

Both WFIKKN1 and WFIKKN2 Have High Affinity for Growth and Differentiation Factors 8 and 11^{*[S]}

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WFIKKN1 and WFIKKN2 are large extracellular multidomain proteins consisting of a WAP, a follistatin, an immunoglobulin, two Kunitz-type protease inhibitor domains, and an NTR domain. Recent experiments have shown that WFIKKN2 protein binds mature GDF8/myostatin and myostatin propeptide and inhibits the biological activity of myostatin (Hill, J. J., Qiu, Y., Hewick, R. M., and Wolfman, N. M. (2003) *Mol. Endocrinol.* 17, 1144–1154). Here we show that the paralogue of this protein, WFIKKN1, also binds to both myostatin and myostatin propeptide and that both WFIKKN1 and WFIKKN2 bind GDF11, the growth and differentiation factor most closely related to myostatin, with high affinity. Structure-function studies on WFIKKN1 have revealed that the follistatin domain is primarily responsible for the binding of mature growth factor, whereas the NTR domain contributes most significantly to the interaction with myostatin propeptide. Analysis of the evolutionary histories of WFIKKN1/WFIKKN2 and GDF8/GDF11 proteins indicates that the functional association of an ancestral WFIKKN protein with an ancestor of GDF8/11 may date back to cephalochordates/urochordates. Although duplication of the corresponding genes gave rise to WFIKKN1/WFIKKN2 and GDF8/GDF11 in early vertebrates, the data presented here suggest that there is significant functional overlap of the paralogous proteins.

By using sensitive homology search and gene finding programs, we have previously identified two closely related multidomain proteins of unknown function: WFIKKN and WFIKKNRP (1, 2). Both proteins (recently renamed as WFIKKN1 and WFIKKN2) contain a WAP domain, a follistatin/Kazal domain, an immunoglobulin domain, two Kunitz-type protease inhibitor domains, and an NTR domain. Because WAP-, Kazal-, and Kunitz-type protease inhibitor modules are frequently found in serine protease inhibitor proteins (3) and the inhibitory N-terminal domain of tissue inhibitors of metalloproteases belongs to the NTR domain family (4), we have suggested that WFIKKNs may be multivalent protease inhibitors that may control the action of different proteases.

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains 1 a supplemental data file, Tables S1 and S2, and supplemental Fig. 1.

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Although the second Kunitz-type protease inhibitor domain of human WFIKKN1 protein was indeed found to inhibit trypsin (and only trypsin) out of a panel of trypsin-related serine proteases (5), structural and evolutionary analyses suggest that trypsin may not be the prime target of this Kunitz domain (6).

Studies on the expression characteristics of WFIKKN1 and WFIKKN2 did not provide obvious clues as to the biological function of these proteins as both genes were expressed in multiple tissues (1, 2). The *WFIKKN1* gene was found to be expressed (in order of decreasing intensity) in pancreas, thymus, liver, kidney, lung, and testis with practically no expression in brain, heart, skeletal muscle, ovary, small intestine, colon, leukocyte, spleen, and prostate. The *WFIKKN2* gene is expressed (in order of decreasing intensity) in ovary, testis, pancreas, brain, and lung but not in heart, liver, placenta, small intestine, colon, leukocyte, spleen and prostate. Thus the main difference between the two genes is that the *WFIKKN2* gene is expressed in brain but not in liver, whereas the reverse is true for the *WFIKKN1* gene.

In the case of fetal tissues, the *WFIKKN1* gene is expressed at the highest level in the lung with weaker expression in skeletal muscle and liver, whereas the *WFIKKN2* gene is expressed primarily in fetal brain, skeletal muscle, thymus, and kidney. Thus, despite significant differences in their expression pattern, the two genes were similar in that both are expressed in fetal skeletal muscle. It is noteworthy in this respect that the WFIKKN2 protein was subsequently identified as a growth and differentiation factor-associated serum protein that binds myostatin, and through this interaction it regulates muscle development (7).

Myostatin (or GDF8)² and BMP-11 (or GDF11) of vertebrates are closely related members of the transforming growth factor β superfamily; mouse myostatin and mouse GDF11 differ by only 11 amino acids in the mature growth factor domain (8, 9). The fact that in the amphioxus *Branchiostoma belcheri* there is a *GDF8/11* gene that is an archetype of both vertebrate myostatins and GDF11s suggests that the gene duplication generating vertebrate *MSTN* and *GDF11* genes occurred relatively recently, after the divergence of cephalochordates from vertebrates (10). Amphioxus GDF8/11 is expressed in adult gills, muscle, and notochords, suggesting that it plays varied roles in the differentiation of these tissues (10).

GDF8 and GDF11, the two descendant of the ancestral *GDF8/11* gene, have diverged significantly in function. In ver-

² The abbreviations used are: GDF8, growth and differentiation factor 8 or myostatin; GDF11, growth and differentiation factor 11 or bone morphogenetic protein 11; BMP-11, bone morphogenetic protein 11 or growth and differentiation factor 11.

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tebrates, myostatin acts primarily as a negative regulator of muscle growth; deletion of myostatin gene or mutations in the myostatin gene cause the increase of skeletal muscle mass as a result of a combination of muscle fiber hypertrophy and hyperplasia (11, 12). Conversely, GDF11 appears to play a crucial role in anterior/posterior patterning of the axial skeleton; GDF11 knock-out mouse displays skeletal defects resulting from abnormal anterior-posterior patterning (13).

It appears that both GDF8 and GDF11 have multiple additional roles. For example, although in mammals myostatin is strongly expressed only in skeletal muscle and weakly in heart, mammary glands, and adipose tissue (8, 14, 15), it can also circulate as an endocrine factor (16), and thus its action is not necessarily restricted to the sites of its synthesis. In harmony with this expectation, myostatin has been shown to play a role in adipocyte and osteogenic differentiation (17–19). Furthermore, the fact that in chicken and fishes myostatins are expressed in most tissues, including brain, testes, ovary, eyes, muscle, intestine, and spleen (20–29), is consistent with the notion that the function of myostatin is not restricted to skeletal muscle. (Additional information on the expression characteristics of myostatin in mammals is available in the Gene Expression Atlas of the Genomics Institute of the Novartis Research Foundation.)

In adult tissues GDF11 is expressed in skeletal muscle, brain, and dental pulp, but during embryogenesis (in addition to its unique role in anterior/posterior patterning of the axial skeleton), it also controls the patterning of mesodermal and neural tissues (9, 30). GDF11 is a negative regulator of chondrogenesis, myogenesis (31), neurogenesis (32, 33), and production of islet progenitor cells (34). GDF11 also regulates kidney organogenesis (35), pancreatic development (36), and rostrocaudal patterning of the developing spinal cord (37). (Additional information on expression characteristics of GDF11 in mammals is available in the Gene Expression Atlas of the Genomics Institute of the Novartis Research Foundation.) The fact that both myostatin (15) and GDF11 (38, 39) are implicated in the regulation of cardiomyocyte proliferation indicates that there is overlap in the functional spectrum of the two growth and differentiation factors.

