Soluble Heparin Binding Epidermal Growth Factor-Like Growth Factor Is a Regulator of *GALGT2* Expression and *GALGT2*-Dependent Muscle and Neuromuscular Phenotypes

Megan L. Cramer,^a Rui Xu,^b Paul T. Martin^{b,c}

^aMolecular, Cellular, and Developmental Biology Graduate Program, The Ohio State University, Columbus, Ohio, USA

^bCenter for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, USA

^cDepartment of Pediatrics, The Ohio State University College of Medicine, Columbus, Ohio, USA

ABSTRACT *GALGT2* (also *B4GALNT2*) encodes a glycosyltransferase that is normally confined to the neuromuscular and myotendinous junction in adult skeletal muscle. *GALGT2* overexpression in muscle can inhibit muscular dystrophy in mouse models of the disease by inducing the overexpression of surrogate muscle proteins, including utrophin, agrin, laminins, and integrins. Despite its well-documented biological properties, little is known about the endogenous regulation of muscle *GALGT2* expression. Here, we demonstrate that epidermal growth factor receptor (EGFR) ligands can activate the human *GALGT2* promoter. Overexpression of one such ligand, soluble heparin-binding EGF-like growth factor (sHB-EGF), also stimulated mouse muscle *Galgt2* gene expression and expression of *GALGT2*-inducible surrogate muscle genes. Deletion analysis of the *GALGT2* promoter identified a 45-bp region containing a TFAP4-binding site that was required for sHB-EGF activation. sHB-EGF increased TFAP4 binding to this site in muscle cells and increased endogenous *Tfap4* gene expression. sHB-EGF also increased muscle EGFR protein expression and activated EGFR-Akt signaling. sHB-EGF expression was concentrated at the neuromuscular junction, and *Hbegf* deletion reduced *Galgt2*-dependent synaptic glycosylation. *Hbegf* deletion also mimicked *Galgt2*-dependent neuromuscular and muscular dystrophy phenotypes. These data demonstrate that sHB-EGF is an endogenous regulator of muscle *Galgt2* gene expression and can mimic *Galgt2*-dependent muscle phenotypes.

KEYWORDS AAV, EGFR, *GALGT2*, HB-EGF, agrin, laminin, muscular dystrophy, neuromuscular junction, skeletal muscle

In a variety of neuromuscular disorders, the primary genetic defect can be overcome not only by gene replacement but also by overexpression of surrogate genes that encode proteins homologous in structure or function to the mutated gene. For example, overexpression of utrophin or integrin $\alpha 7$ can inhibit muscular dystrophy (MD) in the dystrophin-deficient *mdx* model of Duchenne muscular dystrophy (1–5), and overexpression of laminin $\alpha 1$, agrin, or laminin $\alpha 4$ can inhibit MD in the laminin $\alpha 2$ -deficient *dy^w* model of congenital muscular dystrophy 1A (6–8). We have studied a surrogate gene therapy called *GALGT2* (also called *B4GALNT2*). When overexpressed in skeletal muscle, *GALGT2* induces the ectopic overexpression of many surrogate genes and proteins, including utrophin, plectin 1, agrin, laminin $\alpha 2$, laminin $\alpha 4$, laminin $\alpha 5$, integrin $\alpha 7$, and integrin $\beta 1$ (9, 10). The *GALGT2* gene encodes $\beta 1,4$ -N-acetylgalactosaminyltransferase that creates the cytotoxic T cell (CT) glycan Neu5Ac $\alpha 2$ -3[GalNAc $\beta 1$ -4]Gal $\beta 1$ -4GlcNAc β -R on glycoproteins, including α dystroglycan (11–14). Dystroglycan, which comprises α and β chains, is a muscle membrane protein that

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Address correspondence to Paul T. Martin, Paul.Martin@nationwidechildrens.org.

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serves as an essential transmembrane link between the extracellular matrix (ECM) and the F-actin cytoskeleton (15, 16). GALGT2 overexpression increases expression of ECM proteins, including laminin α 4, laminin α 5, and agrin, increases ECM protein binding to α dystroglycan, and increases expression of cytoplasmic F-actin binding proteins that link the cytoskeleton to β dystroglycan, including dystrophin, utrophin, and plectin1 (9, 10, 17–20). In changing the molecular nature of the muscle membrane, *GALGT2* overexpression protects both wild-type (WT) and dystrophin-deficient (*mdx*) skeletal myofibers from eccentric contraction-induced injury and can inhibit the development of muscular dystrophy in four different animal models of the disease (*mdx*, *dy^w*, *Sgca*, and *FKRP^{P448L}*) (14, 18, 19, 21, 22). Many of the surrogate genes and proteins induced by *GALGT2* are, like *GALGT2*, normally confined in expression to the neuromuscular junction (NMJ) and myotendinous junction (MTJ) in adult muscle (23–27). Similarly, like *GALGT2*, many of these genes and proteins are highly expressed in extrasynaptic muscle regions in the early postnatal period (23, 25, 26, 28–34). Thus, by drawing such proteins into an extrasynaptic expression pattern, *GALGT2* is essentially recreating the molecular environment normally seen in a young muscle. As such, *GALGT2* overexpression provides a means of understanding mechanisms controlling the expression of these therapeutic genes and proteins, with regard to not only muscle therapy but also normal muscle development.

Although the potential of *GALGT2* gene therapy has been appreciated and successfully applied in neuromuscular disease models, regulation of endogenous *Galgt2* gene expression in mouse muscle remains poorly understood. Given its potential as a therapeutic, a better understanding of the endogenous mechanisms controlling muscle *Galgt2* expression, as well as the expression of *GALGT2*-inducible muscle surrogate genes and *GALGT2*-dependent muscle phenotypes, is highly warranted, and this is the goal of our current study.

RESULTS

EGFR ligands stimulate the human *GALGT2* promoter in muscle cells. Using C2C12 myotube cultures stably expressing a bp –5870 *GALGT2* promoter firefly luciferase (*Luc2*) reporter, we performed a screen of trophic factors to assess their ability to induce the *GALGT2* promoter (Fig. 1A). Positives were also validated using C2C12 myotubes stably expressing a *GALGT2* promoter *Renilla* luciferase (*hRluc*) reporter (not shown). Many trophic factors, including brain-derived neurotrophic factor (BDNF), glucagon-like peptide 2, ciliary neurotrophic factor (CNTF), nerve growth factor 2.5S (NGF 2.5S), leukemia inhibitory factor (LIF), human growth hormone (HGH), neurotrophin 3 (NT3), peptide YY, glial cell line-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), calcitonin gene-related peptide (CGRP), somatostatin, and neuregulin 1 (NRG1) did not increase luciferase signal when added for 24 h. Concentrations chosen are described in Materials and Methods and exceed or equal the binding affinity of each ligand for their known signaling receptor. Ligands known to activate the epidermal growth factor receptor (EGFR), including soluble heparin-binding EGF-like growth factor (sHB-EGF), epidermal growth factor (EGF), amphiregulin, epiregulin, and transforming growth factor α (TGF α), all induced *GALGT2* promoter activity, as did insulin and insulin-like growth factor 1 (IGF1) (Fig. 1A). We specifically tested sHB-EGF, as the full-length transmembrane form of HB-EGF does not activate EGFR until it is cleaved to generate sHB-EGF by ADAM proteases (35–38). Of the EGFR agonists, only HB-EGF and TGF α are appreciably expressed in skeletal muscle, with HB-EGF showing more specificity for muscle than TGF α , which is expressed in almost all tissues (39). sHB-EGF was able to activate the *GALGT2* promoter in a dose-dependent manner, with saturation occurring at 10 nM (Fig. 1B). sHB-EGF only activated the *GALGT2* promoter luciferase reporter in C2C12 cells if they were first differentiated into myotubes (not shown). Furthermore, in differentiated C2C12 cells, sHB-EGF increased the expression of endogenous *Galgt2* mRNA levels by 10-fold relative to levels for untreated cells (Fig. 1C).

