GDF3 simultaneously antagonizes BMP signaling and activates TGFβ receptor signaling

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Abstract

Growth differentiation factor 3 (*Gdf3*) is a relatively understudied member of the Tgfβ superfamily. GDF3 is highly expressed in stem cell populations and restricts cellular differentiation. Gdf3 expression levels decrease after development only to rise in states of obesity and inflammation. However, the function of GDF3 in adult mammalian biology is contentious. To understand the impact of GDF3 on cellular signaling we explored the cellular responses to either recombinant GDF3 or an inducible genetically-encoded *Gdf3* in C2C12 myoblasts. In addition, we employ fluorescent reporters to simultaneously assay both BMP receptor signaling through a SMAD1/5/8 DNA-binding BMP-responsive element and TGFβ receptor signaling through a SMAD3 DNA-binding element. We find that GDF3 is capable of dose-dependent inhibition of multiple BMP proteins including BMP2, BMP7, BMP9, BMP10, and BMP15. We also find that GDF3 produces a *bona fide* activin-like ligand signaling through the TGFBR2 receptor. Expression profiling with RNA-seq reveals that BMP2-regulated genes are attenuated by the addition of GDF3. Together these results clarify the dual biological roles of Gdf3 in cultured myoblasts.

Introduction

The incidence of obesity and Type 2 diabetes in the United States are at record high levels. The thiazolidinedione (TZDs) class of drugs are powerful insulin sensitizers. TZDs are ligands of the nuclear hormone receptor PPARγ that potently improve insulin sensitivity but their use is limited by significant side effects such as weight gain and increased risk of bone fractures. We recently demonstrated a mechanism wherein the insulin-sensitizing effects of TZDs were achieved in mice without TZD-associated adverse effects. Mice encoding a mutated version of PPARγ that cannot be phosphorylated at the 273rd amino acid (Serine S273) are protected from insulin resistance without exhibiting differences in body weight. This insulin-sensitizing effect was associated with decreased expression of the gene Growth differentiation factor 3 (*Gdf3*). Exogenously increasing GDF3 expression was sufficient to make even lean mice moderately insulin resistant. Together, these findings suggest that increased GDF3 levels may contribute to obesity-induced insulin resistance following PPARγ S273 phosphorylation (Hall et al. Cell Met 2020). However, much is unknown about GDF3, including whether it has clinical relevance for the treatment of type 2 diabetes, obesity, or other indications. In addition, significant aspects of the biology of GDF3 are either unknown or disputed.

While there is strong evidence that Gdf3 is a marker of pluripotent cells and carcinomas, Gdf3's function in differentiated cells is less clear (*1, 2*). Like many TGFβ superfamily proteins, Gdf3's function may be highly cell type and context-dependent. Conflicting research exists in the field about how GDF3 functions – some studies suggest that GDF3 inhibits BMP signals while others suggest that GDF3 activates TGFβ signaling (*3-9*). Both BMP and TGFβ signaling are complex pathways involved in the development and function of multiple tissue systems. Resolving the ambiguity regarding GDF3 function is also critical to realizing its clinical potential in regulating metabolic diseases such as diabetes and obesity.

To study the molecular function of GDF3 in BMP and TGFβ signaling, we created tools to simultaneously interrogate BMP and TGFβ signaling. We show here that using dual fluorescent reporters, that GDF3 can simultaneously inhibit BMP signaling and activate TGFβ signaling. We also show that GDF3 inhibits BMP signaling only in the presence of its endogenous ligands (BMPs) and activation of TGFβ signaling requires TGFβ receptor 2 (TGFBR2). These studies suggest that GDF3 has dual roles to antagonize BMP signaling and to promote activin-like signaling.

Results

Generation of cells expressing a dual fluorescence reporter for simultaneous BMP (BRE) and TGFβ (SBE) signaling.

GDF3 is a member of the TGFβ superfamily. Proteins in this family are translated into full-length pre-proteins that undergo enzymatic cleavage to produce mature peptides (*10*). The mature GDF3 polypeptide lacks a highly conserved cysteine found in nearly all BMPs/GDFs and an additional two cysteines found in TGFβs and Activins (*11, 12*). Phylogenetic analysis of the 114 amino acid mature human GDF3 protein aligned to mature polypeptides of the TGFβ superfamily shows closest amino acid identity to GDF1 at 48% (*11*). The GDF3/GDF1 phylogenetic branch is mapped marginally closer to the mature TGFβ, Activin, GDF15, GDF11, and Myostatin/GDF8 protein branches. The mature GDF3 protein clustered marginally more distantly from BMPs and other GDF proteins (Fig 1A).

BMP signaling promotes phosphorylation of SMAD1, SMAD5, and/or SMAD8. Similarly, TGFβ & Activin signaling promote the phosphorylation of SMAD2, and/or SMAD3. Immunoblotting for protein phosphorylation has served as the backbone for understanding ligand and receptor signaling (*13*). Luminescence reporter assays for BMP signaling have used the Id1 promoter's

BMP-Responsive Element (BRE)(*14*). Likewise, TGFβ signaling has used luminescence driven by the SMAD3-Binding Element (SBE or $CAGA_{12}$) (*15*). The Elowitz group at Caltech recently created a flow cytometry-compatible BMP reporter with the BRE driving Citrine, a yellow fluorescent protein (*16-18*). This has further increased throughput and allowed for a systems level approach to BMP signaling not previously possible. We have extended these studies to generate a red fluorescent TGFβ signaling reporter driven by the SBE (referred to as SBE-RFP henceforth). C2C12 myoblasts were transfected with both the BMP signaling reporter construct (BRE-YFP) and the TGFβ reporter construct (SBE-RFP) (Fig 1B) to generate C2C12 myoblasts stably expressing both constructs (dual reporter cells). To prevent cellular differentiation, our initial experiments were performed in complete C2C12 media containing 20% fetal bovine serum (FBS). We find, as previously published, recombinant BMP2 potently activates the BRE-YFP reporter in a dose-dependent manner (Fig 1C). Similarly, we demonstrate that TGFβ1 potently activates the SBE-RFP reporter in a dose-dependent manner (Fig 1D). While BMP2 has no effect on the SBE-RFP reporter at any dose tested, all the tested doses of TGFβ1 inhibit the BRE-YFP reporter by ~30%. We conclude that the dual reporter system allows us to examine the effect of TGFβ superfamily ligands on both BMP and TGFβ reporters simultaneously.

GDF3 simultaneously inhibits BMP signaling and activates TGFβ signaling in a dose dependent manner.

We next sought to understand the effects of GDF3 in this system. Dual reporter cells were incubated with increasing doses of recombinant mouse GDF3 in complete media. We observe recombinant GDF3 can inhibit the BRE-YFP reporter in a dose-dependent manner (Fig. 1C). GDF3, like TGFβ1, activates the SBE-RFP reporter but requires higher concentrations than TGFβ1; 1000 ng/mL of GDF3 has a similar effect as 1.3 ng/mL of TGFβ1 (Fig. 1D). Considering

that FBS contains a mixture of BMP and TGFβ ligands which could impact signaling, we next decided to test the effects of our recombinant proteins under serum-free conditions.