Similarly to other members of the transforming growth factor β superfamily, GDF8 and GDF11 are secreted extracellular proteins that are produced from precursor proteins by proteolytic processing. After cleavage of a single peptide bond by a furin-type protease, the N-terminal propeptide and the disulfide-bonded homodimer of C-terminal mature growth factor domains remain associated, forming an inactive complex known as the small latent complex (40–42). Active mature growth factors GDF8 and GDF11 may be liberated from the latent complexes through degradation of the propeptide by a variety of proteases; members of the BMP-1/Tolloid family of metalloproteinases are known to play key roles in the cleavage of the propeptides of both myostatin and GDF11 (33, 43). The fact that WFIKKN2 was found to bind and inhibit the activity of both myostatin and GDF11 but not activin and transforming growth factor β (7) indicates that WFIKKN2 may be considered a GDF8/11-specific rather than a GDF8-specific inhibitor.

The close homology of GDF8 and GDF11 is also reflected by the fact that they signal through the same set of receptors. Both myostatin and GDF11 signal through activin type II receptors and receptors of the transforming growth factor β -like signaling pathway (41, 44–46).

The close homology of WFIKKN1 with WFIKKN2 and GDF8 with GDF11 has prompted us to investigate to what extent their function overlap. Here we report that both WFIKKN1 and WFIKKN2 bind myostatin and GDF11 with high affinity suggesting that there is a significant functional overlap of WFIKKN1 and WFIKKN2 proteins. Structure-function studies have shown that the follistatin domain is primarily responsible for the binding of mature growth factors, whereas the propeptide interacts with the NTR domain.

EXPERIMENTAL PROCEDURES

Enzymes, PCR Primers, Proteins, and Bacterial Strains—Restriction enzymes were purchased from Promega (Madison, WI) and New England Biolabs (Beverly, MA). PCR primers were obtained from Integrated DNA Technologies (Coralville, IA). T4 DNA Ligase and Klenow polymerase were New England Biolabs products. For amplification reactions we used *Taq* polymerase from Fermentas (Vilnius, Lithuania); proofreading thermostable polymerases Vent was from New England Biolabs, KodHifi was from Novagen, Merck, and Accuzyme was from Bionline (London, UK). DNA purification was performed with GenElute plasmid preparation kit (Sigma) and Nucleospin extract PCR purification kit (Macherey-Nagel, Duren, Germany). *Escherichia coli* JM109 bacterial strain was used for DNA propagation during DNA manipulation steps, whereas bacterial protein expression was done in *E. coli* BL21(DE3). Mature mouse myostatin and mature human GDF11 were purchased from R & D Systems (Wiesbaden, Germany). Nickel-Sepharose and low molecular weight calibration kit were the products of GE Healthcare; CM5 sensorchips and the reagents for protein coupling to the chips were from Biacore AB (Uppsala, Sweden).

Expression of WFIKKN1 and WFIKKN2 Proteins in Drosophila melanogaster S2 Cells—The cDNA fragment encoding mature human WFIKKN1 protein was amplified with the 5' CGC GAA TTC AGC CAC CCG GGC GTG TGC CCC 3' sense and 5' GAG GTC GAC GTC CTG GAA GCG GTT GAG CAG 3' antisense primers from the plasmid containing the full-length WFIKKN1 cDNA (1). The DNA was ligated into the *Drosophila* expression vector pMT/BiP/V5/His A. The DNA coding for mature WFIKKN1 protein was fused to the BiP secretion signal sequence, and the expression of the recombinant protein was under the control of the metal-inducible metallothionein promoter. The cDNA of the human WFIKKN2 protein (2) was amplified with the 5' GAG TCG ACC CAC GCC GGC ATC TGC CCC AAC 3' sense and 5'CTG GAT CCT CGA GGT GCA AGC CAA GAA ACT C 3' antisense primers from the plasmid containing the WFIKKN2 cDNA and was also ligated into the pMT/BiP/V5/His A *Drosophila* expression vector.

D. melanogaster S2 cells (*Drosophila* Genomics Resource Center, Indiana University, Bloomington, IN) were transfected with 4 μ g of expression plasmid and 16 μ g of pCoHygro selection vector using Cellfectin reagent (Invitrogen) according to

the protocol recommended by the manufacturer. For selection of stable transfectants, cells were suspended and cultured in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 300 $\mu\text{g}/\text{ml}$ hygromycin B (Invitrogen). Stably transformed polyclonal lines were established after 5 weeks of selection with hygromycin B. Except for propagation in serum-free medium, hygromycin B was always included in the media.

For protein induction, stable transfectants were grown in serum-free medium (Invitrogen) to a cell density of $2\text{--}3 \times 10^6/\text{ml}$, and protein expression was induced by adding CuSO_4 at 400 μmol final concentration. After 1 week of induction, the culture was centrifuged, and the conditioned medium was harvested and the cells were suspended in fresh induction medium to start another round of induction. Usually three rounds of induction were performed with the same cells. The medium collected from three rounds of induction was dialyzed against 50 mM Tris, pH 7.5, and applied onto a nickel affinity column (GE Healthcare). The column was washed with 10 column volumes of 20 mM Tris-HCl buffer, pH 7.9, containing 500 mM NaCl and 5 mM imidazole and then with 5 column volumes of 20 mM Tris-HCl buffer, pH 7.9, containing 500 mM NaCl and 30 mM imidazole, and the bound protein was eluted with 20 mM Tris-HCl buffer, pH 7.9, containing 100 mM imidazole. The eluted protein was concentrated on Amicon Ultra-15 centrifugal filter devices and chromatographed on a Sephacryl S-300 column equilibrated with 100 mM ammonium bicarbonate buffer, pH 8.0.

Samples were analyzed by SDS-PAGE under both reducing and nonreducing conditions. Fractions containing recombinant protein were pooled, desalted on a Sephadex G-25 fine column, and lyophilized. Under reducing conditions the secreted WFIKKN1 and WFIKKN2 have electrophoretic mobilities corresponding to apparent molecular masses of ~ 65 and ~ 70 kDa, respectively. The observed molecular masses are higher than the calculated values (58 kDa for WFIKKN1 and 60 kDa for WFIKKN2); these differences may be attributed to glycosylation at the *N*-glycosylation sites predicted at residue Asn⁴⁹³ in the NTR domain of human WFIKKN1 and at residues Asn¹⁸³, Asn³¹⁹, and Asn⁵¹⁹ of human WFIKKN2 protein. N-terminal sequence analysis of the purified recombinant WFIKKN1 protein gave RSISMAQFSHPG, indicating that the secretory signal peptide of the expression vector was cleaved at the predicted cleavage site. In the case of the recombinant WFIKKN2 protein, the N-terminal sequence was GICPNDMN, indicating that the signal peptidase cleaved at an alanine residue, corresponding to Ala⁴³ of WFIKKN2.

