (Fig. 24 A), VITO-2 and YAP-65 (Fig. 24 B) as well as p202 (Fig. 24 C) were observed. One day later (48 hours in culture) a significant up-regulation of MyoD, myogenin, TEF-3 (Fig. 24 A), VITO-2 (Fig. 24 B) was found. The moderate increase of expression at this time point of C2C12 differentiation was found in the case of TEF-5 (Fig. 24 A), p204 and p205 (Fig. 24 C). At the third day of C2C12differentiation, a down-regulation of TEF-1 and MyoD (Fig. 24 A) and VITO-1 (Fig. 24) was noted. Terminally differentiated myotubes displayed a significant up-regulation of TEF-3 (Fig. 24 A) and VITO-2 expression (Fig. 24 B), whereas myogenin, TEF-1 (Fig. 24 A) and VITO-1 (Fig. 24 B) were down-regulated. TAZ and Vgl-4 expression (Fig. 24 B) were not affected during the course of myotubes formation.

The expression levels of TEFs and their co-activators during the course of C2C12 differentiation were referred to the known markers i.e. MyoD and Myogenin as well as interferon inducible p200 family (Liu et al. 2000; Liu et al. 2002; Asefa et al. 2004, Zhu et al. 2004).

4.5. Intracellular localization of VITO1/2 proteins in the various cell lines

Since VITO-1/2 proteins are potential co-activators of TEFs to play their role they have to co-localize in the same compartment of the cells and to take part in the transcriptional machinery have to be translocated to the nucleus. To investigate the localization of VITO-1/2 in different cell lines, VITO-1 and VITO-2 were cloned into the pDsRED-5.1 vector with a N-terminal DsRed tag making possible their visualization. TEF-3 and Vgl-4 were cloned into the pEGFP-C2 vector with a C-terminal EGFP tag (3.2.3.1).

In contrast to the ubiquitously expressed VITO-2, Vgl-4 and TEFs proteins, VITO-1 expression is restricted to the skeletal muscles. For this reason C2C12 cells were chosen to analyze the intracellular localization of VITOs and TEFs. Twenty-four hours after transfection VITO-1, VITO-2, Vgl-4 as well as TEF-3 were found in the cytoplasm (Figs. 25 A,C,E,G). However, 48 hours after transfection they were

translocated into the nucleus where they might play a role in the regulation of transcription (Figs. 25 B,D,F,H).

The 10T1/2 mouse fibroblast cell line is able to convert into myotubes after demethylation or MyoD transfection but does not differentiate to muscle cells without induction. Therefore the localization of VITOs and TEFs was also studied in this cell line. Interestingly, all factors showed an identical behavior as in C2C12 cells. Again 24 hours after transfection all proteins were localized in cytoplasm (Fig. 26 A,C,E,G) followed by translocation into the nucleus 24 hours later (Fig. 26 B,D,F,H).

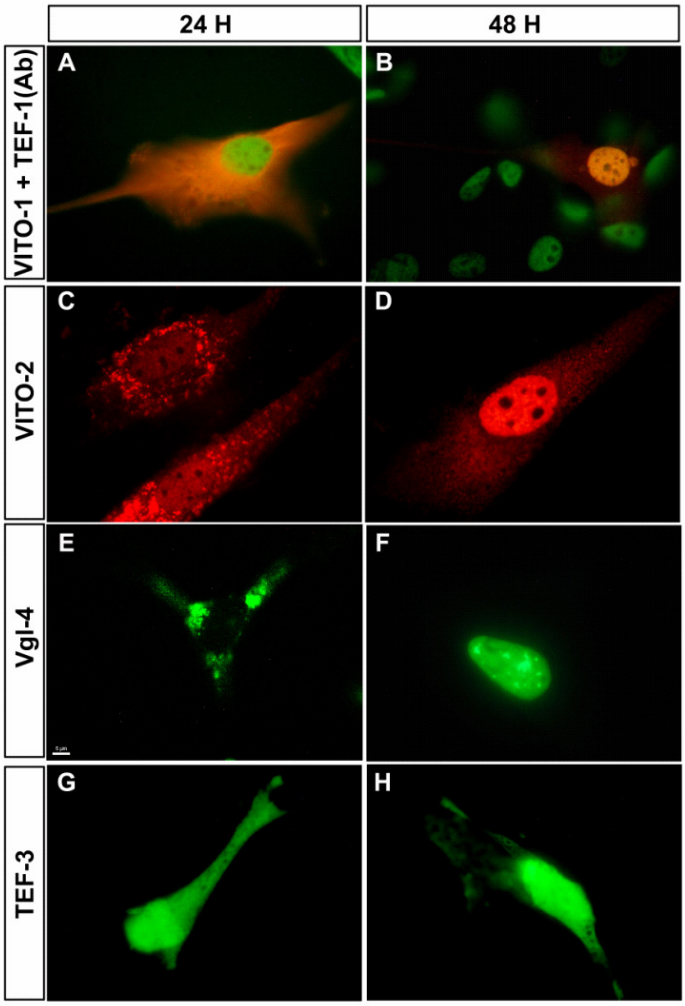


Fig. 25. Localization of VITO-1 (red) and TEF-1 (detected with anti-TEF-1 antibody and Alexa 488) (A,B), VITO-2 (C,D), Vgl-4 (E,F) and TEF-3 (G,H) in C2C12 cells.

The last tested cell line was 293T, in which VITO-1 is not expressed (data not show). It was interesting to disclose whether ectopically expressed VITO-1 might show a similar localization and translocation as in the previously described cell lines. In contrast to C2C12 and 10T1/2 cells, VITO-1 remained in cytoplasm of 293T cells even

48 hours after transfection (Figs. 27 A,B), whereas VITO-2, Vgl-4 and TEF-3 were translocated into nucleus (Figs. 27 C-H). Only cotransfection of VITO-1 together with TEF-3, but not TEF-1 resulted in translocation of VITO-1 to nucleus in 293T cells (Fig. 28).

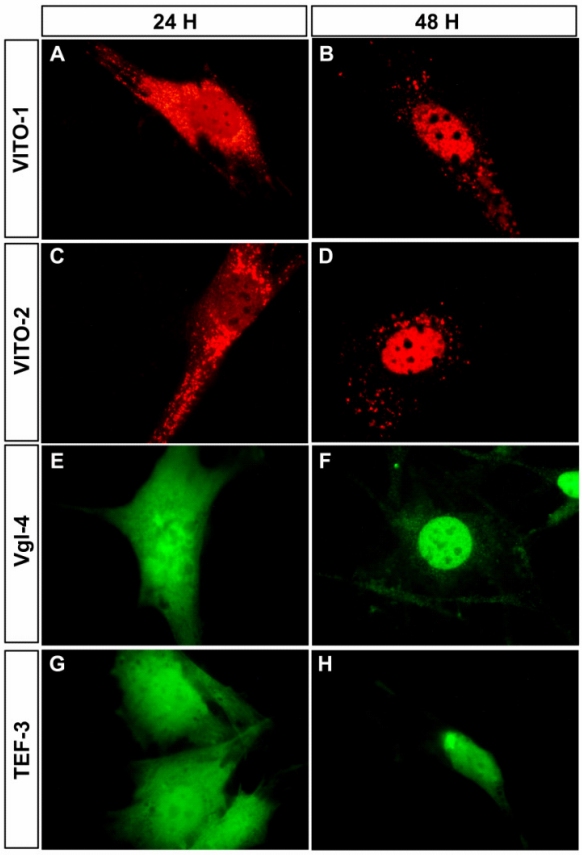


Fig. 26. Localization of VITO-1 (red) (A,B), VITO-2 (red) (C,D), Vgl-4 (green) (E,F) and TEF-3 (green) (G,H) in 10T1/2 cells.

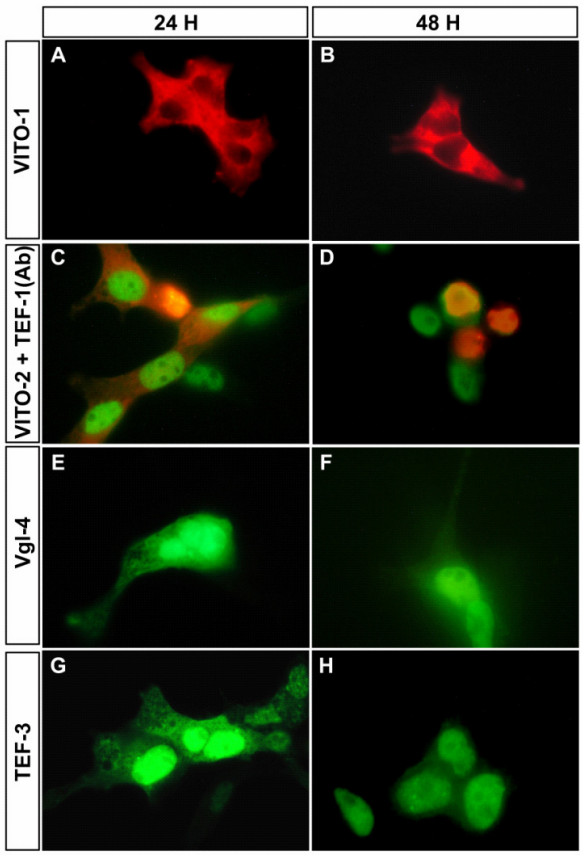


Fig. 27. Localization of VITO-1 (red) (A,B), VITO-2 (red) and TEF-1 (detected with anti-TEF-1 antibody and Alexa 488) (C,D), Vgl-4 (green) (E,F) and TEF-3 (green) (G,H) in 293T cells.

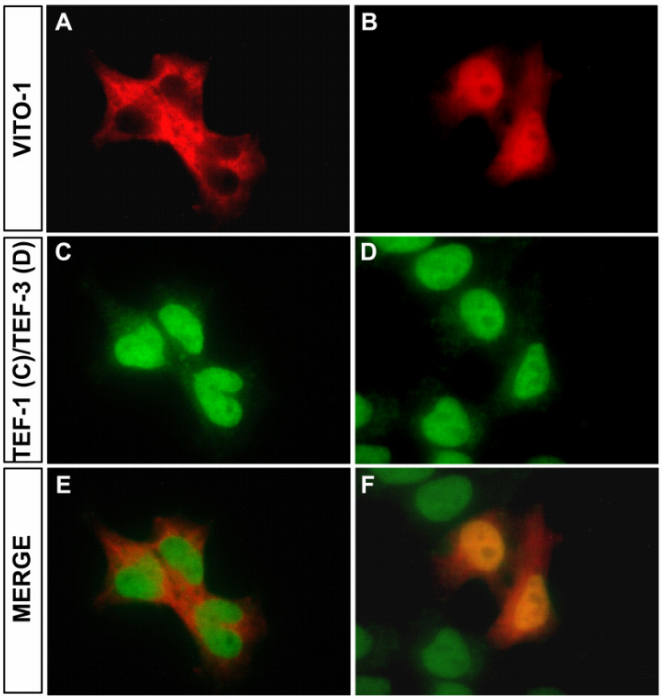


Fig. 28. Colocalization of VITO-1 (red) and TEF-1 (detected with anti-TEF-1 antibody and Alexa 488) (A,C,E), VITO-1 (red) and TEF-3 (green) (B,D,F) in 293T cells 48 hours after transfection.

4.6. VITO-1 but not VITO-2 enhances MyoD-mediated myogenic conversion of fibroblasts

MyoD is known to be a central component of the myogenic network. The fibroblasts cell lines 10T1/2 and 3T3 can adopt muscles characteristics as well as typical muscles cell markers e.g. MyHC when MyoD is ectopically expressed (Taylor et al. 1979; Davis et al. 1987; Braun et al. 1989; Choi et al. 1990). To determine whether VITO-1/2 genes can also play an important role in the muscles specification program, a MyoD mediated transdifferentiation assay of 10T1/2 and 3T3 cell lines was performed. Both cell lines were transfected either by electroporation or by FUGENE 6 reagent according to the manual (ROCHE) with different combinations of plasmids, to express following genes: pEMSV-MyoD alone or together with pCS2-VITO-1/2, pDsRED-VITO-1/2 as well as pCS-VITO-1/2 alone. Transfection efficiencies were normalized to co-transfected p-Hook-LacZ or pEGFP-C2 vectors.

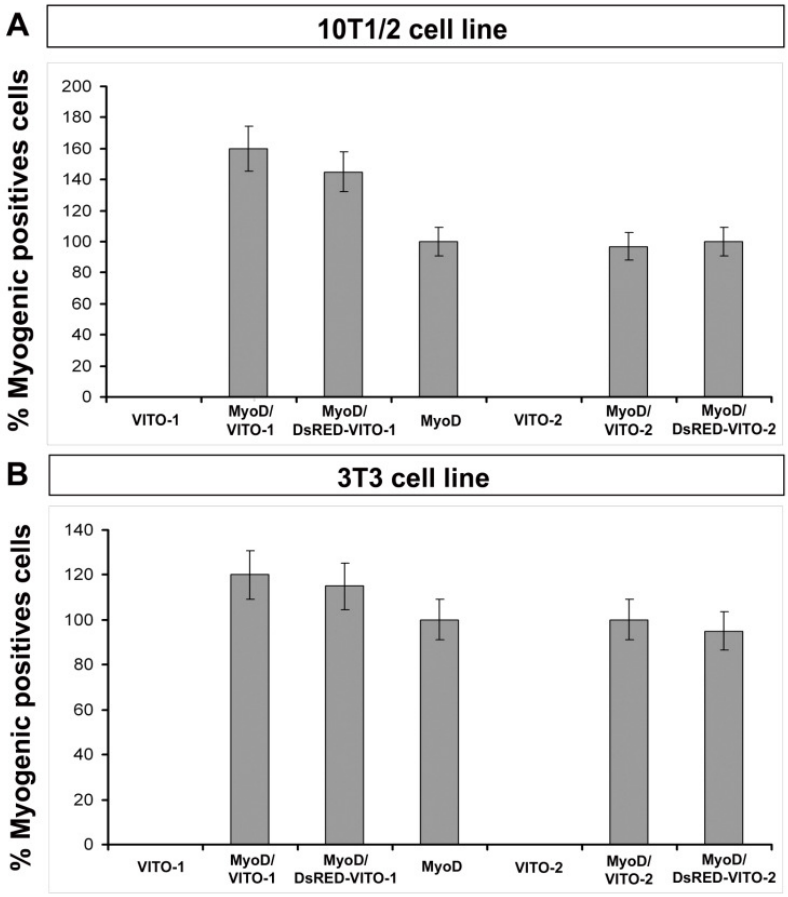


Fig. 29. Results of the myogenic conversion of fibroblasts cell lines 10T1/2 (A) and 3T3 (B). Both cell lines were transfected with the following combinations of plasmids: pCS2-VITO-1, pEMSV-MyoD/pCS2-VITO-1, pEMSV-MyoD/pDsRED-VITO-1, pEMSV-Myod, pCS2-VITO-2, pEMSV-MyoD/pCS2-VITO-2, pEMSV-MyoD/pDsRED-VITO-2. The total amount of DNA was equal in all transfections and supplemented with pCS2 vector if necessary. The number of MF20 or myogenin positive cells obtained after transfection of MyoD was set as 100%. Transfections efficiencies were standardized against β-gal activity or GFP fluorescence.

Transfected cells were counted and normalized against β-gal activity or GFP fluorescence. Cultured cells were stained with MF20 or myogenin antibody followed by detection with DAB or with Alexa 594 depending on the control plasmid (β-gal vector or pEGFP respectively). Both used methods yielded similar results. The number of myogenic cells obtained after transfection was normalized against control plasmids and set as 100% when both cell lines were transfected with MyoD alone. Neither VITO-1 and VITO-2 alone were not able to initiate myogenic conversion of fibroblasts (Fig. 29 A,B). However, expression of native VITO-1 as well as VITO-1 linked to the DsRED tag together with MyoD significantly increased myogenic conversion (Fig. 29). When VITO-1 was used as fused protein to the DsRED tag, myogenic conversion was at the

level of 145% (10T1/2) and 115% (3T3) (Fig. 29 A,B). In contrast, VITO-2 co-transfected together with MyoD was not able to increase myogenic conversion in both tested cell lines (Fig. 29 A,B). Three different plasmids carrying cDNA of VITO-2 were used: pCS2-VITO-2, pDsRED-VITO-2, GAL4-VITO-2.

The same set of experiments was done on 293T cell line. Twenty-four hours after transfection, medium was replaced and cells were growth in 5% HS for 4-5 days. Similarly to fibroblast myogenic conversion, VITO-2 alone or in combination with MyoD was not efficient to induce transdifferentiation of 293T cells (data not show). Surprisingly, when 293T cells were co-transfected with combination of three plasmids i.e. VITO-1, MyoD and TEF-3, they start express muscles cell marker MyHC but they were not able to form myotubes (Fig. 30), even after 7-8 days in culture (data not show).

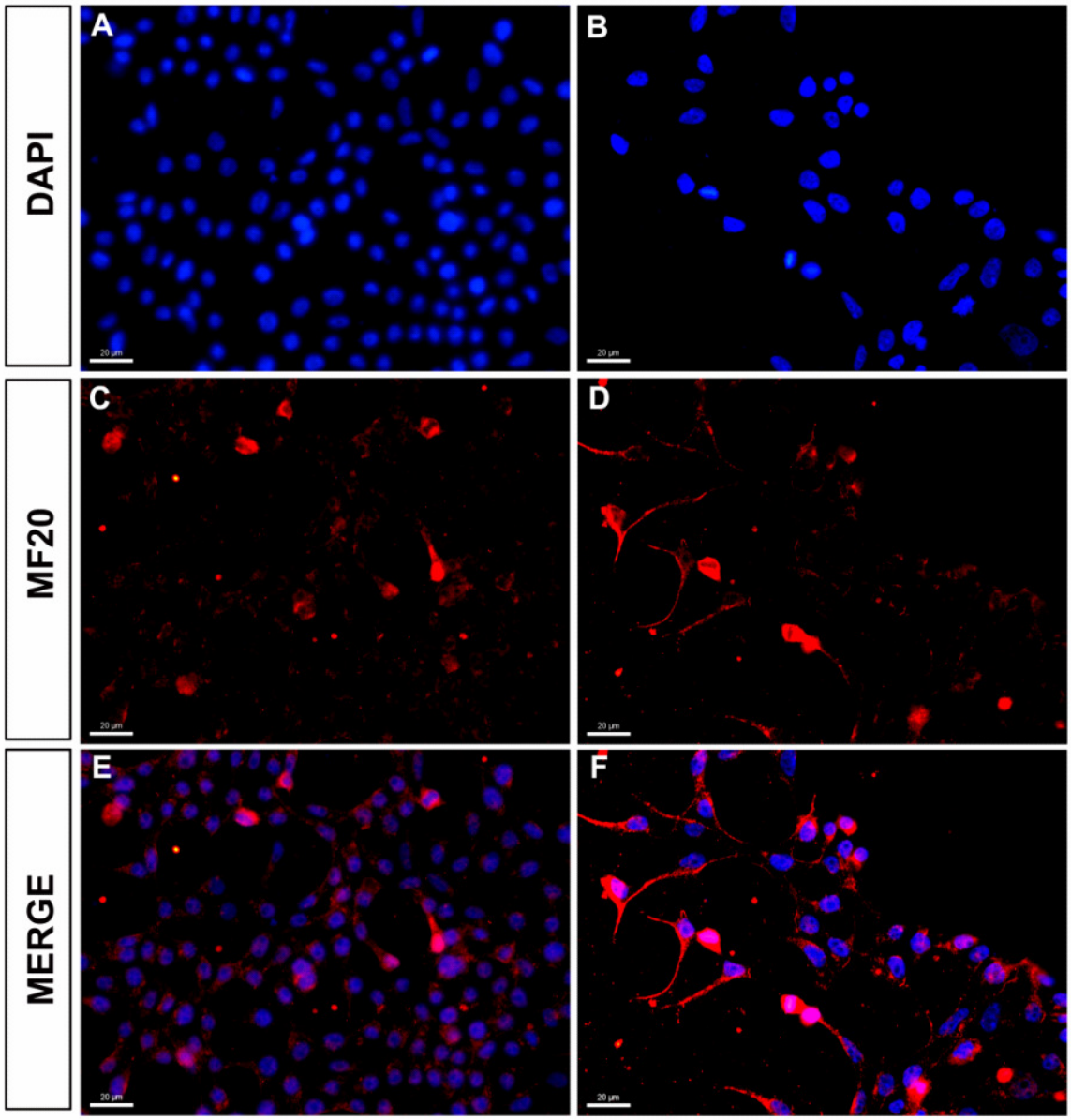


Fig. 30. The myogenic conversion of 293T cell line by co-transfection of MyoD, VITO-1 and TEF-3. After 5 days in culture cells were stained with MF20 antibody (red) (C,D), nucleus were visualized by DAPI staining (A,B), and merged with antibody stained cells (E,F).

In this experiment after 5 days in culture cells were stained with MF20 antibody. No MyHC expression was observed when 293T cells were transfected with MyoD alone, VITO-1/2 alone VITO-1/TEF-3, or VITO-2/MyoD (data not show).

Since VITO-1 showed unique properties to enhance MyoD depended myogenic conversion of fibroblasts the expression profile of VITOs and TEFs in these cell lines had to be verified. TEFs members as well as VITO-1/2 and Vgl-4 products were amplified using specific primers (3.1.7.2).

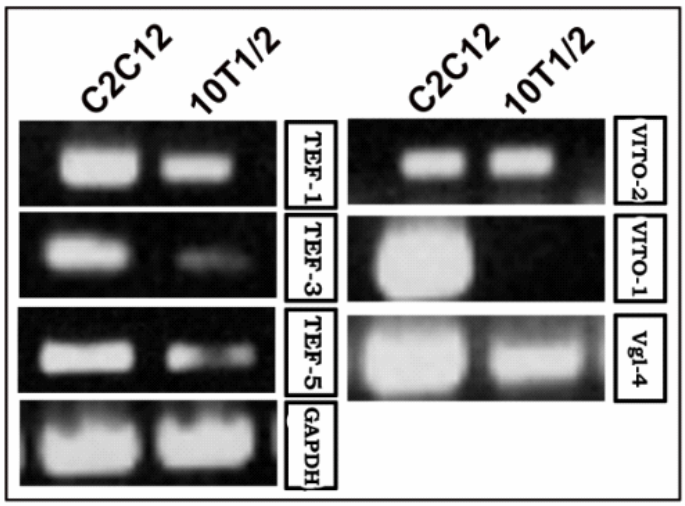


Fig. 31. RT-PCR analysis of TEF-1/3/5, VITO-1/2 as well as Vgl-4 using a total RNA isolated from fibroblast 10T1/2 cell line and myoblasts C2C12 cells. The quality of RNA was monitored by expression of the houkeeping gene mouse GAPDH.

TEF1/3/5 transcripts were detected in both cell lines. Similar results were obtained for VITO-2 and Vgl-4 mRNA. In contrast, only VITO-1 PCR product was not detectable in fibroblast cell line whereas in dividing myoblasts was present (Fig. 31).

4.7. Knockdown of VITO-1/2 genes by RNAi

The requirement of VITO family genes for MyoD mediated conversion of mesenchymal cells suggested an important role in the myogenic differentiation. However only VITO-1 was found to enhance trandifferentiation of fibroblast into

myotubes although VITO-2 is also abundantly expressed during mouse embryonic development and is up-regulated in the terminal step of C2C12 differentiation.