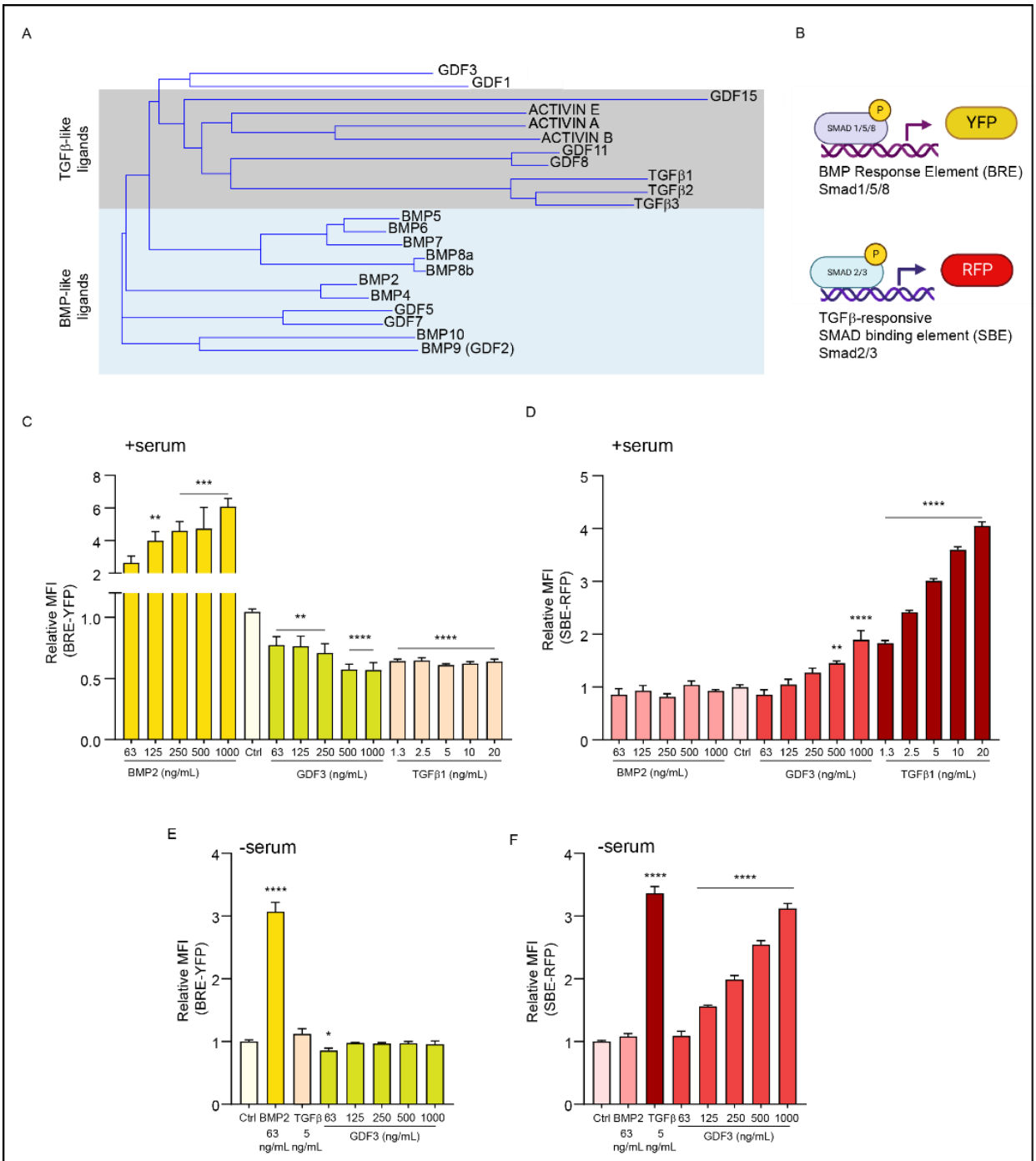

Figure 1: GDF3 simultaneously inhibits BMP signaling and activates TGFβ signaling.
A) Phylogenetic analysis following multiple sequence alignment of mature human GDF3 protein and mature polypeptides of members of the TGFβ superfamily. B) Schematic representations of the BMP responsive element (BRE) driving yellow fluorescent protein (YFP) and a TGFβ responsive Smad binding element (SBE) driving red fluorescent protein (RFP). C-G) C2C12 myoblasts stably expressing both the BRE-YFP and SBE-RFP reporters (dual reporter cells). C) Relative Mean Fluorescence Intensity (MFI) of YFP in dual reporter cells incubated with increasing doses of recombinant BMP2, GDF3, and TGFβ1 in complete media containing 20% fetal bovine serum for 24 hours. D) Relative MFI of RFP in the same cells as C. E) Relative MFI of YFP in reporter cells incubated with recombinant proteins in serum-free media for 24 hours F) Relative MFI of RFP in the same cells as E. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to untreated control (Ctrl).

To do this, the C2C12 reporter cells were incubated in serum-free medium for 4 hours, followed by treatment with recombinant proteins. Under serum-free conditions, recombinant GDF3 had a minimal effect on the BRE-YFP reporter. In contrast, the strong reporter activation of BMP2 was unchanged in the absence of FBS (Fig. 1E). Unlike the effects seen for the BRE-YFP reporter, recombinant GDF3 activated the SBE-RFP reporter more strongly in serum-free conditions (Fig 1F). TGFβ1 also potently activated the SBE-RFP reporter (Fig 1F). These data suggest that Gdf3 can autonomously activate TGFβ signaling. However, Gdf3 requires a serum-derived factor to affect BMP signaling.

GDF3 inhibits multiple BMP signaling ligands

Considering the different effects observed in the presence or absence of serum, we hypothesized that GDF3 may inhibit BMP signaling only in the presence of BMP ligands. We therefore tested the effect of recombinant GDF3 on isolated recombinant BMP2, BMP7, BMP9, BMP10 or BMP15 in serum-free conditions. Indeed, while GDF3 alone has no effect on the BRE-YFP reporter, GDF3 inhibits all BMPs tested in a dose dependent manner (Fig. 2A). These findings suggest that GDF3 is a broad inhibitor of BMP signaling. In addition, while GDF3 can autonomously activate TGFβ reporter activity, GDF3 requires a serum-derived factor to affect BMP signaling.

GDF3 has additive effects with Tgfβ signaling ligands.

We also investigated how GDF3 would interact with TGFβ-like signaling ligands. To do so, we added recombinant GDF3 with either recombinant TGFβ1 or Activin A in serum-free conditions. We find that GDF3 has additive effects on TGFβ reporter activity with both TGFβ1 (Fig. 2B) and Activin A (Fig. 2C). GDF3 in combination with TGFβ1 or Activin A leads to significantly higher SBE-RFP reporter activity than either of the ligands in isolation (Fig 2B and 2C). These results

suggest that GDF3 acts as a TGFβ signaling ligand in the absence of serum or any other ligands. However, these results do not address whether GDF3 is signaling through the same receptors as other Tgfβ family ligands.

