Limb-girdle muscular dystrophy type 2L (LGMD2L) is a myopathy arising from mutations in ANO5; however, information about the contribution of ANO5 to muscle physiology is lacking. To explain the role of ANO5 in LGMD2L, we previously hypothesized that ANO5-mediated phospholipid scrambling facilitates cell–cell fusion of mononucleated muscle progenitor cells (MPCs), which is required for muscle repair. Here, we show that heterologous overexpression of ANO5 confers Ca^{2+}-dependent phospholipid scrambling to HEK-293 cells and that scrambling is associated with the simultaneous development of a nonselective ionic current. MPCs isolated from adult Ano5^{-/-} mice exhibit defective cell fusion in culture and produce muscle fibers with significantly fewer nuclei compared with controls. This defective fusion is associated with a decrease of Ca^{2+}-dependent phosphatidylserine exposure on the surface of Ano5^{-/-} MPCs and a decrease in the amplitude of Ca^{2+}-dependent outwardly rectifying ionic currents. Viral introduction of ANO5 in Ano5^{-/-} MPCs restores MPC fusion competence, ANO5-dependent phospholipid scrambling, and Ca^{2+}-dependent outwardly rectifying ionic currents. ANO5-rescued MPCs produce myotubes having numbers of nuclei similar to wild-type controls. These data suggest that ANO5-mediated phospholipid scrambling or ionic currents play an important role in muscle repair.

Anoctamin 5/TMEM16E facilitates muscle precursor cell fusion

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Introduction

During the past several decades, advances in molecular genetics have greatly accelerated our capacity to identify variants linked to genetic diseases. However, elucidating the processes that lead to pathology lags significantly behind our diagnostic abilities. The limb-girdle muscular dystrophies (LGMDs) are a case in point. In the last decade, 15 new genes have been linked to LGMDs, but how mutations in these genes contribute to their associated diseases remains incompletely understood.

Muscular dystrophies encompass a heterogeneous group of pathologies characterized by progressive skeletal muscle weakness and atrophy. There are now at least 34 genes that have been linked to LGMDs that primarily affect hip or shoulder, girdle, and limb muscles. Here, we focus on LGMD type 2L (LGMD2L), an autosomal-recessive LGMD that is characterized by late onset (onset range, 11–50 yr) with myalgia that is commonly associated with exercise intolerance, progressive muscle weakness/atrophy, and elevated serum creatine kinase (Liewluck and Milone, 2018). LGMD2L was linked to mutations in ANO5 (TMEM16E) in 2010 (Bolduc et al., 2010; Mahjneh et al., 2010; Hicks et al., 2011), but how ANOS participates in muscle function remains a mystery.

Many LGMD genes encode proteins that are involved in maintaining muscle structural integrity. For example, the most common LGMDs, the α-dystroglycanopathies (10 LGMD2-linked genes), are caused by defects in proteins linking the muscle cytoskeleton to the extracellular matrix through the sarcolemma. When this support system is perturbed, it renders the muscle sarcolemma more susceptible to mechanical damage (Endo, 2015). Other LGMD genes, such as those encoding dyserfin (LGMD2B) and caveolin (LGMD1C), are thought to play roles in membrane repair (Bansal and Campbell, 2004; Corrotte et al., 2013). Because muscle is subjected to physical stress during activity, highly sophisticated processes exist to repair damage and regenerate injured muscle (Han and Campbell, 2007; Dumont et al., 2015). Previous work has suggested that mutations in ANOS alter muscle repair processes (Jaiswal et al., 2007; Bolduc et al., 2010; Griffin et al., 2016). For example, we previously characterized an Ano5^{-/-} knockout mouse that recapitulates many features of LGMD2L and demonstrated that this mouse exhibits its defective muscle repair processes, both in vivo and in vitro (Griffin et al., 2016). One mechanism by which muscle fibers are repaired involves fusion of mononucleated muscle progenitor cells (MPCs) with damaged muscle. Just as multinucleated muscle fibers are formed during embryogenesis by the fusion of hundreds to thousands of MPCs (Abmayr and Pavlath, 2012), this process is recapitulated during muscle repair and regeneration as MPCs are recruited to fuse with and repair torn fibers or to...
form new muscle fibers (Collins et al., 2005; Glover and Brown, 2007; Gurevich et al., 2016).

The repair of multinucleated muscle fibers by fusion of mononucleated MPCs involves several steps, including proliferation and differentiation of fusion-competent cells, migration of cells to the site of fusion, recognition and adhesion of fusion-competent cells, and finally cell fusion. We suggest that ANO5 plays a role in regulating or coordinating this process during regenerative muscle repair.