FIG 1 Muscle trophic factor screen of *GALGT2* promoter activity. (A) Trophic factors were added for 1 day to C2C12 myotube cultures containing a *GALGT2* promoter firefly luciferase reporter. Factors in blue indicate ligands that activate the epidermal growth factor receptor (EGFR). Factors in green indicate factors that activate the insulin or insulin-like growth factor receptor. (B) sHB-EGF was added at different concentrations to *GALGT2* promoter firefly luciferase reporter cells. Data are reported relative to those of untreated C2C12 myotube cultures in panels A and B (set to 0). (C) qRT-PCR measures of gene expression for endogenous *Galgt2* following treatment with sHB-EGF. Fold change is reported relative to the level for saline-injected contralateral muscles (set to 1). NGF, mouse nerve growth factor 2.5S. Errors are standard errors of the means (SEM) for $n = 12$ (A and B) and $n = 9$ (C) measures. *, $P < 0.05$; ***, $P < 0.001$.

sHB-EGF activates the human *GALGT2* promoter via a TFAP4-responsive element. We next wanted to define the DNA element or elements in the *GALGT2* promoter responsible for driving elevated luciferase reporter expression in response to sHB-EGF. To do this, we created a series of deletions in the *GALGT2* promoter (Fig. 2A). Deletions up to the last bp, bp -950 , of the 5' end of the promoter had no impact on the ability of sHB-EGF to activate luciferase. Deletions leaving only bp -500 or bp -200 , however, significantly diminished promoter reporter activity (Fig. 2B), suggesting the 450-bp sequence between bp -950 and bp -500 responded to sHB-EGF. We next created five smaller deletions, each approximately 50 bp, within this 450-bp sequence. Of these, only the second deletion (950 Δ 2; deletion of bp -822 to -777) showed a significant decrease in luciferase signal (Fig. 2C). A motif analysis identified only two major transcription factor binding sites in the bp -822 to -777 region (Fig. 2D) (40). One site included two overlapping FOXD3 binding sites at bp -818 to -804 and bp -815 to -801 , while the second site contained overlapping binding sites for TFAP4, LMO2, and myogenin (MYOG) (which binds at E boxes) at bp -798 to -781 , bp -796 to -785 , and bp -793 to -788 , respectively.

FIG 2 Mapping of an sHB-EGF-responsive element on the human *GALGT2* promoter. (A) C2C12 myotube cultures were made bearing a human *GALGT2* promoter firefly luciferase reporter construct with promoter elements ranging from bp -5870 to -200 relative to the *GALGT2* transcriptional start site. Five smaller deletions were also made within the bp -950 *GALGT2* promoter as indicated. (B) bp -5870 , -950 , -500 , and -200 *GALGT2* promoter constructs were assayed for luciferase activity in relative light units (RLU) after the addition of 10 nM sHB-EGF. (C) Each bp -950 deletion construct was compared to unaltered bp -950 . Data are reported relative to untreated C2C12 myotube cultures in panels B and C (set to 0). (D) Predicted transcription factor binding domains in the bp -822 to -777 region of the *GALGT2* promoter. Errors are SEM for $n = 12$ measures per condition in panels B and C. ***, $P < 0.001$.

We next assessed whether the transcription factors predicted to bind to the bp -822 to -777 region could activate the *GALGT2* promoter when overexpressed. To do this, we obtained adenovirus (Ad) constructs that overexpress MYOG, FOXD3, LMO2, or TFAP4. Ad-GFP (green fluorescent protein) was used as a negative control. C2C12 myotube cultures stably expressing the bp -950 *GALGT2* promoter firefly luciferase reporter were treated with each Ad vector either in the presence or absence of sHB-EGF. Neither Ad-GFP, Ad-MYOG, nor Ad-FOXD3 significantly increased promoter activity (relative to untreated cells), while Ad-LMO2 and Ad-TFAP4 did, with Ad-TFAP4 showing the greatest activation (Fig. 3A). Ad-LMO2 and Ad-TFAP4 also significantly activated the bp -950 *GALGT2* promoter in the presence of sHB-EGF relative to sHB-EGF alone (Fig. 3A). In contrast, Ad-LMO2 or Ad-TFAP4 was unable to significantly increase expression in the 950Δ2 construct (Fig. 3B).

We next used short interfering RNAs (siRNAs) targeting mouse *Tfap4* or *Lmo2* to knock down mRNA expression and then retest sHB-EGF induction (Fig. 3C). siRNA targeting *Tfap4* significantly reduced *GALGT2* promoter activity in sHB-EGF-treated cells ($P < 0.01$), while siRNA targeting *Lmo2* and a scrambled *Tfap4* control did not (Fig. 3C). The siRNA targeting *Tfap4* knocked down endogenous *Tfap4* gene expression in both resting and sHB-EGF-treated cells by 58% relative to the relevant control (Fig. 3D). Interestingly, sHB-EGF treatment alone induced *Tfap4* gene expression 1.9 ± 0.3 -fold ($P < 0.001$). siRNA targeting *Lmo2* reduced *Lmo2* gene expression by only 30%, and sHB-EGF did not induce endogenous *Lmo2* gene expression (not shown). Interestingly, both Ad-TFAP4 and Ad-FOXD3 increased endogenous *Galgt2* gene expression in C2C12 myotubes, while Ad-MYOG, Ad-GFP, and Ad-LMO2 did not (Fig. 3E). Ad-TFAP4, Ad-

FIG 3 Characterization of LMO2 and TFAP4 activation of and binding to the *GALGT2* promoter. (A) Adenovirus vectors were used to infect and overexpress *GFP*, *MYOG*, *FOXD3*, *LMO2*, or *TFAP4* in C2C12 myotubes expressing the bp -950 *GALGT2* promoter luciferase reporter in the absence (open bars) or presence (hatched bars) of sHB-EGF. Luciferase reporter activity was measured in relative light units (RLU). (B) C2C12 myotubes stably expressing the -950Δ2 *GALGT2* promoter luciferase reporter were assayed as described for panel A after addition of sHB-EGF or adenovirus (Ad) containing *LMO2* or *TFAP4*. (C) C2C12 myotubes expressing the *GALGT2* promoter were treated with siRNA against *TFAP4*, *LMO2*, or a scrambled negative (neg) control in the presence or absence of sHB-EGF. Luciferase reporter activity was measured in RLU. (D) *Tfap4* gene expression was measured in C2C12 myotube cultures treated with siRNAs for scrambled negative (neg) control or against *Tfap4*, with or without sHB-EGF. (E) Mouse *Galgt2* mRNA was measured in C2C12 cells treated with adenovirus (Ad) containing *GFP*, *MYOG*, *TFAP4*, *LMO2*, or *FOXD3*. (F) Chromatin immunoprecipitation measuring binding of *LMO2* or *TFAP4* to the bp -951 to -726 region of the *GALGT2* promoter in C2C12 myotubes at 24 and 48 h after sHB-EGF addition relative to those of cells not treated with sHB-EGF. Fold change data in panels A to E are reported relative to the level of the untreated control, set to 0. Errors are SEM for $n = 4$ to 8 (A to E) and standard deviations for $n = 3$ (F) measures per condition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

LMO2, Ad-*MYOG*, and Ad-*FOXD3* all induced expression for their respective (human) genes by 15- to 20-fold relative to levels for the same endogenous mouse gene, but Ad-*FOXD3* also increased endogenous mouse *Tfap4* expression by 2.1 ± 0.1 -fold and decreased endogenous mouse *Lmo2* expression to 0.3 ± 0.1 of normal levels. Thus, *FOXD3* overexpression also led to increased expression of *Tfap4* in C2C12 cells. As we

could not detect any expression of *Foxd3* in mouse muscle (not shown), we did not pursue this result further.

Lastly, we determined if sHB-EGF would induce binding of LMO2 or TFAP4 to the bp –951 to –726 region by performing chromatin immunoprecipitation (ChIP) of nuclear lysates from C2C12 myotubes before or after sHB-EGF addition (Fig. 3F). sHB-EGF increased TFAP4 occupancy to this site at 24 and 48 h after sHB-EGF addition. LMO2 binding, in contrast, showed the opposite pattern. Thus, sHB-EGF appears to induce a switch in occupancy of transcription factors in the bp –951 to –726 region of the *GALGT2* promoter, increasing bound TFAP4 at the expense of bound LMO2.

sHB-EGF activates an EGFR phosphorylation cascade in muscle cells. To better characterize the sHB-EGF signaling pathway, we added inhibitors of various signaling pathways to the bp –5870 *GALGT2* promoter reporter muscle cells along with sHB-EGF. All inhibitors were tested in a dose-response curve, and concentrations shown reflect maximal levels of inhibition. Inhibitors of EGFR (tyrophostin; AG-1478), a known sHB-EGF receptor, phosphatidylinositol 3 (PI3) kinase (LY294002), Akt kinase (MK-2206), mTOR (rapamycin), Raf kinase (vemurafenib), and extracellular signal-regulated kinase (ERK; SCH772984) all maximally inhibited sHB-EGF activation by more than 50%, while inhibitors of Src (KX2-391), Bcr/Abl (nilotinib), STAT5, c-Jun N-terminal kinase (JNK; SP600125), and c-myc (10058-F4) did not (Fig. 4A).