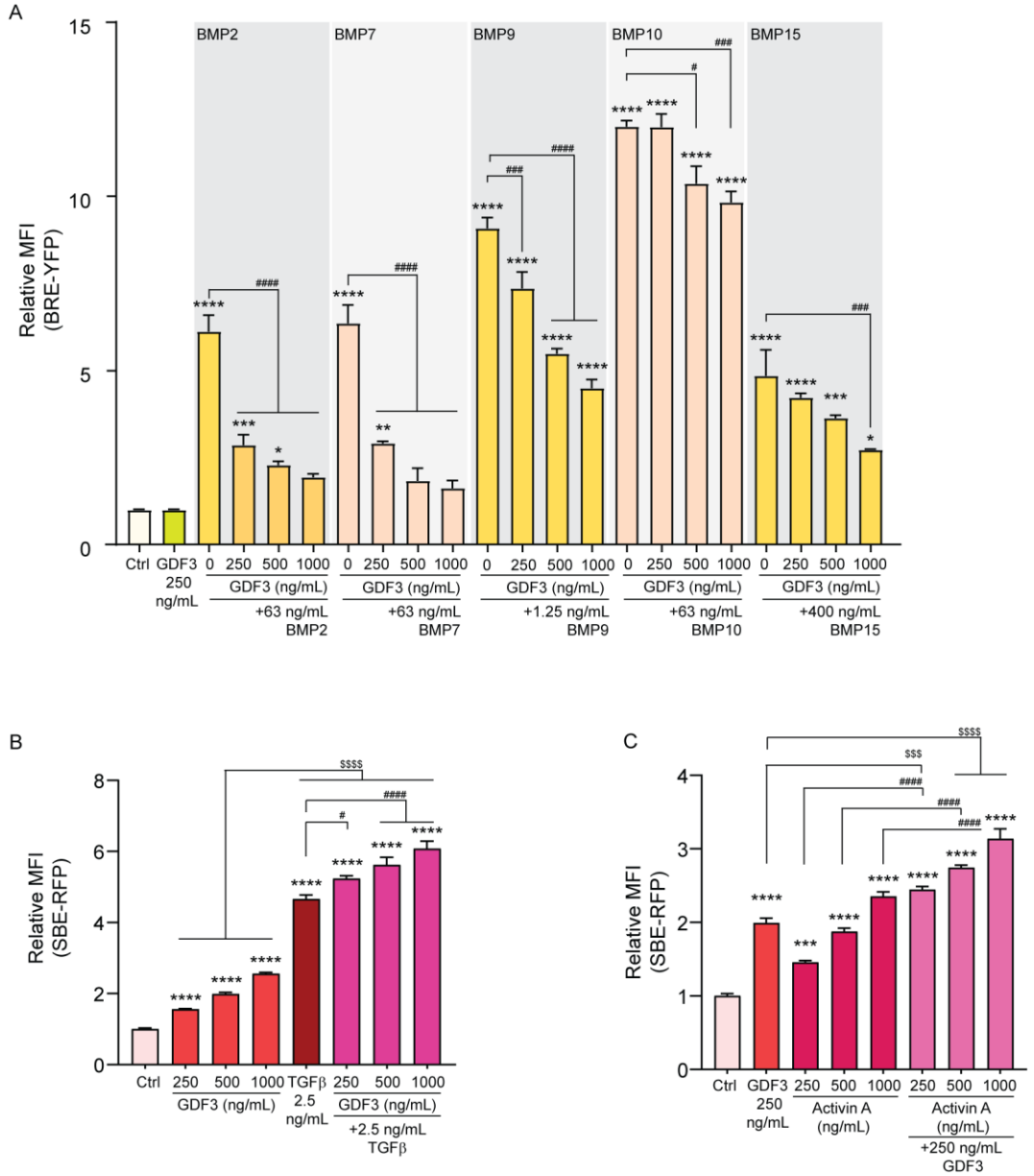

Figure 2: GDF3 inhibits BMP signaling ligands and has additive effects with TGFβ signaling ligands. A-C) C2C12 dual reporter cells incubated with recombinant proteins in serum free media for 24 hours. A) Relative MFI of YFP in cells incubated with increasing doses of recombinant GDF3 and/or recombinant BMP2, BMP7, BMP9, BMP10 or BMP15. B) Relative MFI of RFP in cells incubated with recombinant GDF3 and/or TGFβ1. C) Relative MFI of RFP in cells incubated with recombinant GDF3 and/or Activin A. */ $ /#p<0.05, **/$$/##p<0.01, ***/$$$/###p<0.001, ****/$$$$/#### p<0.0001 when compared to control (*) or GDF3 alone ($) or the BMP or TGFβ signaling ligand (#).

GDF3 inhibits downstream gene expression changes seen in response to BMP2 and shows similar gene expression changes as TGFβ1.

We next sought to validate the effects of recombinant GDF3 on endogenous gene expression by performing RNA-sequencing experiments. Recombinant Gdf3 increased expression of 92 genes (G+) and decreased expression of 126 genes (G-). While TGFβ1 had more pronounced effects to increase expression of more than 1000 genes (T+) and to repress the expression of greater than 1300 genes (T-) beyond the significance threshold. Approximately 44% of GDF3 regulated genes were also significantly co-regulated by TGFβ1 (Fig 3A). Of note, GDF3 at 250 ng/ml had effects smaller in magnitude than that of 2.5 ng/ml TGFβ1 (Fig 3A). This weaker effect of Gdf3 on endogenous gene expression compared to TGFβ1 is consistent with results from our reporter assay experiments (Fig 1F).

Dual reporter cells were also incubated with serum free media alone (Control), with recombinant BMP2, or BMP2 together with GDF3. BMP2 is a potent regulator of transcriptional activity with more than 580 genes significantly upregulated (B+). We find that addition of recombinant GDF3 significantly limited the expression of 47 genes activated by BMP2 (BG+). Similarly, of the more than 570 genes repressed by BMP2 (B-), the decreased expression of 66 genes was blocked with the addition of GDF3 (BG-) (Fig 3B). GDF3 limits the regulation of approximately 10% of the genes controlled by BMP2, suggesting that GDF3 can act as a limited antagonist of BMP2 activity on endogenous gene expression. In our experiments, cells treated with GDF3 had strong downregulation of *Ranbp3l*, a gene implicated in SMAD signaling (Fig 3C). Notable other downregulated genes include *Pik3c2b, Id3, Omd,* and *Peg10*. Among strongly upregulated genes are *Ctla2a*, *Heyl*, and *Vegfc*.

The transcriptional profile of cells treated with GDF3 is consistent with inhibition of muscle cell differentiation. That is, the genes downregulated in GDF3-treated cells overlap with genes upregulated during the differentiation of mouse primary myoblasts, mouse C2C12 myoblasts, or

human myoblasts into myotubes (*19, 20*). Conversely, genes upregulated following GDF3 treatment overlap with the genes that are inhibited during muscle cell differentiation (Fig 3D) (*21*). These results indicate that GDF3's effects reflect changes to the differentiation of C2C12 myoblasts to myotubes.