Expression of Fragments of Human WFIKKN1 in Pichia pastoris—Proteins corresponding to single or two tandem domains of WFIKKN1 were produced by *P. pastoris* expression utilizing the Easy Select protein expression kit and *P. pastoris* GS115 strain (Invitrogen). The second Kunitz-type domain of human WFIKKN1 was produced as described previously (6).

The cDNA fragments coding for the different domains of WFIKKN1 protein were amplified with Accuzyme proofreading *Taq* polymerase in 35 reaction cycles using the plasmid containing the full-length WFIKKN1 cDNA as template. The primers used for the amplifications were as follows: WAP/

sense, 5' CGC GAA TTC AGC CAC CCG GGC GTG TGC CCC 3', and WAP/antisense, 5' GCG TCT AGA GGG AAG CGT GCT GCC ACG CA 3'; WAP-FS/sense, 5' CGC GAA TTC AGC CAC CCG GGC GTG TGC CCC 3', and WAP-FS/antisense, 5' CGC GTC GAC GCT GAG CAC GTG CTT GCA GGG 3'; FS/sense, 5' CGC GAA TTC ACG ACA GCG GCC TCC TGC GAG G 3', and FS/antisense, 5' CGC GTC GAC GCT GAG CAC GTG CTT GCA GGG 3'; WAP-FS-IG/sense, 5' CGC GAA TTC AGC CAC CCG GGC GTG TGC CCC 3', and WAP-FS-IG/antisense, 5' GTG CGT CGA CCC CAG CAG CGT TGC GCG CGG T 3'; IG/sense, 5' GTG CGA ATT CGT TGG GGG TAC GGC CAG CCT C 3', and IG/antisense, 5' GTG CGT CGA CCC CAG CAG CGT TGC GCG CGG T 3'; IG-KU1/sense, 5' GTG CGA ATT CGT TGG GGG TAC GGC CAG CCT C 3', and IG-KU1/antisense, 5' GCT GTC GAC GCC GCG GGC ACA GGC CTG 3'; KU1/sense, 5' GCT GAA TTC GCC GAG TGC CTG CCG GAT 3', and KU1/antisense, 5' GCT GTC GAC GCC GCG GGC ACA GGC CTG 3'; KU1-KU2/sense, 5' GCT GAA TTC GCC GAG TGC CTG CCG GAT 3', and KU1-KU2/antisense, 5' GAG GTC GAC CAC GGG GCA GGC ATC CTC 3'; KU2-NTR/sense, 5' GAG GAA TTC ACC GAC GCC TGC GTG CTG CCT 3', and KU2-NTR/antisense, 5' GAG GTC GAC GTC CTG GAA GCG GTT GAG CAG 3'; NTR/sense, 5' GAG GAA TTC CGC ACA CCG CCC TGC CGC GCC 3', and NTR/antisense, 5' GAG GTC GAC GTC CTG GAA GCG GTT GAG CAG 3'.

In each case the amplified DNA was digested with EcoRI and SalI restriction enzymes and ligated into pPICZ α *P. pastoris* expression vector digested with the same enzymes. 10 μg of plasmid DNA containing the cDNA of the appropriate domain(s) of WFIKKN1 was linearized by SacII digestion, and *P. pastoris* GS115 cells were transformed by electroporation. Genomic DNA was prepared from Zeocin-resistant colonies to check the insertion of the expression plasmids into the yeast chromosome. Verified *P. pastoris* clones were grown in 400 ml of medium containing 2% peptone, 1% yeast extract, and 5% glycerol for 72 h and then the medium was changed to induction medium containing 100 mM potassium phosphate, pH 7.5, 0.2% ammonium sulfate, 0.34% Yeast Nitrogen Base, 0.004% histidine, 0.00004% biotin, and 1% methanol and were shaken for 48 h. The cells were centrifuged, and the supernatant was applied onto a nickel-affinity column. Unbound components were removed by washing the column with 20 mM Tris-HCl buffer, pH 7.9, containing 500 mM NaCl and 30 mM imidazole. The bound recombinant protein was eluted with 20 mM Tris-HCl buffer, pH 7.9, containing 300 mM imidazole and further purified by gel filtration on a Sephadex G-75 column.

Methanol induction of the *P. pastoris* cells transformed with the appropriate expression plasmids leads to the production of proteins containing the individual WAP, FS, KU1, KU2, and NTR domains. Although the integration into the yeast chromosome of the expression plasmids containing the cDNA coding for the IG domain was verified by specific PCR, the clones did not produce the recombinant protein.

SDS-PAGE analysis of the proteins purified from the induction media showed that the recombinant WFIKKN1_WAP and WFIKKN1_KU1 proteins were disulfide-bonded dimers. To overcome this problem of protein dimerization of single

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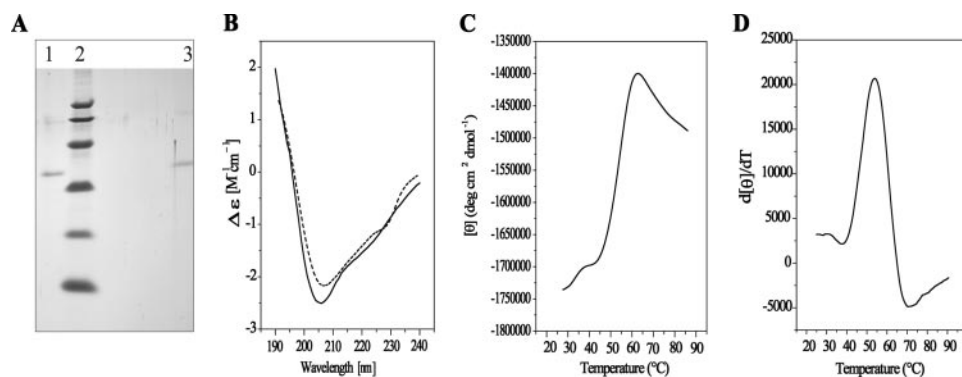


FIGURE 1. Characterization of human myostatin-propeptide expressed in *E. coli*. *A*, SDS-PAGE of refolded recombinant myostatin propeptide. *Lane 1*, reduced myostatin propeptide; *lane 2*, reduced proteins of the low molecular weight calibration kit; *lane 3*, nonreduced myostatin propeptide. *B*, far-ultraviolet circular dichroism spectra of recombinant myostatin propeptide. Spectra were recorded in 10 mM Tris-HCl, pH 8.0, at 25 °C using 0.1 mg/ml protein. The *solid line* indicates the spectrum of the recombinant protein, and the *dashed line* indicates the CDPro-predicted spectrum of a protein consisting of 0.202 regular β -strand, 0.113 distorted β -strand, 0.065 regular helix, and 0.088 distorted helix, 0.217 turn, and 0.315 unordered structure. *C*, temperature dependence of the CD spectra of myostatin propeptide. Changes in the CD of the protein were monitored at 203 nm during the course of heating from 25 to 90 °C at a heating rate of 60 °C/h. *D*, melting temperature was determined by derivative processing of changes in CD (of *C*) using the spectra analysis program for WINDOWS 95/NT version 1.53.04 (Jasco).

domain proteins, we have constructed expression plasmids containing the cDNA of two or three tandem domains of WFIKKN1. The clones transfected with the WFIKKN1_WAP-FS-IG construct did not produce recombinant protein; the WFIKKN1_IG-KU1 protein formed dimers, whereas *P. pastoris* cells containing the expression cassettes for WFIKKN1_WAP-FS, WFIKKN1_KU1-KU2, and WFIKKN1_KU2-NTR produced stable monomers.