Initially, it was thought that ANO5 was an ion channel because the founding members of the 10-gene ANO/TMEM16 family are Ca$^{2+}$-activated Cl$^-$ channels (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008; Pedemonte and Galietta, 2014; Picollo et al., 2015; Whitlock and Hartzell, 2017). However, it is now recognized that many ANO paralogs are not Cl$^-$ channels but have other functions, most notably Ca$^{2+}$-activated phospholipid scrambling (Ca$^{2+}$-PLS; Suzuki et al., 2010; Malvezzi et al., 2013; Brunner et al., 2014; Whitlock and Hartzell, 2017). ANO6 was the first ANO found to exhibit phospholipid scrambling (PLSase) activity, but more recently, two ANO homologues from fungi were found to be PLSases when purified and reconstituted into liposomes (Malvezzi et al., 2013; Brunner et al., 2014; Pelz et al., 2018). One of these scramblases has been crystallized (Brunner et al., 2014), which has greatly informed efforts to elucidate how its functions (Bethel and Grabe, 2016; Jiang et al., 2017). While initial reports suggested that ANO5 was not a PLSase (Suzuki et al., 2013), other evidence suggests that it might elicit Ca$^{2+}$-PLS in certain biological contexts (Gyebu et al., 2015). Moreover, recent evidence has suggested that ANO5-dependent PLS might play a role in the development of gnathodiaphyseal dysplasia (Di Zanni et al., 2018).

PLS is used by many cells as a means of cell–cell communication during a variety of biological processes, including cell fusion (Bevers and Williamson, 2010). The plasma membrane (PM) is composed of two lipid monolayers that exhibit tightly regulated asymmetric lipid organization, with the extracellular leaflet enriched in phosphatidylcholine and sphingomyelin and an intracellular leaflet enriched in phosphatidyserine (PtdSer), phosphatidylinositides, and phosphatidylethanolamine (reviewed in van Meer et al., 2008). When activated by increases in cytosolic Ca$^{2+}$, PLSases form a hydrophilic pathway through the membrane, facilitating the diffusion of polar lipid head groups nonselectively between membrane leaflets, resulting in the loss of leaflet asymmetry (Pomorski and Menon, 2006; Brunner et al., 2016). The loss of this asymmetry has a variety of biophysical consequences, including altered lipid packing and lateral pressure between lipid head groups (Whitlock and Hartzell, 2017). In addition, PLS exposes PtdSer and phosphatidylethanolamine to the extracellular face, where they are recognized by a variety of both soluble and membrane-bound receptors that elicit diverse intracellular signaling cascades in neighboring cells (Bevers and Williamson, 2016). PtdSer exposure via PLS is spatiotemporally linked to muscle fusion (van den Eijnde et al., 1997, 2001; Jeong and Conboy, 2011), both during development and regeneration, and it has been suggested that PtdSer exposure is a signal for cell–cell fusion. Several PtdSer receptors have recently been identified for their role in regulating the fusion of MPCs; Hochreiter-Hufford et al., 2013; Hamoud et al., 2014; Kim et al., 2016; Park et al., 2016).

Here we investigate the function of the ANO5/TMEM16E protein and explore how loss of Ano5 affects muscle cell biology. We begin by studying the function of ANO5 in a heterologous expression system. We find that exogenous ANO5 confers Ca$^{2+}$-PLS to HEK-293 cells. Moreover, we find that ANO5-dependent Ca$^{2+}$-PLS is associated with the development of an ionic conductance. This conductance, like that described for ANO6 and the fungal ANO scramblases atTMEM16 and nhTMEM16 (Malvezzi et al., 2013; Yu et al., 2015; Lee et al., 2016), is ion nonspecific. Next, we investigated the Ca$^{2+}$-PLS activity of fusogenic, primary MPCs and find that the loss of Ano5 perturbs both the Ca$^{2+}$-dependent exposure of PtdSer and PLS-associated ionic currents. Introducing exogenous ANO5 restores Ca$^{2+}$-PLS in Ano5$^{-/-}$ MPCs and rescues the coordination of the fusion in these cells producing muscle fibers with significantly increased myonuclear number. We believe this work suggests a role for ANO5-dependent Ca$^{2+}$-PLS in the coordination of proper MPC fusion to produce multinucleated skeletal muscle fibers.

Abstracts of this work have been published previously (Whitlock et al., 2015, 2016).

**Materials and methods**

**Surface biotinylation**

Surface proteins were covalently biotinylated by 0.5 mg/ml Sulfo-NHS-LC biotin (sulfo succinimidyl-6-[biotin-amido]hexanoate, Pierce) for 40 min on ice. The reaction was quenched with 20 mM glycine for 30 min on ice. Protein lysates were collected in RIPA buffer (TEKNOVA) with protease inhibitors, and one-fifth soluble protein lysate was reserved to evaluate “total” protein. The remaining lysate was incubated with streptavidin-conjugated beads overnight at 4°C to capture biotinylated proteins. Total and biotinylated surface membrane protein pools were evaluated via Western blot.

**Western blot**

Steady-state protein levels were evaluated via SDS-PAGE followed by immunoblot. Antibodies used were mouse anti-ANO clone 85.1 (1:250, Q4KM2; UC-Davis Neuromab, Antibodies Inc.), mouse anti-FLAG (1:1,000, F1804; Sigma), anti-ANO6 (1:1,000, PA5-58610; Invitrogen), and mouse anti-GAPDH (1:1,000, MAB374; Millipore).