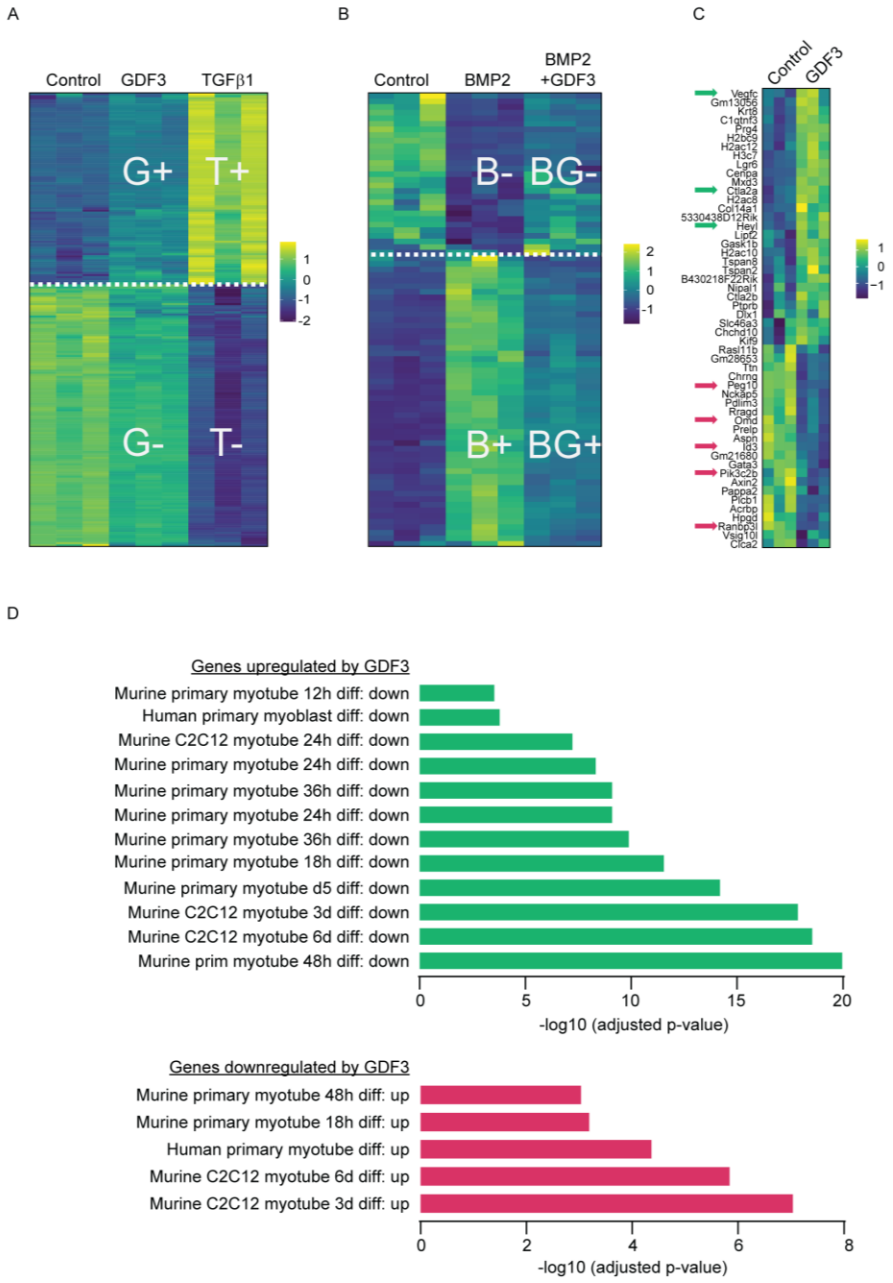

Figure 3: Transcriptional signature of Gdf3, BMP2, and TGFβ1 in C2C12 myoblasts
A) Heat map of RNA sequencing data from C2C12 dual reporter cells incubated in serum free media (Control), with recombinant GDF3, or TGFβ1 in serum free media for 24 hours. B) Heat map of RNA sequencing data from Control cells, cells incubated with recombinant BMP2, or BMP2 and GDF3 in serum free media for 24 hours. C) Heat map for gene expression changes in cells from (A) treated with recombinant GDF3 versus Control. D) SysMyo pathway analysis of RNA sequencing data from GDF3-regulated genes.

GDF3 signaling through ALK5, TGFBR2, and betaglycan

We next sought to understand which receptors transduce GDF3 signals to mediate transcriptional activity and regulation of BRE and SBE reporters. We queried expression levels of BMP and TGFβ receptors and co-receptors in our bulk C2C12 RNA-seq analysis (Fig 4A). The Type I BMP receptors *Alk3* and *Alk2* are highly expressed, and we observe lower levels of *Alk6* mRNA. The Type I TGFβ receptors *Alk5* and *Alk4* are expressed. Surprisingly, *Alk7* is not expressed in these cells, although *Alk7* is frequently annotated as the Gdf3 receptor (*4*). High levels of three Type II receptors are observed, *Tgfbr2*, *Bmpr2*, and *Acvr2a*. Several co-receptors are also expressed in these cells, including *Tgfbr3* (*betaglycan)*. In order to determine which of these receptors transduce GDF3 signals, we employed recombinant soluble Fc-chimeras of receptor proteins as ligand traps (*22*). We predict that ligand traps that bind to and neutralize GDF3 would alter reporter activity. We first investigated whether GDF3's induction of the SBE-RFP reporter could be blocked by ligand traps of the canonical TGFβ receptors TGFBR1 (ALK5), TGFBR2 or the TGFβ co-receptor TGFBR3 (Betaglycan) (Fig 4B). We also tested the effects of the soluble form of the receptor ALK7 (Fig 4C). The Fc-chimeras of ALK5 or ALK7 had no effect on GDF3's induction of the SBE-RFP reporter (Fig 4B-C). The Fc-TGFBR3 was able to block GDF3's effect to a small extent at the highest concentrations tested (Fig 4B). However, the Fc-TGFBR2 was able to completely block the effect of recombinant GDF3 on the SBE-RFP reporter even at the lowest concentration tested (Fig 4B). Our results indicate that GDF3 signaling is diminished by Fc-TGFBR2. To further validate this finding, we created dual reporter cells lacking *Alk5*, *Tgfbr2* or *Tgfbr3* with CRISPR/Cas9 where the mRNA levels of either Alk5, Tgfbr2 or Tgfbr3 were reduced by atleast ~50% compared to controls (Fig S1A). Under serum-free conditions, recombinant GDF3 (Fig 4E) increased SBE-RFP reporter activity in control cells expressing a scrambled sgRNA. In cells lacking *Alk5*, *Tgfbr2* or *Tgfbr3,* GDF3 or TGFβ1-induced SBE-RFP reporter

activity was blocked by up to 70% (Fig 4D and Fig S1B). Together these findings suggest that GDF3 activates TGFβ signaling via Alk5, TGFBR2 and the co-receptor betaglycan.

