Final Report

Title: Biological functions and mechanism of action of WFIKKN1 and WFIKKN2 proteins

OTKA Identifier: 108630

Background

In our earlier work we have identified the genes of two closely related multidomain proteins that were shown to contain a **W**AP-domain, a **F**ollistatin/Kazal domain, an **I**mmunoglobulin domain, two **K**unitz-domains and an **N**TR domain; the genes were designated as *WFIKKN1* and *WFIKKN2* to reflect the domain organization of the encoded proteins (Trexler *et al*., 2001, 2002).

Since WAP-, Kazal-, Kunitz- and NTR-modules frequently serve as protease inhibitors initially we have assumed that these proteins might function as multivalent protease inhibitors. Our studies revealed that WFIKKN proteins inhibit the proteolytic activity of trypsin; the second Kunitz-type domains of WFIKKN proteins were shown to be responsible for their trypsin inhibitory activity (Nagy *et al*., 2003 Kondás *et al*., 2011).

In 2003 Hill and coworkers (Hill *et al*., 2003) have identified the mouse ortholog of WFIKKN2 as a myostatin-binding protein present in normal mouse serum (note that these authors refer to this protein as Growth and differentiation factor-Associated Serum Protein-1, or GASP-1). They have shown that the protein binds both myostatin (GDF8) and GDF11 and block the signaling activity of these growth factors, implicating the protein in the regulation of muscle development (Hill *et al*., 2003).

Myostatin and GDF11 are two closely related members of the TGFbeta superfamily, but the biological roles of GDF8 and GDF11 are markedly different.

Myostatin acts primarily as a negative regulator of muscle growth: deletion of the myostatin gene or mutations in the myostatin gene cause the increase of skeletal muscle mass (McPherron and Lee, 1997a,b; Grobet *et al*., 1997; Kambadur *et al*., 1997; Szabó *et al*, 1998).

GDF11 plays a crucial role in anterior/posterior patterning of the axial skeleton: GDF11 knock-out mice display skeletal defects resulting from abnormal anterior-posterior patterning (McPherron, *et al*., 1999). GDF11 also plays a role in neurodifferention: it acts as an inhibitory signal in neurogenesis of the olfactory epithelium (Wu *et al*., 2003) and inhibits the NGF induced differentiation of PC12 cells to a neural-tissue-like phenotype (Ge at al., 2005).

Similarly to other growth factors of the TGFbeta family, myostatin and GDF11 are produced from large covalent homodimeric precursor proteins by proteolytic processing. In the case of myostatin and GDF11, after cleavage of a single peptide bond by a furin-type protease, the large N-terminal prodomains and the C-terminal growth factor domains remain associated, forming non-covalent „latent" complexes (Thies *et al*., 2001, Ge *et al*., 2005, Harrison *et al*., 2011). Mature, fully active covalent homodimeric growth factors are released from these latent complexes by proteases that degrade the prodomains.

Since Hill and coworkers (Hill *et al*., 2003) have identified WFIKKN2 as a myostatin-binding protein we investigated whether WFIKKN1 also has myostatin-binding affinity. Our studies revealed that:

a.) Both WFIKKN1 and WFIKKN2 bind mature myostatin and GDF11 with high affinity (Kondás *et al*., 2008)
b.) In reporter assays both WFIKKN1 and WFIKKN2 are potent inhibitors of the biological activity of GDF8 and GDF11 (Kondás *et al*., 2008; Szláma *et al*., 2010).
c.) WFIKKN proteins bind myostatin primarily through interactions with the Follistatin domain and the NTR-domain (Kondás *et al*., 2008).
d.) WFIKKN1 (but not WFIKKN2) binds to the prodomain of myostatin, this interaction is mediated by the NTR domain of WFIKKN1 (Kondás *et al*., 2008; Szláma *et al*., 2010, Szláma *et al*, 2013).

e.) Latent myostatin has significant activity and this activity is controlled more efficiently by WFIKKN1 than by WFIKKN2. Our studies have shown that this difference is attributable to the fact that only WFIKKN1 has affinity for the prodomain of latent myostatin (Szláma *et al*, 2013).

