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The *Ubr2* gene is expressed in skeletal muscle atrophying as a result of hind limb suspension, but not *Merg1a* expression alone

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Abstract

Skeletal muscle (SKM) atrophy is a potentially debilitating condition induced by muscle disuse, denervation, many disease states, and aging. The ubiquitin proteasome pathway (UPP) contributes greatly to the protein loss suffered in muscle atrophy. The MERG1a K^+ channel is known to induce UPP activity and atrophy in SKM. It has been further demonstrated that the mouse ether-a-gogo-related gene (*Merg*)1a channel modulates expression of MURF1, an E3 ligase component of the UPP, while it does not affect expression of the UPP E3 ligase *Mafbx*/ATROGIN1. Because the UBR2 E3 ligase is known to participate in SKM atrophy, we have investigated the effect of *Merg1a* expression and hind limb suspension on *Ubr2* expression. Here, we report that hind limb suspension results in a significant 25.6% decrease in mouse gastrocnemius muscle fiber cross sectional area (CSA) and that electro-transfer of *Merg1a* alone into gastrocnemius muscles yields a 15.3% decrease in CSA after 7 days. More interestingly, we discovered that hind limb suspension caused a significant 8-fold increase in *Merg1a* expression and a significant 4.7-fold increase in *Ubr2* transcript after 4 days, while electro-transfer of *Merg1a* into gastrocnemius muscles resulted in a significant 6.2-fold increase in *Merg1a* transcript after 4 days but had no effect on *Ubr2* expression. In summary, the MERG1a K^+ channel, known to induce atrophy and MURF1 E3 ligase expression, does not affect UBR2 E3 ligase transcript levels. Therefore, to date, the MERG1a channel's contribution to UPP activity appears mainly to be through up-regulation of *Murf1* gene expression.

Key Words: Skeletal muscle atrophy; UBR2; E3α-II; ERG1a potassium channel; hind limb suspension; E3 ligase; ubiquitin proteasome pathway

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Skeletal muscle (SKM) can comprise 30-50% of body weight in humans and it functions in body movement, maintenance of posture and balance, soft tissue support, bolster of body entry and exit points and in temperature regulation. The loss of SKM mass and strength is referred to as atrophy and is known to result from unweighting, injury, denervation, disuse, disease (e.g., diabetes, cancer, etc.) and aging.[1-5] Atrophy occurs when there is an imbalance in the processes of protein synthesis and protein degradation that favors protein loss. Numerous cellular pathways can contribute to SKM atrophy, including calpains, cathepsins and the ubiquitin proteasome pathway (UPP).[2,4] The UPP is reported to be responsible for as much as 75% of the protein degradation that occurs during SKM atrophy[2,4,6] It is composed of three basic enzymes: E1 proteins which activate ubiquitin; E2 ubiquitin-conjugating molecules which accept the activated ubiquitin and interact with specific E3 enzymes; and the E3 ligase which binds target protein molecules and catalyzes the transfer of the ubiquitin from E2 to the E3-bound protein.[7,8] A minimum of four ubiquitin molecules are required to be ligated to the target protein before it is degraded by the 26S proteasome. To date, only a few members of the E1 family have been identified (only two E1 enzymes have been detected within the human proteome) and over 50 E2 enzymes have been reported; however, several hundred potential E3 ligases have been described within mammalian genomes. Two main types of E3 enzymes have been described: the "homologous with E6-associated protein C6 terminus" (HECT) domain enzymes and the larger family of

"really interesting new gene" (RING) finger scaffold proteins. A newer described group of proteins with ubiquitin ligase activity, having atypical RING finger moieties,[9] have been described and are often referred to as E4 enzymes. These have been reported to work in concert with E3 proteins to enhance their activity.[10]