4.7.1. VITO-1 and VITO-2 can be knocked-down efficiently using RNAi

To knock down VITO family genes by transient transfections in cell lines a pSUPER vector was used (generous gift from Prof. Agami). pSUPER vector is based on the polymerase III H1-RNA gene promoter and was designed to generate small interfering RNA. Specific sequences against VITO-1, VITO-2 and myogenin were designed according to the principles described by Brummelkamp (Brummelkamp et al. 2002) and are listed in Materials & Methods in 3.1.7.4. To knock down VITO-1 gene, three different sequences were designed, cloned into the pSUPER vector and named pSUPER-VITO-1A, pSUPER-VITO-1B and pSUPER-VITO-1C. The ability to repress VITO-1 transcripts was evaluated by co-transfection of either pSUPER-VITO-1A/B/C with pDsRED-VITO-1 in 10T1/2 cells. Only pSUPER-VITO-1A and pSUPER-VITO-1B were able to down-regulate VITO-1 transcipts fused to the DsRED tag efficiently. The level of DsRED protein was monitored by fluoresce measurement of total protein lysate isolated from transfected cells. Results are presented in Fig. 32 B. The pSUPER-VITO-1B plasmid seemed to be the most efficient (>90%) to knock-down VITO-1 transcripts followed by pSUPER-VITO-1A (>75%). Fig. 32 A illustrates the sequences chosen for the RNAi design.

Since myogenin is known to be indispensable for C2C12 myoblasts differentiation I decided to knock-down myogenin and this served as a positive control for a molecule affecting muscle cells differentiation. Three different sequences were selected and cloned into pSUPER vector. The efficiency to down-regulate myogenin mRNA was monitored by staining with a myogenin antibody. A strong reduction of cells positive for myogenin (>80%) was observed in the case of only one out of three tested plasmids (data not show). The construct, which efficiently down-regulated myogenin expression was called pSUPER-myogenin and used for further experiments.

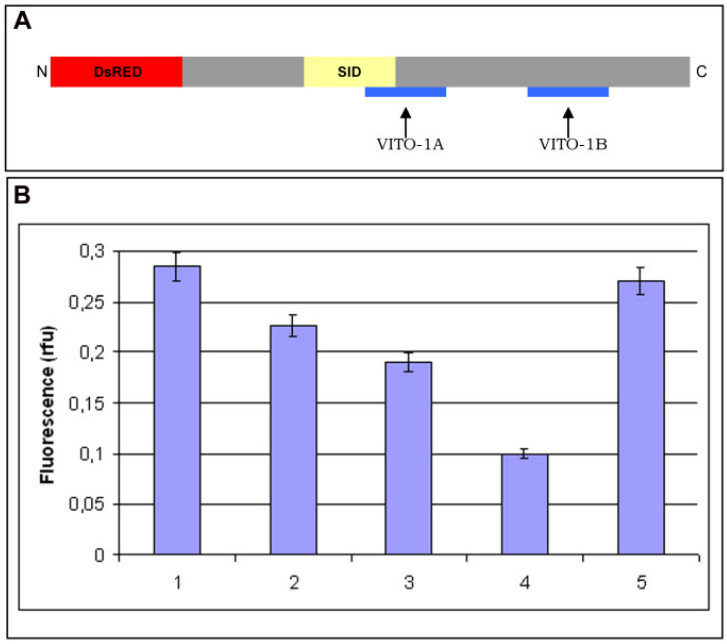


Fig. 32. Position of sequences used to knock-down VITO-1 transcripts. Control plasmid with VITO-1 linked to the N-terminal DsRED tag with SID domain yellow shaded box (A). Cumulative results of the ability do down-regulate VITO-1 transcripts are shown in panel (B); 10T1/2 cell line were transfected with a combination of different plasmids: 1- pDsRED-VITO-1/pSUPER; 2- pDsRED-VITO-1/pSUPER-VITO-1A; 3- pDsRED-VITO-1/pSUPER-VITO-1B; 4- pDsRED-VITO-1/pSUPER-VITO-1A at higher concentration; 5- pDsRED-VITO-1/pSUPER-myogenin.

To select sequences, which are able to down-regulate VITO-2 specifically, a similar approach was chosen. The four sequences within the VITO-2 cDNA, which were selected to construct knock-down plasmids, are listed in chapter 3.1.7.4 of Materials & Methods. Similar to VITO-1, oligonucleotides were cloned into pSUPER vector (3.2.3.1) and named pSUPER-VITO-2A, pSUPER-VITO-2B, pSUPER-VITO-2C and pSUPER-VITO-2D. The efficiency in repression of VITO-2 transcripts was proved by co-transfection of either pSUPER-VITO-2A/B/C/D with pDsRED-VITO-2 in 293T cells. Only pSUPER-VITO-2A and pSUPER-VITO-2B showed satisfying levels of down-regulation of VITO-2 transcipts fused with DsRED tag (Fig. 33). Transfected 293T cells were examined by FACS (3.2.6.6) or by RT-PCR analysis. The strongest reduction of DsRED positive 293T cells was observed with pSUPER-VITO-2B (Figs. 33 B-F). Similar observation was obtained by RT-PCR using specific primers for mouse VITO-2 (Fig. 33 G).

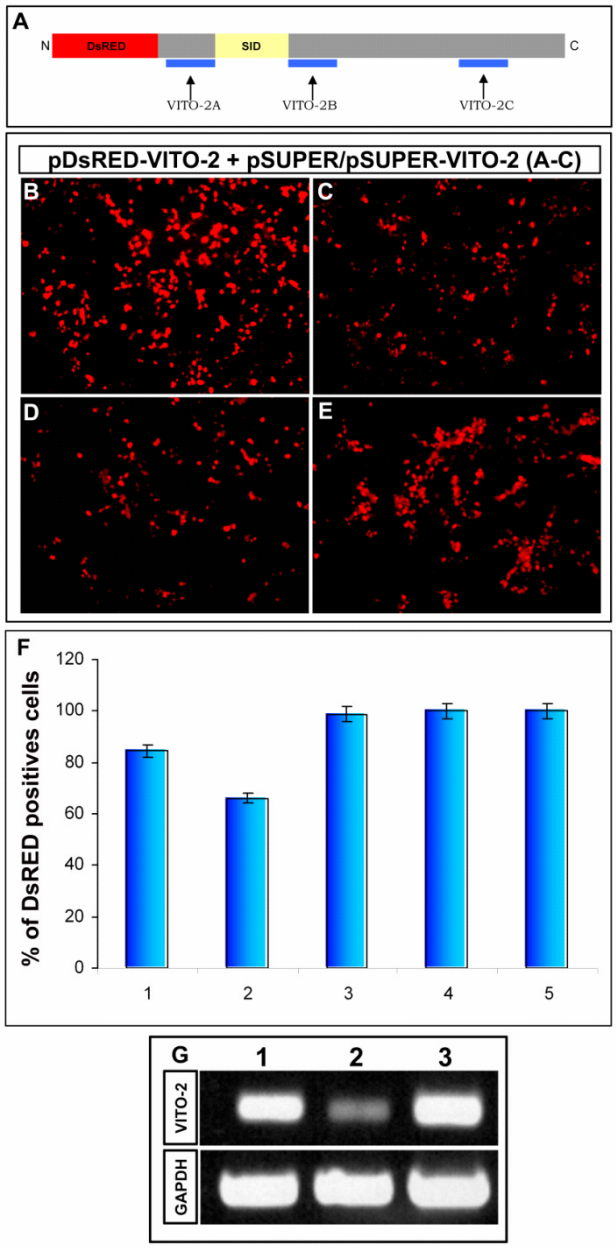


Fig. 33. Positions of selected sequence for RNAi mediated VITO-2 knock-down are shown in panel (A). 293T cells were trasfected with different combinations of plasmids: pDsRED-VITO-2 and pSUPER (B), pDsRED-VITO-2 and pSUPER-VITO-2A (C), pDsRED-VITO-2 and pSUPER-VITO-2B (D), pDsRED-VITO-2 and pSUPER-VITO-2C (E). An equal amount of DNA was used for each transfection using a 1:5 ratio (pDsRED-VITO-2: pSUPER/ pSUPER-VITO-2A/B/C). Cumulative results of FACS analysis of 293T cell lines (B-E) transfected with pDsRED-VITO-2 and all versions of pSUPER-VITO-2 plasmids (F): 1 – pDsRED-VITO-2/pSUPER-VITO-2A; 2- pDsRED-VITO-2/pSUPER-VITO-2B; 3- pDsRED-VITO-2/pSUPER-VITO-2C; 4 - pDsRED-VITO-2/pSUPER-myogenin; 5- pDsRED-VITO-2/pSUPER. Panel G shows RT-PCR analysis of VITO-2 expression on total RNA isolated from 293T cells 48H after transfection with 1- pDsRED-VITO-2/pSUPER-VITO-2A; 2- pDsRED-VITO-2/pSUPER-VITO-2B; 3- pDsRED-VITO-2/pSUPER. An equal amount of RNA was controlled by expression of GAPDH.

4.7.2. Attenuation of VITO-1 but not VITO-2 inhibits MyoD mediated conversion of 10T1/2 and 3T3 cells

In the previous section I showed that VITO-1 but not VITO-2 enhances MyoD mediated transdifferentiation (4.6). This results, however does not answer the question whether either VITO-1 or VITO-2 are required and necessary for efficient conversion of fibroblasts by MyoD. In order to achieve repression of VITO-1 and VITO-2 in MyoD transfected fibroblasts cell lines, an RNAi approach was used. Selection of sequence that can specifically target VITO-1 and VITO-2 transcripts was described in 4.7.1. Additionally, myogenin which is a known factor necessary for terminal differentiation and acts downstream of MyoD (Braun et al. 1989) was also knocked-down using the RNAi technique. Efficiencies of transfected cells were normalized by counting GFP positives cells (when pEGFP-C2 was used as control vector) or β-gal positive cells (when pHook-LacZ was used as control vector). After 4 days of culture in 5%HS 10T1/2 and 3T3 cells were tested for β-gal activity and then stained with MF20 or myogenin antibodies followed by detection with DAB. In case, when p-EGFP-C2 was used for monitoring transfection efficiencies both cell lines were also stained with MF20 or myogenin antibodies and detected with fluorochrom conjugated secondary antibody. Transfection of MyoD alone or co-transfection of MyoD in combination VITO-1 or VITO-2 served as positive control, as described in chapter 4.6. Every set of transfection was made in triplicate.

As expected, inhibition of expression of myogenin in converted 10T1/2 and 3T3 cells by co-transfection of pSUPER-myogenin and MyoD resulted in robust inhibition of myogenic differentiation with 83% reduction for 10T1/2 cells and 44% reduction in case of 3T3 cell line. Knock down of VITO-1 expression by co-transfection of pSUPER-VITO-1A and MyoD let to a 64% and 50% reduction in conversion of 10T1/2 and 3T3 cells to myotubes, respectively. In contrast no reduction of myogenic conversion of 10T1/2 and 3T3 cell lines was observed in case of co-transfection of MyoD and pSUPER-VITO-2B. Statistically not significant differences in the degree of inhibition of converted fibroblasts cells in both cell lines between MyoD/pSUPER-VITO-1A and MyoD/pSUPER-myogenin were noted. Figure 34 shows an example of 10T1/2 converted cells transfected with MyoD together with pCS2-VITO-1 (A,E) MyoD and pSUPER (B,F), MyoD/pSUPER-VITO-1A (C,G) and MyoD/pSUPER-

myogenin (Fig. 34 D,H). The results of the transfection experiments are summarized in Fig. 35.

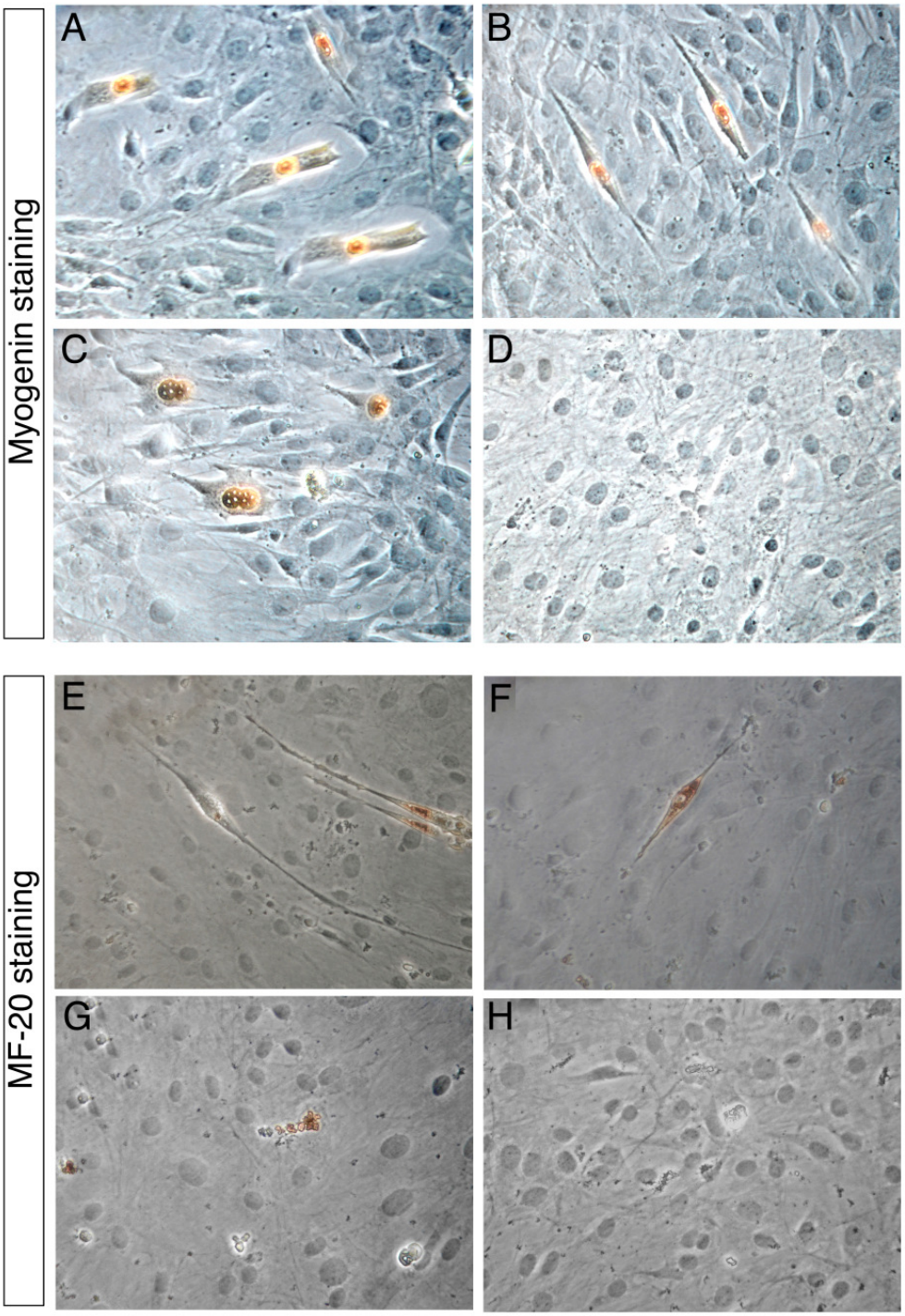


Fig. 34. MyoD mediated myogenic conversion of 10T1/2 cells is modulated by VITO-1 expression levels. Fibroblasts cell line was transfected with pEMSV-MyoD and pCS2-VITO-1 (A,E), pEMSV-MyoD and pSUPER (B,F), pEMSV-MyoD and pSUPER-VITO-1A (C,G), pEMSV-MyoD and pSUPER-myogenin (D,H) and stained after 96 H with antibody against Myogenin (A-D) or MF-20 antibody specific for MyHC (E-H).

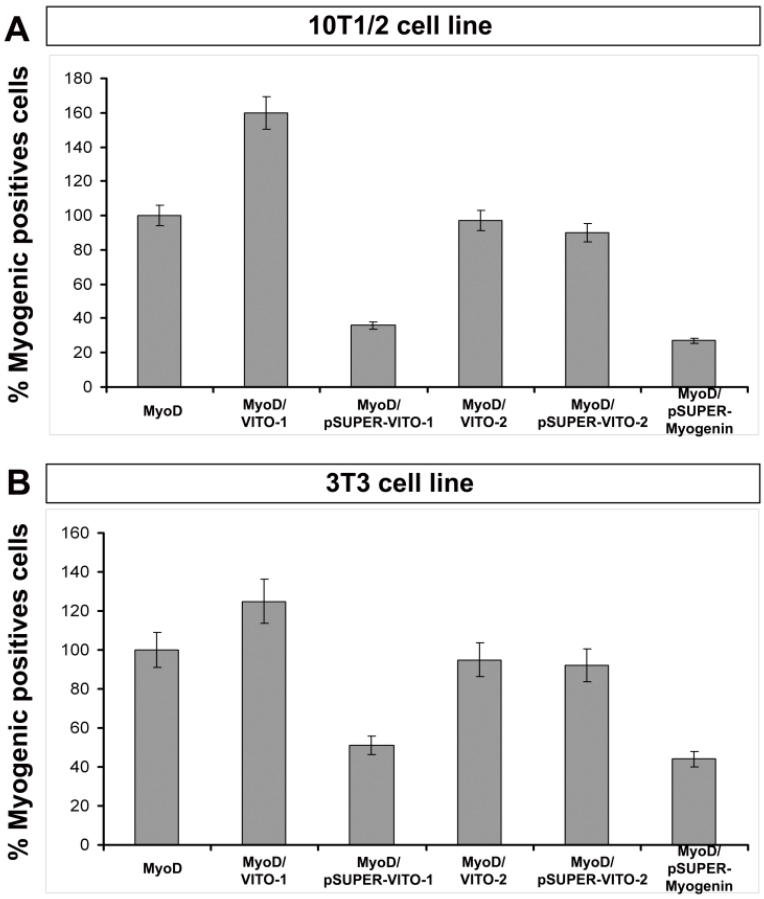


Fig. 35. Summary of myogenic conversion experiments in 10T1/2 (A) and 3T3 (B) cell lines. The number of myogenic cells positive for MyHC or Myogenin was set as 100% when cells were transfected with MyoD only. Co-transfection of pEMSV-MyoD and pCS2-VITO-1 led to an improvement of MyoD mediated conversion in 10T1/2 and 3T3 cell lines up to 160% and 125% respectively. Knock down of VITO-1 in transdifferentiation by co-expression pEMSV-MyoD and pSUPER-VITO-1A plasmids caused a reduction of myogenic cells in 10T1/2 and 3T3 cell lines to 36% and 51% respectively. Similar results were obtained by knocking-down of myogenin in cells expressed pEMSV-MyoD and pSUPER-myogenin in 10T1/2 and 3T3 cell lines to 27% and 44% respectively. In contrast co-transfection of pEMSV-MyoD and pCS2-VITO-2 did not enhance MyoD mediated conversion of 10T1/2 and 3T3 cells. Knock-down of VITO-2 gene using pSUPER-VITO-2A/B did not suppress MyoD mediated conversion of 10T1/2 and 3T3 cell line. Transfections efficiencies were normalized against a co-transfected LacZ expression vector (pHook-LacZ).

4.7.3. Knockdown of VITO-1/2 genes in terminally differentiated C2C12 cell line by RNAi

The requirement of VITO-1, but not VITO-2 for MyoD mediated transdifferentiation of mesenchymal cells suggested an important role of VITO-1 in the

formation of muscles cells. The C2C12 myoblasts is a well established model to analyze myogenic differentiation.

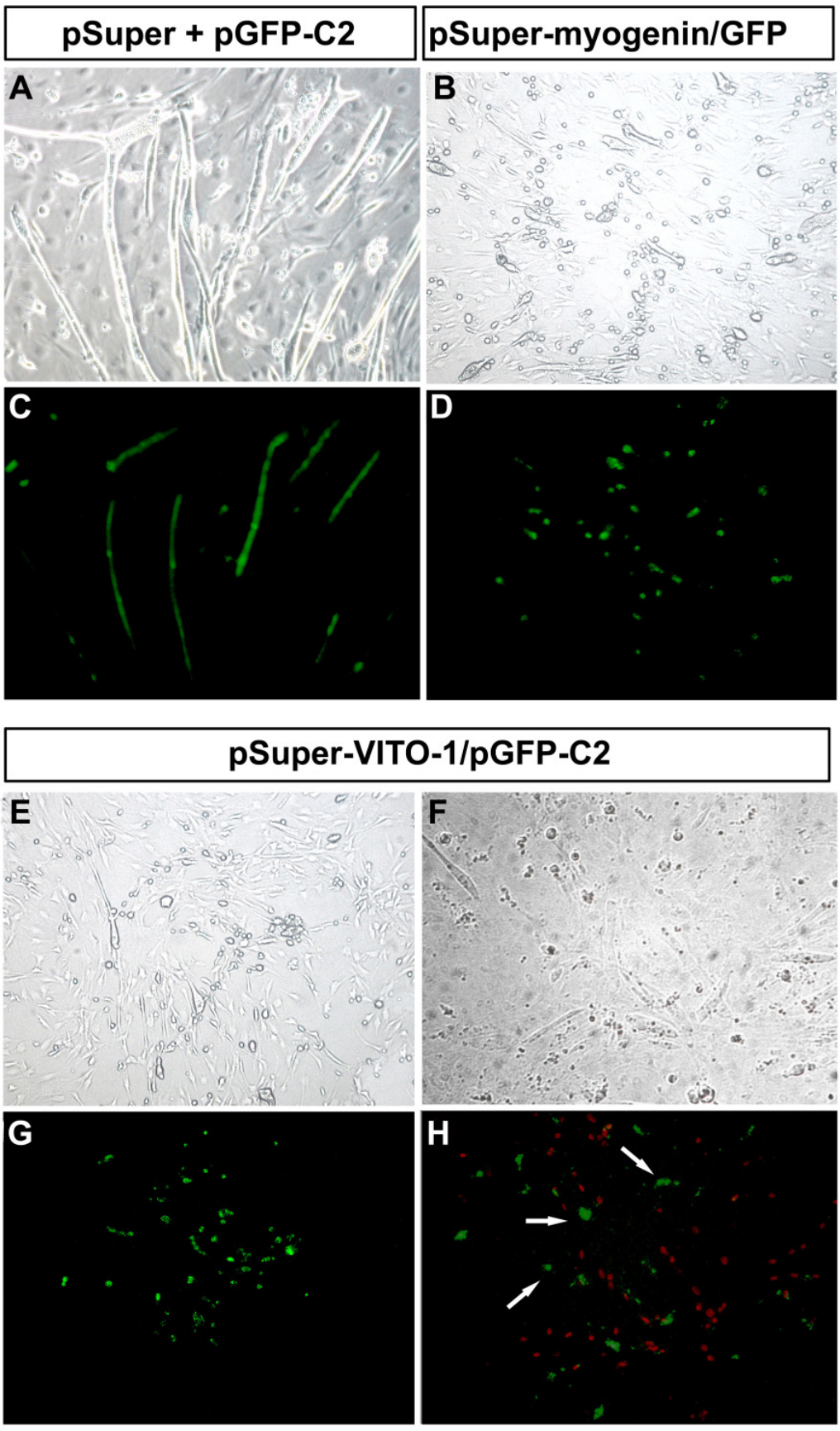


Fig. 36. Knockdown of VITO-1 expression attenuates differentiation of C2C12 muscle cells. C2C12 myoblasts were co-transfected with pSUPER and pEGFP-C2 (A,C), pSUPER-VITO-1 and pEGFP-C2 (E-H), pSUPER-Myogenin and pEGFP-C2 (B,D). Cells were examined for GFP fluorescence to visualize transfected cells (C,D,G,H) and stained with Myogenin antibody detected with rhodamine coupled secondary antibody (merged D,H) or inspected by phase contrast microscopy (A,B,E,F). Note that transfected cells expressing VITO-1 or Myogenin siRNA (green) are negative for Myogenin (red).