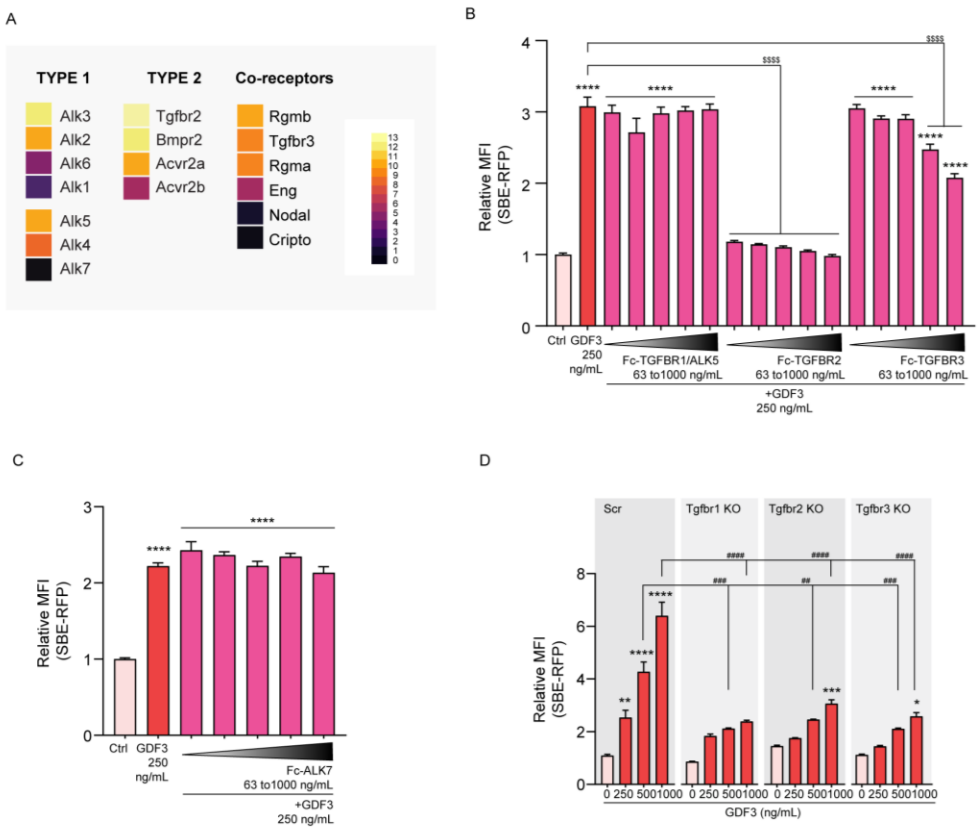

Figure 4: GDF3 activates Tgfβ signaling via TGFBR2.
A) Relative mRNA expression levels of BMP and TGFβ signaling Type1, Type 2 receptors and co-receptors in C2C12 dual reporter cells. B-D) C2C12 dual reporter cells incubated with recombinant proteins in serum free media for 24 hours. B) Relative MFI of RFP in cells incubated with recombinant Fc chimeras of the receptors TGFBR1/ ALK5, TGFBR2 and TGFBR3 and/or recombinant GDF3. C) Relative MFI of RFP in cells incubated with recombinant Fc-ALK7 and/or recombinant GDF3. D) Relative MFI of RFP in control (Scr) cells or in Tgfbr1, Tgfbr2 or Tgfbr3 knock out (KO) cells in response to recombinant GDF3. */$/#p<0.05, **/$$/##p<0.01, ***/$$$/###p<0.001, ****/$$$$/####p<0.0001 when compared to untreated control or Scr (*) or GDF3 alone ($) or between KO and Scr for the same dose of GDF3 (#).

Genetically encoded GDF3 induction has similar effects to recombinant GDF3

Considering the similarity between effects observed with recombinant GDF3 and TGFβ1 and also noting previous concerns about TGFβ contamination in purified recombinant proteins (Olsen et al. Plos One, 2017), we wanted to ensure that our effects were not due to a contamination of a TGFβ protein in our recombinant GDF3 preparations. We designed vectors using the Tet-On inducible gene expression system such that we could induce the expression of blue fluorescent protein (BFP) alone (Control) or the expression of GDF3 and BFP in the presence of the

tetracycline analog doxycycline. We transfected our dual reporter C2C12 myoblasts with either the control or GDF3 encoding vectors and treated these cells with increasing doses of doxycycline. When the cells transfected with the Gdf3-encoding vector were treated with increasing doses of doxycycline under serum-free conditions, we saw a significant increase in Gdf3 mRNA expression (Fig 5A). Gdf3 mRNA remained undetectable in cells expressing the control vector at any dose of doxycycline tested (data not shown). To determine if the gene expression changes seen with recombinant GDF3 hold true with genetically encoded Gdf3, we analyzed the expression levels of target genes regulated by recombinant GDF3. The expression levels of all the genes tested were regulated in the presence of increasing doses of doxycycline, or alternately in the presence of increasing doses of recombinant GDF3 (Fig 5B). Of these, *Id3*, *Ranbp3l*, *Pik3c2b*, *Peg10,* and *Omd* all showed significantly lower gene expression levels in response to doxycycline or recombinant GDF3, while *Vegfc*, *Ctla2a* and *Heyl* showed increased gene expression levels in response to doxycycline or recombinant GDF3 (Fig 5B). We next tested if the induction of genetically encoded Gdf3 could activate our fluorescent reporters. Induction of genetically encoded Gdf3 in serum free conditions increased the activity of the SBE-RFP reporter above 50 ng/mL of doxycycline (Fig 5C), consistent with our previous results with recombinant GDF3 (Fig 1F). Doxycycline induction of *Gdf3* showed no changes in BRE-YFP reporter activity (Fig S1C), consistent with results from recombinant proteins (Fig 1E). However, induction of *Gdf3* with doxycycline was able to attenuate the recombinant BMP2 dependent increase of BRE-YFP reporter activity (Fig 5D). These results are consistent with our reporter assays using recombinant GDF3 and BMP2 (Fig 2A). Doxycycline induction of BFP in cells transfected with the control vector had no effect on either the SBE-RFP or BRE-YFP reporter activities (Fig S1D and S1E). These experiments find similar regulation of endogenous gene expression and reporter gene regulation by recombinant GDF3 and a genetically encoded *Gdf3*, assuaging concerns that the effects of recombinant GDF3 could be an effect of trace amounts of contaminating TGFβ while increasing confidence that Gdf3 does activate TGFβ signaling.

