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Journal
Circulation Research, 118(7)

ISSN
0009-7330

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Publication Date
2016-04-01

DOI
10.1161/circresaha.116.308391

Peer reviewed

HHS Public Access

Author manuscript

Circ Res. Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

Circ Res. 2016 April 1; 118(7): 1125–1142. doi:10.1161/CIRCRESAHA.116.308391.

Biochemistry and Biology of GDF11 and Myostatin: similarities, differences and questions for future investigation

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Abstract

Growth differentiation factor 11 (GDF11) and myostatin (MSTN or GDF8) are closely related members of the transforming growth factor β superfamily (TGFβ) and are often perceived to serve similar or overlapping roles. Yet, despite commonalities in protein sequence, receptor utilization and signaling, accumulating evidence suggests that these two ligands can have distinct functions in a number of situations. GDF11 is essential for mammalian development and has been suggested to regulate aging of multiple tissues, whereas MSTN is a well-described negative regulator of postnatal skeletal and cardiac muscle mass and modulates metabolic processes. In this review, we discuss the biochemical regulation of GDF11 and MSTN and their functions in the heart, skeletal muscle, and brain. We also highlight recent clinical findings with respect to a potential role for GDF11 and/or MSTN in humans with heart disease. Finally, we address key outstanding questions related to GDF11 and MSTN dynamics and signaling during development, growth, and aging.

Keywords

GDF11; GDF8; BMP11; MSTN; TGFβ; muscle

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DISCLOSURES

Harvard University and Brigham and Women's Hospital have filed for intellectual property on GDF11, listing Dr. Wagers, Rubin and Dr. Lee as inventors.

GDF11, also known as bone morphogenetic protein 11 (BMP11), and its homolog MSTN, (also known as GDF8) are closely related members of the TGFβ superfamily[1, 2]. MSTN plays an evolutionary conserved role in antagonizing postnatal muscle growth, limiting both the number and size of individual muscle fibers[1]. Hence, disruption of the *myostatin* gene or targeted inhibition of MSTN protein triggers hyper-muscular phenotypes in many mammals and fish[3-5]. MSTN function also has been implicated in postnatal glucose metabolism and adipogenesis[6]. GDF11, in contrast, plays a broad role during mammalian development, regulating anterior/posterior patterning, formation of the kidney, stomach, spleen and endocrine pancreas, and olfactory neurogenesis[2, 7-11]. GDF11's functions in postnatal tissues are less explored, partly due to the perinatal lethality of *Gdf11*-knockout mice[2, 7], which exhibit homeotic skeletal transformations, cleft palate, and renal agenesis (Table 1). Recent work identified GDF11 as a candidate hormonal regulator of aging in a variety of different organs. Consistent with this function, boosting levels of GDF11 protein in aged mice improves age-related phenotypes in the heart[12], brain[13], and skeletal muscle[14]. In addition, two studies recently implicated GDF11 as a negative regulator of erythroid differentiation in mouse aging and thalassemia models[15, 16].

Further highlighting the differences in MSTN and GDF11, *myostatin* mRNA is predominantly detected in skeletal and cardiac muscle whereas *GDF11* mRNA is detected broadly in numerous tissues[17] and is most abundant in the kidney and spleen[12]. Both GDF11 and MSTN are found in the bloodstream, and while the functional implications of their circulation are still under investigation, their systemic presence implies that these proteins may act as hormonal signals. Given their high sequence similarity, it was expected that many of the features and functions of these two ligands should overlap. However, a growing number of studies have described disparities in their actions, sparking debate over their respective involvement in particular physiological processes. Here, we discuss the molecular properties of GDF11 and MSTN, their roles in regulating different organ systems, and the challenges encountered in studying these proteins, which have contributed to recent controversies regarding their biological roles.

BIOCHEMICAL REGULATION OF GDF11 AND MYOSTATIN

The TGFβ family comprises more than 30 structurally related, yet functionally distinct ligands. This family can be subdivided into three subclasses: the TGFβs, bone morphogenetic proteins (BMPs), and activin/MSTNs. GDF11 and MSTN belong to the activin/MSTN subclass and share 90% sequence identity within their mature, signaling domain. Similar to other TGFβ proteins, both GDF11 and MSTN are synthesized as precursor molecules where an N-terminal prodomain is cleaved from a C-terminal signaling or mature domain by a furin protease (Fig. 1A). The mature ligands are propeller-shaped, disulfide-linked dimers that initiate signal transduction by engaging two Type II receptors and two Type I receptors using convex and concave surfaces, respectively[18] (Fig. 2).

The molecular structure of MSTN has been extensively investigated, including two X-ray crystal structures of MSTN in complex with two known antagonists[19, 20]. In contrast, GDF11 is less well characterized, and much of what is known for MSTN has been inferred for GDF11. However, the unbound X-ray crystal structure of GDF11 was recently

determined revealing the classic propeller-shaped structure with subtle differences between myostatin and GDF11, particularly in receptor binding epitopes[21]. Therefore, while many structural and regulatory mechanisms are shared between these two ligands, growing evidence also points to unique features of GDF11 and MSTN biology.

Role of the Prodomain in Latency and Activation

While mature GDF11 and MSTN ligands share substantial sequence identity, their prodomains are only 52% identical (Fig. 3). Like other TGFβ members, the GDF11 and MSTN prodomains aid in folding of the mature dimeric ligand[22, 23]. However, unlike most TGFβ ligands, GDF11 and MSTN remain tightly bound to their prodomains after cleavage by furin-like proteases[24-29], and are thereby held in a latent state, unable to bind receptors. Ligand activation requires additional cleavage of the prodomain by a Tolloid-like (TLD) metalloproteinase[26, 27]. Compared to other ligands, MSTN is inefficiently processed by furin, leaving a significant amount of unprocessed and presumably inactive protein[24, 25]. However, a SNP for the mutation K153R[30] dramatically improves furin processing, but has no effects on TLD activation[31] (Fig. 3B). Interestingly, this allelic variant was found at higher frequency in two centenarian cohorts, as compared to controls[32], although the implications of this polymorphism in terms of longevity and maintenance of muscle mass and strength have yet to be definitively established[32-41]. While GDF11 has similar furin and TLD recognition sequences, it is not known if sequence variations, especially in the surrounding areas, which are more divergent, alter furin and/or TLD processing of GDF11 (Fig. 3B).

Structural details of the latent state have yet to be described for GDF11 and MSTN. Still, despite differences in mechanism of activation (discussed below), the structure of TGFβ1 in complex with its prodomain[42] offers general insight into the molecular interactions driving latency. The latent structure of TGFβ1 has a ring-like appearance orchestrated by a centrally positioned mature dimer blanketed by the prodomains from each monomer[42] (Fig. 3A). Ligand inhibition is mediated through interactions of the helical N-terminus (α1) with the Type I receptor site and a ‘latency lasso’ where the prodomain wraps around the ligand fingertips toward the Type II receptor site[42]. Consistent with this structure, MSTN prodomain residues 43-115 are necessary and sufficient to inhibit ligand activity[43, 44]. Interestingly, this region contains the TLD proteolytic site[17,18] and is highly conserved with GDF11, suggesting that it serves a similar role in regulating GDF11 (Fig. 3B).

Nonetheless, the mechanism for TGFβ activation differs from that of GDF11 and MSTN[45, 46]. The TGFβ latent complex exists in the extracellular matrix (ECM) covalently bound to the latent transforming growth factor β protein 1 (LTBP1) via N-terminal disulfide linkages, a feature not known to occur for GDF11 and MSTN[47-49]. Additionally, the apex of the TGFβ latent complex interacts with $\alpha_V\beta_{VI}$ integrin[45, 46]. The combination of these two interactions tether the latent TGFβ complex at both ends, such that cellular contractile forces release the mature TGFβ ligand from the prodomain[42, 45, 46]. Although there is no evidence that MSTN or GDF11 latent complexes are covalently bound to a LTBP, these latent complexes do interact with the ECM components LTBP3 and perlecan[25, 50]. The purpose of these interactions remains unknown, but possibly relates to ligand activation, as binding to

LTBP3 can prevent furin processing and overexpression of LTBP3 in skeletal muscle increase muscle mass[25]. Thus, interactions with ECM components may fine-tune the activity of MSTN and GDF11 and dissimilarities in GDF11 and MSTN prodomain sequences (Fig. 3) could allow for unique ECM interactions across tissues.

Receptor Utilization by GDF11 and Myostatin

Similar to the activin-type ligands, both GDF11 and MSTN predominantly utilize the Type II receptors activin receptor kinase II-A (ActRIIA) and ActRIIB and the Type I receptors activin receptor-like kinase 4 (ALK4) and ALK5 to elicit signal transduction via SMADs 2 and 3[28, 51, 52] (Fig. 2B). GDF11 also can signal through an additional Type I receptor, ALK7, although its biological role remains undetermined[51].