A number of E3 ligases have been identified as contributors to atrophy in SKM. Muscle ring finger-1 (MURF1) and Muscle Atrophy F-box (*Mafbx*)/ATROGIN1 are RING finger E3 ligases specific to striated muscle that have been detected in several models of SKM atrophy, including unweighting, and are considered markers of atrophy.[1,2,4] Ubiquitin ring-type (UBR, also referred to as E3α) proteins are RING finger E3 ligases that are also reported to function in atrophic SKM, specifically cachexia-induced atrophy.[11] The UBR box family of RING finger E3 ubiquitin ligases is composed of enzymes which recognize substrate proteins that have an N-degron; that is, the protein to be degraded has: 1) a destabilizing N-terminal residue; and 2) an internal lysine residue for ubiquitination.[7,12,13] These enzymes are often referred to as N-recognins and this pattern of protein degradation, referred to as the N-end rule pathway, relates the identity of the N-terminal amino acid of a protein to its half-life *in vivo*[7,13,14] UBR1 (also E3α-I) was the first E3 ligase implicated in SKM atrophy[14,15] and is believed to bind proteins that commence with unblocked hydrophobic or basic amino acids.[7, 16-19] The UBR2 (also E3α-II) member of the UBR family was described by Varshavsky and co-workers.[17] In 2004, Kwak et al. demonstrated that both the *Ubr1* and *2* homologues are up-regulated in the atrophying SKM of rats bearing malignant tumors.[11] However, it was concluded that the UBR2 isoform is more critically involved in the process because it is more specifically expressed in SKM tissue and is detected at the early onset of atrophy while UBR1 is not. In fact, a recent study suggests that tumor cell-induced up-regulation of *Ubr2* expression in skeletal muscle may be mediated by the p38β-C/EBPβ signaling pathway. [19]

The ether-a-gogo- related gene (ERG)1a K^+ channel is known to be partially responsible for late phase repolarization of the cardiac action potential in many mammalian species, including mice and humans.[21] Our laboratory has demonstrated that this channel is up-regulated in the SKM of mice experiencing atrophy as a result of both hind limb suspension (unweighting) and cancer cachexia.[22] Further, we have demonstrated that electro-transfer of *Merg1a* alone into the SKM of weight bearing mice induces atrophy and, importantly, that electro-transfer of *Merg1a* into mouse SKM up-regulates UPP activity. More recently, we have shown that electro-transfer of *Merg1a* into mouse SKM up-regulates the expression of the E3 ligase *Murf1*, but not *Mafbx*/ATROGIN1.[22] Because of its link to atrophy with both unweighting and cachexia, we asked if *Merg1a* expression would also modulate expression of the *Ubr2* E3 ligase gene. Here, we have determined the time course of *Merg1a* and *Ubr2* expression in response to both hind limb suspension and electro-transfer of the *Merg1a* K+ channel and show that, although *Ubr2* is expressed in response to hindlimb suspension, its expression is not modulated by *Merg1a* expressed alone.

Materials and Methods

Animals. The Purdue Animal Care and Use Committee approved all procedures. ND4-Swiss Webster male mice at 8 weeks age (Harlan-Sprague; Indianapolis, IN) were used in all procedures. Animals were provided food and water *ad libitum*, housed in Purdue University animal facilities on a 12 hour light/dark cycle and monitored by lab animal veterinarians. At completion of all studies, anesthetized animals were humanely killed according to the approved protocol prior to harvest of the gastrocnemius muscles (GMs).

Hind Limb Suspension. Custom suspension cages were assembled as described earlier.[23] Animals were positioned in these cages resting in an approximate 30° head down tilt with their hind limbs raised off of the floor so that they were unable to place any weight on them. Commercial mouse cages were used to house control mice in a natural weight bearing state.

Tissue Sectioning, Staining and CSA Determination. GMs were embedded using OCT™ (Electron Microscopy Sciences; Hatfield, PA), cryo-sectioned (12 μm) and stained for β–galactosidase (LACZ) activity as described previously.[21] Images of sections were captured with a Leaf Micro–Lumina digital camera (Scitex; Tel-Aviv, Israel). The CSA (μm^2) of each muscle fiber was determined using a micrometer slide and Image J software (NIH; Bethesda, MD).

Plasmids. The *Merg1a* clone in pBK/CMV[24] was a generous gift from Dr. Barry London (Cardiovascular Institute, University of Pittsburgh, PA). The CMV-*lacZ* in pNL vector was purchased from the Center Commercial de Gros (Toulouse, France).