We next assessed sHB-EGF induction of EGFR, Akt, Erk1/2, MEK1/2, and C-Raf activity by comparing signals on Western blots using phosphospecific and protein-specific antibodies to each component (Fig. 4B to D). sHB-EGF addition caused a 20-fold increase in phosphorylation of EGFR (pEGFR [Tyr1173]/EGFR, 23.9 ± 1.3 ; $P < 0.05$) within 15 min compared to levels for untreated cells (Fig. 4B). Similarly, phosphorylation of Akt (pAkt [Ser473]/Akt, 19.7 ± 1.6 ; $P < 0.001$) and mTOR (pmTOR [Ser2448]/mTOR, 7.6 ± 1.7 ; $P < 0.05$) was increased (Fig. 4B), as was that of C-Raf (pC-Raf [Ser289/296/301]/C-Raf, 34.3 ± 3.8 ; $P < 0.001$), MEK1/2 (pMEK1/2 [Ser217/221]/MEK1/2, 6.9 ± 0.1 ; $P < 0.001$), and Erk1/2 (pErk1/2 [Thr202/Tyr204]/Erk1/2, 4.0 ± 0.3 ; $P < 0.01$) (Fig. 4C). Activation of all proteins was transient, with most protein phosphorylation reverting to near baseline levels by 60 min after treatment (Fig. 4B and C). Phosphorylation of c-Src was not significantly increased following sHB-EGF treatment (pSrc [Tyr416]/Src, 1.8 ± 0.4 ; $P = 0.37$) (Fig. 4D), consistent with the results of our inhibitor screen (Fig. 4A). These studies support the notion that sHB-EGF activates an EGFR-Akt/mTOR-Mek/Erk kinase signaling cascade in muscle cells (Fig. 4E).

sHB-EGF induces endogenous expression of *Galgt2* and *Galgt2*-inducible surrogate genes in skeletal muscle. We next assessed the ability of sHB-EGF to induce *Galgt2* transcription in mouse skeletal muscle tissue. To do this, we created a recombinant, self-complementary [r(sc)] adeno-associated virus (AAV) vector that would express either full-length human HB-EGF or active sHB-EGF. The AAV9 serotype of viral capsid was used, which allows for robust muscle transgene expression (41, 42). r(sc)AAV9.CMV.HB-EGF or r(sc)AAV9.CMV.sHB-EGF (5×10^{10} vector genomes [vg]) was injected into the gastrocnemius muscles of 8-week-old C57BL/6J mice, with the contralateral gastrocnemius receiving an equal volume of phosphate-buffered saline (PBS) as a control. Injected muscles showed 20,000 to 50,000 vg per μg of genomic DNA, while contralateral control muscles showed two or more logarithms less transduction (Fig. 5A). Because the rAAV9 serotype efficiently crosses the vascular barrier to transduce muscles throughout the body (43, 44), this modest amount of AAV vector was expected in the contralateral muscles even after a focal intramuscular injection, but the extent of transduction was extremely low (no more than 1 vg per 150 nuclei).

Expression of sHB-EGF and HB-EGF was increased between 10- and 20-fold over endogenous mouse *Hbegf* expression at both 4 and 12 weeks after AAV injection (Fig. 5B). Overexpression of full-length, uncleaved HB-EGF was unable to significantly increase *Galgt2* gene expression, while sHB-EGF increased *Galgt2* expression more than 6-fold compared to that of mock-injected contralateral muscles (Fig. 5C). In addition to elevating *Galgt2*, sHB-EGF increased the expression of surrogate muscle genes that can

FIG 4 sHB-EGF stimulates phosphorylation signaling cascades in skeletal muscle cells. (A) sHB-EGF was added to C2C12 myotube cultures in the presence of drugs that block specific signal transduction pathways. Drugs in blue showed significant decreases in sHB-EGF activation of the GALGT2 promoter relative to sHB-EGF alone. GALGT2 promoter luciferase reporter activity was measured in relative light units (RLU). Errors are SEM for $n = 12$ measures per condition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Fold changes are reported relative to the level of sHB-EGF alone, set to 1. (B to D) Western blots of C2C12 myotube protein lysates before or after sHB-EGF addition for 15, 30, or 60 min using antibodies to GAPDH protein (a protein loading and transfer control). Western blots in panel B were additionally probed for members of the Akt signaling pathway, including antibodies to EGFR, Akt, and mTOR protein or antibodies specific to phosphorylated EGFR (at Tyr1173), Akt (at Ser473), and mTOR (at Ser2448). Western blots in panel C were probed for members of the RAF signaling pathway, including antibodies to C-Raf, MEK1/2, and Erk1/2 protein or antibodies specific to phosphorylated C-Raf (at Ser289/296/301), MEK1/2 (at Ser217/221), and Erk1/2 (at Thr202/Tyr204). Western blots in panel D were probed for Src protein or phosphorylated Src (at Tyr416). (E) Diagram illustrating the pathways utilized by sHB-EGF to activate the GALGT2 promoter in skeletal muscle.

have a therapeutic impact on muscular dystrophy, including the utrophin (*Utrn*), agrin (*Agrn*), laminin $\alpha 2$ (*Lama2*), laminin $\alpha 4$ (*Lama4*), and integrin $\beta 1$ (*Itgb1*) genes (9, 10), while full-length HB-EGF did not (Fig. 5C). Interestingly, neither sHB-EGF nor HB-EGF significantly upregulated expression of the dystroglycan (*Dag1*), dystrophin (*Dmd*), laminin $\alpha 5$ (*Lama5*), or integrin $\alpha 7$ (*Itga7*) gene (Fig. 5C), while GALGT2 overexpression can increase expression of these genes in muscle (10). Thus, HB-EGF must be cleaved to its active soluble form in skeletal muscle to increase endogenous *Galgt2* transcription

FIG 5 sHB-EGF induces muscle expression of *Galgt2* and some *Galgt2*-inducible therapeutic genes. (A) qPCR measures of AAV vector genomes in muscles 4 and 12 weeks after treatment with r(sc)AAV9.CMV.sHB-EGF or r(sc)AAV9.CMV.HB-EGF compared to levels for saline-injected contralateral muscles from the same animals. (B) qRT-PCR measures of human *HBEGF* gene expression in r(sc)AAV9.CMV.sHB-EGF- or r(sc)AAV9.CMV.HB-EGF-injected muscles relative to endogenous mouse *Hbegf* gene expression levels in saline-injected muscles at 4 and 12 weeks after treatment. (C) qRT-PCR measures of gene expression for *Galgt2* and other therapeutic surrogate genes (and controls) in muscles 4 and 12 weeks after treatment with r(sc)AAV9.CMV.sHB-EGF or r(sc)AAV9.CMV.HB-EGF. Fold changes are relative to levels for saline-injected contralateral muscles (set to 1). (D) Coimmunostaining of sHB-EGF (green) or of sHB-EGF (green) and Galgt2 protein (red). Merged images on the right show overlapping staining in orange and/or yellow. Scale bar is 25 μ m. Errors are SEM for $n = 4$ (A to C) measures per condition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and can induce the expression of some, but not all, *Galgt2*-inducible therapeutic surrogate genes.

We next analyzed *Galgt2* protein and CT glycan expression in sHB-EGF-injected muscles (Fig. 5D). sHB-EGF was expressed along the sarcolemmal membrane of skeletal myofibers in muscles analyzed at 4 weeks postinjection. CT glycan was highly upregulated along the sarcolemmal membrane in such fibers, as shown by costaining with sHB-EGF protein, while myofibers not overexpressing sHB-EGF did not have elevated CT glycan. As *Galgt2* is a *trans*-Golgi protein (12), it would not be expected to be colocalized with sHB-EGF at the sarcolemmal membrane. *Galgt2* protein staining was, however, highly elevated in many sHB-EGF-overexpressing myofibers (Fig. 5D), consistent with increased *Galgt2* mRNA expression (Fig. 5C). As in cultured myotubes, sHB-EGF overexpression in skeletal muscle also increased *Tfap4* gene expression (1.9 ± 0.1 -fold).

sHB-EGF activates an EGFR-Akt pathway in skeletal muscle. Skeletal muscles treated with sHB-EGF for 4 weeks showed a significant increase in Akt phosphorylation (pAkt [Ser473]/Akt, 2.6 ± 0.2 ; $P < 0.05$) but showed no significant change in phosphorylation for EGFR (pEGFR [Tyr1173]/EGFR, 0.5 ± 0.1 ; $P = 0.501$), mTOR (pmTOR [Ser2448]/mTOR, 0.5 ± 0.1 ; $P = 0.074$), C-Raf (pC-Raf [Ser289/296/301]/C-Raf, 1.5 ± 0.4 ; $P = 0.457$), MEK1/2 (pMEK1/2 [Ser217/221]/MEK1/2, 0.7 ± 0.1 ; $P = 0.251$), or Erk1/2 (pErk1/2