f.) We have also studied the interaction of WFIKKN proteins with several additional members of the TGFbeta family. These studies confirmed that WFIKKN proteins are specific antagonists of GDF8 and GDF11: they have much higher affinity for GDF8 and GDF11 than for other members of the TGFbeta family. SPR measurements indicated that both WFIKKN proteins bind TGFbeta1, BMP2 and BMP4 with moderate affinity ($K_d \sim 10^{-7}$M) and BMP3 and BMP8b with lower affinity. Nevertheless, in reporter assays WFIKKN1 and WFIKKN2 did not inhibit the activities of TGFbeta1, BMP2 and BMP4 even in the micromolar range (Szláma *et al*., 2010). Although WFIKKN proteins do not inhibit the signaling activities of BMP2, BMP4 and TGFbeta1, this does not necessarily mean that the interactions of WFIKKNs with BMP2, BMP4 and TGFbeta1 do not have physiological relevance. Growth factor binding proteins may control the action of growth factors not only by inhibiting their action, but also, by serving as a reservoir for growth factors, may localize their action in the vicinity of the binding proteins and may help to establish growth factor gradients in the extracellular space through physical association.

In harmony with the importance of WFIKKN1 and WFIKKN2 as potent and specific inhibitors of GDF8 and GDF11 (Kondás *et al*., 2008; Szláma *et al*., 2010; Szláma *et al*, 2013), mice lacking WFIKKN1 and WFIKKN2 have phenotypes consistent with increased activity of GDF8 and GDF11 (Lee and Lee, 2013). Wfikkn1$^{-/-}$ mice have posteriorly directed transformations of the axial skeleton, which contrast with the anteriorly directed transformations seen in Gdf11$^{-/-}$ mice. Both Wfikkn1$^{-/-}$ and Wfikkn2$^{-/-}$ mice have reductions in muscle weights and impaired muscle regeneration ability, which are the reverse of what we see in Mstn$^{-/-}$ mice (Lee and Lee, 2013).

Adeno-associated virus mediated delivery of Wfikkn2 gene into the muscles of wild type mice resulted in an approx. 30% increase in muscle mass of the treated animals (Haidet *et al*., 2008). Similarly, transgenic mice overexpressing WFIKKN2 protein have larger muscles compared to wild type animals (Monestier *et al*., 2012).

Aims of the project

1. High-resolution mapping of WFIKKN proteins during embryonic development and in adult tissues

Explanation: Our previous studies on the expression of WFIKKN proteins yielded only a low-resolution expression pattern, but have clearly shown that the tissue expression characteristics of the *WFIKKN1* and *WFIKKN2* genes are markedly different. The fact that *WFIKKN* genes are expressed in a wide variety of tissues suggests that these proteins may fulfill biological roles distinct from regulation of skeletal and muscle development. Correlations between the expression profiles of WFIKKN proteins and the expression profiles of various members of the TGFbeta family may provide insights into their biological roles.

2. Mechanism of action of WFIKKN proteins

Explanation: Myostatin and GDF11 are produced from large precursor proteins by proteolytic processing. We wished to test whether WFIKKN proteins – in addition to their interaction with mature growth factors – might also regulate the liberation of mature growth factors from the precursors.

3. Effect of WFIKKN proteins on muscle mass in tumor cachectic mice

Explanation: Since myostatin expressed by tumor cells contributes to cancer- induced skeletal muscle wasting we wished to test whether treatment of tumor-bearing animals with recombinant WFIKKN1 prevents skeletal muscle wasting in tumor cachexia.

Results

1. High-resolution mapping of WFIKKN proteins during embryonic development and in adult tissues.

The main reason why we intended to characterize the expression profiles of WFIKKN proteins was that we wished to gain insight into the biological roles of WFIKKN proteins, distinct from their role in skeletal and muscle development.

Soon after we have started to work on this part of our project, Lee and Lee published detailed expression profiles WFIKKN1, WFIKKN2, GDF11 and myostatin in adult mice and in mouse embyros (Lee and Lee, 2013). These studies have answered most of the questions described in our original research proposal therefore we had to reconsider the priorities of our research plans.

Sigurgeirsson and coworkers (Sigurgeirsson *et al*., 2013) have found that under dark conditions the expression of WFIKKN2 is significantly upregulated in zebrafish brain, raising the possibility that the protein may play a role in sleep–wake dynamics of zebrafish. We wished to answer the question whether the expression of WFIKKN1 and/or WFIKKN2 is also affected by light/dark cycles in mouse brain samples.