Unlike the BMPs, the TGFβ and activin/MSTN sub-classes exhibit high affinity for the Type II receptor and low affinity for the Type I receptor[18]. The Type II receptors for the BMP and activin sub-classes bind on the concave surface of the fingers whereas TGFβs bind the Type II receptor more distally, towards the fingertips[18]. This positioning facilitates a cooperative binding interaction between the Type II and Type I receptor, as shown by the structure of the TGFβ3:TBRII:ALK5 receptor complex[53]. In contrast, the receptors bind BMPs on opposite sides of the fingers, and thus are unable to interact with one another, as described in the BMP2:ActRIIB:ALK3 complex[54]. The ternary receptor configuration for activin/MSTN has yet to be determined, as detailed binding and structural studies have been hampered by the low affinity for their Type I receptors[18].

Using structural data as a guide to denote the approximate receptor interfaces[53, 55-57], it is likely that GDF11 and MSTN bind Type II receptors similarly since residues in this location are identical (Fig. 4). However, residues in the Type I site, specifically the pre-helix loop and wrist helix (Fig. 4), are divergent between GDF11 and MSTN, suggesting that Type I receptor binding might differ, especially in the utilization of ALK7. Supporting this notion, introduction of the MSTN pre-helix loop into Activin A confers signaling through ALK5[20]. Similar chimeric protein studies should help to reveal the biological consequences of sequence differences between GDF11 and MSTN at the receptor interface. Furthermore, with growing evidence indicating the importance of co-receptors in assembling TGFβ ligand complexes[58-65], further studies are needed to define their roles in GDF11 and MSTN signaling.

Regulation of GDF11 and Myostatin by Extracellular Binding Proteins

Signaling by GDF11 and MSTN is regulated by extracellular binding proteins that are typically thought to function as antagonists. These include follistatin (FS), follistatin-like 3 (FSTL3), decorin, and growth/differentiation factor associated serum proteins 1 and 2 (GASP1, GASP2)[28, 66-70]. Structural studies indicate that two FS or FSTL3 molecules symmetrically embrace the ligand to block both receptor epitopes[19, 20]. FSTL3 and FS similarly contain an N-terminal domain followed by tandem follistatin domains. The N-terminal domain binds in the concave Type I receptor slot where GDF11 and MSTN show the highest divergence[19-21]. However, mutagenesis studies and comparison to other FS-ligand structures indicate that the FS N-terminal domain is highly plastic and can

accommodate diverse Type I interfaces[19, 20, 71-73]. Therefore, sequence differences likely have minimal impact on GDF11 and MSTN antagonism by FS. Sequence differences are also unlikely to impact the increased binding to cell surface-localized heparin/heparin sulfate and the subsequent acceleration of ligand degradation that occurs when FS is bound to MSTN[20]. This interaction is known to regulate MSTN signaling within skeletal muscle[74, 75] and a similar mode of regulation may exist for GDF11. Interestingly, FSTL3 does not bind heparin and therefore readily escapes the cell surface to enter circulation[76]. Thus, the distinct localizations of FS-type antagonists provide an intriguing mechanism for differentially modulating the actions of circulating versus locally produced GDF11 and MSTN.

In contrast to broad TGFβ ligand antagonism by FS-type molecules, GASP1 and GASP2 selectively inhibit GDF11 and MSTN[66, 77-80]. GASP proteins contain six independent domains, a whey acidic protein domain, follistatin domain, Ig-like domain, 2 tandem kunitz domains, and a netrin-like domain[66, 81]. The follistatin domain is the primary driver of ligand antagonism[80]. Despite a similar domain layout, GASP1 binds MSTN in a unique 1:1 ratio, forming an asymmetric complex, whereas GASP2 binds symmetrically in a 2:1 ratio [68], similar to FS or FSTL3[19, 20, 77]. Interestingly, C-terminal truncation of GASP1 induces 2:1 binding and weaker affinity for MSTN, similar to that of GASP2[77, 79, 80]. GASP proteins antagonize signaling by preventing ligand binding to the Type II receptor[79, 82], an intriguing mechanism given that GASP maintains unique specificity for GDF11 and MSTN despite conservation of the Type II receptor epitope among the activin/MSTN subclass. This observation suggests that steric forces and/or additional molecular contacts (e.g. in the Type I epitope) are likely important in defining this unique ligand-antagonist relationship.

In summary, GDF11 and MSTN are regulated by specific and non-specific interactions at nearly every step from biosynthesis to engagement with their cognate receptors. Given that sequence divergence exists between the GDF11 and MSTN prodomains, and to a lesser extent in the mature domains, it remains important to determine if the distinct biological functions of these proteins are driven in part by these molecular differences.

Signaling by GDF11 and Myostatin

Canonical TGFβ signaling is mediated through a series of SMAD proteins. Receptor binding by either GDF11 or MSTN induces phosphorylation and activation of the receptor-regulated SMAD (R-SMAD) proteins SMAD2 and SMAD3. Subsequently, the phosphorylated R-SMAD proteins assemble to form oligomeric complexes with the common SMAD (coSMAD) SMAD4, and this complex accumulates in the nucleus to regulate gene expression through direct and indirect DNA binding (Fig. 2B). Cellular responses to SMAD2/3 activation are highly context-dependent, and the presence or absence of particular transcriptional cofactors, DNA binding partners and chromatin modifiers can dramatically alter the ultimate output of ligand binding[83, 84]. GDF11 and MSTN may also signal through non-canonical (i.e., non-SMAD) pathways, including ERK, JNK, and p38 MAPK (Fig. 2B)[85-88], adding further complexity.

The transcriptional targets of GDF11 and MSTN signaling remain incompletely defined. A recent study compared gene expression changes following stimulation of human primary muscle cells with GDF11 or MSTN[87]. Only a few differentially represented transcripts were

identified, suggesting that GDF11 and MSTN gene regulation may be essentially identical[87]. However, this analysis was limited to a single cell type and neither ligand activated a robust gene-expression signature (the highest observed Fold Change values were only ~4-fold for either ligand). Thus, further experiments are needed to clarify potential differences in the transcriptional output of GDF11 and MSTN signaling in different cell types and physiological contexts.

GDF11 RELATED PATHWAYS IN THE HEART

The incidence of myocardial infarction and heart failure increases with age[89], and aging increases mortality risk with any given infarction event[90]. TGFβ signaling regulates responses to myocardial ischemic injury[91, 92], with recent studies suggesting possible roles for GDF11, MSTN and FSTL3 in the heart.

Myostatin in the heart

MSTN is best known for inhibiting skeletal muscle growth[1, 3], but genetically engineered models have demonstrated an additional function in cardiac tissue. MSTN is expressed in fetal and adult hearts[93] and its expression increases in patients with decompensated heart failure[94] and congenital heart disease[95]. MSTN protein levels rapidly increase after ischemia[96] and its circulating levels increase in mice after TAC-induced hypertrophy[97]. Germ-line inactivation of MSTN does not cause cardiac hypertrophy and does not attenuate cardiac fibrosis in dystrophin-deficient mice, indicating that MSTN does not function in cardiac muscle in a manner similar to skeletal muscle[98]. However, other experiments reveal that MSTN-null mice develop increased heart and body weights. $MSTN^{-/-}$ mice (28-30 month-old) were reported to have increased normalized heart mass at death compared to $MSTN^{+/+}$ and $MSTN^{+/-}$ mice[99]. Aged mice with MSTN deletion have improved fractional shortening, smaller left ventricle diastolic diameters and less fibrosis compared to aged wild-type mice[100]. Heineke and colleagues compared deletion *versus* overexpression of MSTN in cardiomyocytes[97]. MSTN deletion in cardiomyocytes did not prevent left ventricular decompensation under pressure overload, whereas transgenic overexpression of MSTN in the heart inhibited cardiac growth[97]. Inducible genetic deletion of MSTN in adult mouse cardiomyocytes leads to dramatic deterioration of cardiac function and high mortality, as well as increased glycolysis and glycogen storage, revealing the importance of endogenous MSTN for adult cardiomyocyte metabolism[101]. Thus, MSTN likely participates in cardiac growth and metabolism, although the experimental findings have not been as consistent in the heart as in skeletal muscle.

Cardiac effects of GDF11

GDF11 is expressed in cardiac tissue[102] but at lower levels compared to spleen, kidney and skeletal muscle in mice[12]. We reported an anti-hypertrophic effect of GDF11 in aging mice[12]. Using heterochronic parabiosis, we reported reduced cardiac hypertrophy in aging mice that shared a common circulation with young mice. We further devised a sham parabiosis procedure wherein mice were joined but did not share a chimeric circulation. With heterochronic sham parabiosis, hypertrophy in old mice was not reduced, implying the presence of a circulating factor that regulated cardiac size. Proteomic studies identified

GDF11 as a candidate for this anti-hypertrophic effect, and supplementing blood levels in old mice by daily intraperitoneal injection of recombinant GDF11 (rGDF11) protein reduced cardiomyocyte size and heart mass over 4 weeks[12].