Therefore the role of VITO-1 and VITO-2 for differentiation of C2C12 cells was analyzed using a siRNA approach. The siRNA plasmids pSUPER, pSUPER-VITO-1A, pSUPER-VITO-2B and pSUPER-myogenin were transfected separately into C2C12 myoblasts together with a p-EGEFP-C2 vector to distinguish cells, which expressed siRNA from those that had not taken up both plasmids. The ratio between EGFP expressing and siRNA expressing vectors used for transient transfection was 1:10. The number of differentiated myotubes and the expression of Myogenin were examined 48 hours after removal of mitotic stimuli. In all experiments, cells were stained with anti-Myogenin antibody. Co-transfection of p-EGFP-C2 and pSUPER vector did not affect myotubes formation (Fig. 36 A,C). In contrast, Myogenin or VITO-1 knockdown by siRNA efficiently blocked formation of terminally differentiated myotubes (Fig. 36 B,D and Fig. 36 E-H respectively).

Surprisingly knockdown of VITO-2 by co-transfection pEGFP-C2 and pSUPER-VITO-2B in C2C12 myoblasts blocked their terminal differentiation (Figs. 37 A-H, Figs. 38 A-D). This results are in contrast to MyoD mediated conversion of fibroblasts that was not affected by a VITO-2 knockdown. Simultaneous detection of VITO-1 or VITO-2 siRNA expressing cells by EGFP fluorescence and anti-Myogenin antibody staining revealed that Myogenin protein was only present in those C2C12 cells that did not express VITO-1 or VITO-2 siRNA. Similar results were obtained for cells expressing Myogenin siRNA and GFP, which were not terminally differentiated and negative for Myogenin.

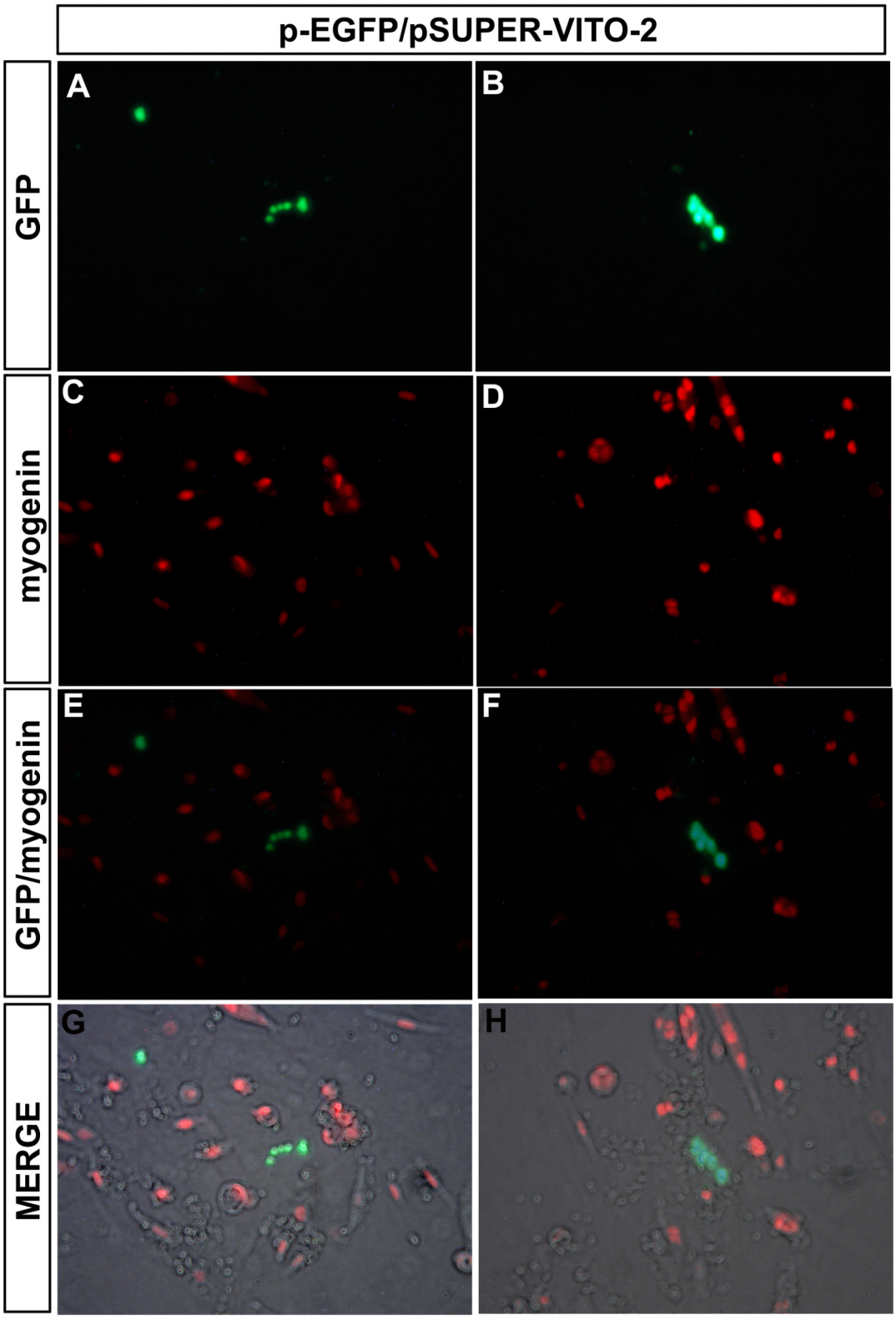


Fig. 37. Knockdown of VITO-2 expression attenuates differentiation of C2C12 cells. C2C12 myoblasts were co-transfected with pSUPER-VITO-2 and pEGFP-C2 (A,B), Cells were examined for GFP fluorescence to visualize tranfected cells and stained with anti-Myogenin antibody (red) (C,D and merged E,F) or inspected by phase contrast microscopy (G,H). Note that transfected cells expressing VITO-2 siRNA (green) are negative for Myogenin (red).

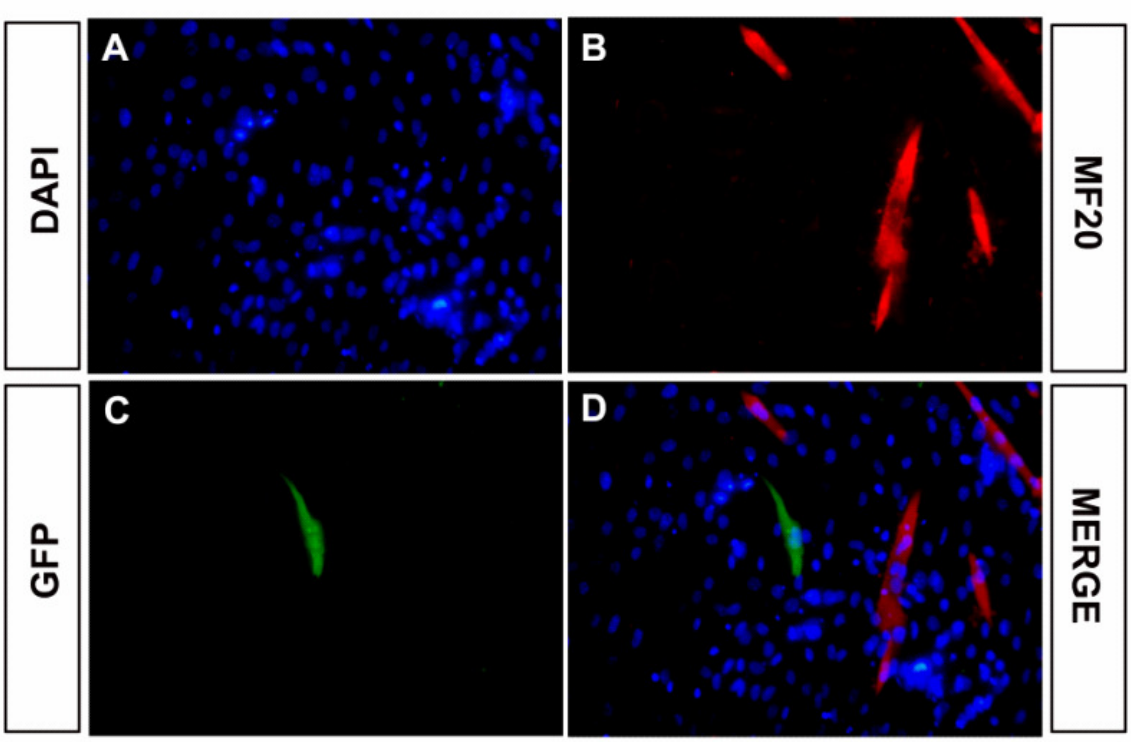


Fig. 38. Knockdown of VITO-2 expression attenuates differentiation of C2C12 cells. C2C12 myoblasts were co-transfected with pSUPER-VITO-2 and pEGFP-C2. Cells were examined for GFP fluorescence to visualize tranfected cells (C) and stained with MF20 antibody (red) (B and merged D). Nuclei were visualized by DAPI staining (A) (blue). Note that transfected cells expressing VITO-2 siRNA (green) are negative for MF20 (red).

The parallel and intermingled existence of cells, which were unable to differentiate might explain why the formation of myotubes was efficiently abrogated, despite the fact that only a subset of C2C12 cells expressed VITO-1 and VITO-2 siRNA.

To analyze whether the expression of VITO-1 and VITO-2 siRNA, resulted in the secretion of proteins that might inhibit differentiation, conditioned medium was examined. Briefly, C2C12 cells were transfected with pSUPER-VITO-1/pEGFP, pSUPER-VITO-2/pEGFP and pSUPER/pEGFP and grown in 2% HS. Medium was replaced every day, collected and fresh medium was added, until myotubes were formed. After transfection of C2C12 cells a second set of these cells were plated and grown in conditioned medium from transfected cells. Non-conditioned fresh medium was used as a control.

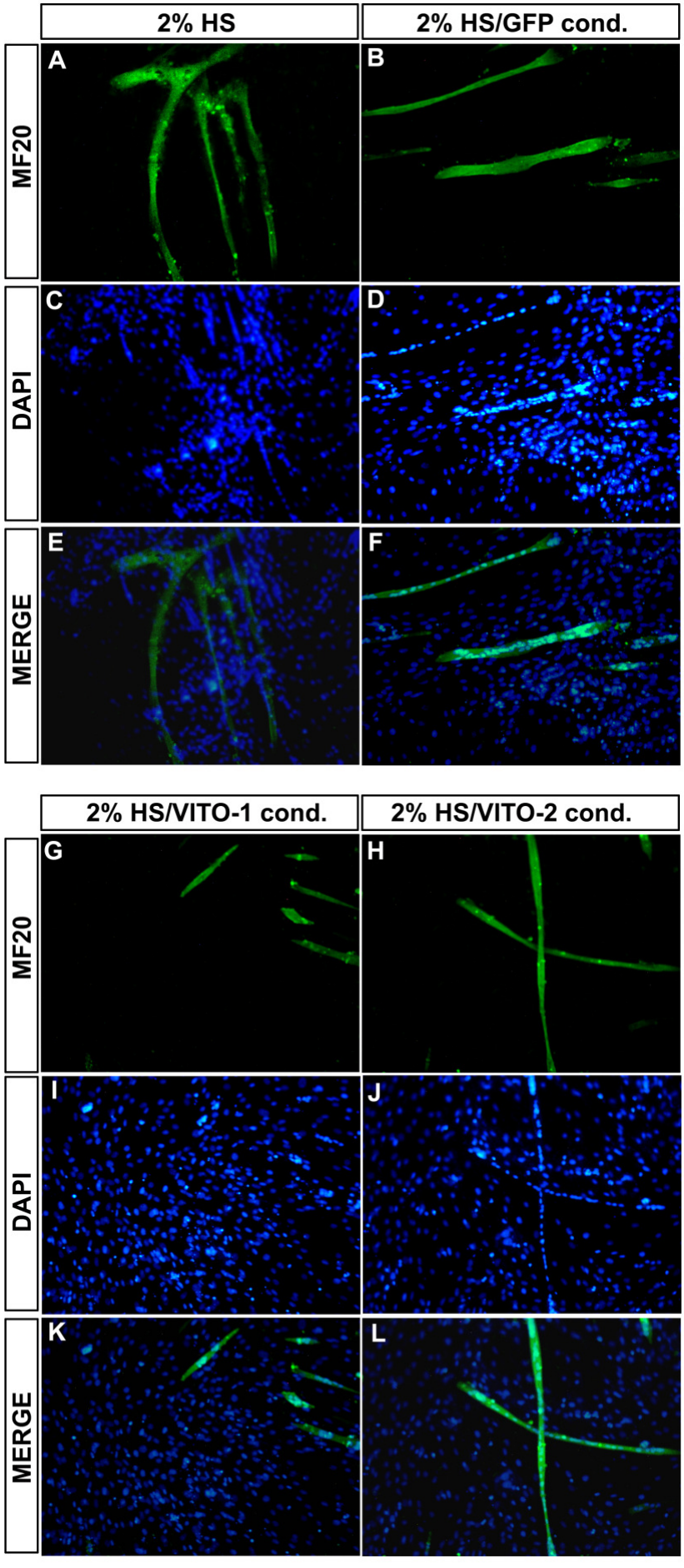

2% HS
2% HS/GFP cond.
MF20
DAPI
MERGE
A
B
C
D
E
F
2% HS/VITO-1 cond.
2% HS/VITO-2 cond.
G
H
I
J
K
L

Fig. 39. C2C12 cells were grown in conditioned medium collected from transfected cells with pSUPER/pEGFP (B,D,F), pSUPER-VITO-1/pEGFP (G,I,K), pSUPER-VITO-2/pEGFP (H,J,L). As a control cells were grown in fresh medium supplemented with 2% HS (A,C,E). Cells were stained with MF20 antibody (green), 72 H after conditioned medium or fresh medium was added. Cells positive for MF20 staining were counted (Fig. 38M).

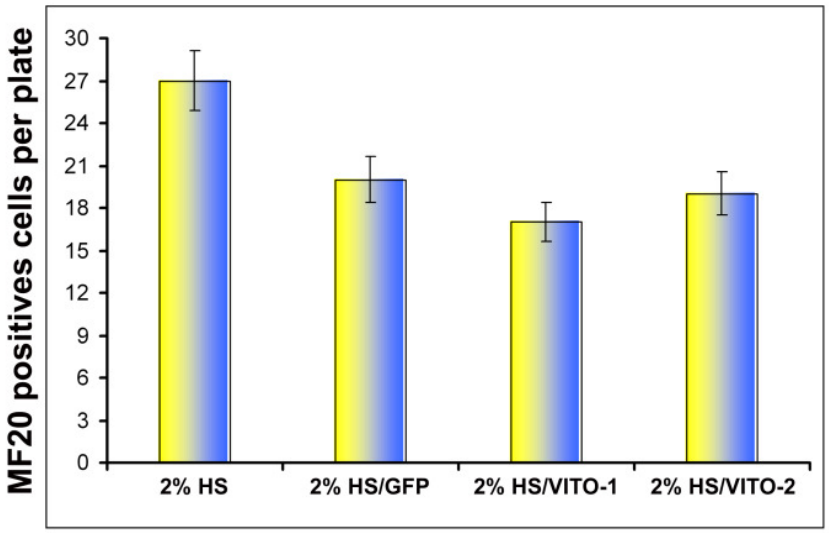


Fig. 39M. Graphical illustration of myotubes formation by C2C12 cells growing in conditioned medium. Cells positive for MF20 staining were counted. Results of three cultures revealed no differences in the ability to form myotubes between different conditioned media. The only statistically significant difference was observed between 2% HS medium and conditioned medium.

72 H after removal of mitotic stimuli cells grown in conditioned medium were stained with MF20 antibody and the number of myotubes were counted. No significant differences in the number of myotubes between conditioned media from cells transfected with pSUPER-VITO-1/pEGFP, pSUPER-VITO-2/pEGFP and pSUPER/pEGFP were observed (Fig. 39). The only reduction in the number of formed myotubes was observed between fresh 2% HS medium and conditioned media (Fig. 39). However, some differences in the size of myotubes were noted when the medium was derived from cells expressing VITO-1 siRNA but not VITO-2 or GFP (Fig. 39).

It should be mentioned, that all attempts to establish a stable C2C12 cell line expressing VITO-1 siRNA failed. During the selection process selected conies stopped to grow, appeared unhealthy and finally died due to programmed cell death.

4.8. VITO-2 is not a direct transcriptional activator

The lack of an intrinsic transactivation domain in the VITO-1 has been demonstrated previously (Maeda et al. 2002; Gunther et al. 2004). To analyze whether VITO-2 contains transactivation domain that might distinguish this gene from VITO-1, a CAT assay was performed. A full length VITO-2 fragment was fused to the yeast GAL4 DNA binding domain (DBD) (Webster et al. 1988; Braun et al. 1990). Wild type GAL4 containing the GAL4 transactivation domain (TAD) and GAL4-VP16 were used as positive controls. VITO-1 fused to yeast DBD domain was used as negative control. The transactivation potential of VITO-2 was analyzed using the GAL4-dependent reporter plasmid pG5E1bCAT (Molkentin et al. 1995). 293T cells as well C2C12 cells were transfected transiently with the reporter plasmid pG5E1bCAT, expression vectors, and RSV-β vector as an internal standard in the ratio 12:3:5 respectively. Transfections efficiencies were normalized against β-galactosidase activities. CAT activity was determined 48 hours after transfection according to manufacture's instructions (Promega). Values presented in Fig.40 were derived from three repetitions of each transfection experiment.

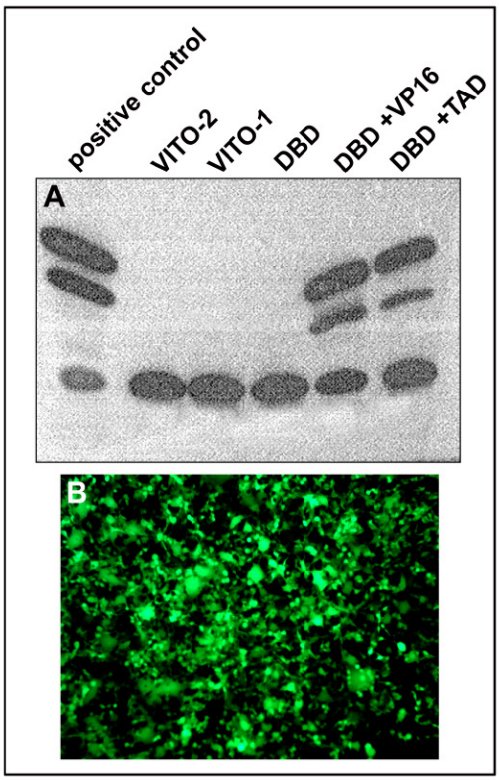


Fig. 40. 293T cells were transfected with reporter vector pG5E1bCAT, the internal standard RSV-β vector together with different expression plasmids GAL4-VITO2,

pCS-DBD-VITO-1. GAL-DBD served as a negative control. GAL-4 DBD-TAD and GAL4-VP16 were used as a positive control. Acetylated derivatives of chloramphenicol were only detected in case of expression of GAL-4 DBD-TAD and GAL4-VP16 plasmids. Acetylated forms of chloamphenicol were separated using thin layer chromatography (TLC). The representative results are shown (A). Transfection efficiency was monitored using pEGFP-C2 vector (B).

No significant VITO-2 dependent transactivation activity was observed (Figs. 40, 41 A,B). The complete region of VITO-2 failed to transactivate expression of CAT driven by GAL4 reporter plasmid in both 293T and C2C12 cell lines.

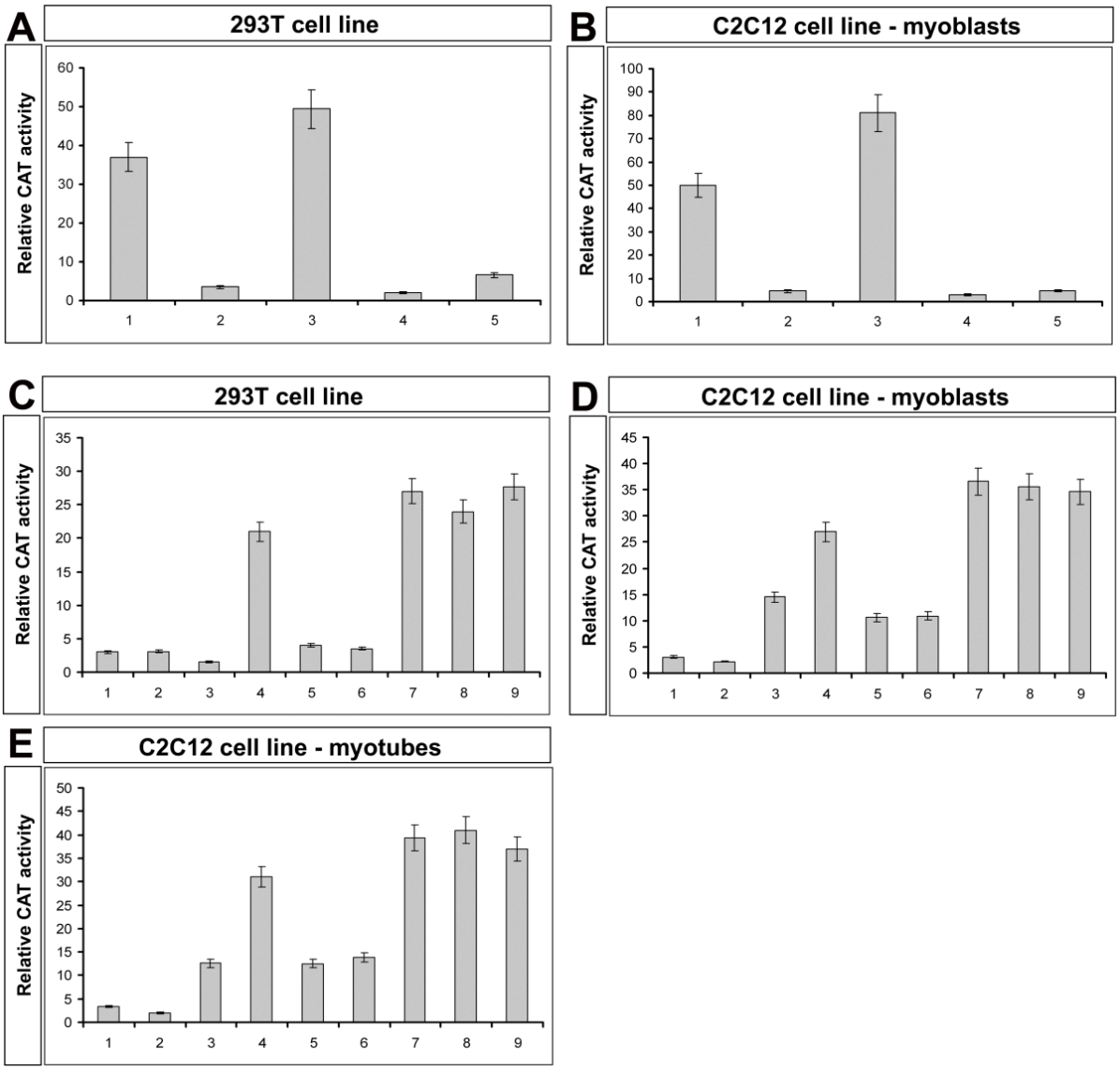


Fig. 41. Cumulative results of CAT assay. 293T (A) and C2C12 myoblasts (B) were transfected with GAL4-DBD-TAD (bar 1 - panel A and B); GAL4-DBD (bar 2 - panel A and B); GAL4-DBD-VP16 (bar 3 - panel A and B); GAL4-VITO-2 (bar 4 - panel A and B); GAL4-VITO-1 (bar 5 - panel A and B) as well as with reporter plasmid and RSV-β vector as internal standard. Panels C-E show cumulative results of CAT activity after transfection of 293T, C2C12 myoblasts

and myotubes. 293T cells (C), C2C12 myoblasts (D) as well as C2C12 myotubes (E) were analyzed for CAT activity after transfecion with reporter plasmid (bars 1,2 in C-E panels) 2MEF2mt-TK alone (bar 1 panels C-E); VITO-2 and MEF2C as well as 2MEF2mt-TK alone (bar 2 - panels C-E). Second reporter plasmid was 2MEF2-TK (bars 3-9 in C-E panels) co-transfected with: 2MEF2-TK alone as negative control (bar 3 - panel C-E); MEF2C (bar 4 - panel C-E); VITO-2 (bar 5 - panel C-E); VITO-1 (bar 6 - panel C-E); MEF2C and VITO-2 (bar 7 - panel C-E); MEF2C and VITO-1 (bar 8 - panel C-E); VITO-2, TEF-1 and MEF2C (bar 9 - panel C-E).