The structural integrity of the monomeric recombinant proteins was verified by N-terminal sequence analysis; the N-terminal sequences of the recombinant proteins were as follows: WFIKKN1_WAP-FS, EAEAEFSHPGV; WFIKKN1_FS, EFT-TAASCEGFV; WFIKKN1_KU1-KU2, EAEAEFAECLPDV; WFIKKN1_KU2-NTR, EAEAEFTDACVL; WFIKKN1_NTR, EFRTPPCRACRL. In all subsequent experiments we used only native proteins that formed stable monomers.

Expression and Characterization of the Propeptide Region of Myostatin—The propeptide of human promyostatin is encoded by all three exons of the gene (47). The regions encoding the propeptide were amplified from human genomic DNA and fused with the following primers: Exon1/sense, 5' CG GAA TTC AAA TGA GAA CAG TGA GCA AAA A G 3', and Exon1/antisense, 5' CTT GCA TTA GAA AAT CAG ACT CTG TAG GCA TGG TAA 3'; Exon2/sense, 5' TTA CCA TGC CTA CAG AGT CTG ATT TTC TAA TGC AAG 3', and Exon2/antisense, 5' GAC CTG TAA AAA CGG ATT CAG CCC ATC TTC TCG TGG 3'; Exon3/sense, 5' CCA GGA GAA GAT GGG CTG AAT CCG TTT TTA GAG GTC 3', and Exon3/antisense, 5' CG GTC GAC CCT TCT GGA TCT TTT TGG TGT G 3'. Amplicons were purified with Nucleospin Extract PCR purification kit (Macherey-Nagel, Duren, Germany) and were linked in a two-step PCR; the first and second exons were linked in a PCR with primer Exon1/sense and primer Exon2/antisense, and then in a second reaction the third exon was linked to this amplicon with primer Exon1/sense and primer Exon3/antisense. The DNA was digested with

EcoRI/Sall restriction endonucleases and ligated into pPR-IBA2A bacterial expression vector digested with the same enzymes.

E. coli BL21(DE3) cells carrying the expression construct pPR-IBA2A/myostatin propeptide were grown in 2TY medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.5) containing 100 μ g/ml ampicillin, and expression of recombinant protein was induced with 100 μ M isopropyl β -D-thiogalactopyranoside. The products were isolated from inclusion bodies, dissolved in 8 M urea, 100 mM Tris, 10 mM EDTA, 100 mM dithioerythritol, pH 8.0, and gel-filtered on Sephadex S-300 equilibrated with the same buffer. Fractions containing the recombinant protein were pooled, rapidly diluted 10 times in 100 mM Tris, pH

7.5, and stirred for 24–48 h at 4 °C. Precipitated protein was removed by centrifugation, and the protein solution was applied onto a 10-ml StrepTactin-Sepharose (IBA BioTAGnology, Göttingen, Germany) column. The column was washed with 5 column volumes of 100 mM Tris, 150 mM NaCl 1 mM EDTA, pH 8.0 buffer, and the bound protein was eluted with the same buffer containing 2.5 mM D-desthiobiotin (IBA BioTAGnology, Göttingen, Germany). The eluted protein was desalted on a Sephadex G-25 column equilibrated with 100 mM ammonium bicarbonate, pH 8.0, and the protein solution was lyophilized. Monomeric myostatin propeptide with the expected molecular mass (31 kDa) was separated from contaminating multimers by chromatography on Sephadex S-300 columns equilibrated with 100 mM ammonium bicarbonate, pH 8.0 (Fig. 1A). N-terminal sequence analysis of the purified recombinant myostatin propeptide gave MAWSHPQFEKGARRDRDGPEFNENSE (where the underlined residues correspond to residues Asn²⁴–Glu²⁸ of myostatin precursor protein).

To study the structural integrity of refolded myostatin propeptide, CD spectra were measured over the range of 190–250 nm by using a Jasco J-720 spectropolarimeter thermostated with a Neslab RT-111 water bath (Fig. 1B). The measurements were carried out in 1-mm path length cells and protein solutions of 0.1 mg/ml in 10 mM Tris-HCl buffer, pH 8.0. Spectra were measured at 25 °C with a 16-s time constant and a scan rate of 20 nm/min. The spectral slit width was 1.0 nm. All measurements represent the computer average of three scans. Secondary structure of the recombinant protein was estimated from the CD spectra with the CDPro software. The spectra of the recombinant protein were also recorded at different temperatures from 25 to 95 °C at 10 °C intervals. Analysis of the CD spectra of refolded recombinant myostatin propeptide indicates that the protein-fold consists of 0.202 regular β -strand, 0.113 distorted β -strand, 0.065 regular helix and 0.088 distorted helix, 0.217 turn, and 0.315 unordered structure. The thermal unfolding of the protein was monitored at 203 nm,

where the difference of the CD spectra recorded at different temperatures was the largest. The heating rate was 60 °C/h. The native structure of the protein collapsed in a highly cooperative fashion, and the melting temperature of the protein was found to be 54 °C (Fig. 1, C and D). The fact that refolded myostatin propeptide binds to myostatin with high affinity (see Table 2) is in harmony with the known affinity of propeptide to mature growth factor domain (40–42) and also supports the structural and functional integrity of the recombinant protein.

Protein Analyses—The composition of protein samples was analyzed by SDS-PAGE under both reducing and nonreducing conditions. The gels were stained with Coomassie Brilliant Blue G-250. The concentrations of the recombinant proteins were determined using the following extinction coefficients: WFIKKN1, 64,440 M⁻¹ cm⁻¹; WFIKKN2, 57,470 M⁻¹ cm⁻¹; myostatin propeptide, 35,200 M⁻¹ cm⁻¹; WFIKKN1_WAP-FS, 16,595 M⁻¹ cm⁻¹; WFIKKN1_FS domain, 10,595 M⁻¹ cm⁻¹; WFIKKN1_KUN1-KUN2, 23,210 M⁻¹ cm⁻¹; WFIKKN1_KUN2-NTR, 23,210 M⁻¹ cm⁻¹; WFIKKN1_NTR, 8855 M⁻¹ cm⁻¹. The extinction coefficients were calculated with the online protein analysis tool, ProtParam (available on line). N-terminal sequencing of the purified recombinant proteins was performed on an Applied Biosystems 471A protein sequencer with an on-line ABI 120A phenylthiohydantoin analyzer.