In contrast, Smith and colleagues reported recently that injections of the same quantity of rGDF11 employed by us in old mice (0.1 mg/kg, injected daily) did not alter cardiac structure or function[103]. These apparently contradictory results may be explained in part by the different sources of rGDF11 and by a potential dose-dependent effect of GDF11. Following the study by Smith and colleagues[103], we performed a dose-response analysis, and demonstrated that for a given protein preparation, the reduction in cardiac mass by GDF11 is dose-dependent; for recent protein preparations with improved quality control of protein concentration, a dose of 0.5 mg/kg reduced cardiac mass in 9 days[104]. Injection of rGDF11 rapidly activates SMAD signaling in cardiac tissue of both young and old mice[104], which together indicate that exogenous GDF11 can regulate cardiomyocyte size and hypertrophy and highlight that doses and protein preparations used could impact the results of *in vivo* studies[105].

Given observations reported previously for genetic manipulation of MSTN expression[88, 98, 99], it is likely that administration of recombinant MSTN could achieve a similar anti-hypertrophic effect, but this has yet to be tested. Furthermore, it remains to be determined if a similar effect can be achieved with other ligands that likewise activate the SMAD2/3 pathway, such as members of the TGFβs or activin sub-classes. Finally, while most studies to date have focused on SMAD activation as a readout of signaling activity, it will be interesting to determine if cross-talk with non-canonical pathways may achieve ligand-specific effects.

FSTL3 in cardiac tissue

FSTL3 is expressed in cardiac tissue[106] and its expression increases in end-stage failing myocardium in humans[107]. Heart mass, left ventricular and systolic pressure, and systolic arterial pressure are increased in FSTL3-deficient mice compared to wild-type mice[108]. In addition, experimental cardiac injury induces myocardial expression of the prosurvival TGFβ ligand, activin A, and one of its antagonist regulators, FSTL3, where it is thought that the relative expression levels of these molecules dictate cell survival following insult. Interestingly, cardiomyocyte-specific deletion of FSTL3 reduces infarct size and apoptosis, suggesting a detrimental effect of endogenous FSTL3 on the heart, while overexpression of FSTL3 inhibits the prosurvival effect of activin A[109]. FSTL3 also regulates cardiac hypertrophy induced by pressure overload[106]. While no differences were seen between hearts of cardiac-specific FSTL3$^{-/-}$ and wild-type mice in standard physiological conditions, FSTL3$^{-/-}$ mice[106] exhibited attenuated myocardial hypertrophy and reduced left ventricular dilatation, and systolic dysfunction and interstitial fibrosis were reduced[106, 110] after transverse aortic constriction-induced pressure overload (TAC). These data suggest that endogenous FSTL3 regulates the heart in many circumstances and that induced expression of FSTL3 may have deleterious effects. It remains to be determined how cardiac insult and upregulation of FSTL3 may impact the heart over time with respect to GDF11 and MSTN levels, especially in the case for older populations where evidence suggests that GDF11

and/or MSTN levels decline with age[104]. Nevertheless, because FSTL3 inhibits multiple TGFβ family ligands[111], cardiac effects of FSTL3 cannot be attributed to a particular ligand interaction at this time.

GDF11 RELATED PATHWAYS IN SKELETAL MUSCLE

Skeletal muscle is composed of multinucleated, non-dividing fibers. Repair of muscle fibers after severe damage invokes the regenerative activities of a unipotent population of muscle stem cells, known as satellite cells, which reside immediately adjacent to myofibers in adult muscle. Aging impairs both the homeostatic maintenance of muscle mass and muscle regenerative potential, and recent studies have focused on the potential role of GDF11-related signaling pathways in these age-associated changes.

Myostatin in muscle homeostasis and repair

Prevailing views on GDF11 function in skeletal muscle and satellite cells have been greatly influenced by analogy to MSTN due to the high homology of their mature, C-terminal ligand domains (Fig. 4). MSTN is expressed almost exclusively in mature and developing muscle and negatively regulates muscle mass. Targeted disruption of MSTN in mice produces a near doubling of adult muscle mass, with an increase in both the size and number of muscle fibers[1] and a shift toward more glycolytic fiber types[1, 112]. Whether the hypertrophic and hyperplastic phenotype of MSTN-null muscle reflects in part a release of MSTN-mediated inhibition on muscle satellite cells remains unclear. While some studies suggest that MSTN inhibits satellite cell proliferation[113, 114], others have reported that it has no impact[115, 116]. Likewise, some groups have found increased numbers of satellite cells in MSTN-null muscle[117], while others report slightly lower numbers[115]. MSTN overexpression and supplementation studies suggest that high levels of MSTN can drive rapid muscle atrophy[118, 119], although more moderate increases in MSTN do not detectably alter muscle mass[120]. Conversely, treatment of mice with the inhibitory MSTN propeptide induces muscle hypertrophy[121].

GDF11 effects in muscle

In contrast to the profound effects of MSTN deficiency, muscle phenotypes are not prominent in GDF11-null mice, which exhibit defects in a variety of mesodermal, endodermal and ectodermal lineages and die within 24 hours after birth[2]. Nonetheless, double mutants lacking both MSTN and GDF11 exhibit increased penetrance of renal, palatal and skeletal abnormalities[7], suggesting that MSTN can compensate to some degree for loss of GDF11 in GDF11-null mice. Studies to similarly assess possible redundancy of GDF11 and MSTN function in regulating muscle mass have been challenging, due to the fact that GDF11-null mice die prior to the age at which MSTN-null mice begin to exhibit muscle phenotypes. However, analysis of mice with muscle fiber specific deletion of GDF11 showed no changes in muscle mass or fiber type, and muscle-specific GDF11 deletion did not exacerbate the phenotype of MSTN-null animals[7]. These results suggest that GDF11 and MSTN may have distinct functions in skeletal muscle, or that GDF11's effects on muscle are mediated by GDF11 protein that is produced by non-muscle tissues.

GDF11 has been implicated as a negative regulator of muscle development by studies in chick embryos and in cultures of differentiating C2C12 cells and primary human and mouse muscle myoblasts. In these systems, GDF11, like MSTN, can block myogenic differentiation[87, 122-124]. Yet, other data suggest that GDF11's role in myogenesis may be more complex. *Gdf11* mRNA levels in mouse skeletal muscle appear to peak during the most rapid phase of postnatal muscle growth and are higher in males than in females, despite males having greater muscle mass[124]. *Gdf11* expression is also increased in the muscles of MSTN-null mice, which nonetheless exhibit accelerated muscle growth and increased muscle mass[124]. These data raise the possibility that GDF11's actions in muscle may not fully overlap with those of MSTN such that GDF11 cannot compensate fully for the loss of MSTN in muscle. Whether this lack of functional redundancy reflects differences in the biochemical properties and signaling activities of GDF11 and MSTN, or differences in their absolute levels in muscle, remains to be determined. Interestingly, ectopic GDF11 can induce expression of FS[122], which as discussed above inhibits both GDF11 and MSTN signaling. Thus, GDF11 can initiate a negative feedback loop that may antagonize its own activity, as well as that of other activin/MSTN ligands. The existence of such a feedback mechanism suggests the likely importance of maintaining signaling by these ligands within a tight physiological range.

Experiments employing heterochronic parabiosis recently implicated GDF11 as a candidate regulator of aging phenotypes in the heart, muscle and brain[12-14]. Consistent with this notion, satellite cells isolated from the uninjured muscle of aged mice that received daily intraperitoneal injection of rGDF11 for four weeks showed improved myogenic activity in *ex vivo* clonal assays and a reduced burden of DNA damage. In addition, aged animals supplemented with rGDF11 showed accelerated recovery from muscle injury in a cryoinjury model[14]. rGDF11 treatment in aged mice also improved neuromuscular junctions and myofibrillar and mitochondrial morphology, and increased average exercise endurance and grip strength without any detectable changes in muscle mass or fiber caliber[14]. Notably, the dosage of rGDF11 in these studies was similar to that reported previously to lack effects on muscle size in an *in vivo* rMSTN dosing study[120]. Interestingly, young mice in this study showed no differences in regenerative capacity following treatment with the same amount of rGDF11, suggesting that GDF11's beneficial effects on muscle were age-dependent.

In contrast to the studies summarized above, a subsequent paper questioned the beneficial effects of rGDF11 on muscle aging, reporting instead that supplementation of rGDF11 in aged mice has no effect and in young mice impairs muscle repair. Yet, it is important to note that the study design employed by this group[87] differed significantly from that in the earlier studies[14]. From a reagent standpoint, the authors used rGDF11 from a different protein source and employed a different dosing regimen. Further, they assessed muscle regeneration in a more severe, cardiotoxin-mediated damage model, which ablates the majority of resident satellite cells[125, 126] and shows distinct temporal and spatial features of regeneration in comparison to cryoinjury[127, 128]. Thus, the different outcomes obtained in the two studies may reflect differences in experimental design, and comparison of the methods employed could provide an avenue for discovering key mechanisms that determine GDF11's *in vivo* effects. For instance, the inability of rGDF11 to accelerate repair in the satellite-cell depleting cardiotoxin injury model[87] suggests that sustaining a sufficient pool of

regenerative satellite cells may be essential for a beneficial effect on regeneration in response to rGDF11. Likewise, the possible negative impact of higher doses of rGDF11 in young mice[87] could indicate that rGDF11 exhibits antagonistic pleiotropy in young versus old animals, or that the precise timing and dosage of rGDF11 administration is critical in determining its *in vivo* effects.