BALB/c mice were held either under normal light/dark cycle or were reared for two weeks in a reverse light cycle and were terminated at the end of a dark period; 8 animals were included in both groups. Three regions of the brains were analyzed: the forebrain region, the middle part containing the thalamus and the hind third of the brain. The expression patterns of WFIKKN genes in the different regions were studied by RT-PCR, by immunohistological examinations and western blots of the extracted proteins.

Expression of both WFIKKN genes were demonstrared by RT-PCR in all three regions of the brain, although the expression levels were very low; the three brain regions did not show significant differences in this respect. Reversing the light/dark cycle increased the expression of WFIKKN1 three-fold in the hind region of the brain, but not in the other two brain regions. These results by RT-PCR could not be confirmed at the protein level, because non-specific background staining with the commercial murine antibody used for the immunohistological experiments was too high. The immunohistological experiments will have to be repeated with more specific antibodies before we could decide whether the results of RT-PCR are confirmed at the protein level.

2. Mechanism of action of WFIKKN proteins

Mature myostatin, similarly to other members of the TGF-β growth factor family, is produced from an inactive precursor protein, promyostatin by multiple steps of proteolytic processing. First, a single peptide bond is cleaved by a furin-type protease, but the N-terminal prodomain and the disulfide-bonded homodimeric growth factor domains remain associated, forming a complex known as the latent complex. Fully active, mature growth factor is liberated from this latent complex through proteolytic degradation of the myostatin prodomain. Members of the BMP-1/Tolloid family of metalloproteinases are known to play key roles in the cleavage of the myostatin prodomain.

Since WFIKKN proteins contain several domain types that have been implicated in inhibition of various types of proteases (Trexler *et al*., 2001), we have speculated that – in addition to their interaction with mature growth factors – WFIKKNs might also inhibit the proteolytic cleavages of promyostatin that lead to the formation of mature, active myostatin (Kondás *et al*., 2011). Although neither WFIKKN1 nor WFIKKN2 had any influence on the peptidolytic activity of furin and BMP1 on small synthetic peptide substrates (Kondás *et al*., 2011), this does not necessarily mean that they have no influence on the activity of these proteases on protein substrates.

2.1. Rate of cleavage of promyostatin by furin is not affected by WFIKKN proteins

Our studies have shown that neither WFIKKN1 nor WFIKKN2 had any influence on the rate of furin processing of promyostatin. The lack of WFIKKNs' influence on the rate of furin-cleavage suggests that the accessibility of the furin-cleavage site of the myostatin precursor is not impaired in WFIKKN/promyostatin complexes. In other words, the interaction of the NTR domain of WFIKKN1 with the prodomain of promyostatin does not shield the furin-cleavage site of the precursor.

2.2. 1. Rate of cleavage of latent myostatin by BMP1 is significantly enhanced in the presence of WFIKKN1 concomitant with cleavage of WFIKKN1

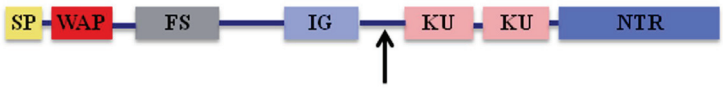
Latent myostatin was digested with BMP1 in the absence and presence of increasing concentrations of WFIKKN1 protein in the presence or absence of heparin. Our studies have shown that the rate of cleavage of myostatin prodomain by BMP1 was significantly enhanced in the presence of WFIKKN1 and this rate enhancement was much more pronounced when heparin was also included in the reaction mixture.

Surprisingly, concomitant with the cleavage of myostatin prodomain, WFIKKN1 was also cleaved by BMP1

These data indicate that the interaction of the NTR domain of WFIKKN1 with the prodomain of latent myostatin increases the susceptibility of the prodomain to BMP1-cleavage but it is not clear whether the enhancer activity is mediated by the NTR domain of full-length WFIKKN1 or of the WFIKKN fragments generated by BMP1-cleavage.