In addition, the ability of VITO-2 to contribute to MEF2C mediated gene activation was investigated. A reporter constructs carrying functional (2MEF2-TK) and mutated (2MEF2mt-TK) binding sites in front of the TK minimal promoter were used (Molkentin et al. 1995). 293T, C2C12 cell lines were transfected with reporter constructs 2MEF2mt-TK (carrying mutated MEF2 binding sites as negative control) and 2MEF2-TK (carrying functional binding sites), RSV-β vector as internal standard and combination of different expression vectors: GAL4-VITO-1/2, GAL4-MEF2C and pXJ40-TEF-1. Transfections efficiencies were normalized against β-galactosidase activities. CAT activity was determined 48 hours after transfection in case of 293T and C2C12 myoblasts cells or 96 hours after transfection in the case C2C12 myotubes. Presented values were derived from triple repetitions of single experiment. Cumulative results of CAT activities are shown in Fig. 41 C-E. Addition of VITO-2 alone or in combination with MEF2C and TEF-1 did not increase activation of the MEF2 reporter by MEF2C.

4.9. Expression of VITO-1/2 genes in the Myf-5 and delta1 knockout mice

To analyze whether VITO1/2 are genetically downstream of Myf-5 in the the hierarchy of regulatory factors controlling myogenesis their expression patterns were examined on the embryos carrying a mutated allele of the Myf-5 gene (generous gift from Prof. Braun) (Braun et al. 1992). Transcripts of VITO-1 and VITO-2 genes were examined by whole mount in situ hybridization at E10.5. VITO-1 transcripts were completely abolished in myotome as well as in the fore limb area, in contrast to wild type embryos. (Figs. 42 A,B). However, VITO-1 transcripts in k.o. Myf-5 embryos were still present in the second pharyngeal pouch as well as in the I and II branchial arches at E10.5. VITO-2 was only detected in wild type embryos at E10.5 in the

myotome (Fig. 42C). In Myf-5 deficient embryos at E10.5, VITO-2 transcripts were completely absent from somites. VITO-2 mRNA was absent in k.o. Myf-5 embryos at E10.5 (Fig. 42 D).

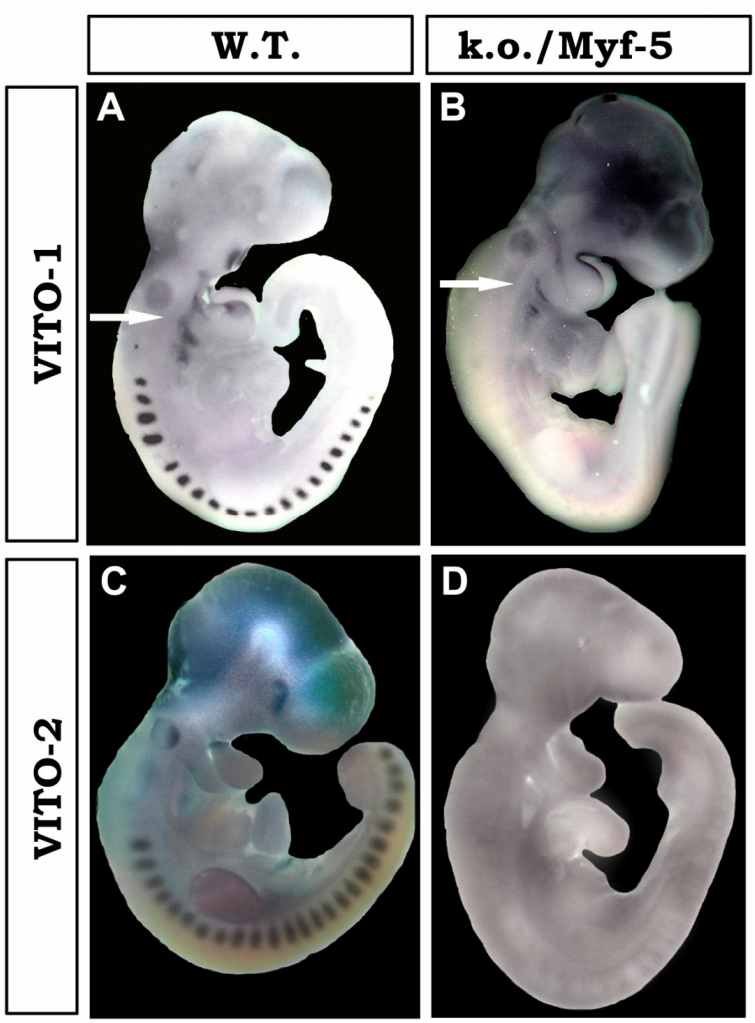


Fig. 42. Whole mount in situ hybridization of m-VITO-1 (A,B) and m-VITO-2 (C,D) in wild type (A,C) and Myf-5 deficient (B,D) mouse embryos at E10.5. Arrows in panels A and B indicate the remaining expression of VITO-1 in the branchial arches.

VITO-1 and VITO-2 transcripts were also examined in the embryos lacking the delta1 gene (dll1) a ligand of Notch (kindly provided by Prof. Gossler). Expression of both genes in embryos carrying mutated allel of dll1 was monitored using whole mount in situ hybridization. Antisense probes of VITO-1 and VITO-2 genes were hybridized to mouse wild type (Fig. 43 A,C) and mutants of dll1 embryos (Fig. 43 B,D) at E10.0. VITO-1 transcripts were not affected in all structures in delta knockout in comparison to wild type situation (Figs. 43 A,B). In dll1 deficient embryos VITO-1 was present in

somites (myotomal part) as well as in branchial arches, as confirmed transversal vibratome sections (data not show).

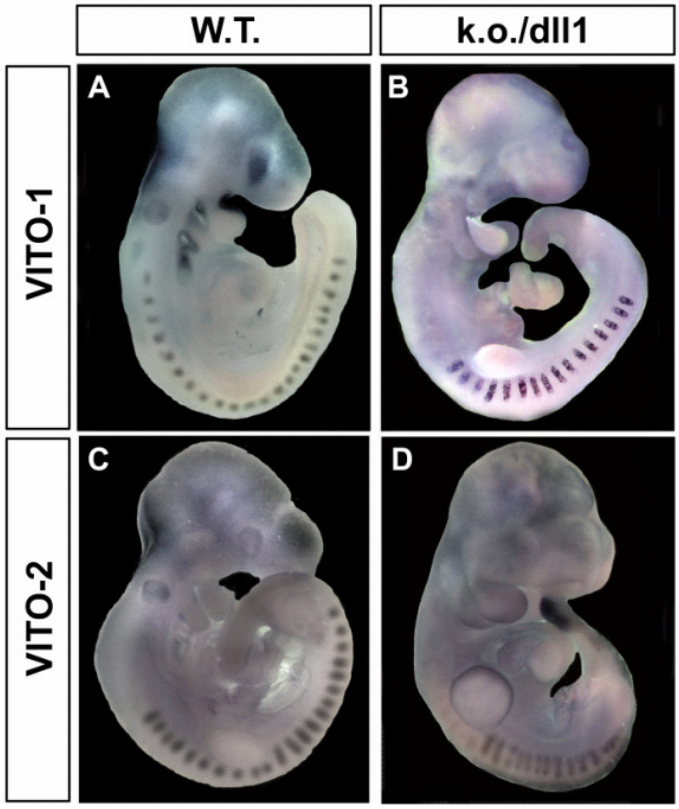


Fig. 43. Whole mount in situ hybridization of m-VITO-1 (A,B) and m-VITO-2 (C,D) of hybridized wild type mouse embryos (A,C) and dll1 deficient embryos (B,D) at E10.5.

Although the expression of VITO-1 was not affected in k.o./dll1 mutant embryos, the pattern of the VITO-2 gene expression was changed (Figs. 43,44). In dll1 mutant embryos the expression of VITO-2 was observed in the caudal part of newly formed somites in the area of the presomatic mesoderm (Fig. 44 A,E) whereas in wild type embryos VITO-2 was found in somite IX or X. To delineate the expression of VITO-2 in the caudal part of somites, vibratom sections were prepared, oriented perpendicularly to the caudal part of the embryo (Fig. 44 A orientation of section is indicated by the arrow). VITO-2 mRNA was abundantly expressed in the presomatic area (Fig. 44 B). In transversally oriented vibratome sections an ectopic expression of VITO-2 was also found in the neural tube of the caudal part of the embryos carrying mutated dll1 allele (Fig. 44 C,D). VITO-2 was never detected in the neural tube of wild type embryos at any developmental stage.

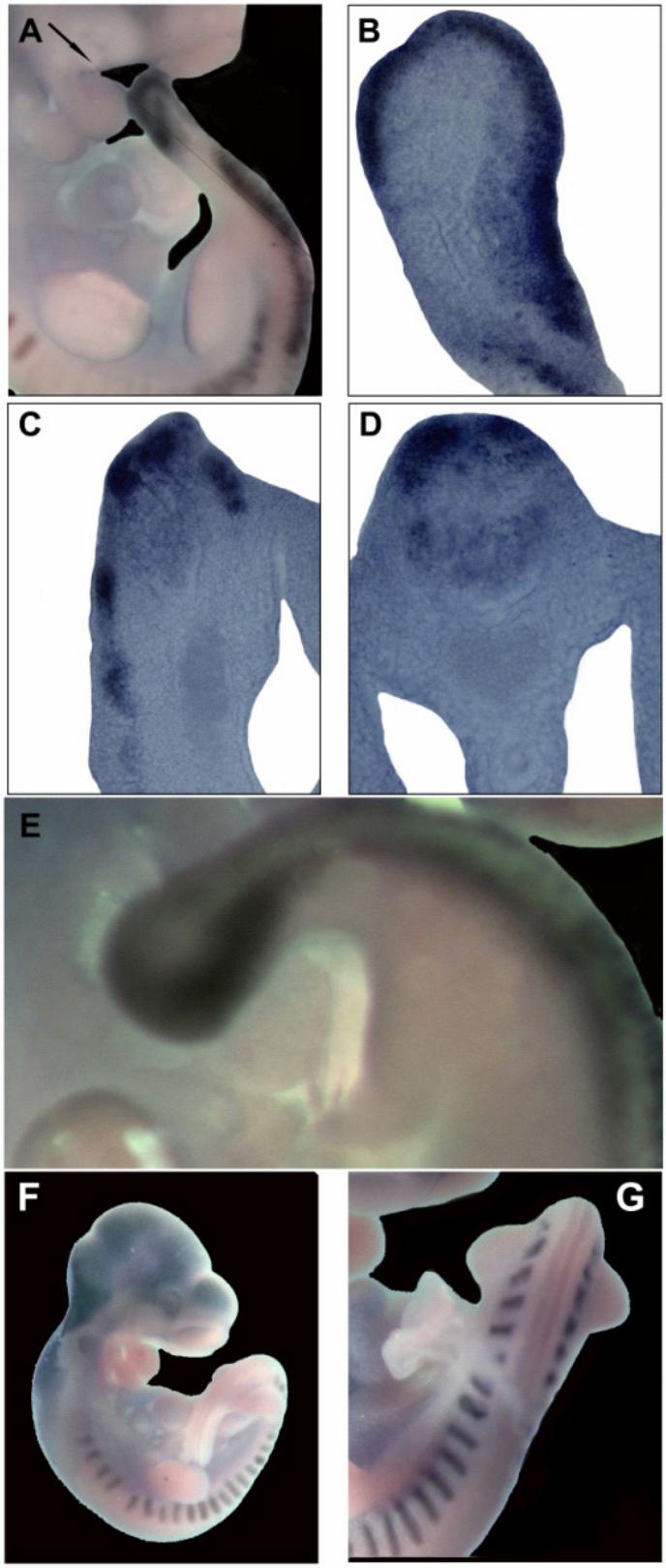
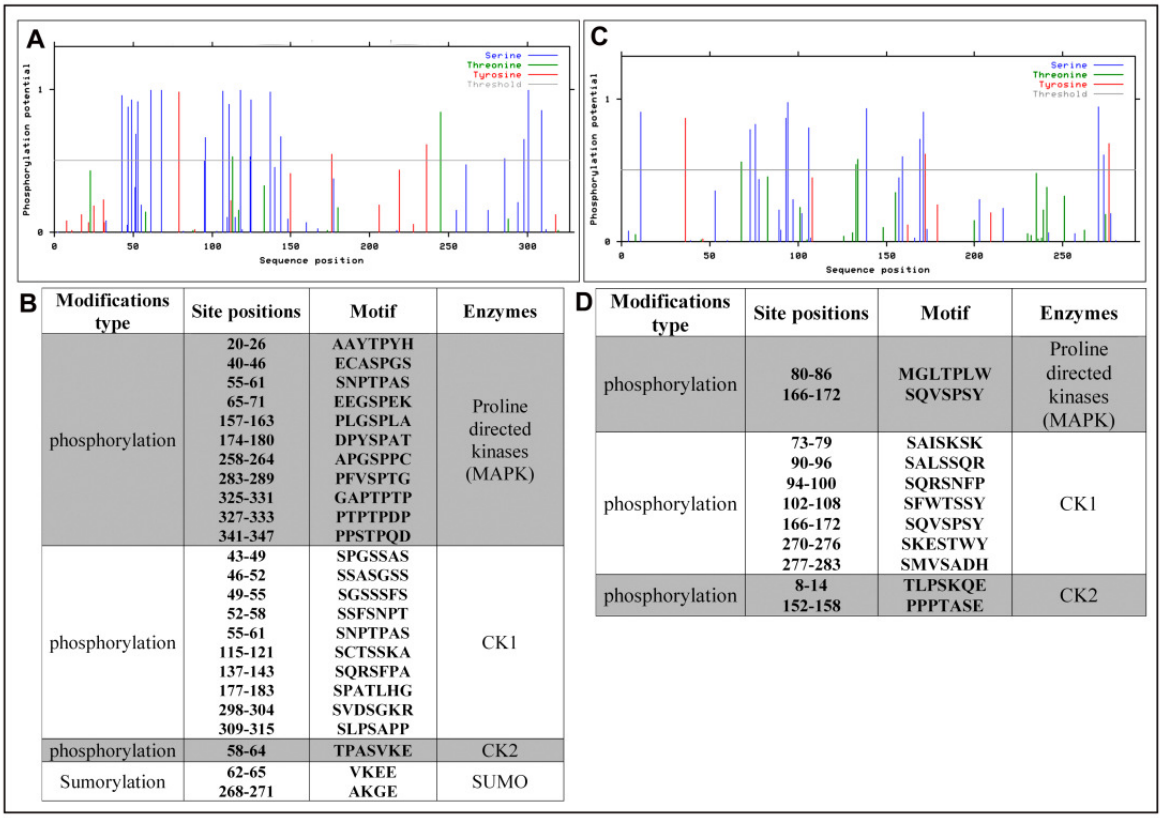


Fig. 44. Whole mount in situ hybridization of m-VITO-2 probe hybridized with k.o./dll1 (A,E) and wild type embryos (F,G). Panels E and F show caudal areas of dll1 and WT embryos, respectively. Vibratome sections indicated an expression of VITO-2 transcripts in the presomatic mesoderm (B). Transversally

oriented vibratoma sections revealed an ectopic expression of VITO-2 mRNA in the neural tube of the caudal part of embryos lacking dll1 (C,D).

4.10. Posttranslational modification of VITO family members

To assess potential posttranslational modifications of VITO-1 and VITO-2 a bioinformatical analysis was performed. Bioinformatical data were obtained using NetPhros 2.0 software at the server of Technical University of Denmark, ELM database and the SUMOplot™ software. VITO-1 and VITO-2 proteins appeared to be good candidates for phospohorylation, mainly at serine residues (Fig. 45 graph A and C, respectively). Additional analysis revealed many potential motifs in both proteins that might be targets for MAPK, CK1 and CK2 kinases. In addition VITO-1 but not VITO-2 protein has also two potential motifs for sumoylation by SUMO modifiers. Potential motifs for phospohorylation and sumoylation of VITO-1 and VITO-2 are assembled in tables B and C respectively (Fig. 45).



B

Modifications type	Site positions	Motif	Enzymes
phosphorylation	20-26 40-46 55-61 65-71 157-163 174-180 258-264 283-289 325-331 327-333 341-347	AAYTPYH ECASPGS SNPTPAS EEGSPEK PLGSPLA DPYSPAT APGSPPC PFVSPTG GAPTPTP PTPTPDP PPSTPQD	Proline directed kinases (MAPK)
phosphorylation	43-49 46-52 49-55 52-58 55-61 115-121 137-143 177-183 298-304 309-315	SPGSSAS SSASGSS SGSSSFS SSFSNPT SNPTPAS SCTSSKA SQRSFPA SPATLHG SVDSGKR SLPSAPP	CK1
phosphorylation	58-64	TPASVKE	CK2
Sumorylation	62-65 268-271	VKEE AKGE	SUMO

D

Modifications type	Site positions	Motif	Enzymes
phosphorylation	80-86 166-172	MGLTPLW SQVSPSY	Proline directed kinases (MAPK)
phosphorylation	73-79 90-96 94-100 102-108 166-172 270-276 277-283	SAISKSK SALSSQR SQRSNFP SFWTSSY SQVSPSY SKESTWY SMVSADH	CK1
phosphorylation	8-14 152-158	TLPSKQE PPPTASE	CK2

Fig. 45. Panels A and C present potential phosphoryations sites of VITO-1 and VITO-2 proteins, respectively. Graphs were obtained using Nephros 2.0 software. Tables B and D show a potential phosphorylation as well as sumoylation sites for

VITO-1 and VITO-2 proteins, respectively including site position, motif and indicated modifiers.

Potential posttranslational modifications of VITO-1/2 proteins were analyzed in a sumoylation assay (3.2.17.). Three different cell lines 293T, 3T3 and C2C12 cells were transfected with a different combination of plasmids expressing proteins of interest. VITO-1 was expressed as a fusion protein with N- and C- terminal Myc tag in the pMT+ vector. VITO-2 protein was fused with N-terminal Myc tag. SUMO-1 was linked with GFP in the expression plasmid pSG5LINK-EGFP-SUMO-1 (generous gift from Prof. Suske). Cells were usually harvested 48 hours after transfection and proteins were isolated as described in Materials & Methods (3.2.17.). Proteins were separated on 8% acrylamide gels, followed by western blotting (3.2.13) followed by detection with anti-c-myc antibody. Western blot analysis revealed no apparent modification of VITO-2 in any tested cell lines (Fig. 46 A,B), since only the major band (VITO-2 linked to Myc tag – 46 kDa) was detected in all transfected cell lines. VITO-1 seemed to be modified most probably by phosphorylation since an additional band, of aproximately 1-2 kDa larger than major band was detected in all cell lines transfected with expression plasmids carrying VITO-1 linked to N- or C- terminal Myc tag (Fig. 46 A,B). The major band derived from unmodified VITO-1 linked to the Myc tag (aproximately 52 kDa) is indicated by arrow (I) and additional upper band by arrow II (Fig. 46 A,B). The additional bands were detected in the 293T, 3T3 and C2C12 cells transfected with VITO-1 alone or in combination with TEF-3 (in case of 293T cells), represent most probably phosphorylated VITO-1 protein. When 293T cells were transfected with VITO-1 together with TEF-3 and SUMO-1, two additional upper bands were identified (Fig. 46 C arrow (III)). The difference between the third band (arrow III) and the lower major band (arrow I) (Fig. 46 C) was estimate of approximately 15-20 kDa. This size diference is compatible with sumoylation of VITO-1 and its phosphorylated version. In addition, 293T cells transfected with VITO-1, TEF-3, SUMO-1 and GAM1 (a known inhibitor of sumoylation), decreased the intensity of the upper band (arrow III) (Fig. 46 C), further supporting that the additional signal (arrow III) is derived from the VITO-1 protein covalently linked to SUMO-1. In order to verify these results, SUMO-1-GFP was detected additionally with anti GFP antibody when 293T cells were transfected with VITO-1, TEF-3, SUMO-1 and GAM1 (Fig. 46 D line 1) and VITO-1, TEF-3, SUMO-1 (Fig. 46 D line 2). In this case western blot analysis revealed a major 25 kDa

band derived from the SUMO-1-GFP fusion protein as well as additional bands (marked with asterisk Fig. 46 D). These additional bands were decreased when cells expressed Gam-1 protein. Although these results support the hypothesis that VITO-1 might be modified by sumoylation additional experiments are necessary. Disruption of the potential sumoylation sites by site direct mutagenesis will prove whether VITO-1 is modified by SUMO.