It is also important to remember that rGDF11's impact on muscle repair *in vivo* likely represents the integrated effects of this growth factor on many different target cells. GDF11 signaling has been implicated in a number of biological processes, including vasculogenesis[13], a process clearly documented to influence the efficiency of muscle repair[129]. Indeed, if the primary actions of GDF11 *in vivo* are on vasculature, this may explain the coordinated responses to systemic administration of this protein seen in skeletal muscle, heart and brain, as each of these organs shows critical dependence on proper vascularization, a process which declines with increasing age[130].

The potential effects of changing levels of MSTN on aging muscle have also been examined. As mentioned above, human genomics studies suggest an association of the K153R polymorphism, thought to increase MSTN activity by enhancing furin processing[31], with longer lifespan[32]. Interestingly, homozygosity for the K153R allele is extremely rare, and most R allele-carrying centenarians are heterozygous for this variant, a genotype that does not appear to alter muscle phenotypes in aged individuals[38, 39, 41]. Studies in mice also suggest that alterations of MSTN signaling may impact lifespan. A recent study of male $MSTN^{+/+}$, $MSTN^{+/-}$ and $MSTN^{-/-}$ mice (n=38-42 mice per group), revealed an increase in both median and maximum lifespan of heterozygous animals, which showed a 30% decrease in circulating MSTN levels, as compared to wild-type controls[99]. In contrast, the median and maximal lifespan of $MSTN^{-/-}$ mice did not differ from wild-type. Replication of this study, with inclusion of female animals, should be very illuminating, particularly as data from human subjects suggests sex-specific differences in the regulation of circulating MSTN levels with age[131].

Analogous studies of lifespan in GDF11 mutant mice are complicated by the developmental phenotypes exhibited by both $GDF11^{-/-}$ and $GDF11^{+/-}$ mice. However, Pazdro and colleagues recently reported that levels of circulating GDF11 are heritable in genetically diverse inbred mouse strains and can be used to predict median lifespan, with higher GDF11 levels at middle age associated with longer lifespan[132]. In addition, an overall positive impact of higher GDF11 and/or MSTN levels on lifespan, as well as muscle function, has been corroborated in invertebrate models. For example, overexpression of *myoglianin,* the fly ortholog of both GDF11 and MSTN, delayed the onset of age-related neuromuscular dysfunction and extended lifespan, while its inhibition hastened neuromuscular decline and caused premature death[133]. Studies in shrimp likewise implicate the ancestral form of GDF11/MSTN in supporting muscle growth and survival[134].

In summary, although more research is clearly needed, evidence across species implicates GDF11 as a candidate regulator of muscle homeostatic and regenerative function during aging, and suggests that raising levels of circulating GDF11 might be helpful for some age-related muscle pathologies. Emerging evidence also implicates both GDF11 and MSTN in

regulating longevity and suggests that even subtle variations in ligand activity can alter phenotypic outcomes. While the mechanisms underlying these effects remain unclear at present, these data raise the possibility that systemic GDF11 could provide a useful biometric for mammalian aging.

GDF11 IN THE BRAIN

TGFβ family ligands have diverse and pleiotropic roles in the development and maintenance of the nervous system, and their effects can vary between ligands, target cell types, and an animal's developmental stage or age. Neurological effects of GDF11 have been best characterized in the developing olfactory epithelium, spinal cord and retina where GDF11 influences the timing and progression of neurogenesis as well as the ratios of different neural subtypes[10, 11, 135]. In the developing olfactory epithelium, GDF11 plays a critical role in an autoregulatory loop, wherein newly born olfactory receptor neurons (ORNs) and their immediate neuronal precursor cells (INPs) signal to neighboring cells to limit the production of more ORNs[10]. ORNs and INPs secrete GDF11, which drives cell cycle exit by inducing upregulation of the cyclin dependent kinase inhibitor $p27^{Kip1}$. Loss of GDF11 augments proliferation of INPs and increases the number of differentiated neurons. Conversely, enhanced activation of GDF11 by genetic deletion of FS leads to sharp decreases in the numbers of both INPs and ORNs. In the spinal cords of mice lacking GDF11, neural progenitor cells fail to exit the cell cycle, the rate of neurogenesis is slowed, the ratios of neural subtypes are altered, and the positional identities of motor neurons are disrupted[135-137]. Interestingly, in the retina, GDF11 also controls the timing of neurogenesis, but through a mechanism that does not involve proliferation[11]. Instead, GDF11 controls the length of time that progenitor cells are competent to produce retinal ganglion cells, and thereby regulates the ratio of RGCs to photoreceptors and amacrine cells.

Compared to its role in the developing nervous system, less is known about the role of endogenous GDF11 in the mature nervous system. Northern blot analysis of tissues from adult rats (3-4 weeks of age) showed high expression of *Gdf11* in dental pulp and brain[17]. In the same study, RNA *in situ* hybridization identified specific areas of *Gdf11* expression in the brain, including the dentate gyrus of the hippocampus, the hypothalamus and the Purkinje cell layer of the cerebellum[17]. It was recently demonstrated that systemic administration of rGDF11 in aged mice alters brain physiology[13]. Heterochronic parabiosis experiments showed that young systemic factors could reverse age-related neurogenic decline by enhancing neural stem cell production and thereby increase adult neurogenesis and olfactory discrimination capacities. Furthermore, youthful blood factors induced remodeling of the aged vasculature, restoring cerebrovascular blood flow to its youthful levels. Systemic injection of rGDF11 recapitulated several beneficial effects of heterochronic parabiosis, including vascular remodeling of the aged blood vessels in the subventricular zone and increased numbers of neural stem cells in this area. rGDF11 also induced the proliferation of brain capillary endothelial cells and activated the SMAD2/3 pathway *in vitro*. The *in vivo* effects of rGDF11 were not as prominent as those seen with heterochronic parabiosis, suggesting that additional factors may be present in the young circulation that exerts these CNS effects[138]. Future studies testing different concentrations of

rGDF11[104], different treatment lengths and co-injection of factors such as IGF-1 or EGF that affect neural stem cell proliferation and/or vascular behavior may clarify this issue.

The CNS effects of rGDF11 are due to exogenous/circulating protein and not to brain-derived GDF11, whose role in the adult/aged brain is not yet understood. It will be important to determine if systemically administered rGDF11 crosses the blood-brain-barrier and acts directly on neurons and neural stem cells, or if its effects on the CNS are a by-product of its cerebrovascular effects. It will likewise be exciting to determine if an auto-regulatory loop exists between circulating and endogenous GDF11 that could amplify its effects. Interestingly, the effects of systemic rGDF11 are consistent with a recent report that constitutive activation of ALK5, a type I receptor for GDF11 and multiple TGFβs, leads to increased adult neurogenesis and higher expression of c-Fos in newborn neurons in the adult hippocampus, more complex dendritic arborization, increased neural activity, and improved performance in memory tests[139]. In addition, TGFβ1 is neuroprotective in mouse models of Alzheimer's disease and excitotoxicity[140, 141], and a recent study also reported that GDF10, which activates similar intracellular pathways as TGFβ1, β2, β3, and GDF11, improves brain vasculature, increases neurite outgrowth, and enhances performance in behavioral assays in a mouse model of stroke[142].

CLINICAL IMPLICATIONS OF GDF11 and MSTN

Olson, Ganz and colleagues investigated whether GDF11 and MSTN might have similar cardioprotective properties in humans to those demonstrated in mice[143]. In 928 archived plasma samples in subjects with stable coronary heart disease (CHD) from the Heart and Soul prospective observational cohort, they measured circulating levels of ligand using modified aptamers as binding reagents[143]. This assay does not discriminate GDF11 from MSTN; thus the measured analyte is referred to here as GDF11+MSTN. The aims of the study were to characterize the association of plasma levels of GDF11+MSTN in humans with 1) age, 2) left ventricular hypertrophy and 3) cardiovascular events and all-cause mortality during a median 8.9 years of follow-up. The key findings were replicated in 971 subjects with stable CHD from the HUNT3 Norwegian cohort, with a median follow-up of 4.5 years.

GDF11, MSTN, and age

Analysis in patients revealed an inverse relationship of plasma GDF11+MSTN to age in the HUNT3 cohort (Fig. 5A), which was also observed in the Heart and Soul cohort[143]. Despite the narrower age range of these human subjects compared to the extremes of age reported in mice[12], significantly lower levels of GDF11+MSTN were detected in older compared to younger individuals in both patient cohorts[143]. This cross-sectional analysis likely underestimates the decline in GDF11+MSTN with age, as GDF11+MSTN is inversely associated with all-cause mortality; thus, surviving older individuals enrolled in these cohorts likely had higher GDF11+MSTN concentrations than those older individuals who had died and whose potentially lower levels could not be captured. Although GDF11+MSTN levels were higher in males compared with females enrolled in this study, an age-related decline in GDF11+MSTN levels was detected in both sexes[143]. In support, a recent study used mass spectrometry to quantify circulating MSTN specifically in human

subjects without chronic disease, and likewise reported higher MSTN concentrations in younger men compared to younger women, but found sex-specific differences in the effects of age on MSTN levels (higher MSTN concentrations in older versus younger women and lower MSTN concentrations in older versus younger men)[131]. Circulating levels of the antagonist FSTL3 increased with age in both sexes[131]. Thus, while this latter study[131] analyzed a much smaller cohort (only 40 individuals of each age/sex), together these reports[131, 143] highlight a potential influence of age, sex, and health status on GDF11+MSTN levels and are consistent with an age-related loss of function of these ligands in humans, either through diminished protein concentrations[143] or increased inhibition of ligand activity[131].