2.2.2. BMP1 cleaves the Arg287–Asp288 bond of WFIKKN1

To characterize the BMP1-cleavage site of WFIKKN1, we have isolated the C-terminal fragment carrying the C-terminal His-tag of the WFIKKN1 protein. N-terminal sequence analysis of the C-terminal fragment revealed that the fragment was generated by cleavage of the Arg287–Asp288 peptide bond, located between the immunoglobulin and the first Kunitz domains of WFIKKN1.



Domain architecture of WFIKKN1 protein.
The name of this protein is an acronym that refers to its constituent WAP-, Follistatin- , Immunoglobulin-, Kunitz-type protease inhibitory domains and NTR domain.The arrow indicates the position of the peptide bond that is cleaved by BMP1.

Cleavage of this peptide bond by BMP1 thus generates WFI1 and KKN1 fragments, corresponding to the N-terminal and C-terminal halves of the protein. The observation that BMP1 cleaves the N-terminal peptide bond of an aspartic residue of WFIKKN1 is in harmony with the strict preference of BMP1 for Asp residues in the P1' position. The importance of Asp288 of WFIKKN1 for its susceptibility to BMP1-cleavage is supported by the fact that its substitution by alanine rendered the protein resistant to cleavage by BMP1.

The BMP1-cleavage site at Arg287–Asp288 of WFIKKN1 is in a flexible low-complexity region that connects the immunoglobulin and the first Kunitz domain of WFIKKN1. It is plausible to assume that the flexibility of this disordered linker region plays a key role in the susceptibility of this peptide bond to BMP1-cleavage.

2.2.3. Rate of cleavage of latent myostatin by BMP1 is not enhanced by the D288A mutant WFIKKN1 in the absence of heparin

To decide whether full-length WFIKKN1 has rate enhancing activity, we substituted the Asp288 residue with alanine to render the protein resistant to BMP1-cleavage. We have found that when latent myostatin was digested with BMP1 in the absence of heparin, the rate of cleavage of myostatin prodomain was practically unaffected by increasing concentrations of D288A mutant WFIKKN1 protein, suggesting that the marked rate enhancement observed in the case of the wild-type WFIKKN1 protein in the absence of heparin is caused by the BMP1-cleavage product KKN1 and not the full-length protein. In harmony with this conclusion, when latent myostatin was digested with BMP1 in the absence of heparin, recombinant KKN1 protein significantly enhanced the rate of cleavage of myostatin prodomain.

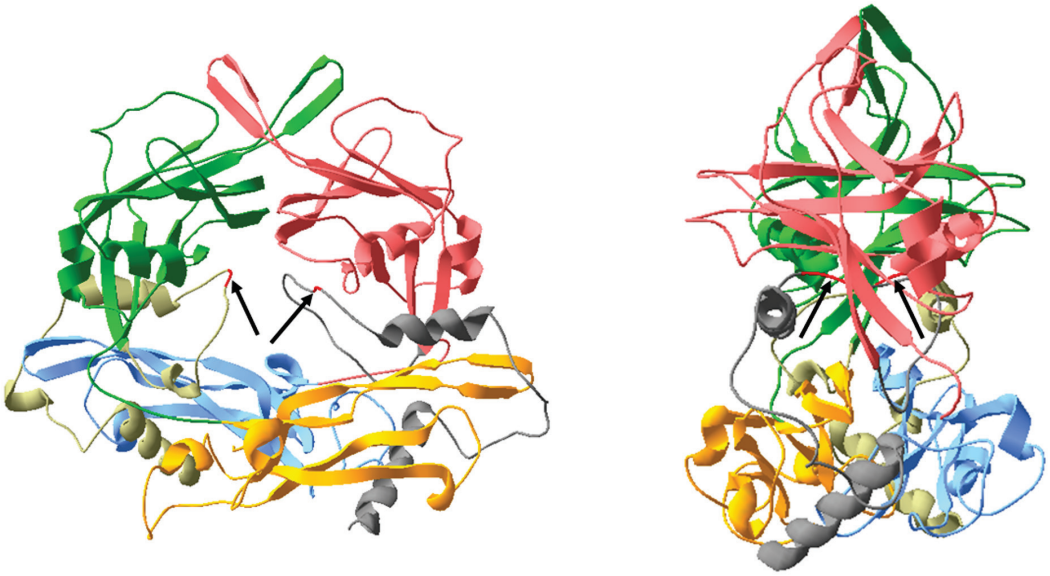
The most plausible explanation for the observation that WFIKKN1 is less active as an enhancer than the KKN1 fragment is that in the compact globular like structure of WFIKKN1 (Walker *et al*., 2015) interdomain interactions render the NTR domain less accessible for interaction

with the myostatin prodomain. According to this explanation, cleavage of WFIKKN1 by BMP1 liberates the KKN1 fragment from these interactions, thereby increasing the accessibility of the NTR domain and favoring its interaction with myostatin prodomain.