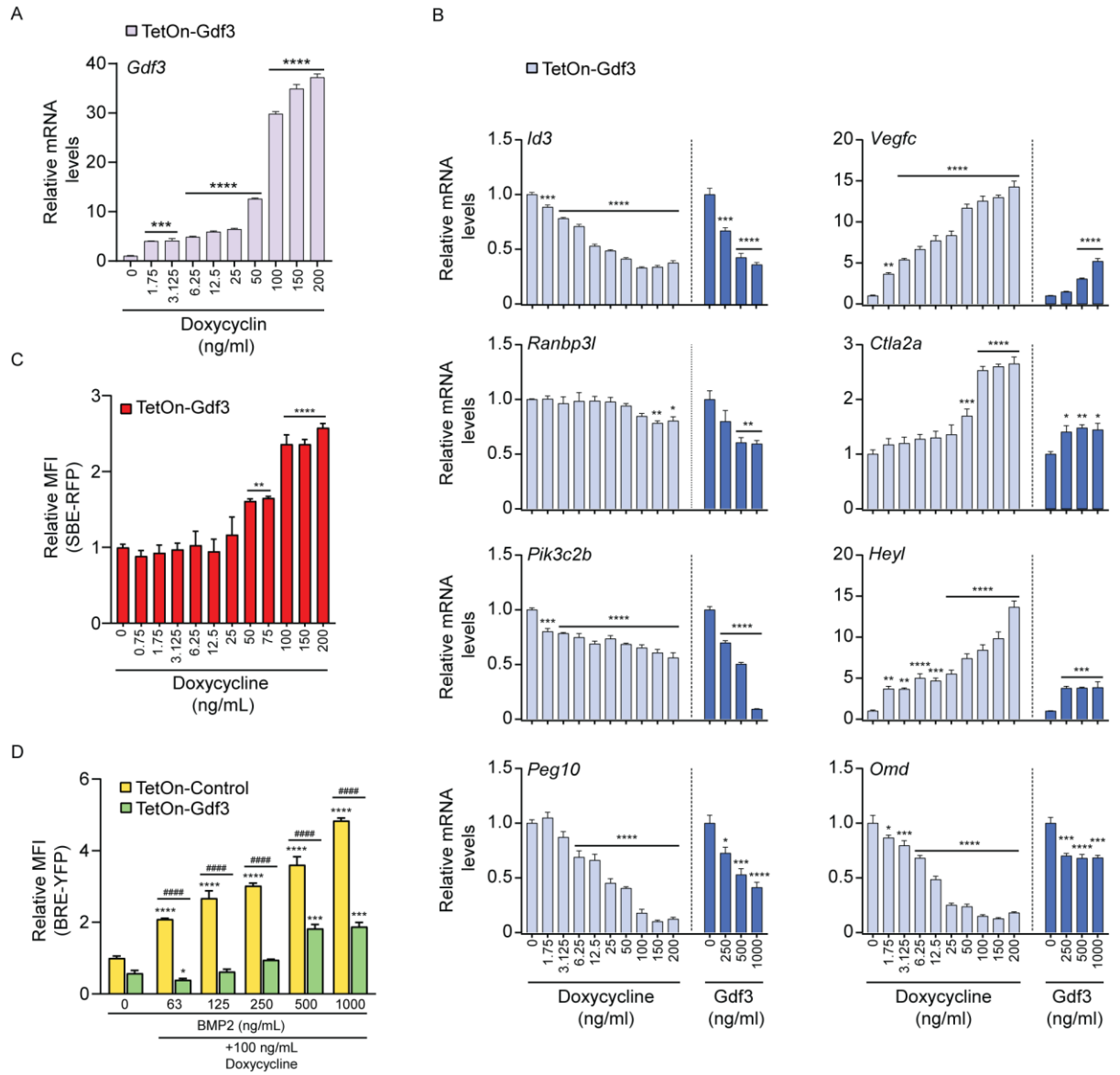

Figure 5: Induction of genetically encoded GDF3 expression has dose dependent effects on BMP and TGFβ signaling
(A-C) C2C12 dual reporter cells were transiently transfected with a plasmid encoding doxycycline inducible mouse Gdf3 and blue fluorescent protein (TetOn-Gdf3) followed by doxycycline treatment in serum free media for 48 hours or incubated with recombiant GDF3. All cells were either harvested for RNA isolation (A, B) or cells expressing YFP, RFP and BFP were sorted via flow cytometry and analyzed for changes in reporter activity (C). A) Relative mRNA fold change of Gdf3 in response to increasing doses of Doxycycline. B) Relative mRNA fold change of Id3, Vegfc, Ranbpl3, Ctla2a, Pi3kcb, Hey1, Peg10 and Omd in response to Doxycycline or recombinant Gdf3. C) Relative MFI of RFP in response to increasing doses of Doxycycline for 48 hours in serum free media. D) C2C12 dual reporter cells were transiently transfected with a plasmid encoding doxycycline inducible BFP (TetOn-Control) or the plasmid TetOn-Gdf3, followed by doxycycline treatment in serum free media for 48 hours and incubations with recombinant BMP2 for 24 hours. Cells expressing YFP, RFP and BFP were sorted via flow cytometry and analyzed for relative MFI of YFP. */#p<0.05, **/##p<0.01, ***/###p<0.001, ****/####p<0.0001 compared to untreated control (*) or compared to the corresponding control incubated with the same dose of recombinant BMP2 (#).

DISCUSSION

Our understanding of the molecular roles occupied by GDF3 have evolved beyond the unilateral control of either BMP or TGFβ signaling into that of a dual regulator (Fig 6). Gdf3's role as a BMP inhibitor was demonstrated in human ES cells, frog embryos, and mouse gene-trap models (*8*). These studies illustrated that both recombinant GDF3 and transfected Gdf3 cDNA can inhibit BMP reporter activity. Contemporaneously, others found that GDF3 acted as a Nodal-like ligand activating the Smad 2/3 pathway similarly to TGFβ in the pre-gastrulation mouse embryo (*6*). These findings led the authors to the "intriguing possibility that GDF-3 acts as a bi-functional protein" with the caveat that endogenous levels of GDF3 inhibit BMP signaling but extremely high, non-physiological levels of GDF3 behave as a TGFβ signaling activator (*9, 12*). Our results support the assertion that Gdf3 is a bi-functional protein. However accurate estimation of endogenous levels of Gdf3 are unknown due to the challenges of antibody based detection systems within the TGFβ-superfamily (*23*). Regardless, we find that Gdf3 has similar potency to recombinant Activin A in our reporter system (Fig 2C). We also demonstrate that the same concentrations of recombinant GDF3 are sufficient to both inhibit BMP signaling and activate TGFβ signaling reporters (Fig 1). These conclusions may help to unify the seemingly incompatible beliefs about the true roles of GDF3 (*3-9*).

In adult mice, *Gdf3* levels are upregulated more than 30-fold in adipose tissue during diet-induced obesity. Much of this expression is derived from adipose tissue macrophages (*7*). We previously showed that *Gdf3* mRNA levels increase with obesity-associated phosphorylation of Serine 273 of the nuclear hormone receptor PPARγ. Insulin sensitizing treatments that block PPARγ S273 phosphorylation also decreased Gdf3 levels. The observed insulin sensitivity was mainly due to an increase in muscle glucose uptake (*7*).

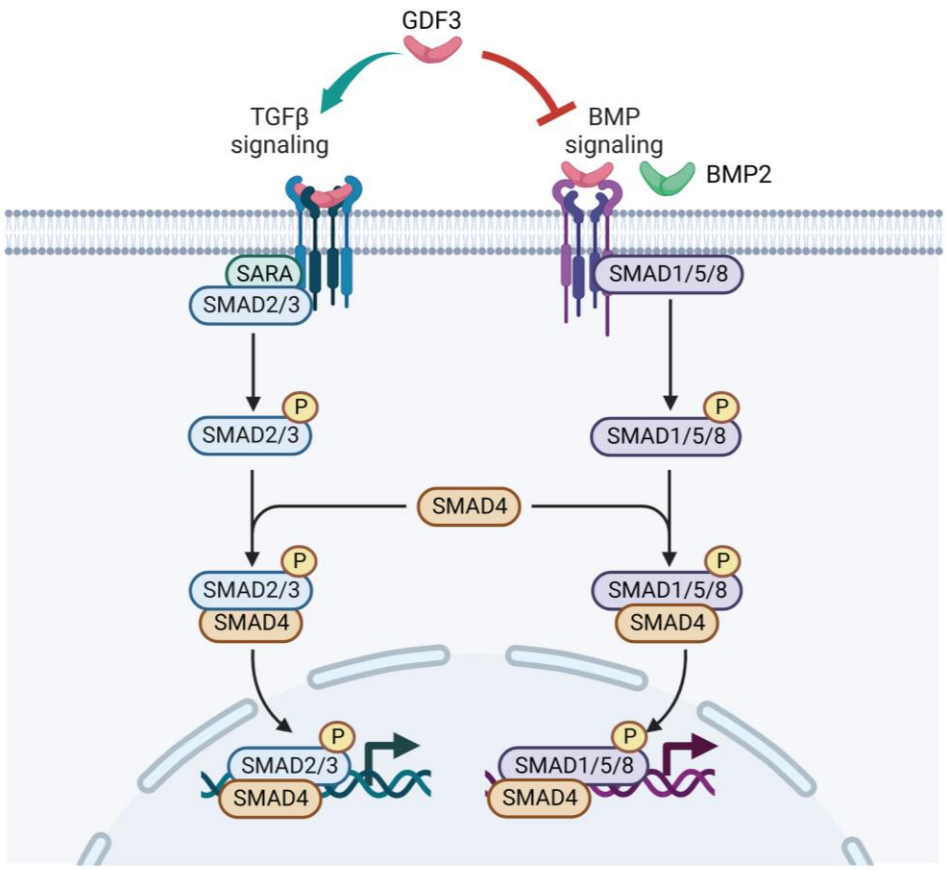

Figure 6: Schematic representation of the role of GDF3 in BMP and Tgfβ signaling
GDF3, a member of the Tgfβ superfamily, acts activates Tgfβ signaling through ALK5, TGFBR2 and Betaglycan receptors. GDF3 potentially antagonizes BMP signaling by binding to BMP receptors. (Made using Biorender.com)