**Cell culture**

T-REx-293 cells (Invitrogen) or HEK293 cells (ATCC) were maintained in modified, high-glucose DMEM (supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin). Primary mouse myoblasts were isolated as described previously (Griffin et al., 2016) from 3-mo-old Ano5$^{-/-}$ or age matched wild-type C57BL/6j mice. Myoblasts were maintained in growth media (Ham’s F10 media [Invitrogen] supplemented with 20% FBS, 5 ng/ml basic fibroblast growth factor, 100 U/ml penicillin G, and 100 µg/ml streptomycin) on bovine collagen–coated plates (Gibco).

**cDNA constructs**

mANO6 (Uniprot: Q6P9J9) was tagged on the C terminus with Clover fluorescent protein and inserted into the pcDNA5/TO

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plasmid (Invitrogen). A codon-optimized cDNA for hANOS (Uniprot: Q7SV66) was synthesized by DNA2.0 (Newark, CA) and tagged on the C terminus with Clover or 3XFLAG and inserted into the pcdNA5/TO plasmid (Invitrogen). For designing chimeras, mANO1 and hANOS cDNAs were aligned using MUSCLE (McWilliam et al., 2013). Chimeras were constructed using overlap extension PCR (Pont-Kingdom, 1997). Chimeras are named X-Y-X, where X is the ANO paralog template whose amino acids are replaced with the aligned amino acids from ANO paralog Y. For ANO151, amino acids 554–588 of ANO1 were replaced with amino acids 530–564 from ANO5, and for ANO515, amino acids 530–564 of ANOS were replaced with amino acids 554–588 from ANO1. PCR primers were designed to engineer complementary overlapping sequences onto the junction-forming ends of PCR products that were subsequently assembled by PCR. PCR-based mutagenesis was used to generate mutations in one or a few amino acids. The protein coding region of all chimeras and mutants were sequenced. Plasmids were introduced into T-REX-293 cells using Lipofectamine 2000, and stable incorporation was selected using hygromycin B (Invitrogen) and blasticidin (Sigma) overnight. Cells were selected using hygromycin B (Invitrogen) and blasticidin (Sigma) according to the manufacturer’s protocol. Protein expression was induced by supplementing cell culture media with 2 µM tetracycline (Sigma) overnight.

**PLS assay**

PLS was assessed by live-cell imaging using the PtdSer binding proteins annexin V–Alexa Fluor 568 or LactC2-mCherry and –Clover, as described previously (Yu et al., 2015). Briefly, cells were washed in nominally Ca2+-free PLS solution (140 mM NaCl, 5 mM KCl, 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM Ca2+–EGTA, 10 mM sucrose, 10 mM HEPES, pH 7.4). For determining ionic selectivity, 140 mM NaCl in the standard extracellular solution was replaced with the indicated concentrations of NaCl, CsCl, or NMDG–Cl as indicated, and the internal solution contained (in mM) 150 NaCl (or CsCl), 1 MgCl2, 5 Ca-EGTA, 0.2 CaCl2, and 1 HEPES, pH 7.4. The osmolarity of each solution was adjusted to 300 mOsm by addition of mannitol. Relative permeabilities of cations relative to Cl− were determined by measuring the changes in zero-current Erev using the Goldman–Hodgkin–Katz equation when the concentration of extracellular ions were changed (dilution potential method) as previously described (Yu et al., 2012):

\[
\Delta E_{rev} = 25.7 \ln \left( \frac{X_0 + Cl^{-} \cdot P_{Cl} / P_{Na}}{X_i + Cl^{-} \cdot P_{Cl} / P_{Na}} \right),
\]

where X is the cation and ΔErev is the difference between Erev with the test solution XCl and that observed with symmetrical solutions. MPCs were evaluated in the same manner as HEK cells with an intracellular solution composed of 146 mM CsCl, 2 mM MgCl2, 5 mM Ca2+-EGTA, 10 mM sucrose, 10 mM HEPES, and 0.2 mM CaCl2, and an extracellular solution composed of 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 15 mM glucose, and 10 mM HEPES, pH 7.4.

**Imaging**

PLS was measured in populations of intact HEK293 cells grown on glass coverslips mounted in Attolfluor chambers (Invitrogen) and imaged at ambient temperature with a Zeiss confocal microscope using a 63× Plan-Apochromat NA 1.4 objective. Binding of annexin V–Alexa Fluor 568 to patch-clamped cells during voltage-clamp recording was imaged with a wide-field Zeiss Axiovert 100 microscope using a 40× NA 0.6 LD-Acroplan objective. Images were acquired with an Orca-Flash 4.0 digital CMOS camera (C11440; Hamamatsu) controlled by Metamorph 7.8 software (Molecular Devices). Images were analyzed using Fiji ImageJ 1.49.