GDF11, MSTN, and cardiovascular and all-cause mortality

A relationship of plasma GDF11+MSTN levels to cardiovascular events (heart failure hospitalization, stroke and myocardial infarction), all-cause deaths and their composite end-point were also demonstrated in 971 subjects in the HUNT3 cohort (Fig. 5B)[143]. In both the HUNT3 and Heart and Soul cohorts, there was a strong, graded relationship between plasma GDF11+MSTN and cardiovascular and mortality outcomes[143]. Participants in the highest quartile of GDF11+MSTN had markedly lower risk of individual types of events and their composite end-point than those in the lowest quartile, both unadjusted (Fig. 5B) and after multi-variable adjustment[143].

GDF11, MSTN, and left ventricular hypertrophy

Of the 928 participants in the Heart and Soul cohort, 368 had left ventricular hypertrophy (LVH) by echocardiography[143]. There was a significant, inverse, graded relationship between GDF11/MSTN and LVH, with 31% of participants in the highest quartile of GDF11/MSTN having LVH compared with 46% in the lowest quartile[143].

In sum, in two large independent cohorts of patients with stable CHD, lower levels of GDF11+MSTN were associated with notably higher rates of incident cardiovascular events and all-cause deaths as well as higher prevalence of LVH. Lastly, GDF11+MSTN levels are lower among older compared to younger individuals. Taken in the context of mechanistic studies in mice, these findings in humans support the hypothesis that GDF11 and/or MSTN protect against adverse cardiovascular events and all-cause deaths[143]. More broadly, these findings support the hypothesis that age-related decline in GDF11+MSTN contributes to cardiovascular aging and mortality in humans[143] as it does in mice.

Future directions for clinical research

A significant interaction was present in the multi-racial Heart and Soul cohort between race and GDF11+MSTN for the composite cardiovascular and all-cause mortality outcome[143]. Whites compared to non-whites had both lower levels of GDF11+MSTN and a stronger link between plasma GDF11+MSTN and adverse outcomes. As noted previously, GDF11+MSTN levels also varied by sex[143]. Accordingly, future studies should investigate in greater detail any racial and sex differences in GDF11 and MSTN levels and their relationship to outcomes.

As discussed above, several endogenous inhibitors of GDF11 have been reported. These inhibitors may antagonize other ligands in the TGFβ family, including MSTN and activin[67, 68, 79]. Heidecker and Ganz investigated the association of GASP1, FSTL3 and FS with cardiovascular outcomes and all cause-mortality in the Heart and Soul and HUNT3 cohorts, using the same archived plasma samples as for the aforementioned GDF11+MSTN analyses[144]. High levels of FSTL3 were associated with increased risk of adverse cardiovascular events and all-cause mortality, and the effect of low levels of GDF11+MSTN and high levels of FSTL3 were additive. Subjects in the least favorable quartile of GDF11+MSTN (lowest) and its inhibitor FSTL3 (highest) had nearly 7-fold increased risk of cardiovascular events and all-cause mortality[144]. Thus, any future studies of human diseases will need to consider the levels of GDF11+MSTN, levels of their inhibitors and their interactions.

Any therapeutic targeting of GDF11 or MSTN in humans must consider its potential adverse effects. GDF11 has been reported to exacerbate anemia in the setting of ineffective erythropoiesis[145]. Blocking GDF11 alleviates anemia in a mouse model of beta-thalassemia and also in humans[146]. Analysis of observational cohort studies may provide much needed clarity whether levels of GDF11 and/or MSTN are associated with any additional adverse outcomes.

CURRENT CONTROVERSIES

The suggestion that GDF11 may serve as an evolutionarily conserved age-dependent hormone in multiple organ systems[12-14, 133], raises possibilities for manipulating this signaling pathway to restore or preserve function to aging tissues. Yet, this provocative notion also presents a new and unexpected role for GDF11 within the TGFβ superfamily, and contrasts with studies of many other TGFβ family proteins in aging, which have frequently concluded that these molecules suppress healthy tissue function during aging, most notably by promoting tissue fibrosis and inflammation[147-150]. Thus, it is not surprising that there has been some skepticism and even controversy surrounding these results. It is likely that much of this controversy reflects the fact that GDF11 is a relatively under-studied member of the TGFβ superfamily, particularly compared to its close relative MSTN, and thus the tools for studying GDF11 biology are still evolving. In addition, given the substantial sequence conservation of GDF11 with MSTN, many have assumed that the functions of these proteins should be identical[7, 87]. Here, we discuss some of these controversies and suggest strategies to resolve the apparent discrepancies to advance our mechanistic understanding of GDF11 functions in organ physiology and aging.

Presence in circulation and direction of change with age of circulating GDF11 protein

In 2013, we reported a significant decline in systemic levels of GDF11 in aged, as compared to young mice[12]. This conclusion was based on an aptamer-driven analysis of serum from young (2 mo.) versus old (24 mo.) mice performed using Somalogics SomaMERs[151], in combination with Western blotting using a monoclonal antibody from abcam, which, at the time, was reported to be specific for GDF11. Subsequent reports from our group[104] and others[87, 143, 152] revealed that the SomaMER and mAb used in these initial studies cross-react with MSTN, and so, while these studies are consistent with a reduction in the

circulating pool of GDF11 and MSTN in aged animals, these data cannot discriminate the relative impact of aging on systemic levels of GDF11 specifically. A report from Egerman and colleagues argued that circulating levels of GDF11 might actually increase with age, based on the results of Western blot, RNA expression, and GDF11-specific immunoassay[87]. However, the Western results reported by this group were based on quantification of a ~25kDa immunoreactive band, the approximate size of the disulfide-linked mature ligand dimer, which was present in samples that had been reduced and denatured prior to Western blot analysis. Puzzled by this observation (since the blotting conditions should have reduced the dimeric ligand to monomer), we reproduced these experiments and confirmed that the ~25kDa band does increase with age. However, we further demonstrated that this band is comprised of serum immunoglobulin, known to increase with age[153], and not GDF11 or MSTN[104]. As expected, under denaturing and reducing conditions, detection of GDF11 and MSTN in serum is limited to the ligand monomer of ~12.5kDa, which declines with age in the Egerman studies[87], as in prior reports[12, 14]. In addition, the RNA expression analysis reported by Egerman et al. was limited to skeletal muscle, which expresses GDF11 at substantially lower levels (2-3 fold) than other tissues (e.g., the spleen and kidney[12], which may represent the predominant sources of circulating GDF11 protein). Finally, their immunoassay results in rats and humans were extremely underpowered (e.g., only 9 individuals “>60 years old” were assessed in the human studies, with no evaluation of health status) and did not yield statistically significant differences for either species.

Studies from this group and one other[103], also suggested that circulating levels of GDF11 may be very low compared to MSTN, leading a third group[154] to argue that changes in circulating GDF11 levels are “mostly irrelevant”. However, published mass spectrometry data clearly demonstrate the presence of GDF11 protein in both human and mouse sera[155], and, many critically important bioactive hormones are present at very low levels (i.e. pg/ml) in circulation, including glucagon, IL6, and TNF-α[156]. Thus, it is difficult to infer biological relevance from protein concentration alone. Future studies to develop highly specific reagents for measuring systemic GDF11 levels using either quantitative mass spectrometry or antibody-based immunoassays that can reliably distinguish GDF11 from MSTN in the blood of both humans and mice, something that has not yet been accomplished[87, 103], will be essential to clarify the overall abundance and age-related changes of circulating GDF11 and MSTN proteins. Adding to the complexity, current methods[131, 154, 157, 158] only measure the ‘total’ amount of protein and cannot account for the status of the ligand in the serum (e.g. free, latent or in complex with an extracellular antagonist). Finally, it is still unclear if GDF11 and MSTN act predominantly as systemic hormones or if they may exert their most potent effects locally through autocrine/paracrine mechanisms, which complicates interpretation of the impact on peripheral tissues of changing levels of these proteins in the blood. Thus, it will be important to clarify which cell types produce GDF11 and what pathophysiological stimuli may alter GDF11 and/or MSTN expression.