Similarly, GDF3 secreted by macrophages infiltrating skeletal muscle after injury can promote skeletal muscle repair and regeneration (*24-29*). The molecular mechanism of how GDF3 affects insulin resistance, muscle glucose uptake, muscle regeneration, and myoblast fusion are still unclear. Considering these phenotypes, we chose to use the myoblast cell line C2C12 for our reporter assays together with recombinant GDF3 to recapitulate how GDF3 secreted from macrophages might affect signaling in muscle cells. In the presence of BMP2, C2C12 cells undergo osteogenic differentiation, while under low serum conditions, C2C12s differentiate into myotubes (*30*). We believe that downstream effectors of both BMP and TGFβ receptor signaling may play crucial roles in the phenotypic effects caused by changing GDF3 expression.

Our studies provide further evidence that GDF3 can broadly inhibit BMP activity. We observe that GDF3 both decreases BMP-stimulated BRE reporter activation and diminishes mRNA expression changes of endogenous BMP2-stimulated genes. These effects are observed both with recombinant mature GDF3 and with the expression of a full-length wild type *Gdf3* cDNA. However, GDF3's inhibition of BMP reporter activity requires either the presence of a BMP ligand or a factor present in fetal bovine serum. These findings are consistent with previous studies where GDF3 could co-immunoprecitipate BMP4, suggesting that GDF3 directly interacts with BMP proteins and prevents the formation of BMP homodimers (*8*). In our experiments, GDF3 was capable of inhibiting reporter activity of all BMPs tested: BMP2, BMP7, BMP9, BMP10, and BMP15. These findings raise the intruiging possibility that GDF3 may block BMP signaling through binding to a shared receptor, such as the BMPR2 or ACVR2A. Given the disparate effects of these signaling molecules in varying tissues, understanding the consequences of obesity-induced GDF3-mediated BMP inhibition and receptor complexes will require further investigation *in vivo*.

In skeletal muscle, myostatin, activins and TGFβ-like signals can inhibit myoblast proliferation and differentiation (*31*). We find that GDF3 shares this biology. We find that GDF3 is sufficient to activate TGFβ-like reporter activity and expression of TGFβ-induced genes. Our experiments suggest that GDF3's SBE reporter activation is dependent on the receptors ALK5, TGFBR2, and betaglycan. The divergent effects of soluble Fc-chimeras and CRISPR/Cas9 mediated gene deletion to diminish GDF3 reporter activity may be caused by the differential affinity for these receptors to GDF3. Hyperactivity of TGFβ signaling is seen in mouse models of Marfan syndrome. Similarly, TGFBR2/SMAD2 signaling defects are associated with increased rates of cardiovascular disorders (*32-35*). Our findings raise the possibility that GDF3 signaling may contribute to cardiometabolic disorders observed in obesity.

RNA sequencing analysis of cells treated with BMP2 together with GDF3 did indeed demonstrate reduced BMP2-induced gene expression of a subset of transcripts compared to BMP2 alone. Our

dataset also showed that GDF3 activates or inhibits similar gene sets as TGFβ1 in the TGFβ signaling pathway but at a weaker level compared to TGFβ1. These endogenous gene expression changes reiterate the findings from our dual fluorescent reporter assays. Moreover, GDF3-regulated transcripts suggest an inhibition of cellular differentiation from myoblasts to myotubes (Fig 3D) (*36*). Among the genes downregulated in the presence of GDF3 were RAN binding protein 3 like (*Ranbp3l)*, Inhibitor of DNA binding 3 (*Id3)*, Osteomodulin (*Omd)*, and Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta (*Pik3c2b)*. *Ranbp3l* has been shown to regulate the nuclear export of Smad1/5/8 in bone marrow derived mesenchymal stem cells. Reduced expression of *Ranbp3l* enhances BMP dependent transcriptional activity and stem cell differentiation whereas increased *Ranbp3l* expression blocks BMP induced osteogenesis of mesenchymal stem cells and promotes myogenic differentiation of C2C12 myoblasts (*37*). *Id3* is a canonical BMP-target gene. *Omd* expression is increased by BMP2 signaling and contributes to osteogenesis (*38*). *Pik3c2b* is a regulator of intracellular trafficking and insulin signaling (*39*). Cytotoxic T lymphocyte-associated protein 2 alpha (*Ctla2a*), Hes related family bHLH transcription factor with YRPW motif like (*Heyl*), and Vascular endothelial growth factor C (*Vegfc*) were among the upregulated genes following GDF3 treatment of myoblasts. *Ctla2a* is a secreted protein that inhibits proliferation and angiogenesis (*40*). *Heyl* is a component of the Notch pathway capable of binding SMAD3 and inhibiting TGFβ signaling (*41*). *Vegfc* is a secreted factor with pleotropic roles in developmental and adult physiology (*42*).

In adult animals, *Gdf3* levels increase with obesity or inflammation. However, further in-depth study of GDF3 biology will be required to help delineate how GDF3's simultaneous antagonism of BMP ligands and activation of Tgfβ receptor signaling leads to various phenotypic effects in mammalian physiology.

Materials and methods

Plasmid vectors

The pHK3-BRE-Citrine (BRE-YFP) fluorescent reporter was a gift from Michael B. Elowitz's lab (Antebi et al. Cell, 2017). To generate the pHK3-CAGA12-mCherry (SBE-RFP), the BRE-Citrine was replaced with CAGA12-mCherry using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs, E5520S). For ease of readability these reporters are referred to as BRE-YFP and SBE-RFP in the manuscript.

The pTetON-TRE-TagBFP2 and pTetON-TRE-mGdf3-P2A-TagBFP2 plasmids were synthesized with codon optimization of mouse Gdf3 cDNA by Vector Builder Inc. GDF3 and/or TagBFP2 (referred to as BFP in the manuscript) expression were induced using the indicated concentrations of doxycycline for the indicated time points.

All transfections were carried out using Lipofectamine 3000 (ThermoFisher Scientific) following the manufacturer's protocol.

Generation of dual reporter cells

C2C12 myoblasts (ECACC, 91031101, C3H female mouse in origin) were transfected with the BRE-YFP reporter plasmid and the piggyback transposase (p-base plasmid) designed to be stably integrated using the piggybac transposon system (*43*). Stable cells were generated by selection with 800µg/mL of Hygromycin B (Sigma-Aldrich, H7772). The cells were again transfected with the SBE-RFP reporter with the piggyback transposase and selected for stable integration of both reporters by three rounds of fluorescence-assisted cell sorting for YFP and RFP double positive cells (dual reporter cells).