**RNA expression analysis**

For real-time PCR, total RNA was collected from MPCs using PureLink RNA kit following the manufacturer’s instructions (Invitrogen). All RNA samples were treated with DNase I (Invitrogen) according to the manufacturer’s protocol to ensure purity. cDNA was generated from total RNA via reverse transcription using SuperScript III reverse transcription (Invitrogen) and random hexamer primers. cDNA was then amplified using the SYBR Select Master Mix reagent (Applied Biosystems) and 2.5 µM of each primer. Real-time PCR reactions were performed and analyzed with StepOnePlus Real Time PCR System (Applied Biosystems), using GAPDH as an internal control. Fold-change of
gene expression was determined using the ΔΔCt method (Livak and Schmittgen, 2001). Multiple independent experiments were performed and analyzed in duplicate. The following qPCR primers were used: synANO5, 5′-CAT GGA GCA CAA CAC CTC CT-3′ (forward), 5′-TTG AGT TCA GCC GCC AGT AG-3′ (reverse); mAno6, 5′-CTT ATC AGG AAG TAT TAC GGC-3′ (forward), 5′-AGA TAT CCA TAG AGG AAG CAG-3′ (reverse); mGapdh, 5′-GGG TCC CAG CTT AGG TTC AT-3′ (forward), 5′-TAC GGC CAA ATC CGT TCA CA-3′ (reverse).

Lentivirus
Synthetic hANO5 was subcloned into a modified version of the lentiviral transfer plasmid LJM1 (19319; Addgene; Sancak et al., 2008) under control of the PGK promoter and flanked by a viral 2A site and eGFP to mark infected cells. The PAX2 (12260; Addgene) packaging plasmid and pMD2.G envelope plasmid (12259; Addgene) were cotransfected into HEK293T cells using Lipofectamine 3000 (Invitrogen; manufacturer’s protocol) along with ANO5 transfer plasmid or scrambled transfer plasmid (1864; Addgene; Sarbassov et al., 2005), and viral supernates were collected at 24, 48, and 72 h after transfection. Viral supernates were filtered (0.45 µm), and lentiviral particles were concentrated using ultracentrifugation. Viral concentrations were estimated by ELISA for p24 to control for consistent infections between experiments and to calculate infectious units (IFUs; 632200; Takara Bio). MPCs were infected at low passage overnight in the presence of 8 µg/ml polybrene in growth media using indicated IFUs.

Results
ANO5 elicits PLS
ANO5 is very closely related to ANO6, with 48% identity in amino acid sequence (Whitlock and Hartzell, 2016). Because ANO6 elicits Ca2+-PLS (Suzuki et al., 2010; Yu et al., 2015), we hypothesized that ANO5 is a PLSase and that perturbations in this activity are associated with changes in skeletal muscle function that contribute to the progression of LGMD2L. To test whether ANO5 is a PLSase, we measured the ability of ANO5 overexpression to confer Ca2+-PLS in HEK-293 cells. We employed HEK293 cells as a model because (1) do not natively express ANO5; (2) exhibit low endogenous Ca2+-PLS activity, as described previously (Yu et al., 2015); and (3) are a good model for measuring ion channel conductances associated with Ca2+-PLS. Previous studies have suggested that ANOS is located in intracellular organelles (Mizuta et al., 2007; Duran et al., 2012) and does not mediate PM scrambling (Suzuki et al., 2013). To confirm that ANOS is present on the cell surface, surface proteins on HEK293 cells transfected with ANOS-3×FLAG were biotinylated using membrane-impermeant Sulfo-NHS-LC-biotin. Bi-
otinylated surface proteins were captured on streptavidin beads, run on SDS-PAGE gels, and Western blots probed with anti-FLAG antibody (Fig. 1b). We found that a small fraction of ANO5 trafficked to the PM. Although ANO5 trafficking in HEK293 cells may not be representative of ANO5 trafficking in muscle, it provides a system to investigate the function of ANO5.

PtdSer exposure was monitored by binding of annexin V–Alexa Fluor 568 in response to elevation of intracellular Ca\textsuperscript{2+} by ionophore-stimulated store-operated Ca\textsuperscript{2+} entry (see Materials and methods; Fig. 1a). Ca\textsuperscript{2+} stimulation elicited PtdSer exposure in the vast majority of cells expressing exogenous ANO5 in contrast to parental HEK293 cells, which do not exhibit this activity (Fig. 1a). ANO5-mediated PLS developed at a rate slightly slower than that produced by ANO6, but both elicited maximal PtdSer exposure in ~10 min (Fig. 1d). We confirmed that the observed PtdSer exposure was Ca\textsuperscript{2+} dependent by using the PtdSer probe LactC2-Clover, which, unlike annexin V, does not require exogenous Ca\textsuperscript{2+} as a cofactor for binding. Treating transiently transfected ANO5-3×FLAG cells with ionophore-stimulated store-operated Ca\textsuperscript{2+} entry (Fig. 1c) did not expose PtdSer as measured by LactC2-Clover binding (Fig. 1d), but subsequent addition of Ca\textsuperscript{2+} rapidly exposed PtdSer. Thus, ANO5-dependent PtdSer exposure requires Ca\textsuperscript{2+}.