It should be noted, however, that in the presence of heparin, the D288A mutant WFIKKN1 protein also has detectable enhancer activity. Similarly, the enhancer activity of KKN1 was more pronounced when heparin was included in the reaction mixture.

2.2.4. Molecular basis of the enhancer activity of KKN1 on BMP1 activation of latent myostatin

To get an insight into the molecular basis of the enhancer activity of KKN1 on BMP1-cleavage of latent myostatin we have analyzed the structural characteristics of the BMP1-cleavage sites of homodimeric latent myostatin. In the homology model of homodimeric latent myostatin (based on the crystal structure of homodimeric pro-TGF-beta1, the two monomers form a disk-like circular structure.

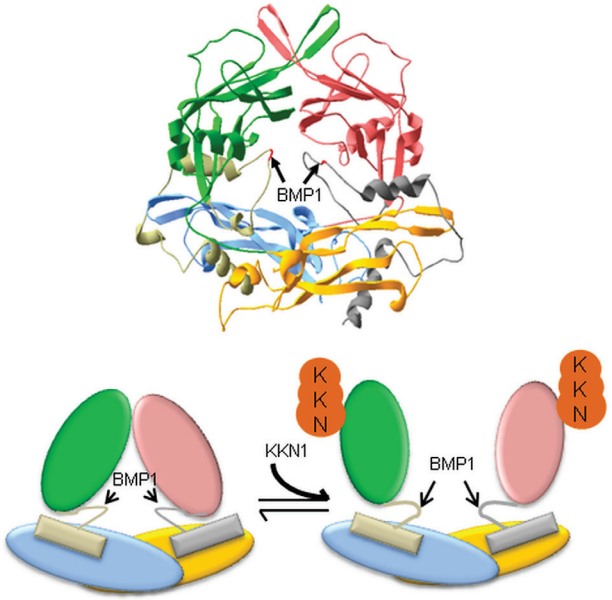


The BMP1-cleavage sites are buried in the center of the homodimer of latent myostatin.
In the homology model of homodimeric latent myostatin the two monomers form a disk-like circular structure. N-terminal parts of the two prodomains (pale yellow - light gray) provide the 'straitjacket' that encircles and shields each growth-factor monomer of the homodimer (light blue - yellow), whereas the C-terminal parts of the two prodomains (green - pink) provide the 'arm domains' that connect the two prodomains in the homodimeric precursor in a 'bowtie'. The loops containing the BMP1-cleavage sites of latent myostatin are buried in the center of the disk-like homodimer, suggesting that they are not readily accessible to cleavage by BMP1. Our data suggest that binding of the NTR domain of KKN1 to the C-terminal subdomains of myostatin prodomains loosens the 'bowtie', shifting the conformational equlibrium from the circular structure to a more open form of latent myostatin making the BMP1 cleavage sites more accessible to the enzyme. The black arrows indicate the position of the BMP1 cleavage sites between residues Arg-98-Asp-99. The positions of Arg-98 and Asp-99 are highlighted in red. The figure in the right-hand panel was generated by 90^{o} clockwise rotation of the homodimer around the y-axis.

N-terminal parts of the two prodomains, consisting of the alfa1 and alfa2 helices, provide the 'straitjacket' that encircles and shields each growth factor monomer of the homodimer, whereas the C-terminal parts of the two prodomains provide the 'arm domains' that connect the two prodomains in the homodimeric precursor. The 'arm domains' consist of three distinct structural regions. A four-stranded beta-sheet (consisting of beta1, beta3, beta6, and beta10 strands and buried by the alfa2, alfa3, and alfa4 helices) is in close proximity of the growth factor monomer of the same polypeptide chain, whereas a four-stranded beta-sheet (consisting of beta2, beta4, beta5, and beta7 strands) and beta-strands beta8 and beta9 extend to link the two arm domains of the homodimeric precursor in a 'bowtie'.