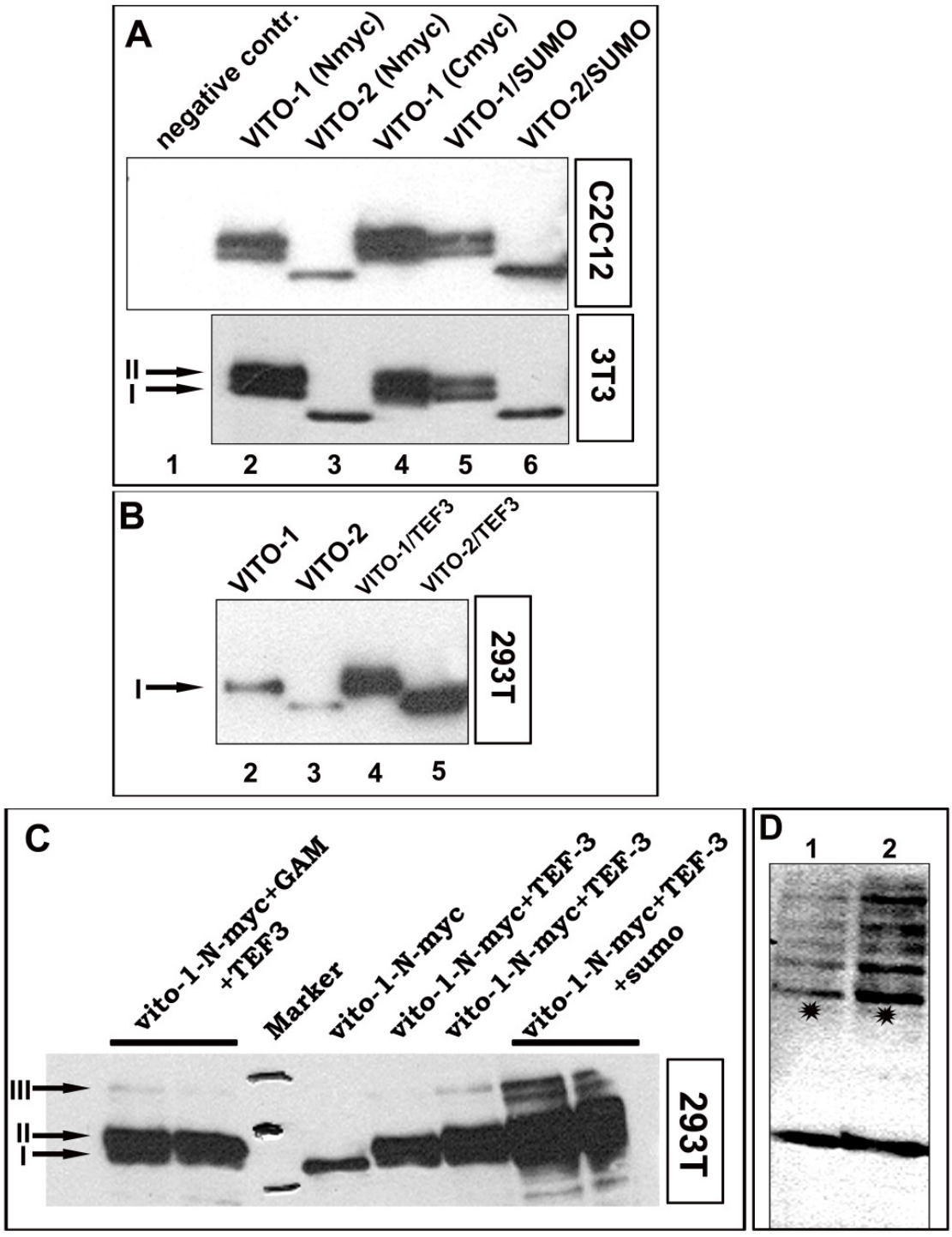


Fig. 46. 3T3 and C2C12 cells were transfected with the different combination of plasmids expressing as follows: 1- negative control (pMT+ vector), 2- VITO-1 linked to the N-terminal Myc tag, 3- VITO-2 linked to the N-terminal Myc tag, 4- VITO-1 linked to the C-terminal Myc tag, 5- VITO-1 linked to the N-terminal Myc tag and SUMO-1, 6- VITO-2 linked to the N-terminal Myc tag and SUMO-1, and Western blot analysis was done. Arrows (I) and (II) indicate major and minor bands of VITO-1 that react with the antibody against the fusion protein (panel A). 293 T cell line was transfected with different combination of the expressing plasmids: line 2- VITO-1 linked to the N-terminal Myc tag; line 3- VITO-2 linked with N-terminal Myc tag; line 4- VITO-1 linked to the N-terminal Myc tag and TEF-3; line 5 - VITO-2 linked to the N-terminal Myc tag and TEF-3 (panel B). Panel C shows a western blot analysis of VITO-1 protein expression in 293 T cell line detected with the anti-myc antibody with different combination of TEF-3, SUMO-1 and GAM1 proteins as indicated. VITO-1 co-expressed with TEF-3, SUMO-1 and GAM1 (line-1 panel D) and TEF-3, SUMO-1 (line- 2 panel D) in 293T cell line. Western blot with the antibody against GFP revealed a decreased amount of the sumoylated VITO-1 protein (asterisk, panel C). Only representative pictures were show. All experiments were done in duplicate.

5. Discussion

5.1. Identification of vestigial and TONDU related co-activators

A better understanding of the transcriptional network will help to define the molecular steps that specify cells during development and in adult life as well as their responsiveness to different stimuli. It will also help to elucidate the molecular program of stem cells, which might open new avenues to manipulate such cells for therapeutic purposes. Major efforts were made to identify individual regulators which are responsible to specify aspects of the transcription network.

The myogenic program is controlled by a complex array of molecules including chromatin modifying enzymes and different transcription factors owing a specific ability to bind activators or repressors (modulators). The ability to activate individual genes might be modulated by posttranslational modifications such as phosphorylation and sumoylation or by binding of molecules which might modify their properties due to conformational shifting. There are three main groups of transcription factors, MRFs, MADS and TEFs, which regulate the fate of skeletal and cardiac muscle cells. The mammalian family of TEFs has been reported to play a decisive role in the regulation of various cell lines and tissues including heart and skeletal muscle. However, the biological activity of TEFs depends on the co-operation with different modulators. Scalloped protein, a Drosophila homolog of TEFs interacts with vestigial to play a crucial role during wing development (Halder et al. 1998; Simmonids et al. 1998; Halder et al. 2001). In vertebrates, molecules such as the human TONDU and its mouse homolog (vestigial related m-vr / Vgl-1) have been identified as homologues of vestigial protein in Drosophila on the base of high similarity within SID domain (Figs. 4,5) (Vaudin et al. 1999). In the work presented here I attempt to identify and characterize the new mouse and human homologues of Drosophila vestigial. Using a subtractive hybridization approach, the first homolog of vestigial in Drosophila and human TONDU protein was identified and named VITO-1 (Mielcarek et al. 2002). Mouse VITO-1 protein shows a similarity of approximately 82% to its human homolog. The same genes were also discovered independently by another group (Maeda et al. 2002). The next homolog of VITO-1 gene was found by virtual screening of the different databases and named VITO-2 due to its high similarity to VITO-1 (Fig. 4).

Mouse and human homologues show high similarity to VITO-1 counterparts. The most conserved region between VITO-1 and VITO-2 genes was identified within SID domain (approximately 95%) (Fig. 5). Interestingly, human TONDU was able to substitute functionally for the vestigial gene in Drosophila (Vaudin et al. 1998). It should be mentioned, that original SID domain of the vestigial protein in Drosophila possesses 85 amino acids (Halder et al. 2001). However, on the basis of the sequence homology within the SID domains of the vestigial, human TONDU and its mouse homolog Vgl-1 as well as VITO-1/2 genes, only 26 but not 85 amino acid are highly conservative within all homologues of vestigial in vertebrate (Fig. 5). On the basis of the sequences analysis I presume that the functional core of SID domain in vertebrates is 26 amino acids long.

Recently a new potential homolog of vestigial was identified and named Vgl-4 (Chen et al. 2004). The authors claimed that this gene is a new homolog of vestigial, TONDU as well as VITO-1/2 genes and possesses two functional SID domains. However the potential SID domains of Vgl-4 have only a 40% similarity to the core consensus of SID domain in vertebrates (Fig. 5). In addition, a phylogenetic analysis of all known homologues of vestigial revealed an enormous distance between the mouse and human Vgl-4 genes and Drosophila vestigial (Fig. 6). The low homology to the core consensus SID domain as well as the minor similarity to vestigial in Drosophila seems to exclude Vgl-4 from the core group of the vestigial and TONDU related genes.

On the basis of the bioinformatics analysis I did not find another genes exhibiting high sequence homology within the SID domain to VITO-1 and VITO-2. It is well known that the structure and function of transcription regulators are often conserved between vertebrates and flies, the Drosophila genome database affords a possibility of finding of ancestral genes of mammalian transcription factors. Although in Drosophila vestigial three domains were described but only SID domain was characterised (Halder et al. 2001). During virtual screening I did not find genes with high homology within two remaining domains in vertebrates. Due to the ubiquitous distribution of TEFs in mouse tissues it can not be excluded that co-activators with lower degree of homology exist and regulate TEFs action in a tissue specific manner.

5.2. Tissue distribution of the VITO family of genes

As described in the chapter 1.3.4, in Drosophila the vestigial gene acts together with scalloped to play a crucial role during wing morphogenesis. In order to be able to interact with each other both molecules have to co-localize in the same compartments and cells to regulate transcription of the target genes. To assess potential functions of VITO-1 and VITO-2 genes, it was necessary to obtain a detailed knowledge about the expression patterns during development and in adult tissues. It has to be emphasised that VITO genes have to co-localize with the potential interaction partners to play a biological function.

As already mentioned another group also published expression pattern of VITO-1, but there are some differences between the obtained in situ hybridization results. For example, the expression of VITO-1 in the pharyngeal clefts and cranial pharynx profile escaped previous attention (Maeda et al. 2002). Identification of VITO-1 transcripts in the Rathkes pouch might suggest a role of VITO-1 in development of the pituitary gland which originates from Rathkes pouch (Mielcarek et al. 2002). In addition, VITO-1 might also play a role in the development of the parathyroid glands and the ultimobranchial bodies due to its expression in the caudal pharyngeal pouches (III and IV) at E10.5, respectively. Maeda and colleagues found at later stage E16.5 VITO-1 mRNA in the hypoglossal muscle of the tongue and branchial arch-derived maxillary and mandibular cartilages (Maeda et al. 2002). To asses the importance of VITO-1 gene during embryogenesis a side by side comparison of the expression profiles of VITO-1 and other key myogenic regulatory factors was made. VITO-1 begins to be present in somites slightly later (E8.75) than Myf-5, approximately at the same time as Myogenin and significantly earlier than MyoD. MEF2C is expressed at the same developmental stage as VITO-1 (Edmondson et al. 1994), while the transcripts of MRF4, TEF-3, MEF2A, MEF2D and TEF-4 occurs later (Hinterberger et al. 1991; Yockey et al. 1996; Edmondson et al. 1994; Yasunami et al. 1995) (Fig. 47). Hence it is possible that functional role in the muscle cells specification in somites is dependent on the interaction with another transcription factor than TEF. This hypothesis is supported by the results published by Maeda. The authors proved the interaction of VITO-1 with MEF2C and showed that this complex can enhance the activation of MEF2C dependent promoters (Maeda et al. 2002). The spatiotemporal overlapping expression of VITO-1 and MEF2C further confirm the possibility that VITO-1 in complex with MEF2C plays

a functional role in somites. Overlapping expression of TEF-5 (a potential interacting partner of VITO genes) and VITO-1 can be seen between E9.5 to E10.5. At this time point, TEF-5 is localized in the hyoid arch and mandibular processes (Brunskill et al. 2001). In the fore limb buds Myf-5 and TEF-5 expressions were first detected at E10.5 followed by (a half day later) expressions of myogenin and MyoD (Sasson et al. 1989; Ott et al. 1991; Yee et al. 1993; Brunskill et al. 2001), while all MEF2 isoforms are present within fore and hind limbs buds at day E11.5 (Edmondson et al. 1994). In addition, TEF-4 mRNA was detected slightly earlier than VITO-1 at E10.0 in the distal portions of the fore and hind limbs buds (Yasunami et al. 1995). Taken together, VITO-1 transcripts in the presumptive muscles forming areas of the limbs overlapped with the expression of TEF-4 as well TEF-5, at the same time point of the embryogenesis. These results of expression patterns might indicate a potential interaction of VITO-1 and TEF-4 or TEF-5 and it can be hypothesised that this plays an important role in the limbs development. The data concerning VITO-1 expression patterns during mouse embryonic development are consistent with the observations published by another group, albeit my results extended the knowledge about its distribution (Figs. 9-11) (Maeda et al. 2002). It is known that skeletal muscle development requires the coordinated expression of numerous transcription regulators and their modulators to control the specification of mesodermal progenitor cells to a muscle fate and the differentiation of those committed myoblasts into functional muscle fibers. Moreover from the expression pattern it might be concluded that VITO-1 plays a role in the specialization of muscle cells but not in the generation of the skeletal muscle cell lineage.

VITO-2 transcripts were detected at E8.5 with abundant expression in the developing midbrain area and rhombomers and vanished at later stages (Fig. 14). Interestingly, TEF-4 is also found at E10.5 along the rhombencephalic roof which originated from the rhombomers making possible interaction of both molecules in these brain structures. However, it is not clear whether TEF-4 is expressed as early as VITO-2 in these areas at E8.5 (Yasunami et al. 1995). There was no transient expression of VITO-2 during mouse embryonic development at later stages as observed for VITO-1. The VITO-2 mRNA is expressed in myotomal part of somites (Figs. 15-17) later than VITO-1 transcipts, between E9.5 – E11.5. In the mouse, the first differentiated muscle cells appear in the somites on about embryonic day E9.0 (Smith et al. 1993). The expression profile of VITO-2 during the course of C2C12 differentiation revealed its

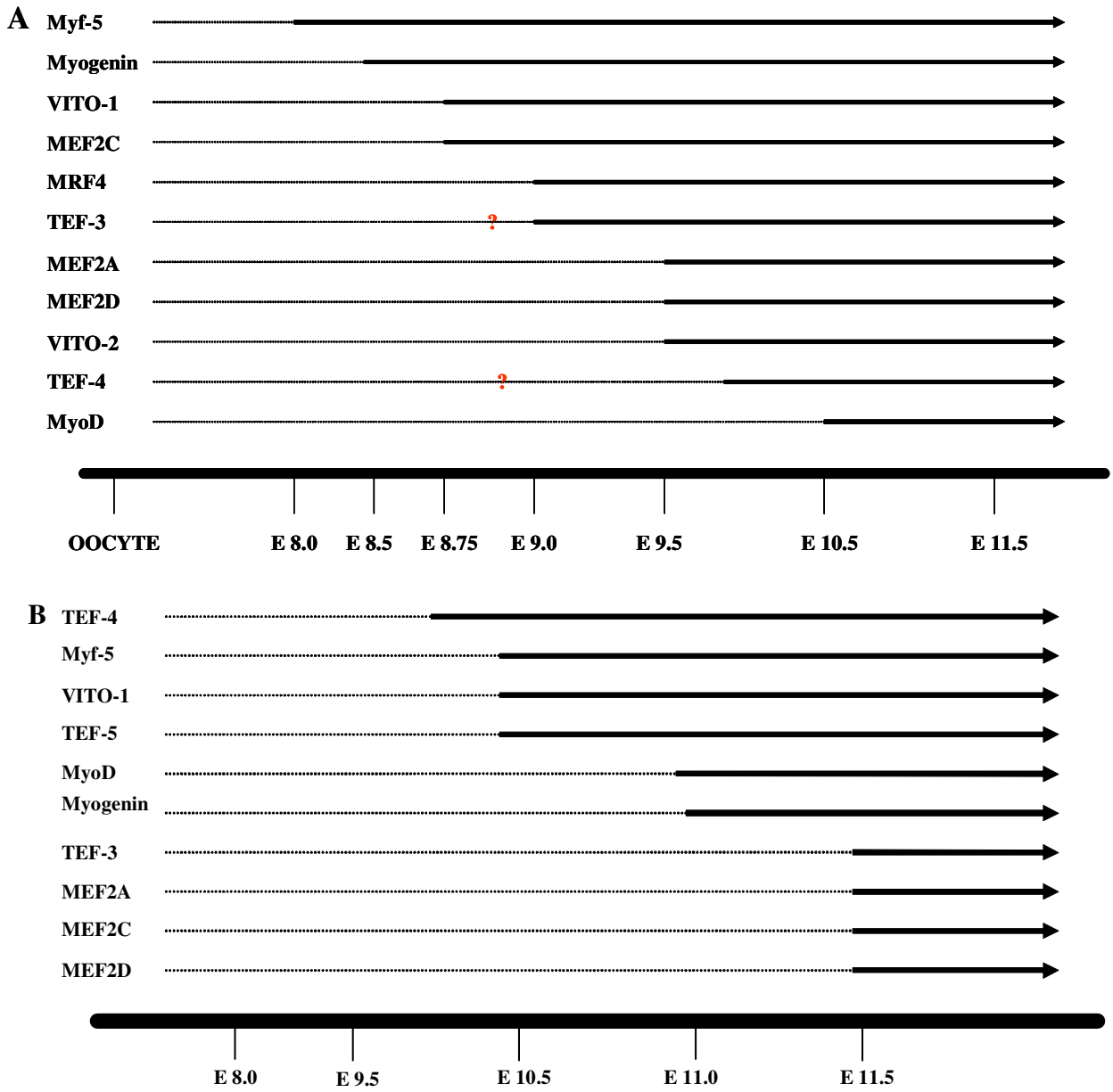


Fig. 47. The expression profile of MRFs, MADS box, TEFs and VITO genes during mouse embryonic development in somites (A) and limbs (B). Question mark – indicate that no data are available (Edmondson et al. 1994, Yasunami et al. 1995, Sasson et al. 1989; Ott et al. 1991; Yee et al. 1993; Brunskill et al. 2001, Hinterberger et al. 1991; Yockey et al. 1996).

up-regulation in the terminally formed myotubes. This is consistent with the expression domain of VITO-2 starting at E9.5 when the first differentiated muscle cells appear in the somites. VITO-2 is expressed at the same time as MEF2A/D transcripts in somites (Edmondson et al. 1994) earlier than TEF-4 and MyoD but later than Myf-5 and VITO-1 (Fig. 47). These finding might indicate that VITO-2 similarly to VITO-1 can co-localize with MEF2 family members or TEF-3 and TEF-4 in somites. In contrast to

VITO-1, VITO-2 transcripts were not detected in the putative muscles forming areas of the fore and hind limbs at any developmental stage. The localization of the VITO-1 and VITO-2 genes in the framework of muscle regulatory genes that are expressed during embryonic developmental stages is shown in Fig. 47.

In the adult tissues, VITO-1 transcripts were found exclusively in the skeletal muscles (Fig. 7). Northern blot as well as semi-quantitative RT-PCR analysis revealed that VITO-1 transcripts were enriched in fast type muscles in mouse (Fig. 8) and human samples (Mielcarek et al. 2002). Such kind of expression in the postnatal life is rather unique among the other known co-activators of the MEFs and TEFs families of genes. Skeletal muscle fibers have the potential to adapt their phynotypic properties to meet different functional demands. As transcription factors (including TEF-1 and TEF-3) have been described as a crucial in this adaptation it is possible that skeletal muscle specific VITO-1 as a potential TEFs co-activator is also involved in this process in adult skeletal muscles (Giger et al 2002). In contrast to the VITO-1 gene other proteins identified as a co-activators of TEFs like YAP65, TAZ and p160 family of genes are rather ubiquitously expressed (data not shown).

VITO-2 a homolog of VITO-1 seems to be ubiquitously expressed in all tested adult tissues based on Northern blot analysis (Fig. 12). In this respect, VITO-2 differs from VITO-1 gene and it is not a skeletal muscle specific gene. Nevertheless, similar to VITO-1, VITO-2 is also expressed more abundantly in fast than in the slow type muscles (Fig. 13). Alike to VITO-1 is also up-regulated in terminally differentiated myotubes in C2C12 myoblasts (Figs. 12, 24). In situ hybridizations disclosed a localization of VITO-2 mRNA in the specific areas of the adult brain (Figs. 19,20). Interestingly, an overlapping expression of VITO-2 and TEF-3 in purkinje cells was found (Fig. 21). Based on these results one might postulate a potential interaction of TEF-3 and VITO-2 in this type of cells. It should be mentioned, however, that others TEFs proteins are also expressed in the adult brain but their expression pattern is still unknown. At the moment no data are available, which would suggest a regulation of neuronal genes by MCAT elements. Nevertheless, it can be assumed that similar mechanism of TEFs binding to the MCATs as described in skeletal and cardiac muscles apply for neurons. The co-expression of VITO-2 and TEF-3 genes in the same cells type is the first evidence, which supports this hypothesis.

Due to the fact that all TEF genes are expressed in the adult heart the obvious question was which co-activator is also expressed in the adult heart. In adult tissues,

VITO-2 was found to be expressed by specific cells in the adult heart (Fig. 12). To investigate the localization of VITO-2 in adult heart in situ hybridization experiments were performed. VITO-2 transcripts were detected in the atrial area in intracardiac ganglia (Fig. 18). The ganglia are composed of different classes of neurons (Richardson et al. 2003). So far, it has been shown, that such cardiac ganglionic plexuses are mainly located at the dorsal surface of the atria. Three major groups of ganglionic plexuses have been identified in the vicinity of the sinoatrial node, atrioventricular node and within the region of the lower pulmonary veins entrance into the left atrium (Cheng et al. 1999). To support the finding that VITO-2 is specifically localized within ganglionic plexuses RT-PCR was performed. VITO-2 mRNA was absent in adult isolated cardiomyocytes (Fig. 18E). So far there is no other vestigial homolog identified in the adult heart. However, human TONDU was initially isolated from the human fetal heart cDNA library (Vaudin et al. 1999). Nevertheless the expression pattern of this gene is still controversial and unclear (Vaudin et al. 1999; Maeda et al. 2002). In this respect VITO-2 can be considered as the first homolog of the vestigial identified in the adult heart but not in the cardiomyocytes and during embryonic heart development. Moreover neurons in different cardiac ganglia control cardiac rate, arteriovenous conduction, and myocardial contractility (Cheng et al. 2004). In this respect, VITO-2 can play a role in the regulation of the conduction system in the adult heart. This presumption can be supported by the fact that transgenic mice overexpressing TEF-3 develop a progressive atria arrythmias with slower conduction velocities across the atria and the ventricular myocardium (Chen et al. 2004).

As it has been already discussed, Vgl-4 was postulated as a homolog of VITO family genes (Chen et al. 2004). The authors showed that in humans Vgl-4 is expressed in the all adult tissues tested. However, they did not evaluate the expression pattern during mouse embryonic development. I found, that transcripts of mouse Vgl-4 homolog are strongly expressed during development at E11.5 onwards (Fig. 22). Mouse homolog of Vgl-4 was found to be expressed ubiquitously in the postnatal life as well as in the myoblasts C2C12 cells. In contrast to VITO-1 and VITO-2 genes, Vgl-4 expression levels were not changed during myotubes formation (Fig. 24). During mouse embryonic development Vgl-4 transcripts were localized in the trabeculated wall of the developing heart at E 15.5 (Fig. 23). Since biochemical data suggest a role of Vgl-4 for co-activating of TEF-1 and MEF2C proteins (Chen et al. 2004) and VITO-1 and VITO-2 are absent in the developing heart, it seems possible, that Vgl-4 is a potential partner

of TEF-1 and MEF2C in the embryonic heart. However the poor conservation of the SID domain does not make a direct interaction likely.

5.3. Nuclear localization of VITO proteins

Since vestigial protein acts as a co-factor for the transcription factor scalloped during wing development in Drosophila, it can be expected that VITO proteins are localized in the nucleus together with TEFs. To address this issue, the intra-cellular co-localization of the VITO-1 and VITO-2 fusion proteins were monitored in different cell lines (Figs. 25-27). In all tested cell lines VITO-1/2 were trans-located into nucleus within 48 hours after transfection. The only exception was VITO-1, which was retained in the cytoplasm of 293T cells even 48 hours after transfection. Co-transfection with TEF-3 but not TEF-1 resulted in the translocation of VITO-1 into the nucleus (Fig. 28). This phenomenon might be explained by a higher affinity of VITO-1 to TEF-3 than to TEF-1. The presence of VITO-1 in the nucleus in C2C12 cells was also reported by another group (Maeda et al. 2002), which postulated that VITO-1 is translocated into the nucleus only after removing mitotic stimuli during C2C12 differentiation. My own results do not support this observation since VITO-1 was translocated into the nucleus without induction of differentiation 36 to 48 hours after transfection. These differences might be attributed to different approaches used to determine the localization. Maeda and colleagues used VITO-1 protein fused to the myc tag followed by its immunodetection. I have used VITO-1 fused to the fluorescence DsRED tag which allows a direct visualization of the target protein. Similar differences were noted in the case of Vgl-4 protein. Both Vgl-4 and TEF-3 linked to GFP tag were also translocated into nucleus in all tested cell lines (Figs. 25-27) without any stimuli. The consequences of the VITO proteins localization in the nucleus will be discussed in chapter 5.9.

5.4. VITO family of genes lack a transactivation domain

It has been shown that VITO-1 can interact with TEF-1 and TEF-3 in vivo and that VITO-1 does not posses an intrinsic transactivation domain. Biochemical data revealed that Vgl-1 gene can interact with TEF-1 and that Vgl-1 is not able to enhance

transcription by itself (Maeda et al. 2002, Gunther et al. 2004). Based on these findings, it seems tempting to speculate that VITO-2 is also an interaction partner for TEF transcription factors as expected by the similarity within the SID domain (Fig. 2). TEFs carry transactivation domains, which are silenced in the absence of co-activators, e.g. VITO-1 and VITO-2. In addition, VITO-2 protein did not contribute to MEF2C mediated activation of MEF2-dependent reporter genes in 293T and C2C12 cell lines (Fig. 41). Taken together, VITO family of genes seem to act as specific modulator of TEF proteins without the ability to activate transcription directly. It has to be emphasized, that VITO proteins represent newly discovered group of proteins which might be different to other known TEFs co-activators (1.3.4). The p160, YAP65 and TAZ proteins are co-activators that contain a transactivation domain. Other TEFs co-activators have been characterized as transcription factors. MAX, MEF2 and SRF might interact with the DNA-binding domain of TEFs as already described in the introduction. In summary, VITO proteins might be characterized as modulators of TEF proteins by changing their structural architecture and activity, alike to vestigial in Drosophila (Halder et al. 2001), without an obvious ability to induce/enhance transcription themselves.