**ANO5 PLS is associated with nonselective ionic currents**

We have previously shown that ANO6 generates ionic currents that develop in parallel with PLS (Yu et al., 2015) and have suggested that this current represents ions that are conducted through the same pathway that conducts lipids (Whitlock and Hartzell, 2016, 2017; Jiang et al., 2017). To determine whether ANO5 also generates ionic currents, we performed whole-cell patch clamp of ANO5-expressing HEK293 cells while simultaneously measuring PLS by annexin V–Alexa Fluor 568 binding. PLS development proceeded at a rate similar to that of ANO6 (Fig. 2a, d). Both PLS and ionic currents developed slowly after elevation of cytosolic Ca\textsuperscript{2+} (Fig. 2a). In general, both current and annexin V binding began to increase several minutes after establishment of whole-cell recording and reached a plateau within 15 min. This is very similar to our previous observations of ANO6 (Yu et al., 2015). Under these patch-clamp conditions, ~90% of ANO5-expressing cells scramble, while 25% of untransfected cells scramble (Fig. 2c). Currents generally exhibited linear I–V relationships (Fig. 2c) with slow activation at positive voltages and some deactivation at very negative voltages.

Ionic selectivity of ANO5 currents was determined by changing extracellular salt concentration, measuring the shift in reversal potential, and calculating the relative ionic permeabilities with the Goldmann–Hodgkin–Katz equation (Barry, 2006). A switch from symmetrical CsCl solutions on both sides of the membrane to a 10-fold lower concentration of extracellular CsCl results in only a modest ~10-mV negative shift in the current’s reversal potential (Fig. 3a). This corresponds to a P\textsubscript{Cs}/P\textsubscript{Cl} permeability ratio of 1.6 (Fig. 3b). A similar value was found for P\textsubscript{Na}/P\textsubscript{Cl} (Fig. 3b). These data suggest that the ANO5 pore is weakly cation
selective. Further, the pore appears to be relatively large, because it is also permeable to the large organic cation NMDG+, which has a mean diameter of >7 Å. These results are in sharp contrast to ANO1, which is highly Cl− selective (Fig. 3 b).

To verify that the currents and PLS were mediated by ANO5 and not caused by up-regulation of another endogenous scramblase, we mutated the scramblase domain of ANO5. We previously identified a 34-amino-acid sequence that we call the scrambling domain (SCRD) in ANO6 that is necessary for Ca2+-PLS (Yu et al., 2015). This sequence is highly conserved in ANO5. When the SCRD domain of ANO5 was replaced with the homologous domain from ANO1 (“ANO515”), PLS and the development of nonselective, scrambling-associated currents were greatly reduced (Fig. 3 c). In contrast, when the ANO5 SCRD was swapped into ANO1 (“ANO151”), it gave ANO1 the ability to scramble lipids and was associated with the development of nonselective, scrambling-associated currents. These results confirm that scrambling in ANO5-transfected HEK293 cells is dependent on ANO5.

MPC fusion and PtdSer exposure is defective in Ano5−/− MPCs

Having shown that ANO5 overexpression confers both PLS and ion currents to HEK293 cells, we then asked whether the loss of ANO5 in MPCs is associated with loss of Ca2+-PLS and associated ionic currents. Previously, we characterized an Ano5−/− mouse model (Griffin et al., 2016) that was created by inserting a “gene trap” exogenous splice acceptor site followed by a premature stop codon between exons 8 and 9 (Fig. 4 a). A similar premature termination of ANO5 is associated with most LGMD2L patients (c.191dupA) and is considered a LGMD2L founder mutation (Hicks et al., 2011). This mouse model recapitulates many aspects of LGMD2L and demonstrates defective cell–cell fusion of isolated MPCs (Griffin et al., 2016). This model exhibits a loss of ANO5 protein in two tissues (skeletal muscle and testis) that typically express high steady-state levels of ANO5 (Fig. 4 b).

MPCs were isolated from the hindlimbs of adult animals and patch-clamped with an internal (pipette) solution containing 200 µM free Ca2+ (Fig. 5 a). Ionic currents were measured by voltage steps, and PtdSer exposure was simultaneously measured by annexin V–Alexa Fluor 568 binding. A majority (>90%) of differentiated wild-type Ano5+/+ MPCs expose PtdSer on the external surface and develop ionic currents characteristic of ANO5 currents after elevating cytosolic Ca2+ by establishing whole-cell recording with Ca2+ in the patch pipette (Fig. 5, a and b). Current amplitude and annexin V–Alexa Fluor 568 binding increased in parallel. The currents had biophysical properties similar to currents in ANO5-transfected HEK293 cells (Fig. 1): they had linear