Effect of GDF11 supplementation on skeletal muscle regeneration in old and young mice

Studies investigating the effects of exogenous rGDF11 on muscle regeneration have reported apparently discrepant conclusions. While we reported[14] that rGDF11 reverses age-specific muscle phenotypes and improves muscle strength, endurance and regenerative potential,

with no discernable effects in young mice, Egerman et al. argued instead that rGDF11 supplementation has no effect in aged mice and may slow regeneration in young mice[87]. As discussed above, these different conclusions likely arise from differences in protein source and experimental design. Of particular importance is the use by Egerman et al. of a more severe cardiotoxin injury model[125, 128], as compared to the milder cryoinjury model applied in our studies[14] and those that originally demonstrated rejuvenation of muscle repair by heterochronic parabiosis[159]. Furthermore, in their studies of young mice, Egerman et al. used a markedly different dosing schedule, shortening the pre-injury treatment window from 28 to 3 days and lengthening post-injury treatment from 7 to 14 days. They also employed an unconventional analysis strategy that selectively focused on tiny "fibers" that lacked apparent nuclei. The reported *in vitro* studies, also, were quite different in design, with one group analyzing cells purified from uninjured muscle[14], and the other cells purified from cardiotoxin-damaged muscle[87], with distinct culture conditions and time points of analysis. Thus, discrepancies in the reported effects of rGDF11 on muscle repair and muscle satellite cells may reflect differences in the recombinant protein itself, in protein dosage and treatment schedule, in the severity or method of muscle damage and the consequent size of the residual muscle stem cell pool, or in the particular culture conditions and analysis strategies employed. Future studies to compare these experimental conditions side-by-side are necessary to identify the key experimental variables that underlie the different outcomes. In addition, the development and use of genetic gain- and loss-of-function models, as well as careful dose-titration assays in mice of different ages and different muscle injury paradigms will be helpful in establishing phenotypic thresholds for the possible context-specific effects of rGDF11 in skeletal muscle.

Effect of GDF11 supplementation on cardiac hypertrophy

Our studies published in 2013[12] and 2015[104] reported an anti-hypertrophic effect of rGDF11 administration by comparing heart weight-tibia length (HW/TL) ratio in treated and control aging mice, while the study by Smith and colleagues reported no effects on the heart[103]. As discussed above, this disagreement may relate to dose-dependent effects of rGDF11 on cardiac mass, but it is also important to emphasize that Smith and colleagues did not observe changes in body weight in rGDF11 injected mice[103], while we reported a significant decrease in body weight in aging mice with exogenous rGDF11[104]. Observations from our laboratories indicate that exogenous rGDF11 decreases body weight in old mice in a dose dependent manner (unpublished observation and[12, 104]). While rGDF11 directly activates cardiomyocyte SMAD signaling in cardiomyocytes[104], it is also possible that the reduction in cardiac size with exogenous rGDF11 *in vivo* is due to an indirect effect of rGDF11. A systemic signal, possibly initiated by a reduction in body size or a change in a specific tissue, is quite plausible. For example, there is clear evidence for cross talk between adipose tissue and cardiac[160, 161] and skeletal[133, 162, 163] tissues. Thus, any interpretation of a direct effect of rGDF11 on the myocardium must be considered in the context of its systemic effects.

In conclusion, GDF11 has emerged as an intriguing candidate in the regulation of vertebrate aging and considerable progress has been made in the analysis of its role in the progression of age-associated disease. Future investigation of GDF11 and MSTN biology and

biochemistry will clarify the similarities and differences in the functions of these proteins and advance our understanding of organismal aging and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Thank you to M. Painter for helpful discussions.

Sources of Funding

This study was supported in part by the National Institutes of Health, the Graduate Dean Fellowship and the Glenn Foundation for Medical Research (R01AG032977, R01AG040019, and R03AG049657 to RTL; R56 AG048917 and R01 AG033053 to AJW; RO1GM114640 to TBT; GD Fellowship to RGW).

Non-standard abbreviations and non-standard acronyms

ActR	Activin Receptor Kinase
ALK	Activin Receptor-like Kinase
BMP11	Bone Morphogenetic Protein 11
CNS	Central Nervous System
EGF	Epidermal Growth Factor
BW	Body Weight
CHD	Coronary Heart Disease
FS	Follistatin
FSTL3	Follistatin-like 3
GASP	Growth and Differentiation Factor-Associated Serum Protein
GDF11	Growth Differentiation Factor 11
GDF8	Growth Differentiation Factor 8
HW/TL	Heart Weight/Tibia Length
IGF1	Insulin-like Growth Factor 1
IL6	Interleukin 6
INPs	Immediate Neuronal Precursors
LTBP	Latent Transforming Growth Factor β Protein
LVH	Left Ventricular Hypertrophy
MSTN	Myostatin
ORNs	Olfactory Receptor Neurons
rGDF11	Recombinant GDF11

SMAD	Mothers Against Decapentaplegic
SNP	Single Nucleotide Polymorphism
TAC	Transverse Aortic Constriction
TGFβ	Transforming Growth Factor β
TLD	Tolloid-like
TNFα	Tumor Necrosis Factor α

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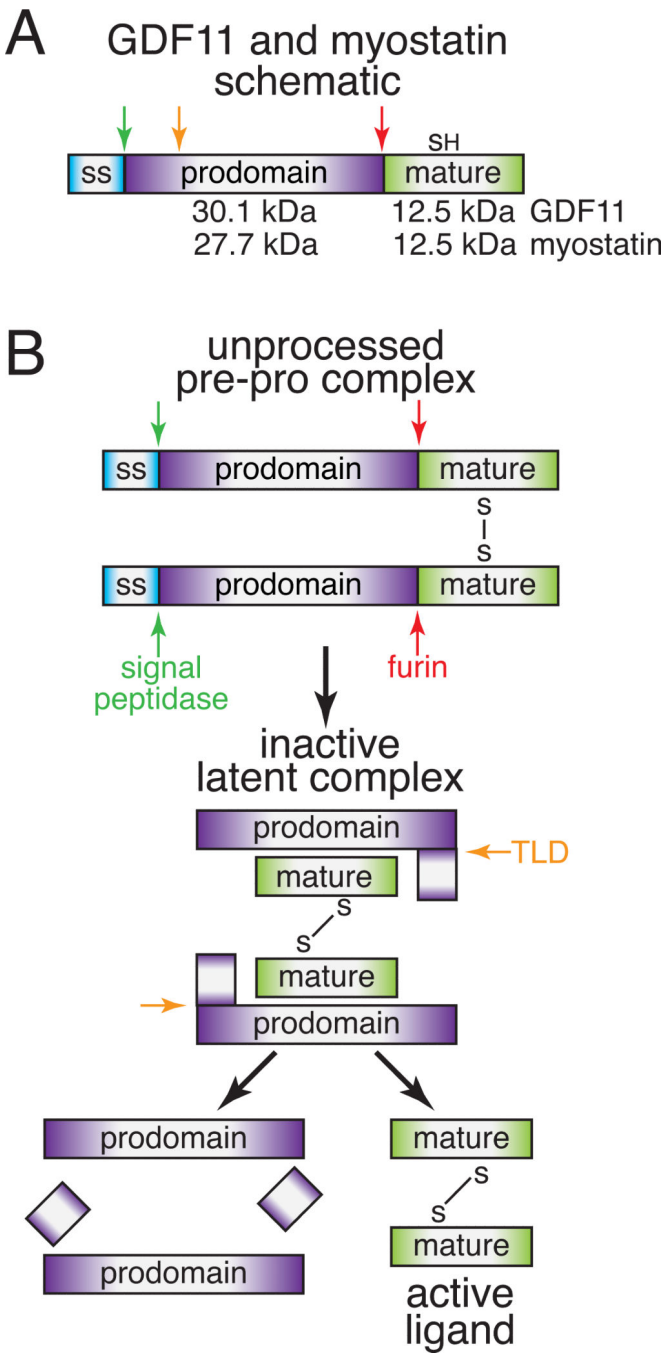

Figure 1. Biosynthesis and proteolytic processing of GDF11 and MSTN

A) Schematic of GDF11/MSTN monomer and relative position of proteolytic sites. B) Ordered proteolytic processing necessary to release an active dimer to elicit signaling.