FIG 6 sHB-EGF stimulates an EGFR-Akt signaling pathway in skeletal muscle tissue. (A) Western blots of protein lysates from muscles treated for 4 weeks with r(sc)AAV9.CMV.sHB-EGF and saline-treated contralateral control muscles probed using antibodies to GAPDH protein (a protein loading and transfer control), antibodies to EGFR, Akt, mTOR, C-Raf, MEK1/2, or Erk1/2 protein, or antibodies specific to phosphorylated EGFR (at Tyr1173), Akt (at Ser473), mTOR (at Ser2448), C-Raf (at Ser289/296/301), MEK1/2 (at Ser217/221), or Erk1/2 (at Thr202/Tyr204). (B) qRT-PCR measures of *Egfr*, *Erbb2*, *Erbb3*, *Erbb4*, and *Akt1* gene expression in muscles from mice treated for 4 or 12 weeks with r(sc)AAV9.CMV.sHB-EGF or r(sc)AAV9.CMV.HB-EGF relative to levels for saline-injected control muscles (set to 1). Errors are SEM for $n = 4$ measures per condition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Wild-type saline-injected or r(sc)AAV9.CMV.sHB-EGF-injected muscles were stained with rhodamine-conjugated α -bungarotoxin to identify nicotinic acetylcholine receptors (AChRs) (red) at the neuromuscular junction and with an antibody to EGFR (green). Merged images on the right show overlapping staining in orange and/or yellow. Scale bar is 25 μm .

[Thr202/Tyr204]/Erk1/2, 0.9 ± 0.1 ; $P = 0.669$) (Fig. 6A). Expression of EGFR protein, however, was increased almost 5-fold after sHB-EGF treatment (EGFR/GAPDH, 5.7 ± 0.6 ; $P < 0.05$) (Fig. 6A), as was mTOR protein (9- $\pm$ 5-fold). Thus, while the pEGFR/EGFR and pmTOR/mTOR ratios did not significantly change, elevated EGFR and mTOR protein expression meant pEGFR and pTOR levels also increased. Mouse *Egfr* gene expression was also significantly increased at both 4 and 12 weeks by sHB-EGF compared to that of the PBS-injected control ($P < 0.001$ for each) (Fig. 6B). Overexpression of full-length transmembrane HB-EGF, in contrast, did not significantly increase *Egfr* mRNA (Fig. 6B). sHB-EGF overexpression also caused a 2-fold increase in *Erbb2* mRNA expression at 4 and 12 weeks postinjection, although this did not reach statistical significance (4 weeks, 2.2 ± 0.5 ; $P = 0.13$; 12 weeks, 2.2 ± 0.3 ; $P = 0.13$), while expression of *Erbb3*, *Erbb4*, and *Akt1* did not change (Fig. 6B). We also immunostained untreated and sHB-EGF-treated muscles with antibodies to EGFR protein. In control muscle, EGFR staining was concentrated at the neuromuscular junction (NMJ), but staining was increased along extrasynaptic regions of the myofiber membrane after sHB-EGF treatment (Fig. 6C). Thus, some facets of EGFR activation and Akt signaling found in muscle cells were present after sHB-EGF overexpression in muscle tissue, but others (MEK1/2, Erk1/2, and C-Raf) were not.

sHB-EGF is required for *Galgt2*-dependent NMJ glycosylation. To stain for sHB-EGF in muscle, we used a function-blocking sHB-EGF-specific monoclonal antibody (MAb 2591). We confirmed the specificity of MAb 2591 by staining Chinese hamster ovary (CHO) cells transfected with a cDNA encoding the full-length transmembrane form of HB-EGF (208 amino acids [aa]) or with a cDNA encoding only sHB-EGF (148 aa). MAb 2591 stained sHB-EGF-transfected cells but not HB-EGF-transfected cells, while polyclonal antibody (PAb) 259 NA, an HB-EGF polyclonal antibody, recognized both

FIG 7 Enrichment of sHB-EGF at the neuromuscular synapse. (A) CHO cells were transfected with cDNAs expressing either soluble HB-EGF (sHB-EGF) or full-length transmembrane HB-EGF. Cells were then immunostained with MAb 2591 (an sHB-EGF-specific function blocking antibody), PAb 259 NA (recognizes full-length HB-EGF protein as well as sHB-EGF), or PAb C18 (recognizes the C-terminal intracellular domain of full-length HB-EGF). Nuclear (DAPI) staining is shown in blue. Scale bar is 50 μ m. WT and HB-EGF-deficient (*Hbegf*^{-/-}) muscle sections were stained with the sHB-EGF-specific antibody MAb 2591 (B), a Galgt2 antibody (C), or an antibody to CT glycan (CT2) (D) (all shown in green). Tissue sections were costained with rhodamine-conjugated α -bungarotoxin (red) to label acetylcholine receptors (AChRs) at the neuromuscular junction. Merged images at the right show staining overlap in yellow or orange. Scale bar is 25 μ m.

sHB-EGF and HB-EGF (Fig. 7A). C18, an antibody to the C-terminal domain of HB-EGF (that only exists in the full-length HB-EGF), recognized HB-EGF but not sHB-EGF (Fig. 7A). In adult skeletal muscle, MAb 2591 stained NMJs (Fig. 7B). sHB-EGF staining coincided with α -bungarotoxin staining, which stains nicotinic acetylcholine receptors (AChRs) in the postsynaptic muscle membrane (Fig. 7B) (45). sHB-EGF muscle staining was eliminated in mice lacking HB-EGF (*Hbegf*^{-/-}) (Fig. 7B). Staining for Galgt2 protein (Fig. 7C) and the CT glycan (Fig. 7D) was reduced, and sometimes eliminated, at NMJs in *Hbegf*^{-/-} muscles, suggesting a requirement, or partial requirement, of HB-EGF. Staining of Galgt2 protein and CT glycan remained present in intramuscular capillaries in *Hbegf*^{-/-} mice, providing an internal staining control (Fig. 7C and D).

Absence of sHB-EGF mimics *Galgt2*^{-/-} neuromuscular junction and muscle phenotypes. Because *Hbegf*^{-/-} muscles had reduced Galgt2 and CT glycan expression, we next determined if loss of *Hbegf* would mimic NMJ or muscle phenotypes found in *Galgt2*^{-/-} or *mdx::Galgt2*^{-/-} mice. *Galgt2*^{-/-} muscles show increased fragmentation of postsynaptic AChRs and an increased localization of AChRs in clathrin-coated endosomes (46). The number of AChR microaggregates in the vicinity of the NMJ was also increased by an order of magnitude in 7-month-old *Hbegf*^{-/-} muscles (Fig. 8A and B). This was equivalent to the pattern and extent of subsynaptic AChR alterations found in 3-month-old *Galgt2*^{-/-} muscles (46). *Galgt2* is also a modulator of skeletal muscle disease severity in *mdx* mice (31). *mdx::Galgt2*^{-/-} muscles show increased size and decreased strength compared to *mdx* muscles. Eight- to 12-month-old *mdx::Hbegf*^{-/-} mice had increased skeletal muscle mass relative to *mdx* mice (Fig. 8C), much like *mdxGalgt2*^{-/-} muscles (31). Muscles in *Hbegf*^{-/-} mice, in contrast, were not significantly different from those of the WT. Forelimb (Fig. 8D) and hindlimb (Fig. 8E) grip strength also trended lower in *mdx::Hbegf*^{-/-} mice relative to that of *mdx* mice, although these changes did not reach statistical significance. *Hbegf*^{-/-} forelimb grip strength was unchanged from that of the WT, while hindlimb grip strength was reduced. When run on a treadmill, *Hbegf*^{-/-} mice were indistinguishable from WT mice ($P > 0.999$), while *mdx* mice showed reduced ambulation ($P < 0.01$ and $P < 0.05$ versus WT and *Hbegf*^{-/-}, respectively) and *mdx::Hbegf*^{-/-} mice were further significantly