Figure 3. ANO5-PLS associated ionic currents are nonselective. (a) Representative whole-cell I–V curves recorded from ANO5-expressing HEK293 cells with 200 µM free Ca2+ in the pipette with external solutions containing 150 mM (black) or 15 mM (red) CsCl. Currents were generated by ramps from -100 to +100 mV. (b) Cation permeability of ANO5 and ANO1 currents relative to Cl− (Pc/Pc) were calculated by changes in reversal potential as shown in panel a. Numbers in or above column indicate the number of cells assayed. Differences between ANO1 and ANO5 were evaluated for each condition using a paired Student’s t test (**, P = 0.0012; ****, P = 0.0001). Differences in cation permeability for ANO5 were compared using a one-way ANOVA (P = 0.2693). (c) Ionic current amplitudes (black) and PLS (red) of HEK293 cells expressing ANO1, ANO5, and ANO1 harboring the ANO5 SCRD (ANO151) and ANO5 harboring the ANO1 SCRD ANO515. Differences in cells scrambling versus not scrambling were compared using a Fisher exact test (**, P = 0.0021; ****, P < 0.0001; ns, P = 0.5658). Numbers below indicate the number of cells assayed. Error bars indicate SEM.

Figure 4. Exogenous “gene trap” knock-in results in loss of ANO5. (a) Ano5 knockout construct. Briefly, a “gene trapping” element was inserted between exons 8 and 9 consisting of an exogenous splice acceptor followed by stop codons (Griffin et al., 2016). (b) Western blot of SDS-PAGE gel from lysates of quadriceps (Quad) or testis from 3-mo-old wild-type or Ano5 knockout mice.
I–V relationships and exhibited slow activation at positive voltages and some deactivation at very negative voltages. In contrast, the majority of Ano5−/− cells (~75%) did not expose PtdSer when patch clamped with Ca2+ in the patch pipette and did not develop PLS-associated ionic currents (Fig. 5, a and b).

ANO5 is not the only scramblase expressed in MPCs. Most notably, MPCs also express significant levels of ANO6. We wondered whether the absence of PLS in Ano5−/− MPCs might be explained by down-regulation of ANO6 in addition to loss of ANO5 expression. However, Western blot showed that ANO6 levels were comparable in differentiated Ano5+/+ and Ano5−/− MPCs (Fig. 5 c). This result demonstrates that the absence of scrambling in Ano5−/− cells is not explained by changes in ANO6 steady-state levels. However, it raises the question why the ANO6 that is present does not mediate scrambling in the Ano5−/− cells (see Discussion).

MPC PLS and fusion are rescued by infection with ANO5 virus
If ANO5 elicits Ca2+-PLS and plays an important role in MPC fusion, then it should be possible to rescue the defective fusion of Ano5−/− MPCs by overexpressing human ANO5. Furthermore, this rescued fusion should be accompanied by rescued Ca2+-PLS and ionic currents. We chose culture conditions that generated robust myotube development following ~36 h of Ano5+/+ MPC differentiation (Fig. 6 a). The ability of cells to form multinucleated myotubes was measured by counting the number of nuclei (DAPI stained) per myotube (phalloidin stained) in Ano5+/+, Ano5−/−, and Ano5−/−/MPCs treated with lentivirus expressing a scrambled sequence or Ano5+/−. MPCs treated with lentivirus expressing a synthetic, codon-optimized hANO5 under control of the phosphoglycerate kinase promoter (Fig. 6 b). Previous investigations have demonstrated that lentivirus is robust in introducing stably integrated transgenes in MPCs (Li et al., 2005). Additionally, unlike promoters that are readily silenced via methylation in muscle (e.g., cytomegalovirus), the phosphoglycerate kinase promoter produces reliable, consistent expression of virally introduced transgenes in muscle cells (Brooks et al., 2004; Jackson et al., 2013). The images (Fig. 6 a), cumulative frequency plot (Fig. 6 e), and frequency histogram (Fig. 6 f) show that Ano5+/− MPCs formed myotubes having more nuclei compared with Ano5−/− MPCs. Most importantly, expression of hANO5 in the Ano5−/− MPCs significantly rescued the fusion defect. For example, only ~25% of the nuclei in wild-type MPCs were found in myotubes having less than five nuclei, but loss of Ano5 resulted in ~50% of the nuclei in myotubes with less than five nuclei (Fig. 6 d). Infection with hANO5 lentivirus significantly reduces the percentage of nuclei in myotubes with less than five nuclei from 50% to 26% (v1.3 infectious units [IFUs]) or 30% (v13 IFUs), very close to the wild-type value. The lentiviral introduction of hANO5 did not change the steady-state level of Ano6 transcript, so the effects of hANO5 rescue are not the result of simply up-regulating Ano6 (Fig. 6 c).

The rescue of fusion by expression of hANO5 was accompanied by the rescue of Ca2+-PLS in Ano5−/− MPCs. Although only ~25% of Ano5−/− MPCs expose PtdSer when stimulated with 200 µM free intracellular Ca2+, >80% expose PtdSer following hANO5 rescue (Fig. 7 a and b). Moreover, hANO5-rescued PLS is accompanied by a nonselective ionic current (Fig. 7 c). These Ca2+-stimulated currents were significantly larger when compared with currents in Ano5+/+ MPCs (P = 0.027; Fig. 7 d). Similar to ANO5-dependent currents in wild-type MPCs, ANO5 rescued currents exhibited a linear I–V relationship (Fig. 7 e).