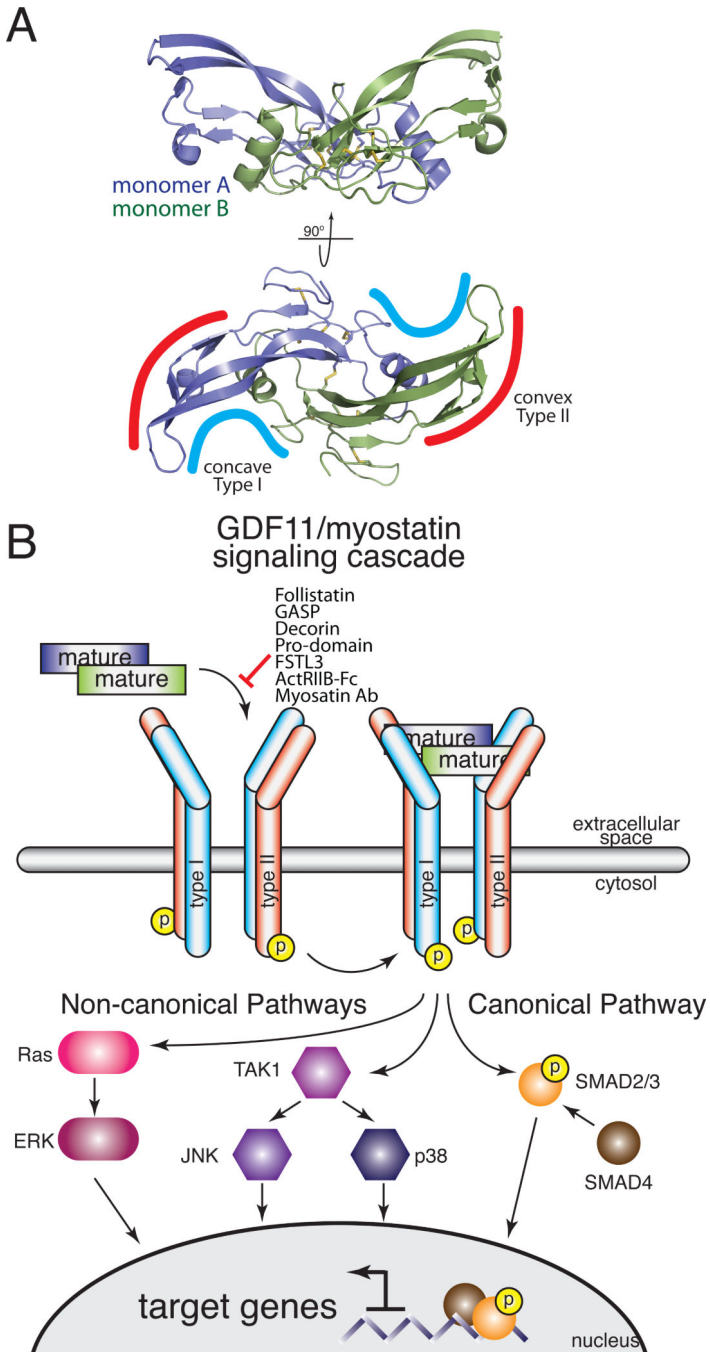

Figure 2. Structure of MSTN and reported elements of GDF11/MSTN

A) The symmetrical MSTN dimer forms two distinct interfaces, concave and convex, for receptor binding (PDB 3HH2[20]). B) GDF11 and MSTN induced canonical and non-canonical signaling. Known extracellular regulators and pharmacological inhibitors of GDF11 and MSTN are listed.

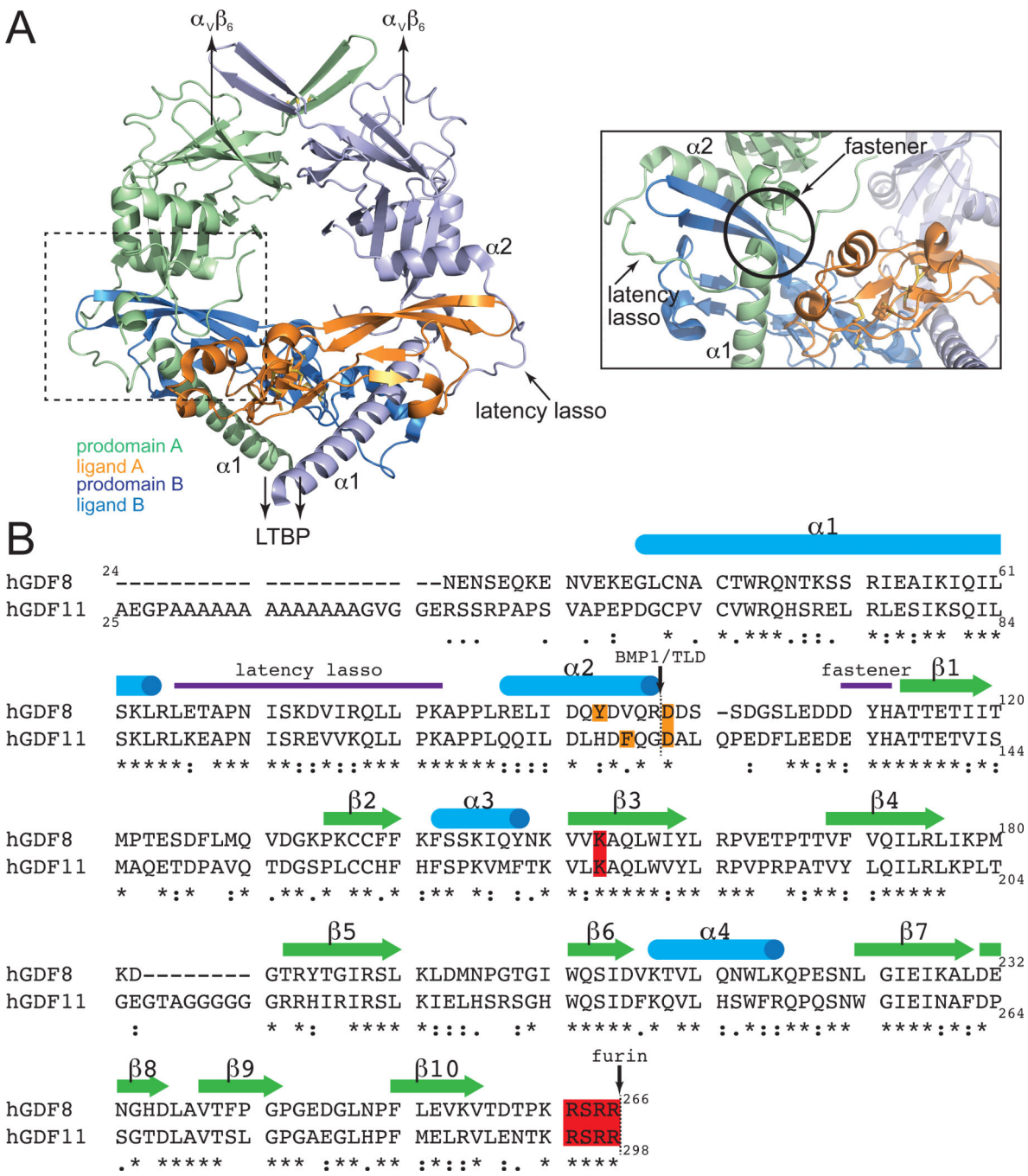

Figure 3. Structural organization of the TGFβ1 prodomain and comparison of GDF11 and MSTN prodomains

A) Structure of the TGFβ1-prodomain latent complex (PDB 3RJR [42]). Key regions identified in the TGFβ prodomain for conferring inhibition of the mature domain are highlighted (dotted box on the left is shown as the inset). B) Sequence alignment of human GDF11 and myostatin prodomains with topology based on the TGFβ structure. Known proteolytic sites and residues important for each proteolytic event are highlighted (furin: red; TLD: orange).

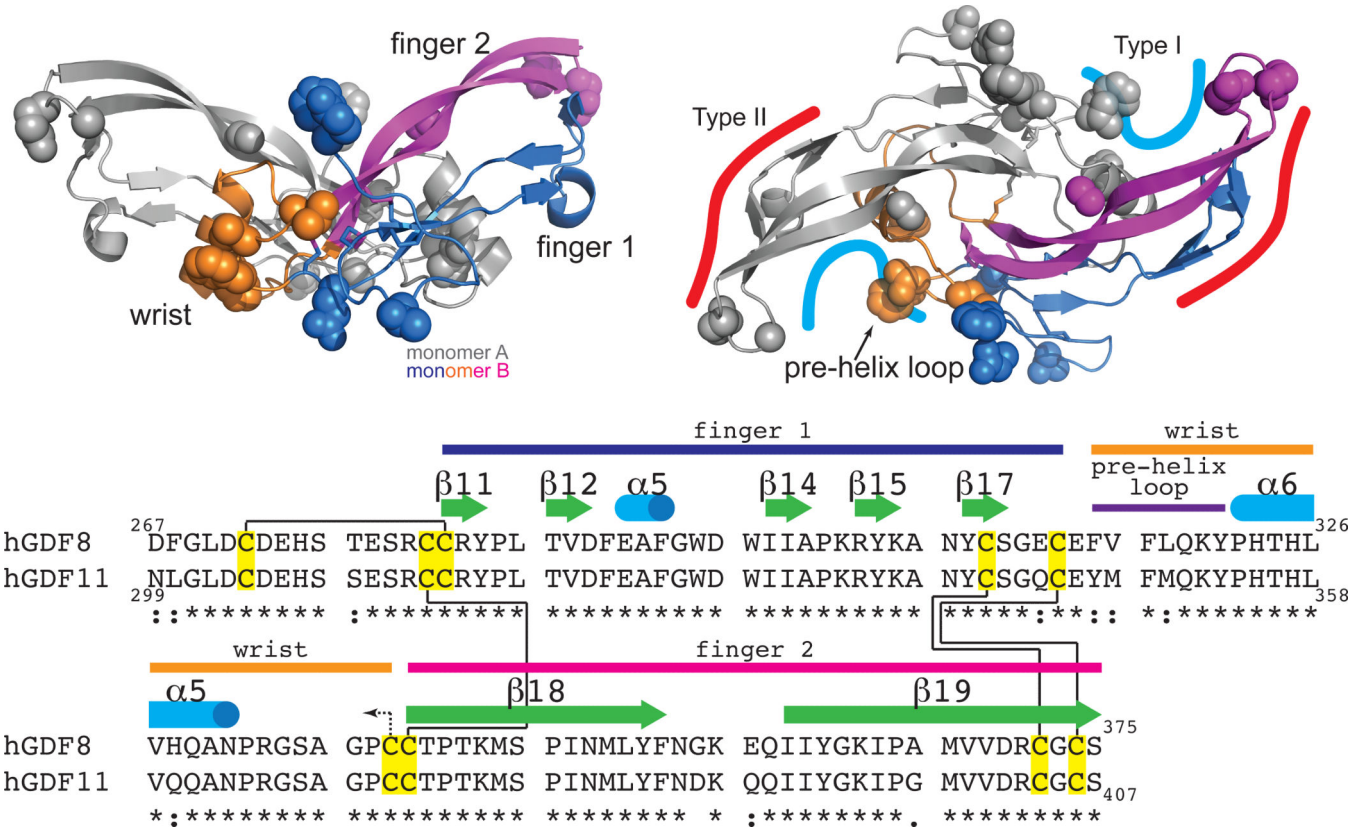

Figure 4. Structural organization and comparison of GDF11 and MSTN mature domains