FIG 8 *Hbegf*^{-/-} mice exhibit *Galgt2*^{-/-} neuromuscular junction and muscle phenotypes. (A) Examples of increased AChR microaggregates at neuromuscular junctions in *Galgt2*^{-/-} (2 panels) and *Hbegf*^{-/-} (4 panels) muscles compared to levels for the wild type (2 panels). Tibialis anterior muscle is shown at 3 months of age for *Galgt2*^{-/-} muscle and at 7 months of age for *Hbegf*^{-/-} and WT muscle. Scale bar is 10 μ m. (B) Quantification of AChR microaggregates in WT, *Galgt2*^{-/-}, and *Hbegf*^{-/-} muscle. WT (n = 45) and *Hbegf*^{-/-} (n = 75) TA muscles were from 7-month-old mice, while *Galgt2*^{-/-} (n = 37) TA muscles were from 3-month-old mice. Data are means $\pm$ SEM. ***, P < 0.001 by one-way ANOVA. (C) Mouse skeletal muscle weights were normalized to total body mass. Abbreviations: TA, tibialis anterior; Gastroc, gastrocnemius; Quad, quadriceps. (D to F) Mice participated in a week-long test of strength and endurance via forelimb grip strength (D), hindlimb grip strength (E), and total run time on a treadmill (F). For panels D to F, data are means $\pm$ SEM for n = 6 (WT), n = 4 (*mdx* and *mdx*:*Hbegf*^{-/-}), and n = 3 (*Hbegf*^{-/-}) measures. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by one-way ANOVA.

diminished relative to *mdx* mice ($P < 0.001$ versus *mdx* mice) (Fig. 8F). Thus, deletion of *Hbegf* in *mdx* mice mimicked dystrophic phenotypes of *Galgt2* deletion. *sHB-EGF* overexpression, however, was very different than *GALGT2* overexpression in *mdx* muscles under certain circumstances. For example, while *GALGT2* overexpression can prevent muscle damage, can reduce fibrosis, can prevent loss of muscle force in *mdx* skeletal muscles, and has an excellent safety profile at high doses (22, 47–49), systemic overexpression of *sHB-EGF* at postnatal day 1 in *mdx* and wild-type mice led to uniform lethality within 3 weeks of treatment (5 out of 5 each).

DISCUSSION

The induction of therapeutic surrogate genes and proteins to inhibit muscle damage associated with muscular dystrophy represents a major opportunity for researchers trying to improve muscle disease outcomes. *GALGT2* gene overexpression is an attractive approach to inducing surrogate therapeutic genes and proteins because it can alter expression of multiple muscular dystrophy modifiers. Such genes include those encoding utrophin (9, 10, 21), which can inhibit muscular dystrophy in the *mdx* mouse model of Duchenne muscular dystrophy (DMD) (1–3), agrin (9, 10, 18), which can inhibit muscular dystrophy in the *dy^w* mouse model of laminin $\alpha 2$ -dependent congenital muscular dystrophy (or MDC1A) (8), plectin 1 (9, 19), which when deleted increases the severity of muscular dystrophy in *mdx* mice (50) and itself causes muscular dystrophy (with epidermolysis bullosa) (51), laminin $\alpha 4$ and $\alpha 5$ (9, 10), which can diminish muscular dystrophy caused by deficits in laminin $\alpha 2$ (7, 52), and integrin $\alpha 7$, which can inhibit muscular dystrophy in *mdx* mice (4, 53). Endogenous regulation of these therapeutic genes and proteins in skeletal muscle, however, remains poorly understood, which is why we undertook the current studies focused on regulation of endogenous muscle *Galgt2* gene expression.

While studies of nerve-derived signals that induce expression of synaptic muscle genes have been fairly extensive, little is known about how muscle-derived factors might induce synaptic gene expression, particularly in the postnatal period when *Galgt2* expression becomes confined to the synapse. Neuregulin 1 (NRG1), for example, is a nerve-derived signal that induces synaptic gene expression in embryonic skeletal muscle, including expression of nicotinic acetylcholine receptor genes (54). *Galgt2*, along with other synaptic proteins, such as utrophin, agrin, laminin $\alpha 4$, and laminin $\alpha 5$, becomes localized to the NMJ in skeletal muscle 2 to 3 weeks after birth (23, 25, 26, 29–31, 34), a later developmental epoch than is seen for many NRG1-regulated genes (55, 56). We identified a group of EGFR ligands (57, 58), including EGF, *sHB-EGF*, amphiregulin, epiregulin, and TGF α , that stimulated muscle *Galgt2* gene expression. NRG1, which does not bind the EGFR but instead binds EGFR family members ErbB3 and ErbB4 (57, 58), in contrast, did not stimulate muscle *Galgt2* expression. Similar to NRG1 receptors ErbB3 and ErbB4 (59), EGFR is localized to the NMJ in adult skeletal muscle, a finding first described by Sanes and colleagues (60) and confirmed in this study. Thus, EGFR ligands and EGFR may be involved in the localization of genes (and proteins) to the NMJ in the postnatal period, akin to NRG1's role in embryonic muscle.

Our studies suggest that *sHB-EGF* controls important postnatal muscle gene expression programs and has important roles in muscle biology. Overexpression of *sHB-EGF* in skeletal muscle not only induced expression of mouse muscle *Galgt2* expression but also upregulated the expression of utrophin, laminin $\alpha 2$, laminin $\alpha 4$, and integrin $\beta 1$. Overexpression of full-length HB-EGF, in contrast, did not produce these changes, confirming the importance of HB-EGF cleavage to its soluble *sHB-EGF* form to mediate EGFR signaling (35, 36, 61). Interestingly, Engvall and colleagues have shown that transgenic overexpression of *ADAM12*, which can cleave transmembrane HB-EGF protein to create active *sHB-EGF*, is therapeutic in *mdx* mice and can increase integrin $\alpha 7$ and utrophin expression (62, 63). Those results may be related to the mechanism we have described here. *sHB-EGF* was concentrated at the NMJ in adult muscle, and deletion of mouse *Hbegf* decreased synaptic expression of muscle *Galgt2* protein and the CT glycan, which is made by *Galgt2* (12, 46, 64). Similarly, deletion of *Hbegf* led to

increased fragmentation of postsynaptic acetylcholine receptors to a degree seen with deletion of *Galgt2* (46), albeit on a slightly different time scale. Moreover, in *mdx* mice, *Hbegf* deletion modulated muscle disease phenotypes in a manner similar to deletion of *Galgt2*. Such phenotypes included increases in muscle hypertrophy and muscle weakness with reduced ambulation. Not all phenotypes, however, were matched between *Hbegf* and *Galgt2*. For example, perinatal systemic overexpression of sHB-EGF was lethal in *mdx* mice, a finding not found with *GALGT2* (14). While this may be due to the use of different promoters in these experiments (cytomegalovirus [CMV] for sHB-EGF and MCK for *GALGT2*), HB-EGF has additional well-described functions not reported with *GALGT2*. For example, *Hbegf*-deficient mice have extensive phenotypes related to cardiac development (61, 65, 66), cardiac hypertrophy (66, 67), tumor cell growth and angiogenesis (57, 68), embryo implantation and placental development (69–71), cognitive function and synaptic plasticity (72, 73), tissue fibrosis (74–77), and intestinal health and regeneration after injury (78–82). Some of these phenotypes have been not reported in *Galgt2*^{−/−} mice, although *Galgt2* deletion can give rise to blood, cardiac, pregnancy, and tumor-related phenotypes (14, 31, 83–85). Similarly, *GALGT2* overexpression in skeletal muscle induced the expression of some surrogate genes not induced by sHB-EGF, including *Lama5* and *Itga7*, while other surrogate genes were induced by both.

Most of the functions described for sHB-EGF correlate with its ability to activate EGFR and downstream pathways, including PI3 kinase, Akt, mTOR, ERK, and MEK, as well as cross talk with IGF receptor 1 (IGFR1) signaling (58, 86). While we have not delineated the entire signaling pathway in muscle, we have shown that sHB-EGF activates the muscle EGFR signaling and increases Akt phosphorylation. sHB-EGF also increases muscle EGFR gene and protein expression, thereby causing the overexpression of its own receptor. This no doubt facilitates the ability of sHB-EGF to induce overexpression of other normally synaptic therapeutic genes in the extrasynaptic muscle membrane. Importantly, while sHB-EGF induces increased Mek1/2 and Erk1/2 phosphorylation in muscle cells, it did not do so in muscle tissue. This suggests that additional regulatory factors or mechanisms are involved in sHB-EGF signaling *in vivo* that are not involved in sHB-EGF signaling in cultured muscle cells.