The loops containing the BMP1-cleavage sites of latent myostatin are buried in the center of the disk-like homodimer, suggesting that they are not readily accessible to cleavage by BMP1. The

most plausible explanation for the enhancer activity of KKN1 is that binding of the NTR domain of KKN1 to the C-terminal subdomains of myostatin prodomains loosens the 'bowtie', shifting the conformational equilibrium from the circular structure toward a more open form of latent myostatin making the BMP1-cleavage sites more accessible to the enzyme.



The KKN1 fragment of WFIKKN1 stimulates BMP1-cleavage of latent myostatin.
A possible explanation for the BMP1-enhancer activity of KKN1 is that it shifts a conformational equilibrium of latent myostatin to a more open form, making its BMP1 cleavage sites more accessible to BMP1.

2.2.5. Heparin-dependence of the activity of WFIKKN1 proteins as enhancers of BMP1-mediated activation of latent myostatin

The heparin-dependence of the BMP1-enhancer activity of WFIKKN1, D288A mutant WFIKKN1 and KKN1 is reminiscent of the heparin-dependence of the BMP1-enhancer activity of procollagen C-proteinase enhancer-1 protein (PCPE-1). According to the model proposed by Bekhouche *et al*. (2010), the enhancer PCPE-1 binds to the substrate procollagen III C-propeptides with high affinity and this PCPE-1/procollagen complex binds to heparin-like glycosaminoglycans via the NTR domain of PCPE-1 as well as via procollagen III. As BMP1 also interacts with heparin, these interactions increase the local concentrations of the reactants facilitating the action of BMP1. We assume that an analogous model might explain the heparin-dependence of the BMP1-enhancer activity of WFIKKN1_D288A and KKN1 proteins, with latent myostatin as substrate. A key aspect of this model is that BMP1, latent myostatin, and WFIKKN1 proteins are all bound to heparin and the increased local concentration of the reactants facilitates the action of BMP1 on latent myostatin. It is well established that latent myostatin and BMP1 have affinity for heparin, and in the present work, we have found evidence that WFIKKN1 proteins also have significant affinity for heparin. Interestingly, KKN1 has higher affinity for heparin than the full-length protein, suggesting that intramolecular interactions of the compact globular WFIKKN1 protein may partially bury the heparin-binding site. In view of these findings, it seems likely that BMP1-cleavage of WFIKKN1 favors the formation of the enhancer complex, not only by increasing the accessibility of the NTR domain for the myostatin prodomain but also by increasing the heparin affinity of the enhancer.

In summary, in the present work we have shown that WFIKKN1 is cleaved by BMP1 generating the C-terminal KKN1 fragment that is a potent heparin-dependent enhancer of BMP1-activation of latent myostatin. BMP1-cleavage of WFIKKN1 protein and latent myostatin are thus synergistic in the sense that they promote the liberation of active myostatin from latent myostatin complex.

Publication: *Szláma G, Vásárhelyi V, Trexler M, Patthy L.(2016) Influence of WFIKKN1 on BMP1-mediated activation of latent myostatin. FEBS J. 283:4515-4527*

3. Effect of WFIKKN proteins on muscle mass in tumor cachectic mice.

Elevated expression of myostatin was found in conditions associated with muscle wasting: muscle dystrophies, sarcopenia and chronic diseases like kidney failure, tumor cachexia, AIDS. In cancer cachexia, Zhou *et al* have reported that blockade of the ActRIIB signaling by the use of a soluble variant of the extracellular domain of the receptor prevented muscle wasting and prolonged survival (Zhou *et al*., 2010). Several members of the TGFbeta growth factor family, e.g. myostatin, GDF11, activin A signal though the ActRIIB and this natural promiscuity of the receptor may have contributed to the withdrawal of the ActRIIB receptor-Fc fusion (ACE-031, Acceleron) from phase II trials (Smith and Li, 2013).