5.5. MyoD mediated conversion of fibroblasts and role of VITO genes

Skeletal, smooth and cardiac muscles as well as adipocytes and many more cell types arise from the same germlayer of the embryo – the mesoderm. A specific fate of the mesodermal cells is determined by the acquisition of a unique combination of the transcription factors. It was shown, that 10T1/2 cells treated with hypomethylating drug 5-azacytidine can trans-differentiate into three different mesenchymal lineages namely, myocytes, chondrocytes and adipocytes (Taylor et al. 1979; Konieczny et al. 1984). MyoD is a member of MRFs family and was initially identified by its ability to convert mesenchymal fibroblasts into the myotubes (Lassar et al. 1986; Davis et al. 1987). In addition, MyoD was found also to drive conversion of other cell types into the myotubes (Weintraub et al. 1989; Choi et al. 1990). The forced expression of other MRF members can also induce the myogenic conversion of non muscles cells with different efficiencies (Braun et al. 1989; Edmondson et al. 1989; Miner et al. 1990). Further studies concentrated on the identification of others factors distinct from MRFs,

which together with them might regulate differentiation of non muscles cells into the myotubes. MEF2C was identified as potential enhancer of the myogenic mediated conversion (Molkentkin et al. 1995). The expression pattern during mouse development and in adult tissues implies that VITO-1 might also play an important role in the myogenic differentiation programme. Interestingly, it has been shown, that VITO-1 is a potent co-activator of TEF-1 and probably also of the MEF2 family genes (Maeda et al. 2002). As shown on Figs. 29 and 34, VITO-1 is able to enhance MyoD mediated conversion of 10T1/2 and 3T3 fibroblasts cells into myotubes. This induction was estimated in the range of 45-60% for 10T1/2 and 20% for 3T3 cells. Similar results were obtained by another group, although they observed a 7 fold enhancement of myogenic conversion (Maeda et al. 2002). Moreover, VITO-1 is not able to activate trans-differentiation of the 10T1/2 and 3T3 cell lines by itself (Fig. 29) indicating that it plays a supportive role, which enhances and modifies a cellular decision imposed by another molecule. In contrast, VITO-2 was not able to enhance myogenic conversion in the both tested cell lines (Fig. 29). The fact that VITO-1 but not VITO-2 is absent in 10T1/2 cells might explain why exogenous expression of VITO-1 but not VITO-2 enhanced the MyoD mediated conversion (Fig. 31). It should also be mentioned that the expression levels of TEF-3 in 10T1/2 cells is very low compared to C2C12 cells whereas VITO-2 is expressed almost at the same level in myoblasts like in fibroblasts (Fig. 31). A semiquantitative RT-PCR analysis of C2C12 cells undergoing terminal myotubes formation revealed that VITO-1 is strongly up-regulated during the first 48 hours of differentiation followed by a reduction of its expression levels, although the expression of VITO-1 in the terminally formed myotubes is still higher than in myoblasts (Figs. 7,24). VITO-2 is strongly up-regulated in the final step of C2C12 differentiation (Fig. 24). Taken together, VITO-1 seems to play a crucial role during early steps of myotubes formation, whereas VITO-2 might be involved in the maturation of myotubes. In this respect VITO-2 function resembles the retinoblastoma (Rb) tumor suppressor protein which is also involved in skeletal myogenesis. During course of the myotubes formation Rb protein became phosphorylated as well its expression level increased around 10 fold (Martelli et al. 1994; Corbeil et al. 1995). Results obtained from ablation of Rb gene in mice have shown its requirement for skeletal muscles formation at terminal stages. It was concluded that a continuous presence of Rb protein is essential for optimal myotubes formation (Huh et al. 2004; Camarda et al. 2004). Despite the fact that VITO-2 and Rb protein seem to share a role

in the formation of myotubes, the functional role of both molecules are fundamentally different. Rb protein is a key regulatory factor of the cell cycle G1-S phase transition (Stevaux et al. 2002), while VITOs are involved rather in co-activation of transcription.

Interestingly, increased amount of VITO-1 is correlated with the up-regulation of TEF-1, TEF-3 and TEF-5 expression which also seem to be important for the initial steps of myotubes formation. Surprisingly newly identified co-activators of TEFs, namely Vgl-4, TAZ and YAP65, do show constant expression profiles (Fig. 24) and described in chapter 1.3.4 of introduction.

In addition, VITO-1 but not VITO-2 improves myogenic conversion of 293T cells (human embryonic kidney cell line) (Fig. 30). Upon co-transfection of MyoD, VITO-1 and TEF-3, 293T cells started to express the muscle cell marker MyHC but they did not form myotubes. Forced expression of MyoD or TEF-3 alone in 293T cells did not result in the induction of the MyHC expression. This data are consistent with the observation, that MyoD alone is not able to activate myogenic programme in CV1 cell line (an African green monkey kidney derived cell) (Weintraub et al. 1989; Weintraub et al. 1991). In addition, it has been shown that VITO-1 is absent in 10T1/2 cells converted by ectopically expressed MyoD (Maeda et al. 2002), which might suggest that VITO-1 is involved in a supplementary pathway promoting muscle differentiation.

The current work demonstrated the requirement of VITO-1 but not VITO-2 for myogenic conversion of fibroblasts. The requirement of VITO-1 for MyoD mediated myogenic conversion was compared to the demand for myogenin. Interestingly, the ablation of myogenin expression resulted in a robust inhibition of myogenic differentiation similar to the inhibition achieved by VITO-1 siRNA (Fig. 35). On the other hand, knockdown of VITO-2 using siRNA assay as tool did not cause inhibition of myogenic conversion in both tested cell lines (Fig. 35). VITO-2 is already expressed in 10T1/2 and 3T3 cell lines, which corresponds to the broad expression profile of VITO-2. The expression profile of VITO-2 during the course of C2C12 differentiation revealed its up-regulation in terminally formed myotubes. Moreover during the mouse embryonic development VITO-2 starts to be expressed in somites when differentiated muscle cells already appear. Hence, one might conclude that VITO-2 gene is rather important for the terminal stages of C2C12 cell line differentiation, but not for the myoblasts specification.

5.6. VITO family of genes are required for C2C12 myoblasts differentiation

The requirement of VITO-1 and VITO-2 for the formation of muscle cells was analyzed in the myoblast C2C12 cell line. Knockdown of VITO-1 expression resulted in an inhibition of myogenin expression and an obstruction of C2C12 myotube formation (Fig. 36). Identical results were obtained using an antisense morpholino approach to knockdown VITO-1 gene in chicken (Chen et al. 2004). C2C12 cells positive for anti-VITO-1-morpholino did not form myotube and were negative for MyHC. In addition it was shown that the absence of VITO-1 prevents MyHC expression in chicken limb muscle precursors (Chen et al. 2004).

The role of the VITO-2 gene for muscle formation was also tested in C2C12 cells. Knockdown of VITO-2 gene using siRNA efficiently attenuated formation of terminally differentiated myotubes (Figs. 37,38). These data are consistent with the expression profile of VITO-2 during C2C12 differentiation but rather differ from observation obtained in MyoD mediated conversion of fibroblasts. As already mentioned VITO-2 might be linked to the terminal formation of the myotubes rather than to muscle specification or initiation of the muscle programme. It can not be excluded that myotubes formed by MyoD mediated conversion differ depending on the presence or absence of VITO-2, while MyHC expression is not affected. VITO-2 might also be an important co-activator during later maturation steps, which are essential for muscle formation. It was observed that C2C12 cells lacking VITO-1 gene were more often mono-nucleated in contrast to cells deficient in VITO-2 gene. Those cells formed clusters and were multi-nucleated (2-3 nucleuses) (Figs. 36-38). Knockdown of VITO-1 and VITO-2 in C2C12 myoblast prevented muscle differentiation but it was not clear whether the inhibition of myotubes formation was solely cell autonomous or whether depletion of VITO gene expression led to changes in the expression of secreted molecules, which stimulate differentiation in an autocrine fashion. To solve this question C2C12 cells were grown in conditioned medium derived from C2C12 cells expressing VITO-1 and VITO-2 siRNA. No differences between conditioned siRNA medium in comparison to the control medium were observed. This finding suggests that abrogation of VITO-1 and VITO-2 expression shuts off genes which are regulated by both co-activators and which are indispensable for differentiation (Fig. 39).

It should be also emphasized, that C2C12 cells lacking VITO-1 gene were unable to form myotubes and that prolonged repression of VITO-1 expression

stimulated programmed cell death. This putative function of VITO-1 in cell survival was indicated by results obtained from C2C12 myoblasts, which stably expressed VITO-1 siRNA. A similar observation was made for vestigial in Drosophila. Cells lacking this gene underwent extensive cell death in the imaginal discs of wings regions (O'Brochta et al. 1983). On the other hand ectopic expression of vestigial led to extensive cell proliferation (Kim et al. 1995; van de Bor et al. 1999; Delanoue et al. 2004) a finding that was not observed in mammalian cells.

It is generally accepted that MyoD and Myf-5 are necessary for the specification of muscles cells whereas myogenin is recognized as a critical factor for terminal differentiation. Recently distinct roles of MyoD and Myogenin were shown by their ability to activate early and late genes, respectively (Cao et al. 2006). It has to be emphasized that forced expression of MyoD is not sufficient to promote a coordinated network of skeletal myogenensis for example in the conversion of smooth muscle to skeletal muscle (Graves et al. 2000). In addition as reviewed in the introduction part of the presented study, promoters of various genes are composed of different regulatory elements. Their regulation depends on a coordinated network involving various families of transcription regulators. It seems therefore an oversimplification to ascribe the initiation and maturation of the myogenic programme to a single transcription factor such as MyoD (Miller et al. 1985, Smith et al. 1993, Cusella de Angelis et al. 1992, Miller 1992). Ongoing studies indicate that VITO-1 plays also crucial role in the muscle specification by enhancement of MyoD mediated conversion, which emphasis the role of VITO family in muscle development (Fig. 48).

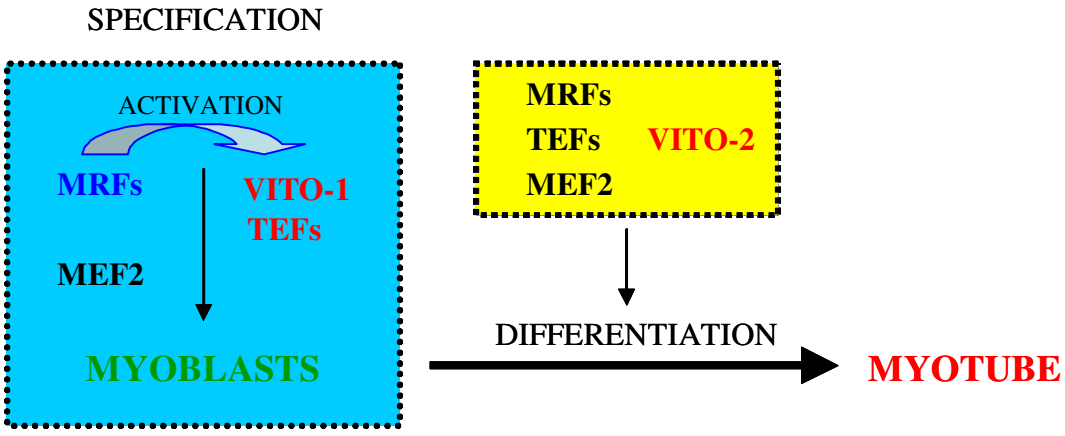


Fig. 48. Position of VITO family of genes during myotubes formation.

5.7. VITO genes are differently regulated by the Notch pathway

The vestigial protein of Drosophila together with its interacting partner scalloped is implied in wing disc development in flies (see 1.3.4). This gene is regulated by two intronic enhancers. Enhancer element vgBE mediates transcription of vestigial under control of the Notch pathway at the boundary region (Williams et al. 1994; Kim et al. 1996). A second regulatory element named vgQ (quadrant enhancer) governs vestigial expression under the Dpp pathway (Kim et al. 1996; Kim et al. 1997). An obvious question was whether VITO-1 and VITO-2 as homologues of vestigial in vertebrate are also under control of the Notch pathway. Delta1 is one of at least five known ligands of Notch (Bettenhausen et al. 1995a) and is closely related to delta in Drosophila (Bettenhausen et al. 1995b). Delta1 is expressed in the presomatic mesoderm, myotome, spinal ganglia and spinal nerves during mouse development (Bettenhausen et al. 1995b; Hrabe de Angelis et al. 1997) as well as in the arterior neural folds of presumptive midbrain and forebrain (Bettenhausen et al. 1995b). Since VITO-2 showed a partially overlapping expression it did not seem unlikely that VITO-2 might be regulated in this period of time by delta1. At E8.0, delta1 deficient embryos lose epithelial somites in the caudal part, show fused spinal ganglia and nerves and an irregular morphology at axial structures (Hrabe de Angelis et al. 1997).

Expression of VITO genes was examined in delta1 deficient embryo. VITO-1 transcripts were not changed in comparison to wild type embryos (Fig. 43) while the expression of VITO-2 was deregulated and an ectopic expression in presomatic mesoderm was found (Figs. 43, 44). VITO-2 transcripts were detectable in delta1 knockout in the caudal part of the neural tube. Based of those findings it can be concluded that VITO-2 but not VITO-1 is under control of the Notch pathway. It is generally assumed that the Notch pathway is involved in the differentiation of different cell types (Artavanis-Tsakonas et al. 1999). It was shown that Notch signalling suppresses myotubes differentiation of C2C12 myoblasts by CBF-1 dependent and independent pathways (Shawber et al. 1996; Nofzinger et al. 1999). In addition it was shown that the intracellular domain of Notch can block specifically the transcriptional activity of MEF2C (Wilson-Rawls et al. 1999). As mentioned above, VITO-2 is expressed in differentiated somites. Overexpression of delta1 in chicken somites inhibited MyoD in the myotome which resulted in a block of skeletal muscle differentiation (Hirsinger et al. 2001) without inhibition of Myf-5 and Pax-3 (Delfini et

al. 2000). Hence, it seems possible that Notch signalling affects VITO-2 although it is not clear at the moment whether VITO-2 is a direct target of Notch signalling. Interestingly, in wild type embryos, TEF-4 is expressed in the area of presomatic mesoderm (Yasunami et al. 1995). Hence, the ectopic expression of VITO-2 in the praxial mesoderm in delta1 mutants might be linked to Notch mediated repression of TEF-4/VITO2 activity. In addition it should be mentioned that TEF-4 and YAP65 control the expression of the Pax-3 gene in the dorsal neural tube (Milewski et al. 2003).

5.8. Lack of VITO-1/2 expression in Myf-5 mutant mice

Due to the fact that both VITO-1 and VITO-2 genes are expressed later than Myf-5 gene in the myotomal part of somites, it seems likely that expression of VITO-1/2 is controlled by Myf-5. Mice carrying mutated Myf-5 allele display normal skeletal muscles but die due to severe rib defects (Braun et al. 1992). Whole mount in situ hybridization revealed that the expression of VITO-1 and VITO-2 genes was absent from the myotome of Myf-5 deficient mice at E10.5. These findings might indicate that VITO family genes are a direct target of Myf-5 during specification of the myogenic precursor cells or myoblasts. In mice lacking Myf-5 gene between E8.0-E11.5, no myotomal cells expressing known muscle markers were detected (Braun et al. 1992; Arnold et al. 1993). On the other hand, VITO-1 was still detectable in the branchial arches of mice lacking Myf-5. These data indicate that expression of VITO-1 in the brachial arch is not dependent on Myf-5. It can be explained by the fact that VITO-1 is not expressed in muscle precursor cells. Hence, it is a non muscle expression domain, which might be independent of Myf-5. This can be supported by the facts that Myf-5 was also found in branchial arch and limb buds where VITO-1 expression is preserved in Myf-5 deficient embryos (Patapoutian et al. 1993, Kablar et al. 1997, Zweigerdt et al. 1997; Hadchouel et al. 2000; Buchberger et al. 2003) Interestingly, the vestigial amount of the Myf-5 transcripts was also found in the presomatic mesoderm in chicken (Kiefer et al. 2001) as well in mouse (Kopan et al. 1994; Cossu et al. 1996). As it was mentioned above, VITO-2 is regulated by Notch pathway. On the other hand its transcripts also depend on the Myf-5, it might be possible that also Myf-5 is regulated by ligand of Notch - delta1. However, there is no evidence, which can support this hypothesis.

5.9. VITO-1 but not VITO-2 is target of SUMO modifier

The activity of the transcription network is also affected by the posttranslational modification of its components. Well known modifications of transcription machinery proteins are phosphorylation, sumoylation, acetylation, methylation, ADP-ribosylation and many others. Bioinformatics analysis of VITO-1 and VITO-2 amino acids sequences revealed many possible target sites for phosphorylation and sumoylation (Fig. 45). SUMO proteins are encoded by three different genes in vertebrates (reviewed by Hilgarth et al. 2004; Johnson 2004; Hay 2005). SUMO proteins bind covalently to the lysine residue in the consensus core sequence ψKxE (ψ-hydrophobic amino acid; x-any amino acid). Sumoylation of proteins modifies their stability, leads to changes in the subcellular localization and inhibit their ubiquitination (Hershko and Ciechanover, 1998). A sumoylation assay was performed to address the issue whether VITO proteins might be regulated by sumoylation. It became clear that VITO-2 was not modified in all tested cell lines (Fig. 46). This observation was consistent with bioinformatics data, since no potential sequence for SUMO modifications was found. Furthermore, despite the fact that VITO-2 protein contains several potential phosphorylation sites, no evidence for a phosphorylation of VITO-2 was found in all tested cell lines. It seems possible that none of the pathways that might utilize these potential phosphorylation sites are active in the cells used in this study. In contrast, VITO-1 protein was most probably phosphorylated in 293T, 3T3 and C2C12 cell lines since an additional band was observed with the MW shifted of approximately 1-2 kDa. VITO-1 might also be sumoylated by SUMO proteins since two potential sites for SUMO in VITO-1 amino acids sequence were identified (Fig. 45). Preliminary results obtained from sumoylation assay 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 (Fig. 46). The biochemical data were further supported by co-transfection experiments using VITO-1 and GAM1. Addition of GAM1 protein led to inhibition of the global sumoylation machinery (Colombo et al. 2002) and efficiently inhibited formation of several VITO-1 bands. However, this finding needs to be verified by the generation of the mutated versions of VITO-1 protein. It is interesting to note that Drosophila homolog of VITO-1 is also sumoylated. It was shown that sumoylation of vestigial enhances an activation of vg-responsive reporter promoter (Takanaka et al.

2005). Such conservation in protein modification between distant species is rather unique.

In mammals it was reported that sumoylation results in an enhancement of the transcription activity in the case of GATA4 (Wang et al. 2004) but not MEF2C (Gregoire et al. 2005). However it is generally believed that SUMO modifiers play a role of the transcription repressors (Gill, 2005; Hilgarth et al. 2004). Recently it was shown that SUMO-1 protein is down-regulated in C2C12 myoblasts undergoing terminal differentiation (Riquelme et al. 2006). At the moment it is hard to predict whether sumoylation of VITO-1 results in an enhancement or a reduction of VITO-1 activity. However, transient overexpression of SUMO-1 during the course of C2C12 differentiation led to attenuation of the myotubes formation and absence of MyHC (Fig. 49A). This observation suggests that SUMO proteins might covalently bind an activators and this way inhibit transcriptional activity of TEFs. On the other hand, sumoylation of MEF2C might block an interactions between this transcription factor with its activators leading to the inhibition of MEF2C function (Fig. 49 B,C). To verify this hypothesis, additional experiments are necessary to perform.

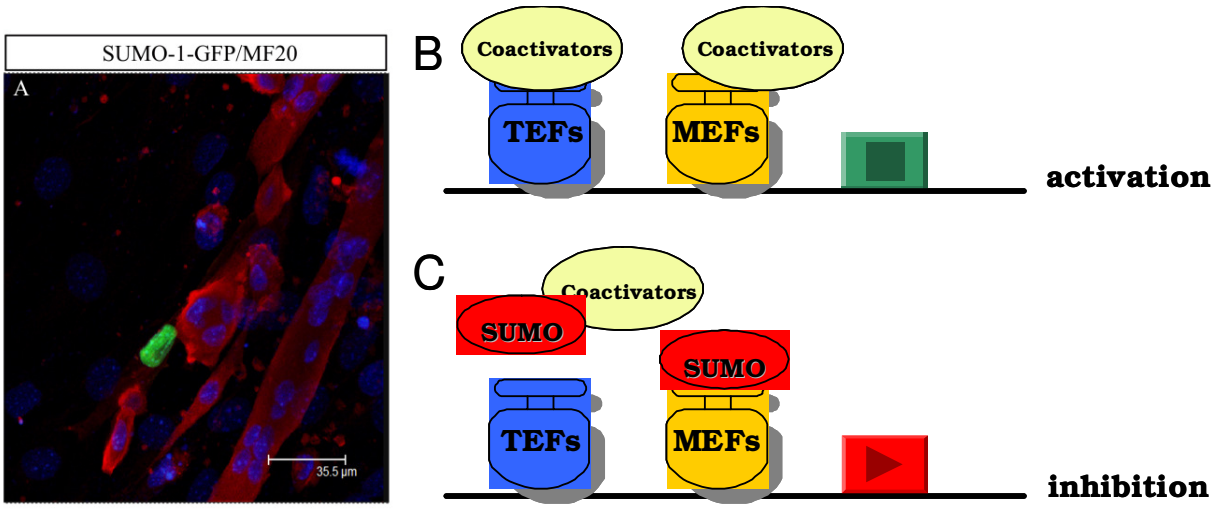


Fig. 49. Panel A - transient overexpression of SUMO-1-GFP (green) in C2C12 myoblasts inhibits their terminal differentiation and absence of MyHC expression (red). Nuclei were visualized by DAPI staining (blue). Panels B and C present a hypothetical mechanism of myogenic programme regulation by sumoylation of key transcription factors and their coactivators.

6. Summary

The myogenic program is controlled by a complex molecular machinery including chromatin modifying enzymes, different transcription factors and their co-activators. The mammalian family of Transcription enhancer factors (TEF) are essential molecules which control transcription in various cell types. They can bind to specific *cis*-regulatory elements within promoter regions of many different genes. Although it is known that their biological properties can be modulated by co-activators or co-repressors, only some components of this transcriptional machinery have been characterized so far. In addition none of the known TEF genes are exclusively expressed in cardiac or skeletal muscle lineage to account for muscle specific expression of MCAT dependent genes. In the work presented here I have identified and characterized additional components of the regulatory complex that modulates activity of TEFs.

Using a subtractive hybridization approach, the first homolog of vestigial in Drosophila and human TONDU protein was identified and named VITO-1. Another member of the VITO gene family was found by virtual screening of the different databases and named VITO-2 due to its high similarity to VITO-1. Both genes are characterized by the presence of a SID domain, which has been described in Drosophila vestigial and human TONDU proteins. Expression pattern of both genes was examined by Nothern Blot, RT-PCRs and in situ hybridization. VITO-1 is expressed in the somitic myotome from E8.75 of mouse embryonic development onwards. In addition, transient domains of VITO-1 expression during embryogenesis were found in the branchial arches, Pharyngeal pouches and clefts, cranial pharynx and Rathkes pouch. In adult tissues its expression was restricted to skeletal muscles and identifying VITO-1 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.