Discussion
ANO5 is probably a PLSase
Here, we demonstrate that like ANO6, heterologous overexpression of ANO5 in HEK293 cells elicits Ca2+-PLS (Suzuki et al., 2010, 2013; Yu et al., 2015). Mutations in the ANO5 SCR domain destroy its scrambling activity, while this same region confers Ca2+-PLS to the calcium-activated chloride channel ANO1. Moreover, MPCs isolated from mice lacking Ano5 exhibit a significant loss of Ca2+-PLS that can be rescued with the introduction of exogenous

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ANO5. Although these results strongly suggest that ANO5 is a PLSase, this conclusion will remain tentative until the protein has been purified and reconstituted. A previous report suggested that transient transfection of cells with Ano5 did not support PLS (Suzuki et al., 2013). These results may differ from our findings for a number of reasons. While Suzuki et al. (2013) used a transiently transfected thymocyte cell model that expressed murine ANO5 at very low levels, we evaluated the Ca²⁺-PLS competence of a codon-optimized human ANO5 in a stably transfected human cell line and confirmed that ANO5 partially trafficked to the PM (see Materials and methods).

As has previously been described for other ANO scramblases (nhTMEM16, afTMEM16, and ANO6; Suzuki et al., 2010; Malvezzi et al., 2013; Brunner et al., 2014; Yu et al., 2015; Jiang et al., 2017), ANO5-dependent Ca²⁺-PLS is tightly associated with the simultaneous development of nonselective or weakly cation-selective transport through the plasma membrane. Furthermore, ANO5-dependent Ca²⁺-PLS is associated with a nonselective inward current that is partially blocked by the PLS inhibitor Arachidonyl trifluoroethylamide (AraT; Mazambane and Whitlock, 2015).

Figure 6. Exogenous ANO5 expression rescues Ano5−/− MPC fusion. (a) Representative images of MPC fusion after ~36 h differentiation for Ano5+/+, Ano5−/−, and Ano5−/− cells rescued with 1.3 IFUs of ANO5 virus. Scale bars, 300 µm. (b) Quantitative PCR evaluation of steady-state ANO5 transcript levels, following viral infection, normalized to Gapdh. Error bars indicate SEM. (c) Quantitative PCR evaluation of steady-state, endogenous Ano6 levels normalized to Gapdh. (d) Frequency of nuclei in fibers containing less than five nuclei. Ano5+/+ represents three independent experiments with three independently isolated cell lines (>6,500 nuclei). Ano5−/−, ANO5 1.3 IFU, and ANO5 13 IFU represent five independent experiments, with three independently isolated cell lines (>7,000 nuclei per condition). Error bars represent SEM. Significance was determined by one-way ANOVA with Dunnett correction (***, P = 0.0003; **, P = 0.0013). (e) Cumulative frequency of nuclei per fiber size. Nuclei per fiber distributions were evaluated by fitting the data for each replicate in each condition to a single exponential. The exponential constants for each condition were statistically compared to Ano5−/− using a one-way ANOVA with Dunnett correction (**, P = 0.0095 and *, P = 0.04). (f) Raw frequency histogram representation of data. The values on the x-axis represent the center of the bins for number of nuclei per fiber. Error bars represent SEM.
ionic currents that we believe may be a consequence of membrane disturbances created by lipid translocation.

**Loss of ANO5 is associated with defective MPC fusion**

The loss of ANOS-dependent Ca\(^{2+}\)-PLS and PLS-associated ionic currents is associated with a significant decrease in the ability of MPCs to produce myotubes having many nuclei. Although fusion is clearly perturbed, it is not abolished in the ANO5\(^{-/-}\) cells. This suggests the possibility that the initial fusion events are not impaired, but the accretion of subsequent fusion events is decreased.

In *Drosophila melanogaster*, myotube formation occurs in two steps. The primary fusion events occur between two different types of mononucleated cells: founder cells and fusion-competent myoblasts. The myoblasts then increase in mass and nuclear number by secondary fusion events that use slightly different machinery (Kim et al., 2015). It is not clear whether vertebrates also use different mechanisms for early and late fusion, but we propose that ANO5 regulates or coordinates fusion steps that lead to the accumulation of large numbers of nuclei in the muscle fibers.

The perturbation in fusion could occur at one of several steps, including proliferation and differentiation of fusion-competent cells, migration of cells to the site of fusion, recognition and adhesion of fusion-competent cells, and formation of the fusion pore between cells (Chen and Olson, 2004; Abmayr and Pavlath, 2012). While we cannot distinguish between the role of ionic currents and PLS in the MPC fusion process because the two activities are tightly linked, we prefer the hypothesis that PLS is a key event because PtdSer apparently plays an integral role (van den Eijnde et al., 1997, 2001) in myoblast fusion. Prolonged exposure (5 d) to the PtdSer-binding protein, annexin V, or PtdSer antibody during myoblast fusion significantly inhibits the formation of myotubes. Additionally, liposomes of PtdSer, but not phosphatidylcholine, stimulate myoblast fusion (van den Eijnde et al., 2001; Jeong and Conboy, 2011). Moreover, it has recently been reported that the PtdSer receptors BAI1 and STAB2 participate in myoblast fusion (Hochreiter-Hufford et al., 2013; Park et al., 2016). Because PLS is known to alter the biophysical properties of cell membranes such as curvature and electrostatics (Whitlock and Hartzell, 2017), it is tempting to propose that PLS may play a role in overcoming the energy barriers for leaflets of fusing membranes to merge together. ANO5 ionic currents could also be important in regulating myoblast fusion by regulating membrane potential or in-