WFIKKN proteins are more specific inhibitors of myostatin therefore we studied the effect of WFIKKN1 on muscle mass of cachectic animals. C26 colon tumor bearing mice were treated with 10 mg/kg doses of WFIKKN1 solution two times a week for 4 weeks. During the treatment the volume of the tumors were regularly determined and at the end of the experiment the weight of the animals, the mass of the Soleus, Tibialis anterior (TA) and extensor digitorum longus (EDL) muscles, the weight of the tumors and the fat content were measured. The results revealed that WFIKKN1-treatment had no significant effect on any of the parameters determined.

Deviation from the workplan

1. High-resolution mapping of WFIKKN proteins during embryonic development and in adult tissues.

The main reason why we intended to characterize the expression profiles of WFIKKN proteins was that we wished to gain insight into the biological roles of WFIKKN proteins, distinct from their role in skeletal and muscle development.

Soon after we have started to work on this part of our project, Lee and Lee published detailed expression profiles WFIKKN1, WFIKKN2, GDF11 and myostatin in adult mice and in mouse embyros (Lee and Lee, 2013). **These studies have answered most of the questions described in our original research proposal therefore we had to reconsider the priorities of our research plans.**

2. Mechanism of action of WFIKKN proteins

2.1. Influence of WFIKKN1 and WFIKKN2 on the conversion of the GDF11 precursor to mature growth factor.

According to our original research plan we wished to study the influence of WFIKKN1 and WFIKKN2 on the conversion of both promyostatin and pro-GDF11 to mature growth factors. A basic requirement for these studies is the production of native recombinant precursor proteins.

Since ProGDF11 and promyostatin are closely related proteins, we trusted that proGDF11 protein and GDF11 prodomain might be produced by the same procedures that we have successfully used for the production of promyostatin and myostatin prodomain (Szláma *et al*., 2013). The cDNAs coding for human proGDF11 and GDF11 prodomain were cloned into the same bacterial expression vector that was used for promyostatin and myostatin prodomain expression and E coli. Although we succeeded in identifying conditions where both proteins were expressed in inclusion bodies of E coli BL21(DE3) cells, we have failed to find conditions to refold the proteins to yield native, soluble proGDF11 and GDF11 prodomain suitable for funnctional studies.

Our failure with the E. coli expression systems has led us to test the Drosophila melanogaster expression system. S2 cells were cotransfected with the expression vectors containing the cDNAs of proGDF11 and GDF11 prodomain and pCoHygro selection vector, but we failed to find stable transfectants producing the recombinant proteins. **In the absence latent GDF11 we could not test whether it is similar to latent myostatin in as much as WFIKKN1 also influences its BMP1-mediated conversion to mature growth factor.**

2.2. Studies on mutant promyostatins

Our observation that the interaction of WFIKKN1 with the prodomain of latent myostatin plays a significant role in the regulation myostatin activity (Szláma *et al*, 2013) and activation of latent myostatin (Szláma *et al*, 2016) raised the possibility that some natural myostatin mutations of the prodomain region may affect this interaction. **Although studies on mutant promyostatins were not included in our original research proposal we initiated studies to clarify the molecular basis of the phenotypic consequences of some common promyostatin mutants.**

K153R and A55T mutants of human promyostatin

Since myostatin plays an important role in regulating muscle and fat mass and in maintenance of physical strength, polymorphic variations might be expected to influence muscle strength and physical state in healthy and sick individuals. K153R and A55T are the most common myostatin polymorphisms, with an allele frequency of 0.19 and 0.12, respectively, in African Americans. Several studies have found an association of the R153 allele with lower strength in older women, and with accelerated sarcopenia (Seibert *et al*., 2001; Corsi *et al*., 2002). A study among Asian Indians in north India found that the T55 and R153 variants of the myostatin gene predispose to obesity, abdominal obesity and low lean body mass (Bhatt *et al*., 2012). In a study with non athletic young men, Santiago *et al.* found that the K153R polymorphism affects the ability to produce 'peak' power during muscle contractions: men with the KR genotype had a worse performance in vertical jumps compared with those with the wild-type KK genotype (Santiago *et al*, 2011). A recent study of Li *et*

al., has found an association between the two polymorphisms and the strength training-induced muscle hypertrophy among men of Han Chinese ethnicity (Li *et al*., 2013). Studies on the myostatin variants of extremely old Spanish and Italian people found that the frequency of the variant R153 allele was significantly higher in centenarians than in controls (González-Freire *et al*., 2010; Garatachea *et al*., 2013).