Electro-transfer. Mice were anesthetized using 10 μl/g body weight of xylazine (1 mg/ml) and ketamine (9 mg/ml) in sterile saline according to an approved protocol. Hind limbs were shaved and the both GMs were injected with expression plasmid (see Study 2 below) in 50 μl sterile saline. Eight electrical pulses at 200V/cm for 20 ms at 1 Hertz were applied to all lower limbs (GMs) with an ECM 830 ElectroSquare Porator (BTX; Hawthorne, NY). This method results in gene transcription and translation in SKM in our laboratory.[22,23,25]

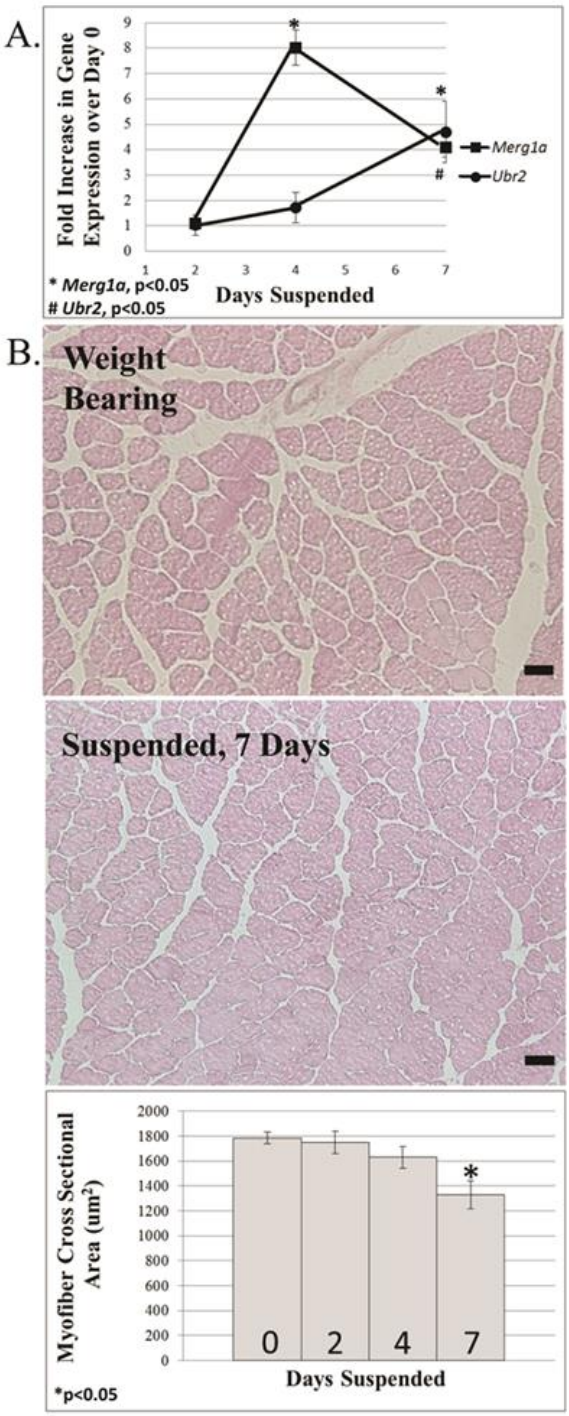

Figure 1. Hind limb suspension causes SKM atrophy and up-regulation of both Merg1a and Ubr2 E3 ligase expression.

Real Time PCR. Trizol reagent (Invitrogen; Carlsbad, CA) was used to extract total RNA from GMs according to manufacturer's instructions. Phenol/chloroform extraction and ethanol precipitation sequentially followed the extraction. Contaminating DNA was degraded by two 10 min treatments with DNase I (ProMega; Madison, WI) and the DNase was subsequently heat inactivated. A GoScript™ Reverse Transcriptase Kit (ProMega; Madison, WI) was used to reverse transcribe cDNA using an Eppendorf Mastercycler Personal (Hauppauge, NY). PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Inc.; Gaithersberg, MD) was added to the PCR reaction (per manufacturer's instructions) and primers (see Table 1) for the gene of interest were added to the samples (in triplicate) while primers for an appropriate "housekeeping" gene (the 18S ribosomal subunit) were added to identical samples (in triplicate). A BioRad MyiQ single Color Real time PCR Detection System (BioRad; Hercules, CA) was used to detect SYBR Green fluorescence as a measure of amplicon. Sample CT values were normalized to (subtracted from) the CT values of the 18S "housekeeping" gene and the number 2 was raised to a power equal to the difference between the sample CT values of the 18S subunit and the gene of interest.[22,23,25]

Study 1 Experimental Design. Three groups of five mice each were hindlimb suspended[22] for either 2, 4 or 7 days. A fourth group of five mice were allowed to remain weight bearing and were used as day 0 controls (n=20). After the assigned control or suspension duration, each group of mice was killed according to the approved protocol and the GMs were harvested and flash frozen in liquid nitrogen. The left GMs from all mice were prepared for real time PCR and thus assayed for expression of *Merg1a* and *Ubr2* genes (see above). Normalized sample CT values for the Day 0 control were averaged and the fold increase in gene expression for each gene per mouse was determined by calculating the ratio of each daily mouse sample gene expression value to the average Day 0 value.