We have also shown that addition of sHB-EGF to muscle cells stimulates increased occupancy of the *GALGT2* promoter by TFAP4. TFAP4 overexpression is sufficient to activate the *GALGT2* promoter and requires a specific binding site to do so. LMO2 overexpression could also stimulate the same site on the *GALGT2* promoter, but it was less potent than TFAP4. Intriguingly, addition of sHB-EGF to muscle cells decreased LMO2 occupancy at the same time that it increased TFAP4 occupancy in ChIP experiments. As such, sHB-EGF may increase *GALGT2* expression by activating a switch from a less active transcription factor (LMO2) to a more active one (TFAP4). sHB-EGF also increased endogenous *Tfap4* gene expression, which may further facilitate increased TFAP4 occupancy on the *GALGT2* promoter. Like sHB-EGF, *TFAP4* overexpression induced mouse *Galgt2* gene expression in muscle cells. Surprisingly, *FOXD3* overexpression also did this. *FOXD3*, however, may act via TFAP4, as *FOXD3* overexpression also increased endogenous mouse *Tfap4* gene expression (while decreasing *Lmo2*). Also, unlike that of *Tfap4*, *FoxD3* expression was not detectable in adult mouse skeletal muscle. The fact that there is also an E-box within the TFAP4/LMO2 binding site suggests that myogenin (MYOG) also activates *GALGT2* expression, but we found no inductive effects from myogenin overexpression. Goldman, Yao, Schaeffer, and others have previously shown that myogenin protein is downregulated in skeletal muscle by muscle activity in a calcium- and histone deacetylase (HDAC) 4- and/or 9-dependent manner (87–92). As such, one would not expect appreciable myogenin to be present in extrasynaptic regions of a functioning muscle cell, where sHB-EGF was overexpressed in our experiments. The activation of TFAP4, however, may provide a mechanism for late-developing synaptic genes to maintain gene expression even in the presence of muscle activity by activating transcription from sites that might normally be occupied by myogenin. TFAP4 may also offset suppression of *GALGT2* expression by promoter

methylation, as is known to occur in gastrointestinal cancers and cancer cell lines (93, 94).

These experiments provide the first evidence that endogenous muscle proteins can activate *Galgt2* gene expression in a manner that mimics its own ability to induce downstream therapeutic genes that can inhibit forms of muscular dystrophy. This work should provide avenues to understanding how to exploit endogenous muscle signaling pathways to induce expression of therapeutic muscle genes for these disorders.

MATERIALS AND METHODS

Mice. All mice were kept and used in accordance with protocols approved by the Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children's Hospital (Columbus, OH). C57BL/6J and *mdx* mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Hbegf*^{-/-} mice were kindly provided by Gail Besner (The Research Institute at Nationwide Children's Hospital, Columbus OH). *Hbegf*^{-/-} mice were bred with *mdx* mice to create stable double knockout mice (*mdx::Hbegf*^{-/-} mice). *Galgt2*^{-/-} mice were provided by the Consortium for Functional Glycomics and originally generated by John Lowe (Genentech, South San Francisco, CA).

Cell lines. The mouse C2C12 cell line was grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) containing 20% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) and differentiated in DMEM containing 2% horse serum (Thermo Fisher Scientific). The human endothelial kidney (HEK293) cell line was maintained in DMEM containing 10% FBS. The CHO cell line was maintained in DMEM-F-12 medium (Gibco, Gaithersburg, MD) containing 10% FBS. All cell lines were cultivated in the presence of 100 U/ml penicillin and 100 µg/ml streptomycin (Pen/Strep; Mediatech).

AAV vectors. The cDNA region encoding the human sHB-EGF protein and the cDNA encoding the full-length transmembrane human HB-EGF were subcloned into a self-complementary (sc) AAV vector designed by Douglas McCarty (Pfizer, Raleigh, NC) (95). Both AAV vectors contained a cytomegalovirus (CMV) promoter and a simian virus 40 (SV40) polyadenylation sequence. r(sc)AAV9.CMV.sHB-EGF and r(sc)AAV9.CMV.HB-EGF vectors were made by the Viral Vector Core at Nationwide Children's Hospital using the triple transfection method in HEK293 cells (96) and were purified using iodixanol gradients and anion-exchange chromatography (97). For sHB-EGF, the human *HBEGF* coding sequence contains only the first 148 amino acids (soluble form). For HB-EGF, the human HBEGF sequence contains all 208 amino acids (full-length transmembrane form) (98).

GALGT2 promoter luciferase reporter plasmids. All constructs are depicted in Fig. 2A. The bp -5870 human *GALGT2* promoter was isolated by PCR from genomic DNA and ligated into the pGL4.14[*luc2*/Hygro] vector (Promega, Madison, WI) containing the firefly luciferase gene at the XhoI and BglII restriction sites in the polylinker region. bp -950, -500, and -200 deletion constructs were amplified and purified from the bp -5870 plasmid by PCR. Generation of serial deletions in the bp -950 construct were also made by PCR using the ultrahigh-fidelity PfuUltra II fusion HS DNA polymerase (Agilent Technologies, Santa Clara, CA) for DNA amplification via methods previously described (99). All plasmids were verified by DNA sequencing. Plasmids were transfected into C2C12 myoblasts using Effectene (Qiagen, Germantown, MD) and selected with 200 µg/ml hygromycin B (Mediatech). Control cells were transfected with a promoterless firefly luciferase plasmid as a negative control. Positive results were also confirmed using the human *GALGT2* promoter cloned into a pGL4.76[*hRLuc*/Hygro] vector (Promega) and selected with hygromycin B, with promoterless controls also generated as described above. Successfully transfected C2C12 cells were differentiated into myotubes following 6 days in differentiation media. All lines formed healthy myotubes under these conditions.

GALGT2 promoter luciferase reporter trophic factor screen. Reporter assays were performed in C2C12 myotube cultures stably expressing the bp -5870 *GALGT2* promoter firefly luciferase reporter. These cells were plated in 96-well plates and became confluent 24 to 48 h after plating. When cells had reached confluence, fusion medium (DMEM containing 2% horse serum and Pen/Strep) was added to cells for 4 days, followed by treatment with trophic factors in serum-free DMEM for 24 h. Trophic factors screened included recombinant human epidermal growth factor (EGF; 100 ng/ml) (R&D Systems, Minneapolis, MN), recombinant soluble human heparin-binding EGF-like growth factor (sHB-EGF; 100 ng/ml) (R&D Systems), recombinant human amphiregulin (50 ng/ml) (Sigma-Aldrich, St. Louis, MO), recombinant mouse epiregulin (100 ng/ml) (Sigma-Aldrich), recombinant human transforming growth factor-α (TGFα; 100 ng/ml) (Sigma-Aldrich), recombinant human insulin-like growth factor 1 (IGF1; 100 ng/ml) (Sigma-Aldrich), insulin (solution from bovine pancreas; 3 µg/ml) (Sigma-Aldrich), recombinant human brain-derived neurotrophic factor (BDNF; 100 ng/ml) (EMD Millipore, Burlington, MA), recombinant human ciliary neurotrophic factor (CNTF; 100 ng/ml) (EMD Millipore), mouse nerve growth factor 2.5S (NGF 2.5S; 100 ng/ml) (EMD Millipore), recombinant mouse leukemia inhibitory factor (LIF; 50 ng/ml) (EMD Millipore), synthetic glucagon-like peptide 2 (10 µM) (Abbiotec, San Diego, CA), human growth hormone (HGH; 100 ng/ml; from pituitary gland) (EMD Millipore), recombinant human neurotrophin-3 (NT-3; 100 ng/ml) (R&D Systems), synthetic human peptide YY (100 nM) (Sigma-Aldrich), recombinant human glial cell line-derived neurotrophic factor (GDNF; 50 ng/ml) (Sigma-Aldrich), recombinant human hepatocyte growth factor (HGF; 100 ng/ml) (Sigma-Aldrich), human calcitonin gene-related peptide (CGRP; 1 µg/ml) (Sigma-Aldrich), synthetic human somatostatin (1 µM) (AbD Serotec, Raleigh, NC), and human neuregulin 1 (NRG1; 10 nM) (R&D Systems). Firefly luciferase activity was determined by following the manufacturer's instructions using the Bright-Glo luciferase assay system (Promega) in a GloMax 96

microplate luminometer (Promega). Relative light units (RLU) in trophic factor-treated cells were normalized to levels for untreated cells.

Transcription factor binding motif analysis. Potential transcription factor binding sites in the human *GALGT2* promoter were analyzed using TFBIND gene tool software (<http://tfbind.hgc.jp/>) with a cutoff value of 0.85 (40).

Ad-mediated gene overexpression. Ads containing human *FOXD3* (NM_012183), *LMO2* (BC034041), *TFAP4* (BC010576), *MYOG* (BC053899), or GFP were purchased from Applied Biological Materials (Richmond, BC, Canada). Ad was amplified in HEK293 cells per the manufacturer's instructions. Expression of Ad-GFP in C2C12 myotubes was used to determine the necessary multiplicity of infection (MOI) to attain >80% infection efficiency without cell death and to serve as a negative control in reporter assays. Following 4 days of differentiation, Ad was added to *GALGT2* promoter luciferase reporter C2C12 cultures stably expressing either the bp –950 or the –950Δ2 *GALGT2* promoter construct in serum-free medium at MOIs of 2, 4, 8, 16, 32, and 64 for 48 h in the presence or absence of sHB-EGF. Firefly luciferase activity was determined by following the manufacturer's instructions using Steady-Glo luciferase assay systems (Promega, Madison, WI) in a GloMax 96 microplate luminometer. An MOI of 32 was required by all Ad constructs, except Ad-*FOXD3* (MOI of 16), to generate maximal firefly luciferase activity without toxicity.