The K153R and A55T mutations affect the prodomain region of myostatin, a region that shields the growth factor domains in both promyostatin and latent myostatin. In principle, structural changes in this region may affect the rate of furin cleavage at the prodomain-growth factor domain boundary, the rate of BMP1 cleavage within the prodomain, the interactions contributing to the stability of the prodomain-growth factor complex as well as the interaction of myostation prodomain with WFIKKN1.

In order to provide information about the possible structural and functional consequences of the A55T and K153R substitutions we have produced the recombinant mutant promyostatins and studied the influence of the substitutions on various biochemical properties of the mutant proteins.

Our preliminary studies on A55T promyostatin indicate that the mutation does not affect the rate of cleavage of mutant promyostatin by furin, the rate of cleavage of mutant latent myostatin by BMP1 or the activity of latent myostatin. Studies on the possible influence of the mutation on the expression characteristics of A55T mutant myostatin are in progress.

The most common polymorphism of the human myostatin gene, the K153R polymorphism, shows significant association with lower muscle strength and obesity. Since this is just the opposite of what we observe in the case of mutations leading to loss of myostatin activity, it seemed plausible to assume that the K153R mutation might increase myostatin activity. To address this question, we have studied the molecular properties of recombinant myostatin precursor carrying the K153R mutation and compared its properties with those of the wild-type protein. Our studies have shown that the K153R substitution significantly increases the rate of furin cleavage but has no effect on the activity of latent myostatin or its cleavage by BMP-1. Since latent myostatin is known to have significant activity (Szláma *et al*, 2013), an increase in the amount of furin-activated myostatin and concomitant increase in myostatin activity may explain the observed association of K153R mutation with lower muscle strength and obesity.

We suggest that the association of the K153R mutation with extreme longevity is also explained by increased myostatin activity. It is noteworthy in this respect that the myostatin ortholog of Drosophila, myoglianin, has been recently shown to control longevity: its overexpression in muscle extends lifespan and delays systemic aging of flies by acting on muscle and adipocytes (Demontis *et al*., 2014), raising the possibility that myostatin may also regulate lifespan in mammals (Patel and Demontis, 2014).

Publication: *Szláma, G., Trexler, M., Buday, L., Patthy, L, (2015) K153R polymorphism in myostatin gene increases the rate of promyostatin activation by furin FEBS Lett. 589(3):295-30.*

The *Mstn*$^{cmpt\text{-}Dl1abc}$ mutant promyostatin

Studies on the hypermuscular Compact mouse, selected for high body weight and protein content, have shown that the Compact phenotype is caused by a 12-bp deletion in the myostatin gene (Szabó *et al*., 1998). This deletion, however, did not provide a simple explanation as to how it leads to loss of myostatin activity. This mutation (denoted *Mstn*$^{Cmpt\text{-}dl1Abc)}$ in the prodomain region of the myostatin precursor does not result in premature termination and does not affect the integrity of the growth factor domain.

In principle, the *Mstn*$^{Cmpt\text{-}dl1Abc}$ mutation present in the prodomain region might lead to decreased myostatin activity if it interferes with any of the steps leading to the production of mature myostatin from promyostatin: it might interfere with the folding of the precursor and/or the formation of the covalent homodimer, preventing its secretion or it might impair the liberation of mature myostatin from the precursor and/or the latent complex.

We wished to examine these possibilities therefore we produced and studied the molecular properties of promyostatins carrying the *Mstn*$^{Cmpt\text{-}dl1Abc}$ mutation. Our studies on recombinant proteins

expressed in bacterial expression systems have shown that the *Mstn*$^{cmpt\text{-}Dl1abc}$ deletion causes misfolding of promyostatin. In mammalian expression systems misfolding of *Mstn*$^{cmpt\text{-}Dl1abc}$ promyostatin impaired its secretion, suggesting that the hypermuscular phenotype of Compact mouse is due to a severe decrease in the level of extracellular myostatin precursor. Based on these findings we suggest that the modifier loci that affect the expressivity of the Compact phenotype (Varga *et al*., 2003; 2005) may encode constituents of the Unfolded Protein Response as they may influence the fate of mutant promyostatin.

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