Surface Plasmon Resonance Analysis—Surface plasmon resonance measurements were performed on a BIAcore X (GE Healthcare) instrument. Proteins to be immobilized were dissolved in 50 mM sodium acetate, pH 4.5, at a final concentration of 500 nM (GDF11), 800 nM (WFIKKN1), or 1.5 μM (WFIKKN1_KU2-NTR, WFIKKN1_NTR, myostatin), and 50 μl of these solutions were injected with a 5 μl/min flow rate for 10 min on a CM5 sensorchip activated by the amine coupling method according to the instructions of the manufacturer.

For interaction measurements, 80-μl samples containing different concentrations of proteins were injected at a flow rate of 20 μl/min, followed by wash with buffer at a flow rate of 20 μl/min. Binding and washes were performed in 20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.005% Tween 20, pH 7.5 buffer. After each cycle the chips were regenerated with 20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.005% Tween 20, pH 7.5 buffer, containing 8 M urea. All experiments were repeated at least three times. Control flow cells were prepared by executing the coupling reaction in the presence of coupling buffer alone. Control cells were used to obtain control sensorgrams showing nonspecific binding to the surface as well as refractive index changes resulting from changes in bulk properties of the solution. Control sensorgrams were subtracted from sensorgrams obtained with immobilized ligand. To correct for differences between the reaction and reference surfaces, we have also subtracted the average of sensorgrams obtained with blank running buffer injections.

The kinetic parameters for each interaction were determined by globally fitting the experimental data with BIAevaluation software 4.0, and the closeness of the fits was characterized by the χ^2 values. Only fits with χ^2 values lower than 5% of the R_{\max} were accepted (48). In the majority of cases the data were fitted to a model of 1:1 Langmuir interaction. In the case of the inter-

actions of WFIKKN1_KU2-NTR, WFIKKN1_NTR, and myostatin propeptide with myostatin, the data could be best fitted with the BIAevaluation model for “two-state reaction (conformation change).” In some experiments WFIKKN1 and WFIKKN2 were mixed in different ratios, and different concentrations of these solutions were injected on sensorchips containing immobilized GDF8 or GDF11. The kinetic parameters for the interaction of the two competing proteins with immobilized ligands were determined by globally fitting the experimental data with BIAevaluation software 4.0 using the BIAevaluation model for “heterogeneous analyte (competing reactions).”

Identification of WFIKKN-related Proteins in Various Vertebrate and Invertebrate Chordates—Homologues of WFIKKN1 and WFIKKN2 proteins were identified, and their phylogenetic relationship was analyzed as described in supplemental file 1.

RESULTS AND DISCUSSION

WFIKKN1 Binds Myostatin with High Affinity—Using an affinity purification-based approach, Hill *et al.* (7) have identified WFIKKN2 as a myostatin-binding protein present in normal mouse and human serum but found no evidence for the related WFIKKN1 protein. Based on this negative result, the authors (7) suggested that WFIKKN1 probably has a different biological specificity or that it is not present in serum.

To test the former possibility, we have immobilized recombinant mouse myostatin on the surface of sensorchips CM5 and performed surface plasmon resonance measurements with different concentrations of human WFIKKN1, human WFIKKN2, or mixtures of the two proteins. As shown in Fig. 2A, WFIKKN1 protein showed high affinity for immobilized myostatin. The kinetic association and dissociation curves fitted to the model of 1:1 binding kinetics and the K_d value for binding of WFIKKN1 to myostatin was calculated to be 3.35×10^{-8} M (Table 1). It should be noted, however, that the affinity of WFIKKN1 for myostatin is lower than that of WFIKKN2; the K_d value for binding of WFIKKN2 to myostatin (Fig. 2C) was calculated to be 2.86×10^{-10} M (Table 1).

Both WFIKKN1 and WFIKKN2 Bind GDF11 with High Affinity—To investigate whether there are differences in the ligand specificity of WFIKKN1 and WFIKKN2, we have immobilized recombinant human GDF11 on the surface of sensorchips CM5 and performed surface plasmon resonance measurements with different concentrations of human WFIKKN1, human WFIKKN2, or mixtures of the two proteins. As shown in Fig. 2, B and D, both WFIKKN1 and WFIKKN2 showed high affinity for immobilized GDF11. The kinetic association and dissociation curves fitted to the model of 1:1 binding kinetics, and the K_d values for binding of WFIKKN1 and WFIKKN2 to GDF11 were calculated to be 2.25×10^{-9} and 1.64×10^{-10} M, respectively (Table 1).

WFIKKN1 Binds Myostatin Primarily through Its Follistatin Domain—It has been shown previously that myostatin binds to the activin-binding protein, follistatin, and the follistatin-related protein, FLRG, with high affinity (41, 42), raising the possibility that the follistatin domains are likely candidates for these interactions. To identify the regions of WFIKKN1 that contribute to its interaction with myostatin, we have character-