VITOs act as specific modulators of TEF proteins without the ability to activate transcription directly. In this respect VITO proteins represent a distinct group of proteins, which exhibit unique features not found for other TEFs co-activators, which have been characterized as both transcription factors and transcriptional co-activators. Functional analysis of VITOs presented in this work demonstrates that VITO-1 is able to enhance MyoD mediated conversion of 10T1/2 and 3T3 fibroblasts cells into myotubes. VITO-1/2 are not able to activate trans-differentiation of 10T1/2 and 3T3 cell lines by themselves indicating that they play a supportive role, which enhances and modifies a cellular decision imposed by other molecules. The importance of VITO-1 in the myogenic cell specification was further supported by the results showing that VITO-1 together with MyoD and TEF-3 induced MyHC expression in 293T cells. MyHC positive cells were not able to form typical myotubes. In contrast, VITO-2 was unable to enhance myogenic conversion in all cell lines tested. Ablation of myogenin expression during MyoD mediated conversion resulted in a robust inhibition of myogenic differentiation similar to the inhibition achieved by VITO-1 siRNA. On the other hand, knockdown of VITO-2 using siRNA assay did not cause inhibition of myogenic conversion of fibroblasts. During mouse embryonic development VITO-2 starts to be expressed in somites when differentiated muscle cells are already present. The requirement of VITO-1 and VITO-2 for the formation of muscle cells was analyzed in the myoblast C2C12 cell line. Disruption of VITO-1 expression resulted in an inhibition of myogenin expression and an obstruction of C2C12 myotubes formation. Knockdown of VITO-2 gene in C2C12 cells using siRNA also efficiently attenuated formation of terminally differentiated myotubes. VITO-2 might be linked to terminal differentiation of myotubes rather than to the initiation of the muscle programme.

In the present work I also investigated posttranslational modifications of VITO-1/2, i.e. phosphorylation and sumoylation. VITO-2 was not modified in all tested cell lines. VITO-1 protein was most probably phosphorylated and probably also sumoylated by SUMO proteins in 293T, 3T3 and C2C12 cell lines.

Taken together spatiotemporal expression pattern in somites during mouse embryogenesis of both homologues together with dynamic changes in expression levels during the course of C2C12 myoblasts differentiation to myotubes as well as the results obtained from MyoD mediated conversion and knockdown of VITO-1/2 during

fibroblasts conversion and myogenesis clearly indicate that VITO-1 is indispensable for the specification of myoblasts, whereas VITO-2 might play a role for the formation of the terminally differentiated myotubes.

7. Zusammenfassung

Die Myogenese wird von einer komplexen molekularen Maschinerie kontrolliert, welche sowohl chromatin – modifizierende Enzyme, als auch verschiedene Transkriptionsfaktoren und deren Koaktivatoren beinhaltet. Die Genfamilie der TEFs umfasst essentielle Moleküle, die die Transkription in verschiedenen Zelltypen von Säugerorganismen kontrollieren. Sie binden an spezifische *cis*-regulatorische Elemente innerhalb der Promoterregionen vieler verschiedener Gene. Obwohl bekannt ist, dass ihre biologischen Funktionen durch Koaktivatoren oder Korepressoren moduliert werden können, sind bislang nur wenige solcher akzessorischer Komponenten identifiziert und charakterisiert worden. Verblüffenderweise wird keines der bekannten TEF-Gene ausschließlich in Herz oder Skelettmuskelzellen exprimiert, was es schwierig macht, die muskelspezifische Expression TEF- abhängiger Gene zu erklären. In der hier vorgelegten Arbeit konnte ich zusätzliche Komponenten des regulatorischen Komplexes, welche die Aktivität von TEFs moduliert, identifizieren und charakterisieren.

Mit Hilfe eines substraktiven Hybridisierungs Anzatzes wurde das erste Homolog von vestigial in Drosophila und des humanem TONDU-Proteins, identifiziert und VITO-1 getauft (basierend auf der Homologie zu dem Drosophila und dem humanen Protein). Ein weiters Mitglied der VITO-Genfamilie wurde durch einen virtuellen Screen von verschiedenen Datenbanken gefunden und aufgrund seiner starken Homologie zu VITO-1 als VITO-2 bezeichnet. Beide Gene werden durch die Anwesenheit einer SID-Domäne charakterisiert, welche in vestigial und TONDU Proteinen beschrieben wurde. Die Expressionmuster beider Gene wurden mit Hilfe von Northern Blots, RT-PCRs und in situ Hybridisierungen untersucht. VITO-1 wird ab dem murinen Entwicklungsstadium E8.75 im somitischen Myotom exprimiert. Zusätzlich konnten transiente Expressionsdomänen während der Embryogenese in den Kiemenbögen, Schlundtaschen und –wülsten im kranialen Pharynx und in der Rathkesschen Tasche nachgewiesen werden. In adulten Geweben ist die Expression von VITO-1 auf die Skelettmuskulatur beschränkt, was VITO-1 als ersten bekannten TEF-Koaktivator identifiziert, welcher die Aktivität der TEFs gewebespezifisch moduliert. VITO-2 wird von E8.5 an in dem prospektiven Bereich des Mittelhirns, und ab Tag E9.5 im Myotom und in den Somiten exprmiert. Im Gegensatz zu VITO-1 wird VITO-2 in adulten Geweben ubiqutär exprimiert und überlappt mit der Expressionsdomäne von

TEF-3. Die Analyse der VITO-1/2 Expression in delta-1 und Myf-5 defizienten-Embryonen legte die Vermutung nahe, dass VITO-2 nicht aber VITO-1, unter der Kontrolle des Notch-pathway steht und die VITO Genfamilie ein direktes Ziel von Myf-5 während der Spezifizierung von myogenen Vorlauferzellen und Myoblasten ist. Allerdings konnte VITO-1 weiterhin in den Kiemenbögen von Myf-5 defizienten Embryonen nachgwiesen werden, was darauf hinweist, dass VITO-1 nicht in Muskelvorläuferzellen der Kiemenbögen exprimiert wird.

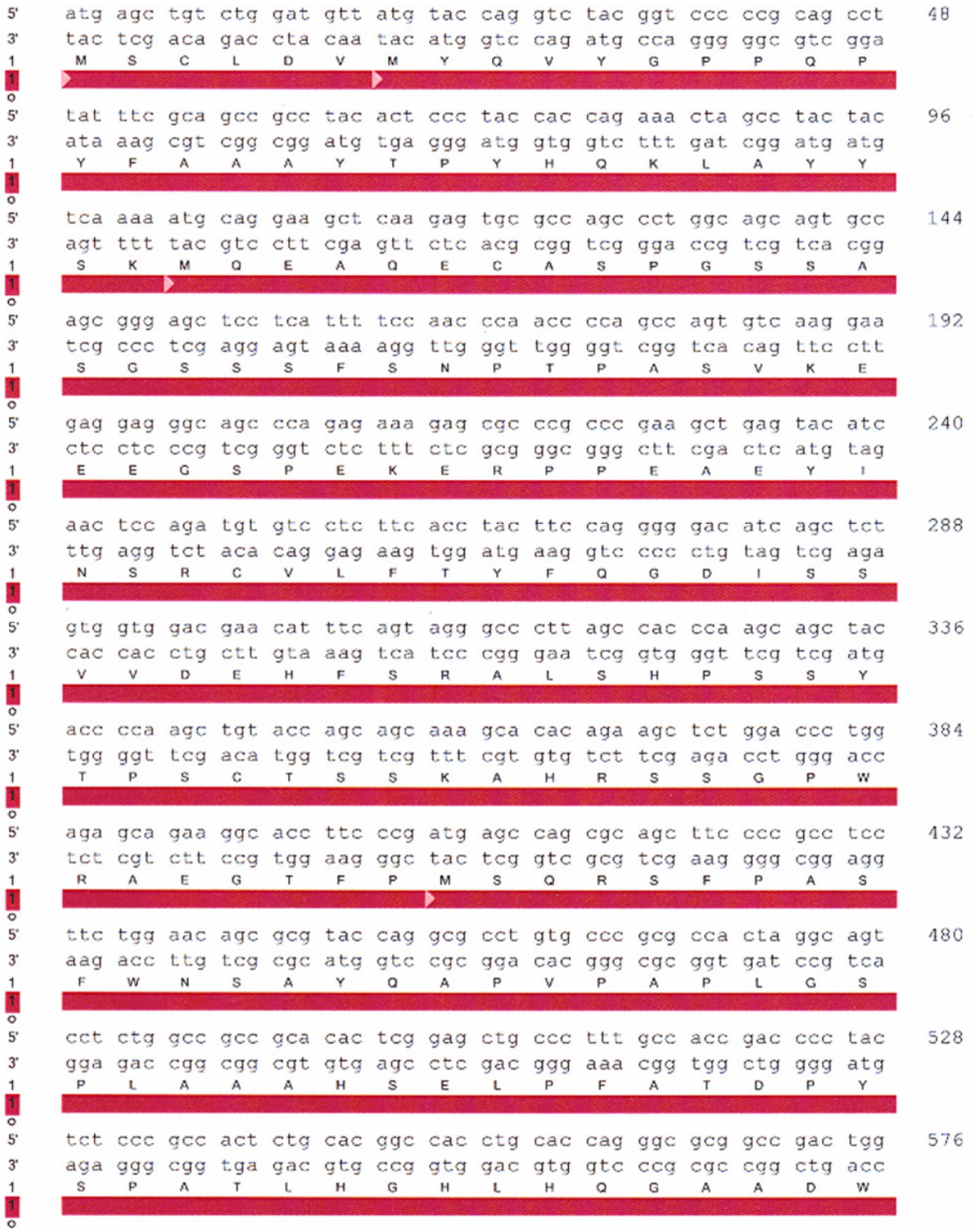
VITOs fungieren als spezifische Modulatoren der TEF Proteine, ohne die Fähigkeit, die Transkription direkt zu aktivieren. Die funktionelle Analyse der VITOs, die in dieser Arbeit beschrieben werden, demonstrieren, dass VITO-1 in der Lage ist, die MyoD-vermittelte Konversion von 10T1/2 und 3T3 Fibroblasten in Muskelzellen zu verstärken. Weder VITO-1 noch VITO-2 waren befähigt, die Transdifferenzierung dieser Zelllinie in Muskelzellen von sich aus zu bewirken. Dieses legt den Schluss nahe, dass VITO-Gene eine unterstützende Rolle bei Zellenscheidungen mittels Verstärkung und Modifizierung anderer Moleküle spielen, die das Zellschicksal festlegen. Die Wichtigkeit von VITO-1 für die Aktivierung muskelspezifischer Transkriptionsvorgänge wird durch Experimente verdeutlicht, die zeigen, dass VITO-1 zusammen mit MyoD und TEF-3 die MyHC-Expression in 293T Zellen induziert. Im Gegensatz zu VITO-1 konnte VITO-2 die myogene Konversion in keiner der getesten Zelllinien verstärken. Interessanterweise resultiert die Ablation der Vito-1 Expression während der MyoD-vermittelten myogenen Konverssion in einer robusten Unterdrückung der myogenen Differenzierung, ähnlich wie bei der siRNA-vermittelten Suppresion der Myogenin Expression. Andererseits konnte die Unterdrückung der VITO-2 Expression mit Hilfe von siRNA die myogene Unwandlung von Fibroblasten nicht verhindern. Während der murinen Embryogenese beginnt die Expression von VITO-2 in den Somiten, wenn differenzierte Muskelzellen bereits vorhanden sind. Die Notwendigkeit von VITO-1 und VITO-2 für die Bildung von Muskelzellen wurde in der Myoblasten C2C12 Zelllinie untersucht. Die Unterdrückung der VITO-1 Expression resultierte hier in einer Inhibition der Myogenin Expression und der Behinderung der C2C12 Myotubenbildung. Auch die siRNA vermittelte Supression der VITO-2 Expression in C2C12 Zellen schwächte die Formation terminal differenzierter Muskelfasern effizient ab. Wahrscheinlich ist VITO-2 eher mit der terminalen Muskelfaserndifferenzierung in Verbindung zu bringen als mit der Induktion der Myogenese.

In der vorliegenden Arbeit habe ich zudem die posttranslationelle Modifikation von VITO-1/2, durch z.B. Phosphorylierung und Sumorylierung untersucht. VITO-2 wurde in keiner der getesteten Zelllinien modifiziert. Dagegen wird das VITO-1 Protein höchstwahrscheinlich phosphoryliert und vermutlich auch durch SUMO Proteine in 293T, 3T3 und C2C12 Zelllinien sumoyliert.

Zusammenfassend zeigte das räumlich-zeitliche Expressionmuster der beiden Homologe in der Somiten während der murinen Embryogenese, zusammen mit den dynamischen Veränderungen im Expressionslevel während der Differenzierung von C2C12 Myoblasten zu Muskelfasern eine wichtige Funktion von Vito-1 und Vito-2 bei der Muskelzellentwicklung an. Die Stimulation der MyoD-vermittelten Konversion von Fibroblasten durch Vito-1 sowie die Notwendigkeit der Expression von VITO-2 bei der terminalen Differenzierung in vitro lassen vermuten, dass VITO-1 unentbehrlich für die Spezifikation von Myoblasten ist, wohingegen VITO-2 eine Rolle in der Formation terminal differenzierter Muskelfasern zu spielen scheint.

8. Appendix

8.1. Sequences of mouse and human VITO-1/2 genes and their alignments



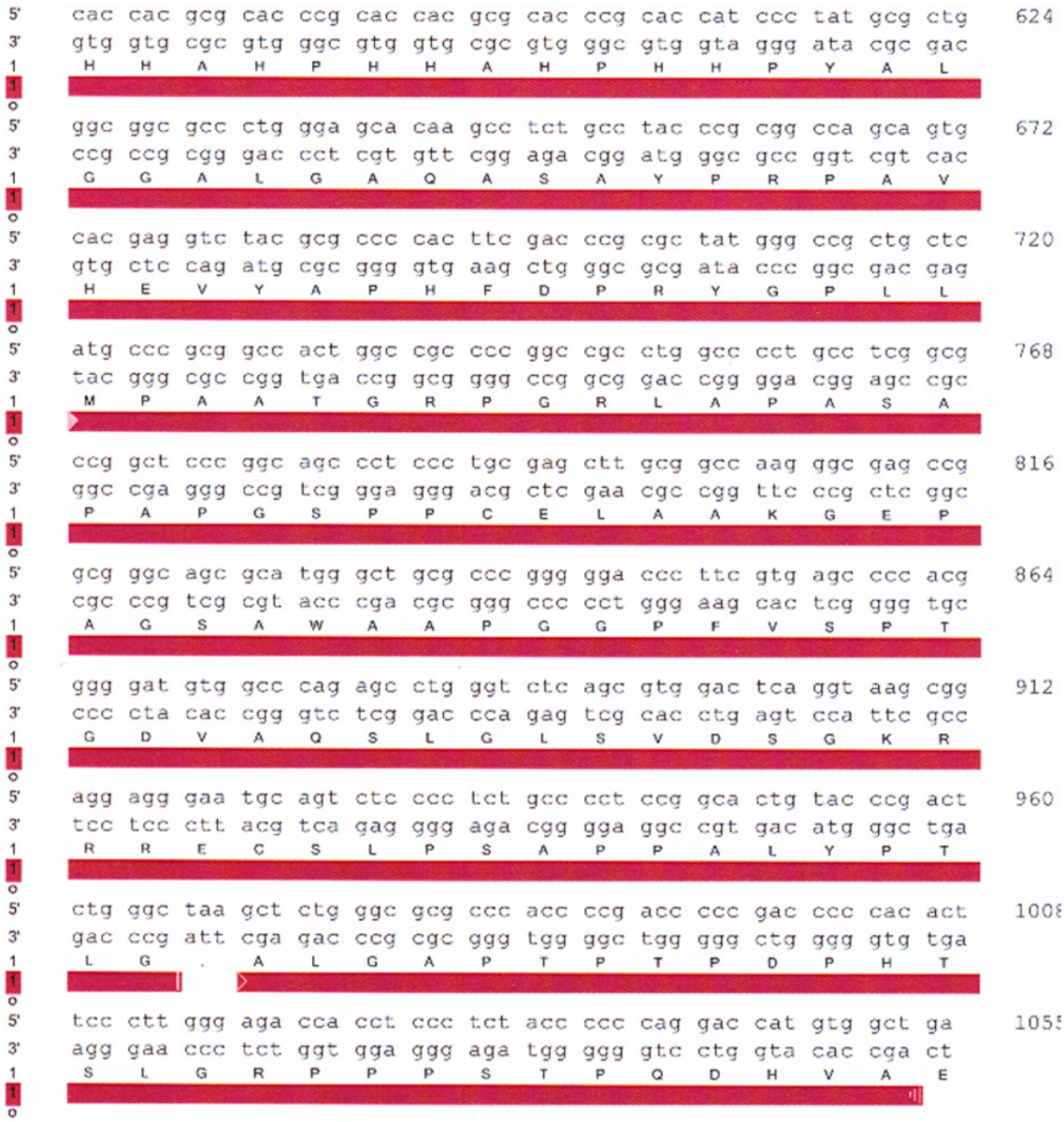


Fig. 8.1.1. Coding region of the mouse VITO-1 gene displayed as nucleotide and amino acid sequences (DNAStar software).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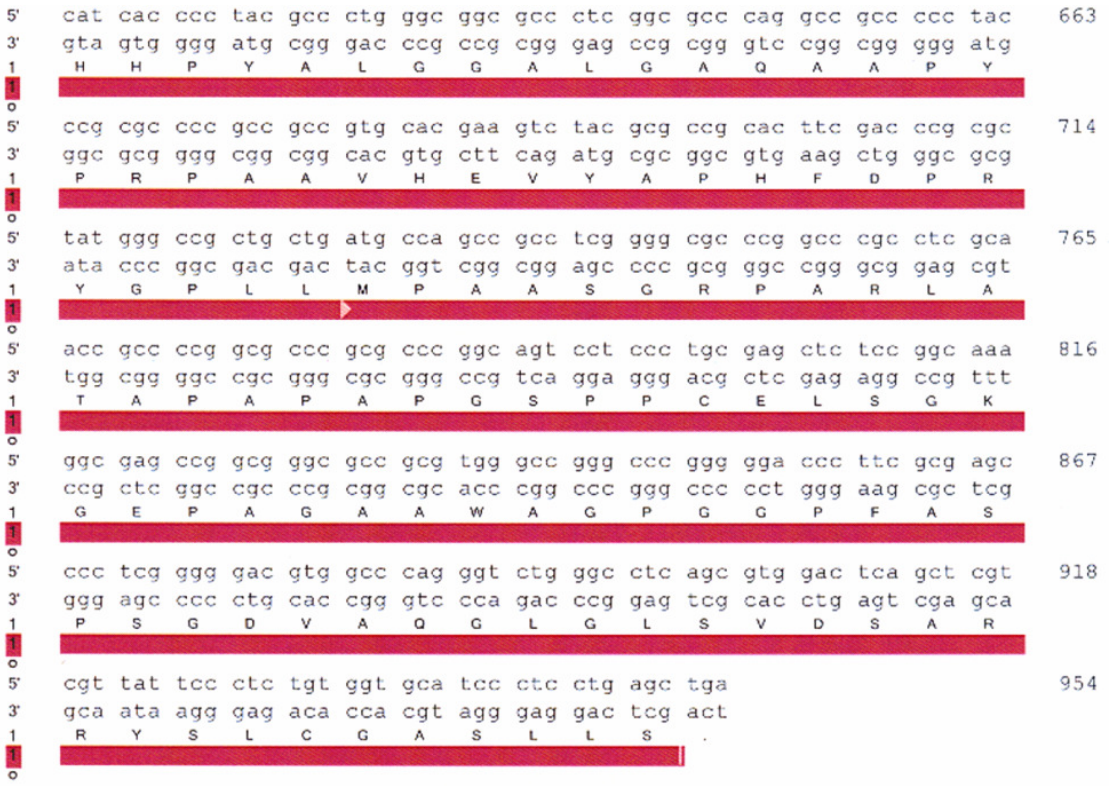
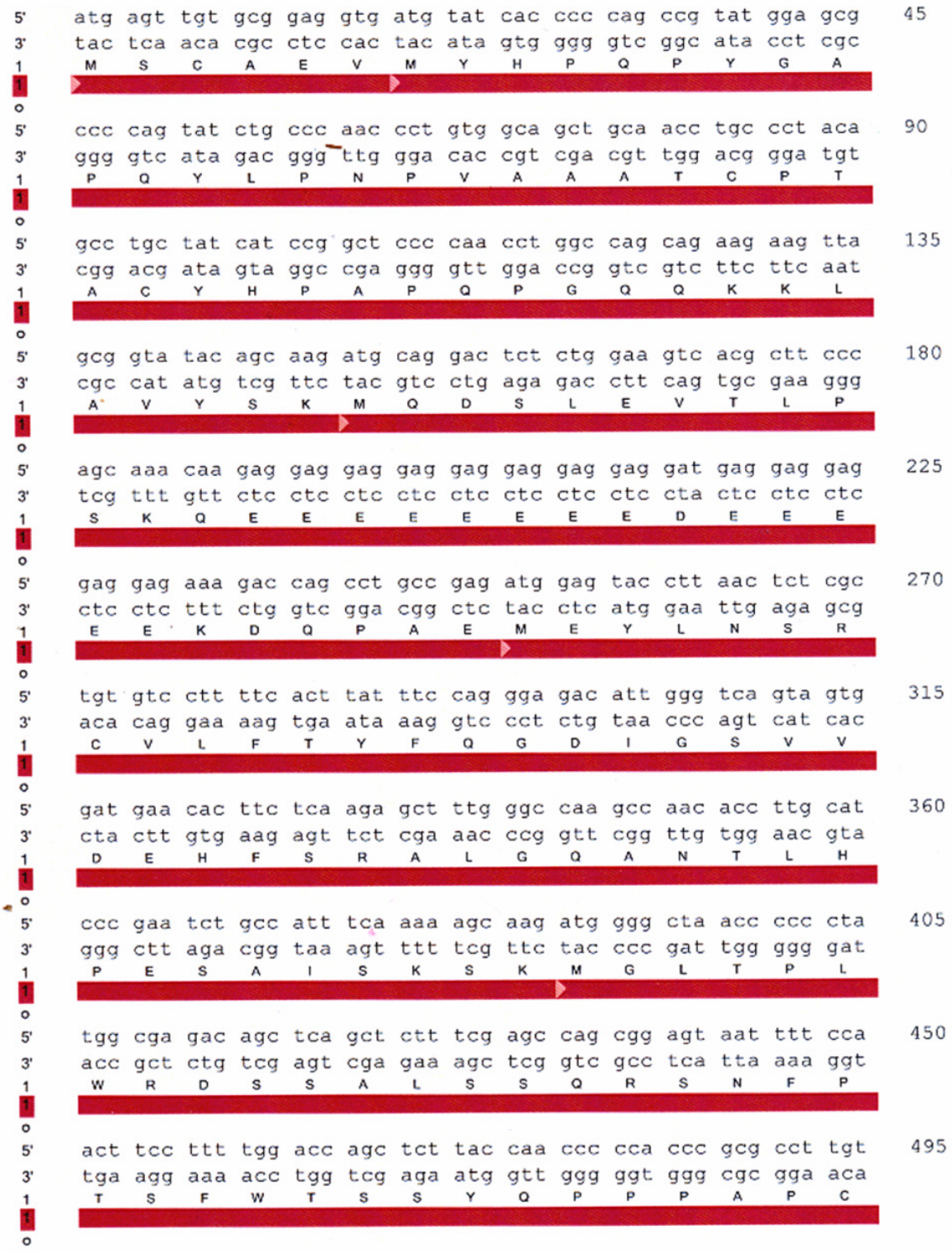