**Figure 7.** Exogenous ANO5 expression rescues Ano5\(^{-/-}\) MPC Ca\(^{2+}\)-PLS. (a) Simultaneous whole-cell patch clamp and annexin V–Alexa Fluor 568 binding as an indicator of Ca\(^{2+}\)-PLS of primary MPCs differentiated for ∼24 h. Images on the left show bright-field micrographs of patched MPCs. Images at the center and right show annexin V binding. Scale bar, 10 μm. (b) Quantification of Ca\(^{2+}\)-PLS in MPCs. Statistical significance was measured using a Fisher exact test (*, P = 0.024). (c) Representative traces of ionic currents from MPCs isolated from the same mouse with or without ANO5 virus rescue following 10 min of whole-cell patch clamp with 200 μM Ca\(^{2+}\) intracellular solution. > denotes 0 nA. 20mV voltage steps from −60 mV to 140 mV. (d) Quantification of ionic currents from cell lines isolated from two 3-mo-old mice with and without viral rescue (*, P = 0.027, paired t test). (e) Current–voltage relationship ANO5 rescued Ano5\(^{-/-}\) MPCs from panel d.
tracellular Ca$^{2+}$ concentration. Another possibility is that PLS is secondary or parallel to the primary event.

These data provide additional insights into our previous observation that the ability of muscle to regenerate in vivo in response to cardiotoxin injury is greatly diminished in this Ano5$^{-/-}$ knockout model (Griffin et al., 2016). Although it is possible that ANO6 has other functions within the cell, the fact that the loss of ANO5-dependent Ca$^{2+}$-PLS and associated currents is correlated with diminished MPC fusion competence strongly suggests that these functions of ANO6 contribute to the perturbed muscle repair and ANO5 myopathy phenotype observed in our model. Although we have focused on the role of ANO5 in coordinating MPC fusion, it is possible that ANO6 also facilitates muscle repair in other ways. In particular, repair of isolated muscle fibers after laser damage, a process that does not rely on progenitor cell fusion (Cooper and Head, 2015), is diminished in the Ano5$^{-/-}$ mouse (Griffin et al., 2016). Nevertheless, our observations here, our previous characterization of a LGMD2L-like phenotype in this model, and data from other investigators demonstrating PtdSer exposure in MPC fusion (van den Eijnde et al., 1997, 2001; Hochreiter-Hufford et al., 2013; Park et al., 2016) support the hypothesis that the loss of ANO5-dependent Ca$^{2+}$-PLS likely contributes to the development and/or progression of LGMD2L by perturbing MPC fusion coordination.

**Subcellular location of ANOS and its role in scrambling**

ANO5 appears to be located largely in intracellular membranes, both in muscle (Mizuta et al., 2007; Xu et al., 2018) and in transfected HEK293 cells (our data). If so, how does it mediate PLS on the PM? One possibility is that the small amount of ANO5 on the PM is sufficient. Another possibility is that PM scrambling is a consequence of membrane or lipid trafficking from intracellular membranes. There have been suggestions in the literature that scrambling involves membrane trafficking (Mirnikjoo et al., 2009; Lee et al., 2013). Furthermore, ANO-dependent Ca$^{2+}$-PLS is associated with the release of extracellular vesicles (Sims et al., 1989; Comfurius et al., 1990; Fujii et al., 2015). This raises the possibility that ANOS influences muscle cell repair through mechanisms that are not localized exclusively at the PM. In this regard, both exocytosis and endocytosis have been implicated to be important in myoblast fusion (Kim et al., 2015), and it has been suggested that extracellular vesicles may play a role in muscle development and regeneration (Demonbreun and McNally, 2017; Guescini et al., 2017), although this remains unproven.

**Why is Ca$^{2+}$-PLS defective in Ano5$^{-/-}$ MPCs despite ANO6 expression?**

It has been suggested that ANOs 3, 4, 5, 6, 7, and 9 are PLSases (Suzuki et al., 2013). Certainly, there is good evidence that different ANOs are expressed in a tissue-specific manner, but most tissues express multiple ANO scramblases (Suzuki et al., 2013). Muscle expresses both ANO5 and ANO6 (in addition to ANO8 and ANO10), but surprisingly Ano5$^{-/-}$ MPCs exhibit defective Ca$^{2+}$-PLS despite the presence of ANO6. These data suggest that despite their similar functions, ANO5 and ANO6 play different roles and/or are regulated differently in muscle cells. Another possibility is that ANO5 is not itself a scramblase, but a scram-
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