WFIKKN1 Binds Both Myostatin and GDF11

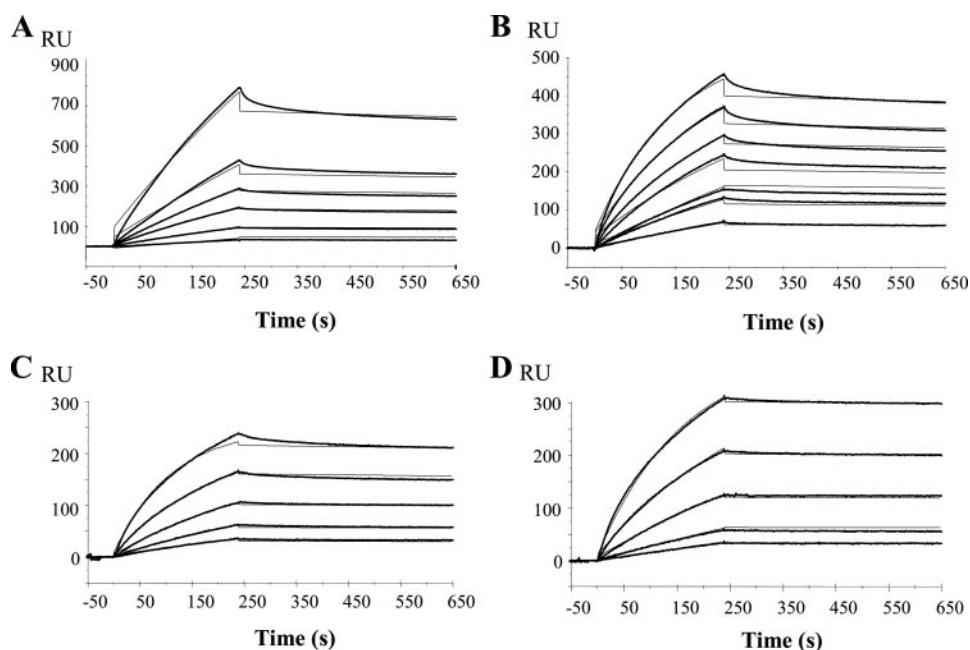


FIGURE 2. Characterization of the interaction of WFIKKN1 and WFIKKN2 with GDF8 and GDF11 by surface plasmon resonance assays. Sensorgrams of the interactions are as follows. *A*, WFIKKN1 (25, 50, 100, 150, 200, and 400 nM) with myostatin; *B*, WFIKKN1 (12.5, 25, 37.5, 50, 100, and 150 nM) with GDF11; *C*, WFIKKN2 (2.5, 5, 10, 20, and 40 nM) with myostatin; *D*, WFIKKN2 (3, 6, 12.5, 25, and 50 nM) with GDF11. Various concentrations of WFIKKN1 or WFIKKN2 in 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.005% Tween 20 were injected over myostatin or GDF11 immobilized on CM5 sensorchips. For each type of experiment, one set of representative data of three parallels are shown.

TABLE 1

Kinetic parameters of the interaction of myostatin and GDF11 with WFIKKN1 and WFIKKN2

The rate constants of the association and dissociation reactions and the equilibrium dissociation constants of the interactions were determined from surface plasmon resonance measurements with the BIAevaluation software 4.0.

Interacting proteins	K_d value	k_a	k_d
Myostatin ^a -WFIKKN1	3.35×10^{-8}	2.91×10^3	9.73×10^{-5}
Myostatin ^a -WFIKKN2	2.86×10^{-10}	2.24×10^5	6.39×10^{-5}
GDF11 ^a -WFIKKN1	2.25×10^{-9}	4.3×10^4	9.69×10^{-5}
GDF11 ^a -WFIKKN2	1.64×10^{-10}	1.22×10^5	2.00×10^{-5}

^a These proteins were immobilized on sensorchips.

TABLE 2

Kinetic parameters of the interaction of myostatin with myostatin propeptide (ProMyo) as well as myostatin and ProMyo with WFIKKN1 protein and its fragments

The rate constants of the association and dissociation reactions and the equilibrium dissociation constants of the interactions were determined from surface plasmon resonance measurements with the BIAevaluation software 4.0. In the case of the interaction of myostatin with myostatin propeptide, WFIKKN1_KU2-NTR and WFIKKN1_NTR, the data were fitted with the BIAevaluation model for a two-state reaction (conformation change), and in all other cases the data fitted to a model of 1:1 Langmuir interaction.

Interacting proteins	K_d value	k_a	k_d
Myostatin ^a -ProMyo	2.35×10^{-8}	7.75×10^3	1.86×10^{-3}
Myostatin ^a -WFIKKN1	3.35×10^{-8}	2.91×10^3	9.73×10^{-5}
Myostatin ^a -WFIKKN1_WAP-FS	6.68×10^{-8}	2.98×10^3	1.99×10^{-4}
Myostatin ^a -WFIKKN1_FS	2.71×10^{-7}	1.34×10^3	3.62×10^{-4}
Myostatin ^a -WFIKKN1_KU2-NTR	2.55×10^{-6}	7.85×10^3	3.95×10^{-2}
Myostatin ^a -WFIKKN1_NTR	2.38×10^{-6}	7.00×10^4	2.49×10^{-2}
ProMyo-WFIKKN1_KU2-NTR ^a	2.44×10^{-7}	6.18×10^3	1.51×10^{-3}
ProMyo-WFIKKN1_NTR ^a	1.60×10^{-7}	4.44×10^3	7.09×10^{-4}
ProMyo-WFIKKN1 ^a	4.85×10^{-7}	5.03×10^3	2.44×10^{-3}

^a These proteins were immobilized on sensorchips.

ized the interactions of myostatin with recombinant proteins containing its follistatin domain (WFIKKN1_FS), its NTR domain (WFIKKN1_NTR), as well as proteins containing the

WAP and follistatin domain (WFIKKN1_WAP-FS), the two Kunitz domains (WFIKKN1_KU1-KU2), or the second Kunitz and NTR domains (WFIKKN1_KU2-NTR).

Surface plasmon resonance analysis of the binding of various WFIKKN1 fragments to the surface of the sensorchip containing immobilized mouse myostatin revealed that the affinity of various fragments decreased in the order WFIKKN1_WAP-FS > WFIKKN1_FS > WFIKKN1_KU2-NTR ~ WFIKKN1_NTR (Table 2) with no interaction being detectable in the case of WFIKKN1_KU1-KU2 protein. Based on these analyses, it appears that WFIKKN proteins bind mature growth factors primarily through interactions with the follistatin domain.

WFIKKN1 Binds Myostatin Propeptide through the NTR Domain—Because WFIKKN2 protein was shown to bind not only mature myostatin but also the

propeptide region of myostatin (7), we investigated the interaction of WFIKKN1 and myostatin propeptide by surface plasmon resonance measurements. Solutions of human myostatin propeptide were injected onto the surface of CM5 sensorchip containing immobilized WFIKKN1 protein, and analyses of the sensorgrams confirmed that the propeptide region of myostatin binds to WFIKKN1 independently from mature myostatin with a K_d value of 4.85×10^{-7} M (Table 2).

The panel of recombinant proteins containing different fragments of WFIKKN1 protein was used to identify the region(s) that participate in the interaction of WFIKKN1 with the propeptide region of myostatin. Kinetic characterization of the interaction of myostatin propeptide with these proteins was determined by surface plasmon resonance measurements using CM5 sensorchips with immobilized WFIKKN1_WAP-FS, WFIKKN1_KU1-KU2, WFIKKN1_KU2-NTR, and WFIKKN1_NTR proteins. Evaluation of the sensorgrams recorded after injection of myostatin propeptide solutions revealed that the propeptide region of myostatin was bound only to WFIKKN1_KU2-NTR and WFIKKN1_NTR. Assuming 1:1 binding, the calculated equilibrium dissociation constants for interaction of myostatin propeptide with WFIKKN1_KU2-NTR and WFIKKN1_NTR were 2.44×10^{-7} and 1.60×10^{-7} M, respectively (Table 2).

Identification of WFIKKN-related Proteins—Searches of public data bases identified full-length WFIKKN-related proteins from urochordates (*Ciona intestinalis*), all groups of vertebrates (lamprey, bony fishes, frog, chicken, and mammals) but not from nonchordate animals. The domain organization of WFIKKN-related proteins of vertebrates was identical; they all contained WAP, follistatin, immunoglobulin, two Kunitz, and

NTR domains. Evolutionary analyses of the WFIKKN-related proteins of vertebrates have revealed that they show clear affinities to either WFIKKN1 or WFIKKN2. The genomes of chicken and mammals were found to contain single *WFIKKN1* and *WFIKKN2* genes, but in the case of completely sequenced fish genomes (*Fugu rubripes*, *Tetraodon nigroviridens*, and *Danio rerio*), there was evidence for a single *WFIKKN1*- and two *WFIKKN2*-related genes. (In the case of the partially sequenced lamprey genome, we could identify two complete *WFIKKN1* genes, but evidence for *WFIKKN2* genes could not be found.)