Flow cytometry

C2C12 dual reporter cells were plated in a 96 well cell culture plate to reach 50 to 70% confluency the next day in complete medium (DMEM high glucose with 20% heat inactivated fetal bovine serum (iFBS) and penicillin and streptomycin. Cells were then incubated with the specified concentrations of recombinant proteins in complete medium. If cells needed to be serum starved, the medium was replaced with serum starvation medium (DMEM high glucose with 0.2% fatty acid free bovine serum albumin and penicillin and streptomycin) for 4 to 6 hours the day after plating followed by incubation with recombinant proteins in serum-free media for 24 hours.

For the induction of genetically encoded GDF3 and or control TagBFP2, dual reporter cells were transiently transfected with either the pTetON-TRE-TagBFP2 or the pTetON-TRE-mGdf3-P2A-TagBFP2 plasmids in 10 cm cell culture dishes. After an overnight incubation in complete medium, these cells were split into a 96 well cell culture plate. After another overnight incubation in complete medium, these cells were serum starved for 4 hours and then treated with doxycycline in serum-free media for the indicated time points. The cells were then processed for flow cytometry.

Briefly, the medium from the 96 well plate was removed and cells were washed with PBS. Cells were then dissociated with 0.25% Trypsin EDTA in a cell culture incubator for approximately 5 minutes. Trypsin was neutralized with PBS containing 3% iFBS and followed by spinning the plate in a refrigerated centrifuge at 500g for 10 minutes with a swinging rotor. The supernatant was dumped and FACS buffer (PBS + 0.3% BSA) containing 1:10000 LIVE/DEAD Fixable Far Red Dead Cell Stain (Thermo Fisher Scientific, #L34973) was added to the cells for 15 minutes on ice. The plate was then centrifuged again, the medium was removed and the plate was washed with FACS buffer and centrifuged again. The supernatant was removed again and each well was incubated with 200uL of FACS buffer. These cells were then analyzed using the CytoFLEX flow Cytometer (Beckman Coulter) and the CytExpert acquisition and analysis software. Briefly, single

unstained C2C12 cells and single fluorescent controls were used to set the gains and gates for each fluorescent channel. Cells were gated to analyze 1000 live single cells from each well. The mean fluorescence intensity (MFI) of either YFP or RFP of the live cells from each well was used for the subsequent analysis. For the experiments with doxycycline induced BFP or GDF3 and BFP expression, the MFI of YFP or RFP was measured from cells expressing YFP, RFP and BFP.

Generation of TGFβ signaling receptor knock out cell lines

sgRNAs targeting each TGFβ receptor were obtained from ThermoFisher Scientific with BbsI overhangs and cloned into the Cas9 expressing vector pX459 (Addgene) based on the protocol from the Feng Zhang lab (*44*). Briefly, the px459 vector was digested with BbsI and dephosphorylated and purified. The sense and antisense oligos for each target were annealed and phosphorylated. The oligos were then ligated with the linearized dephosphorylated pX459 vector and transformed into competent bacterial cells and plated onto LB agar plates with ampicillin. Multiple colonies were screened for each sgRNA clone and the plasmid sequence was verified by PCR and/or sequencing. One positive clone for each target was maxi prepped and used to generate the stable knock out cells. The sequences of the sense oligos used to generate these knock out cells are listed in Table S1.

Dual reporter cells were transfected with the sgRNA targeting *Tgfbr1*, *Tgfbr2*, or *Tgfbr3* and selected for stable integration using Puromycin (2µg/mL). The stable cells were validated by RTqPCR of the target gene compared to stable cells transfected with a non-specific scrambled sgRNA.

RNA sequencing

Dual reporter cells were plated in 12 well plates to reach 70% confluence overnight. The cells were then incubated in serum starvation media for 4 hours followed by incubations with recombinant proteins in serum starvation media for 24 hours. The cells were then washed with PBS and lysed in Tri-reagent and RNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research, # R2050) following the manufacturer's instructions. Total RNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, # ND-2000). mRNA was enriched from 400ng of total RNA using the NEBNext rRNA Depletion Kit v2 (NEB, # E7400X) to remove ribosomal RNA. First stand was synthesized using the Random Hexamer Primer (Thermo Fisher Scientific, # SO142) and Maxima Reverse Transcriptase (Thermo Fisher Scientific, #EP0742). Second strand was synthesized using NEBNext mRNA Second Strand Synthesis Module (NEB, #E6111L). Sequencing adaptors were added by tagmentation and amplified for 12 cycles using the Nextera XT DNA Library Preparation Kit (Illumina, # FC-131). Libraries were pooled at a final concentration of 1.35 pM, and 36bp x 36bp paired end reads were sequenced on an Illumina NextSeq500 instrument. Sequencing reads were demultiplexed using bcl2fastq and aligned to the mm10 mouse genome using HISAT2 (a fast spliced aligner with low memory requirements) (*45*). PCR duplicates and low-quality reads were removed by Picard (https://broadinstitute.github.io/picard) and filtered reads were assigned to the GRCm38 genome modified to minimize overlapping transcripts and quantified using featureCounts (an efficient general purpose program for assigning sequence reads to genomic features (*46*). Differential expression analysis of the data was performed using EdgeR (*47*).

Analysis of gene sets regulated by Gdf3 was performed with Enrichr. This resource introduced us to Sysmyo, a large collection of signatures of gene expression changes in muscle. (*36, 48*).

RTqPCR

Dual reporter cells were plated in 6 well plates and treated as mentioned in the figure legends. Cells were lysed in Tri-reagent and RNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research, # R2050) following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using cDNA generated with the High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, # 4368813) with SYBR Select Master Mix (Applied Biosystems, # 4472920) on the QuantStudio 6 Real-Time PCR System (Applied Biosystems). Relative mRNA expression was determined by the 2^-ddCt method normalized to TATA-binding protein (TBP) levels. The sequences of primers used in this study are listed in Table S2.

Phylogenetic analysis

Phylogenetic analysis of human TGFβ superfamily proteins was performed with ClustalW (*49*). All sequences were retrieved from the UniProt using the database's annotation for the mature chain. Alignments were performed with the blosum62mt2 matrix, ignoring positions with gaps. Human GDF3 was used as the starting profile for alignment.

Statistical Analyses

Data were analyzed using Prism software (GraphPad Software) and are expressed as mean ± SEM. All data were assumed to have a normal distribution and two-tailed Student's t tests, one-way ANOVA with Newman-Keuls Multiple Comparison test, or two-way ANOVA were used to compare means between groups as indicated in the figure legends; $p < 0.05$ was considered significant.

Table 1. Recombinant proteins

Recombinant protein	Species	Catalog No	Company
GDF3	Mouse	9009-GD-010	R&D Systems
BMP2	Mouse	N/A	Vicki Rosen lab (HSDM)
BMP7	Mouse	5666-BP-010	R&D Systems
BMP9	Human	3209-BP-010	R&D Systems
BMP10	Mouse	6038-BP-025	R&D Systems
BMP15	Human	5096-BM-005	R&D Systems
TGFβ1	Human	T7039	Millipore Sigma
Activin A	Human/ Mouse/ Rat	338-AC-010	R&D Systems
Fc-BMPR1A/ ALK3	Human	2406-BR-100	R&D Systems
Fc-BMPR1B/ ALK6	Human	505-PR-100	R&D Systems
Fc-BMPRII	Human	811-BR-100	R&D Systems
Fc-TGFBRI/ ALK5	Human	3025-BR-050	R&D Systems
Fc-TGFBRII	Human	341-BR-050	R&D Systems
Fc-TGFBRIII	Mouse	5034-R3-050	R&D Systems
Fc-ALK7	Rat	577-A7-100	R&D Systems

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