ChIP analysis. C2C12 myoblasts expressing the bp –5870 *GALGT2* promoter firefly luciferase reporter were plated in 10-cm dishes and differentiated in fusion medium for 6 days as described above. Once differentiated into myotubes, cells were treated with human recombinant sHB-EGF protein for 24 h or 48 h. Cross-linking and harvesting of cells was performed using the Magna ChIP A/G kit from EMD Millipore per the manufacturer's instructions. Chromatin was sheared using 8 cycles of 3 s on/15 s off at power setting 0.5, repeated 7 times on a Misonix sonicator 3000 (Misonix, Farmingdale, NY), to generate DNA fragments with a mean size between 200 and 1,000 bp. Sonicated DNA was precleared and then incubated with LMO2 antibody (sc-10497; Santa Cruz Biotechnology), TFAP4 antibody (HPA001912; Sigma-Aldrich), or IgG control (12-371; EMD Millipore) overnight at 4°C by following Magna ChIP A/G protocol instructions (EMD Millipore). Quantitative PCR (qPCR) in a 7500 real-time PCR system (Applied Biosciences, Foster City, CA) with SsoAdvanced universal SYBR green supermix (Bio-Rad, Hercules, CA) was used to quantify DNA fragments pulled down with the antibodies compared to evaluation of input DNA, which was not incubated with antibodies. Primers were created to stably include a region along the *GALGT2* promoter that was 150 to 250 bp in length: bp –951 to –726 (forward, 5'TTAAAGATTCTTGCCGGTCGTG; reverse, 5'CGCTCAAATGATTCTCCACCT).

siRNA knockdown of transcription factors. C2C12 myoblasts expressing the bp –5870 *GALGT2* promoter firefly luciferase reporter were plated in either 96-well or 6-well dishes and differentiated in fusion medium for 6 days as described above. Once differentiated, cells were treated with recombinant human sHB-EGF and/or siRNA targeting transcription factors in serum-free DMEM for 48 h. siRNA was added 24 h prior to the addition of sHB-EGF at doses of 5 nM, 10 nM, or 20 nM and included siRNA against mouse LMO2 (SR403448), TFAP4 (SR411368), or a universal scrambled negative control (OriGene, Rockville, MD). Treated cells were monitored closely to obtain maximal knockdown of targeted transcription factor without resulting in cell death. *GALGT2* promoter activation was measured by reporter firefly luciferase activity by following the manufacturer's instructions using Steady-Glo luciferase assay systems (Promega, Madison, WI) in a GloMax 96 microplate luminometer. Cell lysates were also collected to measure the resulting mRNA expression of targeted transcription factors to ensure siRNA selectivity and activity by semiquantitative reverse transcription-PCR (qRT-PCR).

sHB-EGF inhibitor screen. C2C12 myoblasts expressing the bp –5870 *GALGT2* promoter firefly luciferase reporter were plated in 96-well plates, becoming confluent after 24 to 48 h. At this time, cells were switched to fusion medium for 2 days, followed by 2 days in fusion medium that had been precleared with heparin-agarose beads (Sigma-Aldrich) to remove endogenous heparin-binding proteins. Cells were then treated with recombinant human sHB-EGF and/or inhibitors of signaling proteins in serum-free DMEM for 24 to 48 h. Inhibitors were added 1 h prior to the addition of sHB-EGF at a range of pharmacologically relevant doses and included EGF receptor (EGFR) inhibitor (tyrphostin AG-1478; 200 nM) (Cell Signaling, Danvers, MA), phosphoinositide 3-kinase (PI3K) inhibitor (LY294002; 5 μM) (EMD Millipore), protein kinase B (Akt) inhibitor (MK-2206; 5 μM) (Selleck Chemicals, Houston, TX), mammalian target of rapamycin (mTOR) inhibitor (rapamycin; 100 nM) (EMD Millipore), Raf kinase inhibitor (vemurafenib/PLX4032/RG7204; 3 μM) (Selleck Chemicals), extracellular signal-regulated kinase (ERK) inhibitor (SCH772984; 10 μM) (Selleck Chemicals), Src inhibitor (KX2-391; 5 nM) (Selleck Chemicals), Bcr/Abl inhibitor (nilotinib/AMN-107; 1 μM) (Selleck Chemicals), STAT5 inhibitor (25 μM) (EMD Millipore), JNK inhibitor (SP600125; 25 μM) (EMD Millipore), or c-myc inhibitor (10058-F4/CAS 403811-55-2; 10 μM) (EMD Millipore). Drug concentrations shown provided maximum levels of inhibition without causing cell death. Firefly luciferase activity was determined by following the manufacturer's instructions using Steady-Glo luciferase assay systems (Promega, Madison, WI) in a GloMax 96 microplate luminometer.

Western blot analysis. (i) Muscle cells. C2C12 myoblasts were plated in 12-well plates and differentiated as described above. Cells were then either left untreated or treated with 10 nM recombinant human sHB-EGF in serum-free DMEM for 15, 30, or 60 min. Medium was then removed and cells were rinsed briefly with cold PBS. Cells were incubated for 30 min at 4°C in lysis buffer containing 1% NP-40, 50 mM Tris, 140 mM NaCl, 1 mM EDTA, cOmplete EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN), and PhosSTOP phosphatase inhibitors (Roche). Cell lysates were then collected using cell scrapers, rocked gently at 4°C for 45 min, and centrifuged at 21,000 × *g* at 4°C for 10 min. Supernatant was collected, and the resulting protein concentration was measured by bicinchoninic acid assay (Thermo Fisher Scientific). Protein (25 μg) was boiled at 100°C for 10 min in NuPAGE LDS sample buffer (Thermo Fisher Scientific) containing 0.1 M β-mercaptoethanol (BME; Fisher Scientific), separated on a

Bolt 4 to 12% Bis-Tris plus gel (Thermo Fisher Scientific), and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 and then probed overnight with antibodies to EGFR (2232; Cell Signaling), phosphorylated EGFR (Tyr1173) (53A5) (4407; Cell Signaling), Akt (9272; Cell Signaling), phosphorylated Akt (Ser473) (9271; Cell Signaling), mTOR (2972; Cell Signaling), phosphorylated mTOR (Ser2448) (2971; Cell Signaling), MEK1/2 (9122; Cell Signaling), phosphorylated MEK1/2 (Ser217/221) (41G9) (9154; Cell Signaling), Erk1/2 (p44/42 mitogen-activated protein kinase [MAPK]) (137F5) (4695; Cell Signaling), phosphorylated Erk1/2 (p44/42 MAPK) (Thr202/Tyr204) (9101; Cell Signaling), C-RAF (9422; Cell Signaling), phosphorylated C-RAF (Ser289/296/301) (9431; Cell Signaling), Src (36D10) (2109; Cell Signaling), phosphorylated Src (Tyr416) (D49G4) (6943; Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; G9545; Sigma-Aldrich, St. Louis, MO). After washing, blots were probed with horseradish peroxidase-coupled secondary antibody (Jackson ImmunoResearch Laboratories), washed again, and developed using an enhanced chemiluminescence (ECL; Lumigen, Southfield, MI) method according to the manufacturer's instructions.

(ii) Muscle tissue. Frozen skeletal muscle blocks were cut on a cryostat (20 μ m/section), and muscle tissue was digested in lysis buffer (mentioned above), also using metal lysis beads with shaking four times at 30 Hz for 30 s each time (TissueLyser II; Qiagen). Samples were then rocked gently at 4°C for 3 h and centrifuged at $21,000 \times g$ at 4°C for 10 min. The supernatant was collected, and protein (80 μ g) was prepared and immunoblotted using antibodies mentioned above, except for EGFR (A-10) (sc-373746; Santa Cruz Biotechnology).

(iii) Band quantification. ImageJ 1.46r software (National Institutes of Health, Bethesda, MA) was used to analyze relative band density in both cell culture and skeletal muscle samples using methods we have previously described (100). Phosphorylated protein expression was normalized to total protein expression and then compared to levels for untreated control lysates. Total protein expression was normalized to the loading control (GAPDH) and again compared to untreated control lysates.