A single gene homologous with vertebrate *WFIKKN* genes was identified on chromosome 10q of the urochordate *C. intestinalis*. The fact that several ESTs and a full-length cDNA (AK112345) originate from this genomic region indicates that it is a functional gene. The WFIKKN homolog of *C. intestinalis*, however, differs from vertebrate proteins in that it lacks an immunoglobulin domain. Phylogenetic analyses of domains shared by WFIKKN-related proteins of urochordates and vertebrates have revealed that the *Ciona* protein is basal to WFIKKN1 and WFIKKN2 branches (data not shown), indicating that the gene duplication of the ancestral *WFIKKN* gene occurred in Vertebrata after their divergence from Urochordata, their closest invertebrate relatives (49).

Analyses of the genomes of fishes and mammals have shown that they all contain single genes orthologous with GDF11. As for myostatin, there is only one myostatin gene in chicken and mammals, whereas in the completely sequenced genomes of the fishes *F. rubripes*, *T. nigroviridens*, and *D. rerio*, we found evidence for the presence of two myostatin-like genes. This finding is in harmony with the conclusion of Biga *et al.* (50) that there are two myostatin genes in zebrafish. Because in the amphioxus *B. belcheri* there is a *GDF8/11* gene that is basal to vertebrate myostatins and GDF11s, it is clear that the gene duplication(s) generating vertebrate *MSTN* and *GDF11* genes occurred relatively recently, after the divergence of cephalochordates from vertebrates (10).

The data obtained by the evolutionary analyses of WFIKKN-related proteins and GDF8/11-related proteins thus suggest that the duplication of the ancestral *GDF11/GDF8* gene and the duplication of the ancestral *WFIKKN1/WFIKKN2* gene occurred at about the same time, following the divergence cephalochordates from vertebrates. Although the descendants of the ancestral *GDF8/GDF11* and *WFIKKN1/WFIKKN2* genes diverged significantly in function, the data presented here indicate that there is still extensive functional overlap: both WFIKKN1 and WFIKKN2 bind both GDF8 and GDF11.

Structure, Function, and Conservation of Different Domains of WFIKKN-related Proteins—Comparison of the sequences of the different WFIKKN proteins also provides some insight into the structural or functional importance of its various domains. As shown in supplemental Fig. 1, there are significant differences in the degree of conservation of the different domains of WFIKKN proteins. The fact that the sequences of the follistatin and NTR domains are highly conserved from lamprey to mammals probably reflects their importance for binding growth and differentiation factors, as shown in this study. Conversely, the observation that the first Kunitz domain shows very low

sequence conservation is in harmony with our finding that this domain does not contribute to binding myostatin or myostatin propeptide, but it also suggests that it is not critical for any other function WFIKKN proteins may have. On the other hand, the remarkable conservation of the WAP, immunoglobulin, and the second Kunitz domains suggests that these domains may be critical for the structural integrity of these multidomain proteins or may be involved in interactions with proteins other than growth and differentiation factors.

Similarities and Differences of the Functions of WFIKKN1 and WFIKKN2 Proteins—Our observation that both WFIKKN1 and WFIKKN2 bind GDF8 and GDF11 with high affinity suggests that the biological function of WFIKKN1 and WFIKKN2 may be similar. Nevertheless, the fact that there are significant differences in their affinity for these growth and differentiation factors (see Table 1) indicates that they are functionally not equivalent.

The classical routes for the evolution of the function of paralogues include subfunctionalization (where paralogues partition the ancestral function) and neofunctionalization (where one of the paralogues retains the ancestral function although the other acquires a new function). Based on the evolutionary history of *WFIKKN* genes, it appears that the function of the ancestor of WFIKKN1 and WFIKKN2 (such as the single WFIKKN protein of invertebrate chordates) was to bind the common ancestor of GDF8/GDF11 (such as the GDF8/11 protein of amphioxus). Following duplication of the *GDF8/11* gene in early vertebrates the functions of GDF8 and GDF11 diverged significantly (see Introduction). If WFIKKN paralogues followed a simple subfunctionalization pathway one may have expected a partition of binding functions, *e.g.* that one of the WFIKKN paralogues binds GDF8, the other binds GDF11 with higher affinity. In contrast with this expectation, WFIKKN2 was found to have significantly higher affinity than WFIKKN1 for both GDF8 and GDF11 (see Table 1).

In view of the fact that there are major differences in the expression patterns of human WFIKKN1 and WFIKKN2 (1, 2), it is possible that subfunctionalization occurred at the level of expression pattern rather than at the level of ligand specificity. In other words, it is the expression pattern of WFIKKN1 and WFIKKN 2 that determines which of them may be responsible for the control of the activity of GDF8 or GDF11 in a given tissue. For example, because WFIKKN2 and GDF11 (but not WFIKKN1 and GDF8) are expressed in adult brain, in this tissue the function of WFIKKN2 may be to regulate the action of GDF11. Conversely, because WFIKKN1 and GDF11 (but not WFIKKN2 and GDF8) are expressed at a high level in liver, in this tissue WFIKKN1 may play a crucial role in regulating the action of GDF11.

REFERENCES

1. Trexler, M., Bánayai, L., and Patthy, L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3705–3709
2. Trexler, M., Bánayai, L., and Patthy, L. (2002) *Biol. Chem.* **383**, 223–228
3. Rawlings, N. D., Morton, F. R., Kok, C. Y., Kong, J., and Barrett, A. J. (2008) *Nucleic Acids Res.* **36**, D320–D325
4. Bánayai, L., and Patthy, L. (1999) *Protein Sci.* **8**, 1636–1642
5. Nagy, A., Trexler, M., and Patthy, L. (2003) *Eur. J. Biochem.* **270**, 2101–2107