Fig. 8.1.2. Coding region of the human VITO-1 gene displayed as nucleotide and amino acid sequences (DNAStar software).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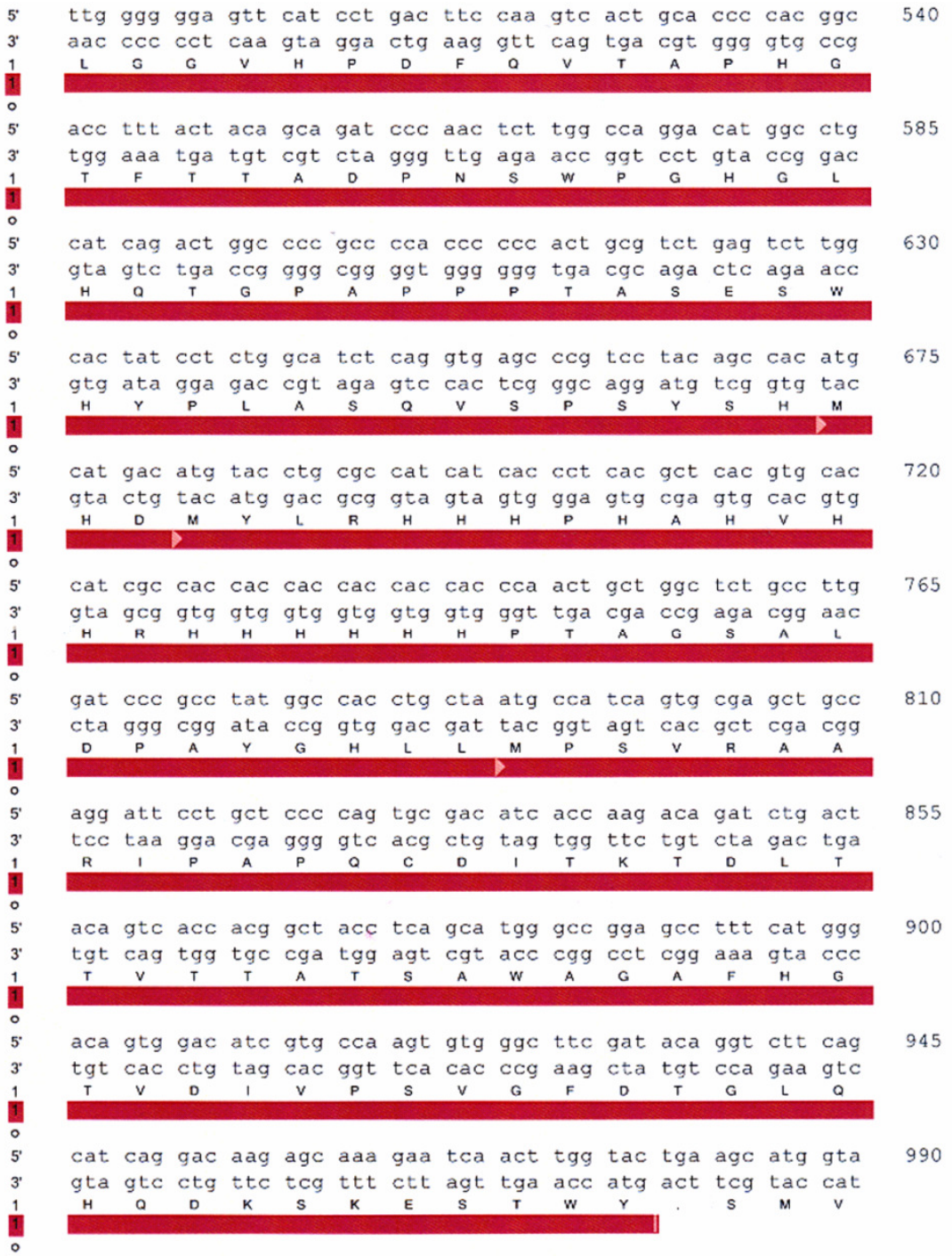
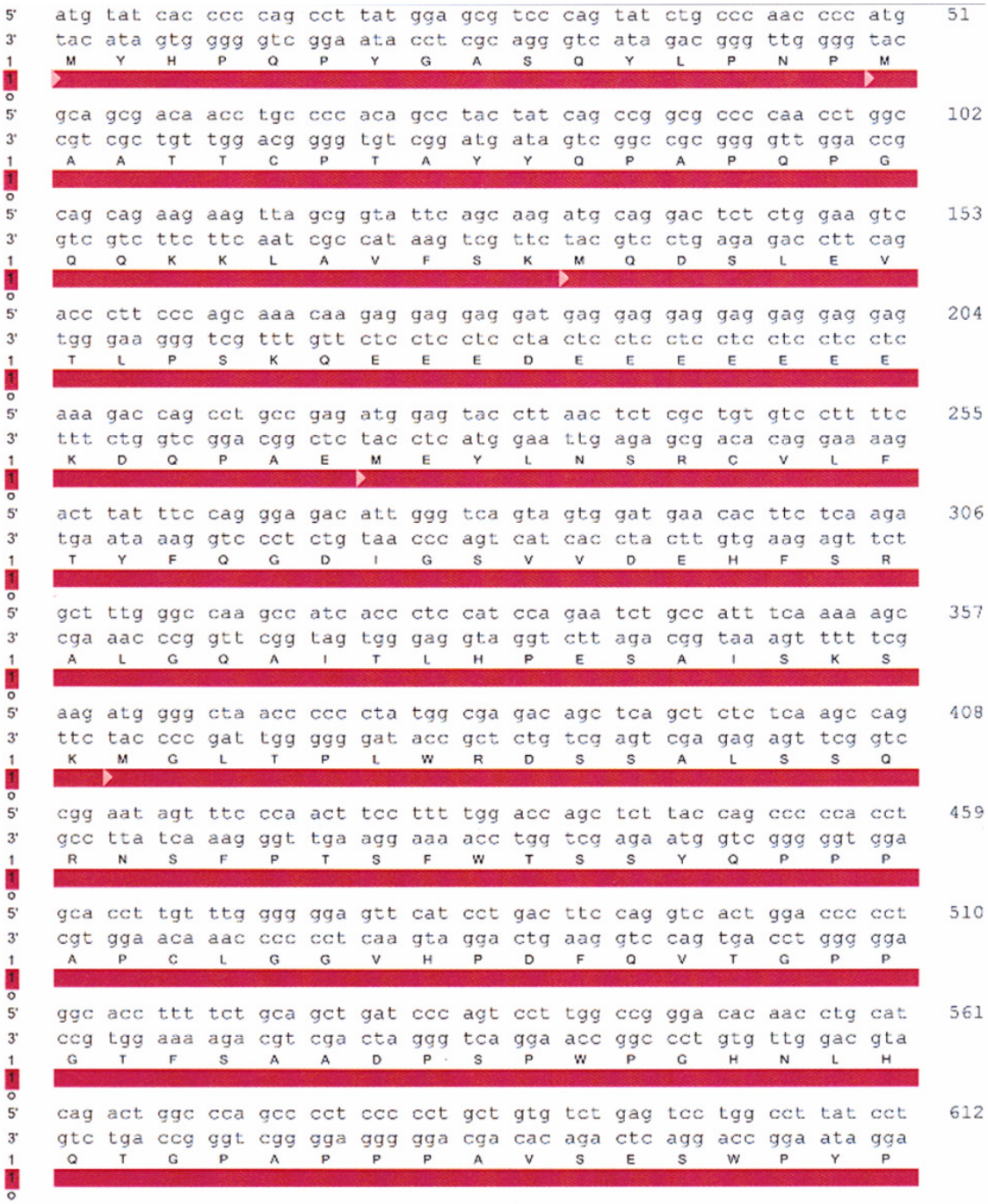


Fig. 8.1.3. Coding region of the mouse VITO-2 gene displayed as nucleotide and amino acid sequences (DNAStar software).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3' gtc gtc ttc ttc aat cgc cat aag tcg ttc tac gtc ctg aga gac ctt cag
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Fig. 8.1.4. Coding region of the human VITO-2 gene displayed as nucleotide and amino acid sequences (DNAStar software).

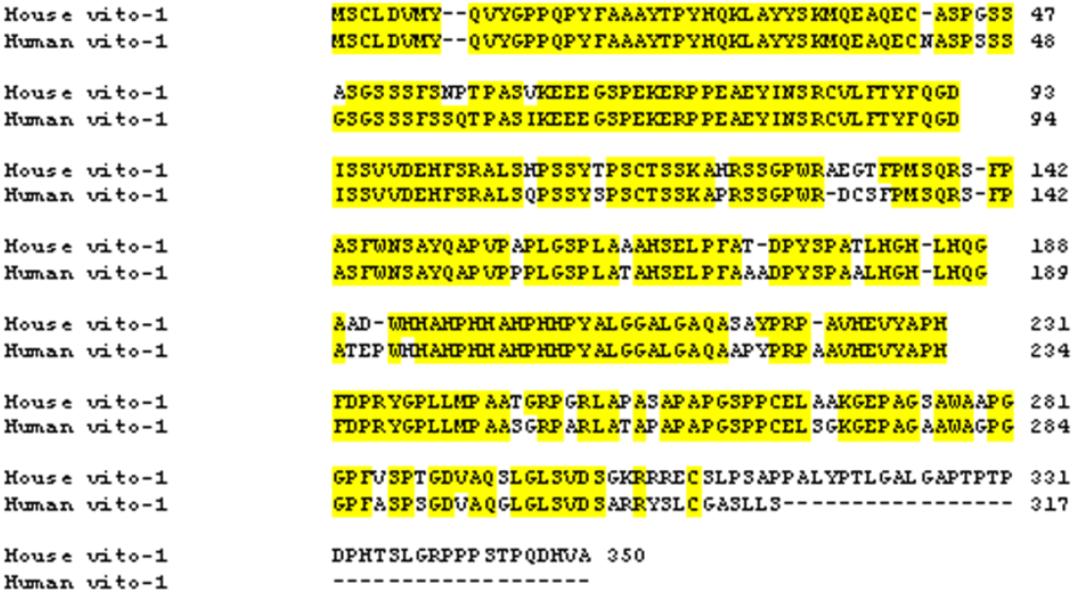

Fig. 8.1.5. Alignment between mouse and human VITO-1 genes on the protein level using the Clustal W method.

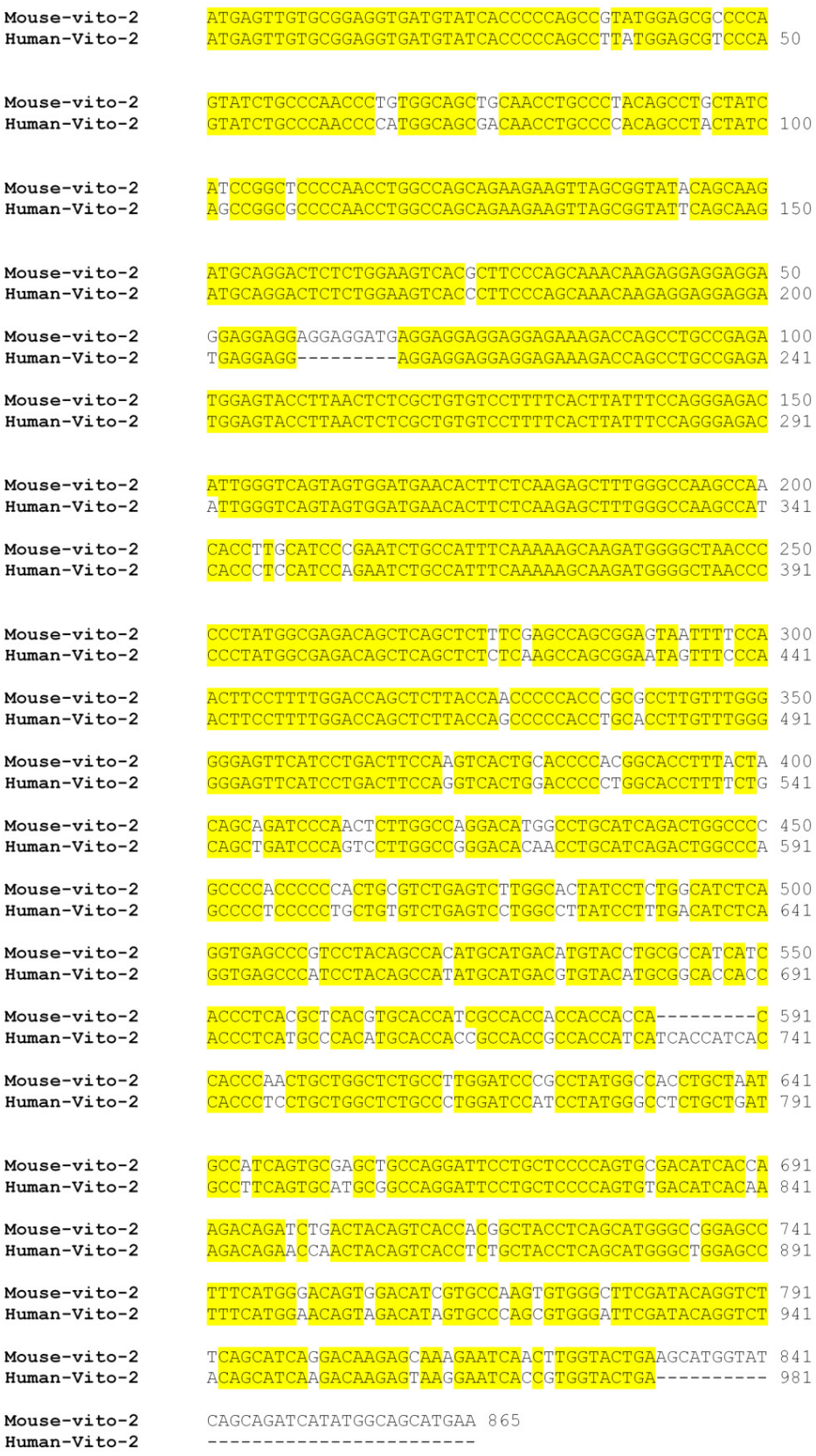

Fig. 8.1.6. Alignment between mouse and human VITO-2 genes on the DNA level using the Clustal W method.

```
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Mouse vito-2            MSCAEVMYHPQPYGAPQYLPNPVAAATCPTACYHPAPQPGQQKKLAVYSK 50
Human vito-2            MSCAEVMYHPQPYGASQYLPNPMAATTCPTAYYQPAPQPGQQKKLAVFSK 50

Mouse vito-1            ASGSSSFSNPTPASVKEEEGS----PEKERPPEAEYINSRCVLFTYFQGD 93
Human vito-1            GSGSSSFSSQTPASIKEEEGS----PEKERPPEAEYINSRCVLFTYFQGD 94
Mouse vito-2            MQDSLEVTLPSKQEEEEEEEDEEEEEKDQPAEMEYLNSRCVLFTYFQGD 50
Human vito-2            MQDSLEVTLPSKQEEEDEEEE---EEEKDQPAEMEYLNSRCVLFTYFQGD 97

Mouse vito-1            ISSVVDEHFSRALSHPSSYTPSCTSSKAHRSSGPWRAEGTFPMSQRS-FP 142
Human vito-1            ISSVVDEHFSRALSQPSSYSPSCTSSKAPRSSGPWR-DCSFPMSQRS-FP 142
Mouse vito-2            IGSVVDEHFSRALGQANTLHPESAISKSKMGLTPLWRDSSALSSQRSNFP 100
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Mouse vito-2            TSFWTSSYQPPPAPCLGGVHPDFQVTAPHGTFTTADPNSWPGHGLHQTGP 150
Human vito-2            TSFWTSSYQPPPAPCLGGVHPDFQVTGPPGTFSAADPSPWPGHNLHQTGP 197

Mouse vito-1            -----AAD-WHHAHPHHAHPHHPYALGGALGAQASAYPRP-AVHEVYAPH 231
Human vito-1            -----ATEPWHHAHPHHAHPHHPYALGGALGAQAAPYPRPAAVHEVYAPH 234
Mouse vito-2            APPPTASESWHYPLASQVSPSYSHMHD-MYLRHHHPHAHVHHRH---HHH 196
Human vito-2            APPPAVSESWPYPLTSQVSPSYSHMHD-VYMRHHHPHAHMHHRHRHHHH 246

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Human vito-1            GPFASPSGDVAQGLGLSVDSARRYSLCGASLLS----------------- 317
Mouse vito-2            AGAFHGTVDIVPSVGFDTGLQHQDKSKESTWYSMVSADHMAA-------- 286
Human vito-2            AGAFHGTVDIVPSVGFDTGLQHQDKSKESPWY------------------ 326

Mouse vito-1            DPHTSLGRPPPSTPQDHVA 350
Human vito-1            -------------------
Mouse vito-2            -------------------
Human vito-2    ----------
```

Fig. 8.1.7. Alignment between mouse and human VITO-1/2 genes on the protein level using the Clustal W method.

8.2. Curriculum Vitae

DATE OF BIRTH: 21 September 1976
STATUS: married
NATIONALITY: Polish

EDUCATION:

1995-2000	Student of Adam Mickiewicz University of Poznań, Faculty of Chemistry, Bioorganic Chemistry Specialization (G.P.A.: 4,5/5,0); Master of Science Thesis: Modified bases in nucleic acids. Kinetin – properties and reactions (5,0/5,0)

Major: Chemistry
Minor: Molecular Biology

RESEARCH EXPERIENCE:

1997-1999	volunteer work in Biochemistry of tRNA Laboratory at the Institute of Bioorganic Chemistry, Polish Academy of Sciences.
1999-2000	preparation of the Master of Science Diploma at the Institute of Bioorganic Chemistry, Polish Academy of Sciences.
2000-2001	PhD student at the Institute of Bioorganic Chemistry Polish Academy of Sciences - not finished.
XII 2001 – I.2005	PhD student at the Martin Luther University Halle - Wittenberg, Medicine Faculty, Institute of Physiological Chemistry
II-2005-VI-2005	PhD student at the Martin Luther University Halle - Wittenberg, Medicine Faculty, Institute of Physiological Chemistry; Max-Planck Institute of Heart and Lung Research – Bad Nauheim.

VII. 2005 – VI. 2006 PhD student at the University Klinik Giessen – Marburg, Max-Planck Institute of Heart and Lung Research – Bad Nauheim.

VII- 2006 PhD student at the Max-Planck Institute of Heart and Lung Research – Bad Nauheim.

CONFERENCES AND COURSES

1. Polish School of Chemistry organized by Academic Federation of Chemistry Students:
   5th Polish School of Chemistry, Murzasichle (1996), poster: "New faces of fullerens" **Mielcarek M**., Ozdowy P.
   6th Polish School of Chemistry, Szklarska Poreba (1997), lecture: "Oxygen – friend or enemy" **Mielcarek M**.
   7th Polish School of Chemistry, Zajaczkowo (1997), lecture: "N^6-furfuryladenine as new modified bases of DNA" **Mielcarek M**., Barciszewski J.
   9th Polish School of Chemistry, Smrek (1998), lecture: "Application of high pressure in chemistry and biology" **Mielcarek M**., Barciszewski J.
   11th Polish School of Chemistry, Szklarska Poreba (1999), lecture: "A new methods in protein biosynthesis in vivo and their application in biotechnology and medicine" **Mielcarek M**., Jarmolowski A., poster: "A molecular mechanism of kinetin actions" **Mielcarek M**., Clark, B.F.C., Barciszewski J.
   12th Polish School of Chemistry, Puławy (2000) lecture: "Biological catalyst of Diels-Alder reaction" **Mielcarek M**., Barciszewski J., poster: "Kinetin prevents tRNA cleavage with OH radicals" **Mielcarek M**., Clark, B.F.C., Barciszewski J.
2. I Congress Polish Society of Cosmetic Chemists "Science for beauty", Warsaw, Poland (1998), poster: "A possibility of kinetin application in cosmoceutical industries" **Mielcarek, M**., Barciszewski, J.
3. I Congress of Biotechnology, Wroclaw, Poland (1998), poster: "Kinetin – biological properties and commercial applications" **Mielcarek, M**., Barciszewski, J., Clark, B.F.C.
4. 18th tRNA Workshop "tRNA 2000" Cambridge, UK (2000), poster: "Kinetin inhibits tRNA degradation with hydroxyl radicals" **Mielcarek, M.,** Clark, B.F.C., Barciszewski, J.

5. Polish-American Conference Physico-Chemical Methods in Biotechnology and Material Science, Poznan, Poland (2000), poster: "Kinetin inhibits tRNA degradation with hydroxyl radicals" **Mielcarek, M**., Barciszewski, J.
6. Course: RNA: Biochemistry & Biotechnology FEBS, Poznan, Poland (1998)
7. Forum "Horizon Chimie", Paris, France (1999) organized by ECPM, ENSCP, ENSIC, ESPCI
8. International Conference "Nucleic Acids and Their Constituents: Chemical Evolution underlying Biological Evolution" Poznan, Poland (1998)
9. International Conference "Molecular Architecture of Evolution: Primary and Secondary Determinants" Poznan, Poland (2000), poster: "Identification kinetin in human urine" **Mielcarek, M**., Barciszewski, J.

PUBLICATIONS:

1. **Mielcarek, M**., Barciszewski, J., (1998) Chemical modification of DNA (in Polish), in: On the frontier of Chemistry and Biology, Koroniak, H., Barciszewski, J., Markiewicz, W.T., Ziemnicki, K., Eds., A. Mickiewicz University Press, Poznan, Poland, pp 217-242
2. Barciszewski, J., **Mielcarek, M**., Barciszewska, M.Z., (1999) Modified nucleosides of nucleic acids. Occurrence and properties of kinetin (in Polish), in: On the frontier of Chemistry and Biology, Koroniak, H., Barciszewski, J., Eds., A. Mickiewicz University Press, Poznan, Poland, pp 77-111
3. Barciszewski, J., **Mielcarek., M**., Stobiecki, M., Siboska, G., Clark, B.F.C., (2000) Identification of 6-furfuryladenine (kinetin) in human urine. *Biochem. Biophys. Res. Commun.* 279, 69-73
4. **Mielcarek, M**., Barciszewska, M.Z., Salanski, P., Stobiecki, M., Jurczak, J., Barciszewski, J., (2002) Native transfer RNA catalyzes Diels-Alder reaction. *Biochem. Biophys. Res. Commun.* 294, 145-148

AFFILIATIONS:

Member of Federation of Polish Biochemical Society (FEBS Constituent Society)

8.3. Publications and scientific activity in congresses during PhD studies

8.3.1. Publications

1. **Mielcarek, M.,** Gunther, S., Kruger, M., Braun, T., (2002) Vito-1, a novel vestigial related protein is predominantly expressed in the skeletal muscle lineage. *Mech. Dev.* 119S, S269-S274
2. Gunther, S.*, **Mielcarek, M**.*, Kruger, M., Braun, T., (2004) Vito-1 is an essential cofactor of TEF-1 dependent muscle specific gene regulation. *Nucl. Acid Res*., 32, 791-802.
3. **Mielcarek, M**., Piotrowska, I., Kostin, S., Schneider, A., Gossler, A., Braun, T., VITO-2, a novel member of VITO family genes depends on Myf-5 during early embryogenesis. (manuscript in preparation)
4. **Mielcarek, M**., Kostin, S., Piotrowska, I., Braun, T., Distinct role of VITO genes in muscle formation. (manuscript in preparation)

8.3.2. Presentation

I Myores Annual Meting Rome, Italy (2005), poster: “Vito proteins are essential new cofactors of the muscle regulatory programme” **Mielcarek, M**., Braun, T.,

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Erklärung

Hiermit erkläre ich, daß ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt und die den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bad Nauheim, September 2006 Michal Mielcarek