AAV-induced overexpression of HB-EGF and sHB-EGF. The gastrocnemius muscle on the left side of 5-week-old male C57BL/6J mice was injected with 5×10^{10} vector genomes of r(sc)AAV9.CMV.HB-EGF or r(sc)AAV9.CMV.sHB-EGF in a volume of 50 μ l sterile PBS using a 0.3-ml insulin syringe near the midpoint of the muscle. Muscles on the contralateral (right) side of the mouse were mock injected with an identical volume of sterile PBS. At 4 or 12 weeks postinjection, mice were sacrificed and dissected. Gastrocnemius muscles were embedded in optimal cutting temperature (O.C.T.) compound (Fisher Scientific, Pittsburgh, PA) and snap-frozen in liquid nitrogen-cooled isopentane.

qPCR of AAV vector genomes. TaqMan qPCR was used to quantify AAV vector genome copies in treated gastrocnemius muscles and saline-treated control muscles. Frozen skeletal muscle blocks were cut on a cryostat (20 μ m/section), and DNA was extracted from these shavings using the Qiagen DNeasy blood and tissue kit (Germantown, MD). DNA purity and quantity were measured using an ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific). qPCR was performed in a 7500 real-time PCR system with TaqMan gene expression master mix (Thermo Fisher Scientific). A vector-specific primer/probe set (forward, 5'ATCCGGTGGTGGTGCAGT; probe, 5'/56-FAM/TCCTCAGTG/ZEN/GATGTTGCCTT/3IABkFQ; reverse, 5'CAGCAGCTTCATACGGCC) was used to amplify a portion of the vector DNA encompassing the 3' end of the SV40 intron and the 5' end of the human *HBEGF* cDNA. No amplification of the endogenous mouse *Hbegf* was observed in control samples using this primer/probe set. The plasmid used to make r(sc)AAV9.CMV.sHBEGF was linearized with ClaI, repurified, and utilized to generate a standard curve from 50 to 5 million copies in log increments. The correlation coefficient of the standard curve always equaled or exceeded 0.99. Copy number was reported as vector genomes per microgram of genomic DNA assayed. Each sample was measured in duplicate, and values for each cohort were averaged.

qRT-PCR. Relative mRNA transcript levels were assessed by qRT-PCR using the $2^{-\Delta\Delta CT}$ method for both C2C12 cells and skeletal muscle (101). C2C12 myotube cultures were rinsed in ice-cold PBS and then collected in TRIzol (Thermo Fisher Scientific) using cell scrapers. Frozen skeletal muscle blocks were cut on a cryostat (20 μ m/section). Total RNA from cells or tissue was extracted, purified, and reverse transcribed as previously described (100). Samples underwent qRT-PCR in a 7500 real-time PCR system with TaqMan gene expression master mix, using eukaryotic 18S rRNA (Thermo Fisher Scientific) as an internal control. Primer/probe sets were designed (PrimerQuest DNA software) and synthesized (Integrated DNA Technologies, Coralville, IA) to recognize *Homo sapiens HBEGF* (forward, 5'GTGAC TTGCAAGAGGCAGAT; probe, 5'/56-FAM/TATCCTCCA/ZEN/AGCCACAAGCACTGG/3IABkFQ; reverse, 5'GTGCTCCTCTGTTTGGT), *Mus musculus Hbegf* (forward, 5'AGGACTTGAAGGGACAGA; probe, 5'/56-FAM/AGTTGCTTT/ZEN/CTCCTCCAAGCCACA/3IABkFQ; reverse, 5'CCCATTCTTTCTTTGCTTGG), and *Mus musculus Galnt2 (B4Galnt2)* (forward, 5'GCCCACGAGAGATTCTTATT; probe, 5'/56-FAM/AT AATTACA/ZEN/CCCGGGCAAGAGCCC/3IABkFQ; reverse, 5'TCCTTTGGTGGTGTCTTACTT). Premade primer/probe sets for *Mus musculus Egfr* (Mm.PT.58.28853978), *ErbB2* (Mm.PT.58.29549739), *ErbB3* (Mm.PT.30061803), *ErbB4* (Mm.PT.58.16591892), and *Akt1* (Mm.PT.58.8333433) were purchased from Integrated DNA Technologies. *Mus musculus* primers for *Lama2*, *Lama4*, *Lama5*, *Itga7*, *Itgb1*, *Dmd*, *Dag1*, *Utrn*, and *Agrn* were purchased and used as previously described (10). Probes for human transcription factor genes *TFAP4* (Hs01558245_m1), *LMO2* (Hs00153472-m1), *FOXD3* (Hs00255287_s1), and *MYOG* (Hs01072232_m1) were purchased from Applied Biosystems. Mouse *Myog* probes (Mm00446194_m1) were purchased from Applied Biosystems. Mouse *Tfap4* (Mm.PT.58.5559446) and *Lmo2* (Mm.PT.58.21550807) probes were purchased from IDT. Premade primer pairs for *Mus musculus Lmo2* (MP207520), *Tfap4* (MP217129), and *Foxd3* (MP204962) were purchased from OriGene (Rockville,

MD). Relative mRNA levels were averaged for each cohort and compared to levels for PBS-injected muscle.

Immunofluorescence staining of CHO cells and skeletal muscle. (i) CHO cells. CHO cells transfected with expression vectors for sHB-EGF or HB-EGF were grown on gelatin-coated glass coverslips using previously described methods (102). Cells were washed in PBS, fixed in 4% paraformaldehyde (PFA) for 10 min, and then fixed further with 4% PFA and 0.1% Triton X-100 for 5 min. After washing, cells were incubated with antibodies that recognize sHB-EGF (MAb 2591; R&D Systems), sHB-EGF and HB-EGF (PAB 259 NA; R&D Systems), or the intracellular C-terminal domain of HB-EGF protein (C18; Santa Cruz Biotechnology, Dallas, TX). After washing, cells were incubated with appropriate fluorophore-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), washed again, and then mounted in ProLong Gold antifade mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific).

(ii) Skeletal muscle. Unfixed frozen muscle blocks were cut on a cryostat into 8- to 10- μ m sections. To probe for HB-EGF, staining was performed as described above with MAb 2591 and costained with rhodamine-conjugated α -bungarotoxin (B35451; Invitrogen, Carlsbad, CA). To probe for Galgt2 and CT glycan, slides were blocked in 3% bovine serum albumin (BSA) and then stained with a monoclonal antibody to CT glycan (CT2) or a polyclonal antibody to Galgt2 (PAB20841; Abnova, Walnut, CA). After washing in PBS, sections were incubated with appropriate fluorophore-conjugated secondary antibodies and rhodamine-conjugated α -bungarotoxin. After staining, all sections were mounted as described above. For EGFR staining, sections were fixed as described above, blocked with Mouse-On-Mouse (M.O.M.) blocking reagent (Vector Labs, Burlingame, CA) followed by a second block with 3% BSA, incubated with a monoclonal antibody to EGFR (A-10) (sc-373746; Santa Cruz Biotechnology), washed, and incubated with fluorescein isothiocyanate-conjugated secondary antibody along with rhodamine-conjugated α -bungarotoxin.

All immunofluorescence staining was visualized with a Zeiss Axioskop2 plus epifluorescence microscope using fluorophore-specific filters, and representative images were captured with a Zeiss AxioCam MRCS camera (Carl Zeiss Microscopy, Jena, Germany). All images comparing individual stains were time matched using identical exposure settings across different experimental conditions.

Quantification of AChR microaggregates in skeletal muscle. Quantification of AChR microaggregates based on α -bungarotoxin staining of NMJs was done as previously described (46), using 8- μ m-thick cryostat cut sections of the tibialis anterior muscle. A total of 45, 37, or 75 synapses were analyzed for C57BL/6J, *Galgt2*^{-/-}, and *Hbgef*^{-/-} muscles, respectively.

Grip strength and ambulation treadmill. Forelimb and hindlimb grip strength were assessed in mice using a grip strength meter (Columbus Instruments, Columbus, OH). Measurements were averaged from 5 forelimb repetitions and 10 hindlimb repetitions each day; mice were tested once per day for a week, and the daily measurements were averaged to produce a final forelimb and hindlimb measurement. To measure ambulation, mice were run on a treadmill with a 10° decline (Treadmill Simplex II; Columbus Instruments). The mice ran for 1 min at 5 m/min, which increased by 1 m/min each minute until a maximum of 15 m/min and then remained at that speed for an additional 20 min. The time that the mice remained on the treadmill, up to a total time of 30 min, was recorded. After 2 days of training, mice were tested once per day for 5 days, and the daily measurements were averaged to produce a final measurement of treadmill running time before exhaustion.

Statistics. The significance in variance between cohorts was determined by one- or two-way analysis of variance (ANOVA), where appropriate, followed by Bonferroni's multiple-comparison test. Kaplan-Meier survival plots were analyzed via the log-rank test.

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