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6. Liepinsh, E., Nagy, A., Trexler, M., Patthy, L., and Otting, G. (2006) *J. Biomol. NMR* **35**, 73–78
7. Hill, J. J., Qiu, Y., Hewick, R. M., and Wolfman, N. M. (2003) *Mol. Endocrinol.* **17**, 1144–1154
8. McPherron, A. C., Lawler, A. M., and Lee, S. J. (1997) *Nature* **387**, 83–90
9. Gamer, L. W., Wolfman, N. M., Celeste, A. J., Hattersley, G., Hewick, R., and Rosen, V. (1999) *Dev. Biol.* **208**, 222–232
10. Xing, F., Tan, X., Zhang, P. J., Ma, J., Zhang, Y., Xu, P., and Xu, Y. (2007) *Dev. Genes Evol.* **217**, 549–554
11. McPherron, A. C., and Lee, S. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12457–12461
12. Lee, S. J., and McPherron, A. C. (1999) *Curr. Opin. Genet. Dev.* **9**, 604–607
13. McPherron, A. C., Lawler, A. M., and Lee, S. J. (1999) *Nat. Genet.* **22**, 260–264
14. Ji, S., Losinski, R. L., Cornelius, S. G., Frank, G. R., Willis, G. M., Gerrard, D. E., Depreux, F. F., and Spurlock, M. E. (1998) *Am. J. Physiol.* **275**, R1265–R1273
15. Sharma, M., Kambadur, R., Matthews, K. G., Somers, W. G., Devlin, G. P., Conaglen, J. V., Fowke, P. J., and Bass, J. J. (1999) *J. Cell Physiol.* **180**, 1–9
16. Zimmers, T. A., Davies, M. V., Koniaris, L. G., Haynes, P., Esquela, A. F., Tomkinson, K. N., McPherron, A. C., Wolfman, N. M., and Lee, S. J. (2002) *Science* **296**, 1486–1488
17. Artaza, J. N., Bhasin, S., Magee, T. R., Reisz-Porszasz, S., Shen, R., Groome, N. P., Meerasahib, M. F., and Gonzalez-Cadavid, N. F. (2005) *Endocrinology* **146**, 3547–3557
18. Feldman, B. J., Streeper, R. S., Farese, R. V., Jr., and Yamamoto, K. R. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 15675–15680
19. Hamrick, M. W., Shi, X., Zhang, W., Pennington, C., Thakore, H., Haque, M., Kang, B., Isales, C. M., Fulzele, S., and Wenger, K. H. (2007) *Bone (Elmsford)* **40**, 1544–1553
20. Østbye, T. K., Galloway, T. F., Nielsen, C., Gabestad, I., Bardal, T., and Andersen, Ø. (2001) *Eur. J. Biochem.* **268**, 5249–5257
21. Roberts, S. B., and Goetz, F. W. (2001) *FEBS Lett.* **491**, 212–216
22. Roberts, S. B., and Goetz, F. W. (2003) *Mol. Cell Endocrinol.* **210**, 9–20
23. Kocabas, A. M., Kucuktas, H., Dunham, R. A., and Liu, Z. (2002) *Biochim. Biophys. Acta* **1575**, 99–107
24. Garikipati, D. K., Gahr, S. A., and Rodgers, B. D. (2006) *J. Endocrinol.* **190**, 879–888
25. Garikipati, D. K., Gahr, S. A., Roalson, E. H., and Rodgers, B. D. (2007) *Endocrinology* **148**, 2106–2115
26. Ye, H. Q., Chen, S. L., Sha, Z. X., and Liu, Y. (2007) *Mar. Biotechnol. (NY)* **9**, 262–272
27. Helterline, D. L., Garikipati, D., Stenkamp, D. L., and Rodgers, B. D. (2007) *Gen. Comp. Endocrinol.* **151**, 90–97
28. Kubota, K., Sato, F., Aramaki, S., Soh, T., Yamauchi, N., and Hattori, M. A. (2007) *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **148**, 550–555
29. Sundaresan, N. R., Saxena, V. K., Singh, R., Jain, P., Singh, K. P., Anish, D., Singh, N., Saxena, M., and Ahmed, K. A. (2008) *Res. Vet. Sci.* **85**, 86–91
30. Nakashima, M., Toyono, T., Akamine, A., and Joyner, A. (1999) *Mech. Dev.* **80**, 185–189
31. Gamer, L. W., Cox, K. A., Small, C., and Rosen, V. (2001) *Dev. Biol.* **229**, 407–420
32. Wu, H. H., Ivkovic, S., Murray, R. C., Jaramillo, S., Lyons, K. M., Johnson, J. E., and Calof, A. L. (2003) *Neuron* **37**, 197–207
33. Ge, G., Hopkins, D. R., Ho, W. B., and Greenspan, D. S. (2005) *Mol. Cell Biol.* **25**, 5846–5858
34. Harmon, E. B., Apelqvist, A. A., Smart, N. G., Gu, X., Osborne, D. H., and Kim, S. K. (2004) *Development (Camb.)* **131**, 6163–6174
35. Esquela, A. F., and Lee, S. J. (2003) *Dev. Biol.* **257**, 356–370
36. Dichmann, D. S., Yassin, H., and Serup, P. (2006) *Dev. Dyn.* **235**, 3016–3025
37. Liu, J. P. (2006) *Development (Camb.)* **133**, 2865–2874
38. McKoy, G., Bicknell, K. A., Patel, K., and Brooks, G. (2007) *Cardiovasc. Res.* **74**, 304–312
39. Nomura, T., Ueyama, T., Ashihara, E., Tateishi, K., Asada, S., Nakajima, N., Isodono, K., Takahashi, T., Matsubara, H., and Oh, H. (2008) *Biochem. Biophys. Res. Commun.* **365**, 863–869
40. Thies, R. S., Chen, T., Davies, M. V., Tomkinson, K. N., Pearson, A. A., Shakey, Q. A., and Wolfman, N. M. (2001) *Growth Factors* **18**, 251–259
41. Lee, S. J., and McPherron, A. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 9306–9311
42. Hill, J. J., Davies, M. V., Pearson, A. A., Wang, J. H., Hewick, R. M., Wolfman, N. M., and Qiu, Y. (2002) *J. Biol. Chem.* **277**, 40735–40741
43. Wolfman, N. M., McPherron, A. C., Pappano, W. N., Davies, M. V., Song, K., Tomkinson, K. N., Wright, J. F., Zhao, L., Sebald, S. M., Greenspan, D. S., and Lee, S. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15842–15846
44. Oh, S. P., Yeo, C. Y., Lee, Y., Schrewe, H., Whitman, M., and Li, E. (2002) *Genes Dev.* **16**, 2749–2754
45. Rebbapragada, A., Benchabane, H., Wrana, J. L., Celeste, A. J., and Attisano, L. (2003) *Mol. Cell Biol.* **23**, 7230–7242
46. Andersson, O., Reissmann, E., and Ibáñez, C. F. (2006) *EMBO Rep.* **2006**, 831–837
47. Gonzalez-Cadavid, N. F., Taylor, W. E., Yarasheski, K., Sinha-Hikim, I., Ma, K., Ezzat, S., Shen, R., Lalani, R., Asa, S., Mamita, M., Nair, G., Arver, S., and Bhasin, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14938–14943
48. Lin, X., Takahashi, K., Campion, S. L., Liu, Y., Gustavsen, G. G., Peña, L. A., and Zamora, P. O. (2006) *Int. J. Mol. Med.* **17**, 833–839
49. Delsuc, F., Brinkmann, H., Chourrout, D., and Philippe, H. (2006) *Nature* **439**, 965–968
50. Biga, P. R., Roberts, S. B., Iliev, D. B., McCauley, L. A., Moon, J. S., Collodi, P., and Goetz, F. W. (2005) *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **141**, 218–230