MSTN dimer is shown where one monomer colored to show three sub-divisions of the ligand (finger 1: blue; wrist: orange; finger 2: magenta), which correspond to the colors in the sequence alignment below. Residues that differ between GDF11 and myostatin are shown as spheres. Differences are localized predominantly to the Type I site. Topology in the sequence alignment is an extension of the topology shown in Figure 3 and delineated by the structure of myostatin (PDB 3HH2[20]). Cysteines (yellow) and corresponding intramolecular (solid black line) and intermolecular (dotted line) disulfide linkages are shown.

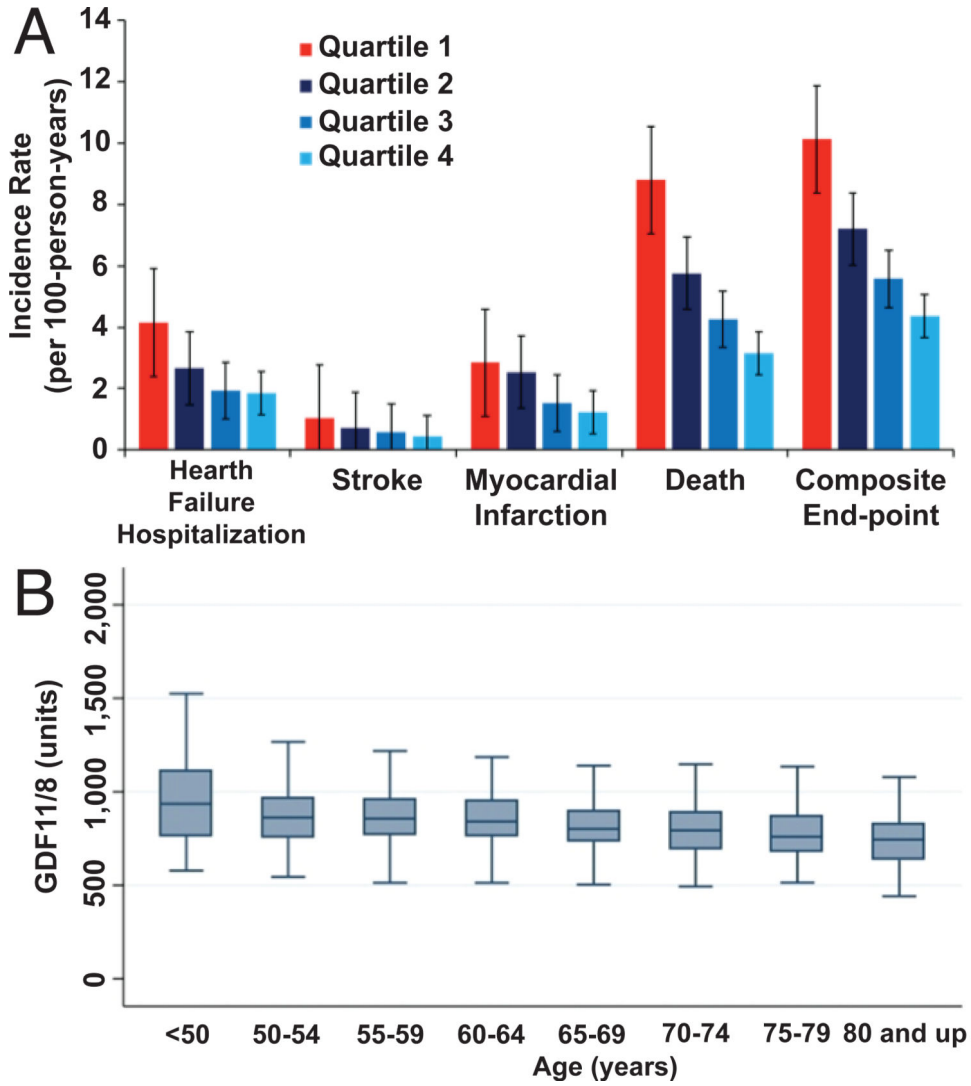

Figure 5. GDF11/MSTN in humans

A) Incidence of heart failure hospitalization, stroke, myocardial infarction, all-cause death, and their composite end-point in the HUNT3 cohort, unadjusted, stratified by quartile of GDF11+MSTN. Quartile 1 is the referent quartile with the lowest GDF11+8 concentrations. P-values for trend are <0.001 for heart failure, death, and composite end-point, 0.004 for stroke, and 0.02 for myocardial infarction. B) GDF11+MSTN levels by age in HUNT3 cohort, unadjusted. Inner line = median, box 25th-75th percentile, outer whiskers denote adjacent value 1.5 times height of box. Units = relative fluorescence units. P-value is < 0.001. This figure is reproduced from Olson et al.[143] with permission from the European Heart Journal.

Table 1

Comparison of developmental expression patterns and phenotypes in GDF11- and Myostatin-deficient mice.

Tissue/Phenotype	**MSTN**	**GDF11**	
Predominant expression pattern	Developing and adult skeletal muscle[1]	Primitive streak and tail bud Expressed in developing limb buds[2, 7, 17, 164]	
	MSTN KO	**GDF11 KO**	**MSTN/GDF11 DKO**
Premature lethality	No[1, 7]	Yes – perinatal[2, 7]	YES – born at expected ratio but none born alive[2]
Bone	NR	Anterior homeotic transformation of the axial skeletal (transformation of posterior vertebrae to anterior identity) via altered HOX gene expression on A/P axis[2]; Increased frequency of cleft palate[7]	More severe homeotic transformations than GDF11KO All have cleft palate; Additional skeletal defects, including limb defects (extra forelimbs, shortened limbs), Digit patterning defects (sixth digit)[7]
Kidney	NR	Most have Renal agenesis[165]	All have renal agenesis[7]
Pancreas	NR	Reduced pancreas size due to exocrine hypoplasia; 2-4 fold increase in endocrine progenitor cells by E18[166] Increased number of islet progenitors[8]	NR
Olfactory epithelium	NR	Increased number of olfactory neurons and neuronal progenitors[10]	NR
Retina	NR	Increased number of retinal ganglion cells and reduced number of retinal amacrine cells and photoreceptors[11]	NR
Skeletal muscle	Myofiber hyperplasia and hypertrophy[1, 112, 167]	None reported (perinatal lethality)	NR (perinatal lethality)
Stomach	NR	Two-fold reduction in the thickness of gastric wall with reduced number of characteristic folds[8]	NR
Fat	Increased BW with decreased lipid content, decreased serum lipid and triglyceride levels[6, 102, 168]	None Reported– but analysis limited due to perinatal lethality	NR (perinatal lethality)
Heart	Increased HW and BW[169]	NR (perinatal lethality)	NR (perinatal lethality)
	Conditional MSTN KO in skeletal myofibers only (in MLC-cre X MSTN-flox)	**Conditional GDF11 KO in skeletal myofibers only (in MLC-cre X GDF11-flox mice)**	**Conditional KO of GDF11 and MSTN in skeletal myofibers only**
Adult skeletal muscle	Two-fold increase in young adult muscle mass (due to hyperplasia and hypertrophy); not apparent at birth (emerges postnatally); more glycolytic fibers (IIB)[7]	No increase in young adult muscle mass; no change in fiber type[7]	Same as MSTN conditional KO; no increase in phenotypic severity[7]
	Conditional MSTN KO in cardiac myocytes only	**Conditional GDF11 KO in cardiac myocytes only**	**Conditional KO of GDF11 and MSTN in cardiac myocytes only**
Cardiac myocytes	Did not prevent left ventricular decompensation after TAC[97]. Cardiac hypertrophy and hearth failure, but cardiac function is restored after several weeks[88, 101]	N/A	N/A

GDF11 and MSTN exhibit many similarities in their biosynthesis, regulation, receptor utilization, and intracellular signaling pathways. Yet, the consequences of loss of *Gdf11* or *MSTN* expression in mice are phenotypically distinct. Comparative analysis suggests only partial functional redundancy. See text for details. NR - not reported