Study 2 Experimental Design. All GMs of 20 mice (4 groups of 5 each) were injected with 10 μg *LacZ* and 20 μg *Merg1a* expression plasmid and were subjected to electro-transfer as described above. A group of 5 mice each was killed at days 0, 2, 4 and 7 after electro-transfer[22,23,25] and the GMs from all legs were harvested and flash frozen in liquid nitrogen. The GMs from the left legs were cryo-sectioned and stained for LACZ activity as a marker for gene expression while right legs were prepared for real time PCR as described above. Because a greater abundance (2X) of *Merg1a* than *LacZ* expression plasmid was injected, we assume that all myofibers staining for LACZ reporter activity also express *Merg1a* and that those myofibers not staining for LACZ activity are not expressing plasmid.

Statistics. Data were analyzed by ANOVA using a completely randomized design. When significant differences were found, means were separated by Fisher's Protected Least Significant Difference. All data were analyzed using the General Linear Model Procedure of SAS (SAS Institute Inc.; Cary, NC). All statements of significance are $p \leq 0.05$.

Results and Discussion

Study 1. Hind limb suspension causes SKM atrophy and up-regulation of both Merg1a and Ubr2 E3 ligase expression.

Similar to earlier studies conducted in our laboratory,[22] hindlimb suspension caused decreases in myofiber CSA at Day 2 (2%) and Day 4 (8.7%), but the decreases are not statistically significant (Figure 1B). The 25.6% decrease at Day 7, however, is statistically significant. Also, similar to other studies we have conducted,[23] hind limb suspension does not result in a statistically significant increase in *Merg1a* K^+ channel expression at Day 2; however, it does result in a significant 8-fold increase in the expression of the *Merg1a* K^+ channel by Day 4 (Figure 1A). The level of *Merg1a* K^+ channel transcript decreases from that of Day 4 by Day7 although it remains significantly higher (4.1 fold) than Day 0.

Study 2. Ectopic expression of the Merg1a K+ channel gene induces atrophy, but not expression of the gene encoding the Ubr2 E3 ligase. Reminiscent of earlier work in our laboratory,[22] myofiber CSA drops by 3.1% (Figure 2B) after 4 days of *Merg1a* ectopic expression. Myofiber CSA drops by a statistically significant 15.3% after seven days of *Merg1a* expression, demonstrating that *Merg1a* expression induces SKM atrophy. In response to injection of *Merg1a* expression plasmid into GM followed by electro-transfer, *Merg1a* transcript is produced as shown earlier.[22,23,25] Levels of this transcript increase 1.7-fold by Day 2 and then to a statistically significant higher 6.2 fold level by Day 4. The *Merg1a* transcript level drops by day 7, but is still a significant 3.8 fold higher than it was at Day 0 (Figure 2A). In contrast, *Ubr2* gene expression does not increase in response to ectopic *Merg1a* gene expression. SKM atrophy occurs as a result of an imbalance in protein synthesis and protein degradation which ultimately favors protein loss and muscle deterioration. The UPP, comprised of E1, E2 and E3 enzymes, is reported to be responsible for a large portion of the protein degradation that occurs in atrophying muscle.[1-6] The E3 ligases MURF1 and *Mafbx*/ATROGIN1 are ring finger E3 ligases specific to striated muscle which contribute greatly to skeletal muscle atrophy induced by numerous factors.[1-4]

Our laboratory reported that the *Merg1a* K^+ channel is expressed at high levels in the GMs of mice experiencing atrophy as a result of both hind limb suspension and cancer cachexia while it is basically undetected in the matched muscles of appropriate control animals.[22] We have also shown that the atrophy resulting from hind limb suspension can be inhibited by ectopic expression (resulting from electro-transfer) of the dysfunctional *Merg1a* mutant, DN-*Merg1a*, in the GMs as well as by treatment with astemizole, a known pharmacological ERG1 channel blocker. Further, electro-transfer of the *Merg1a* K^+ channel gene into mouse GM induces atrophy and, importantly, increases UPP activity.[22] The mechanism(s) by which MERG1a modulates UPP activity is not known; however, we recently demonstrated that *Merg1a* expression does not modulate expression of

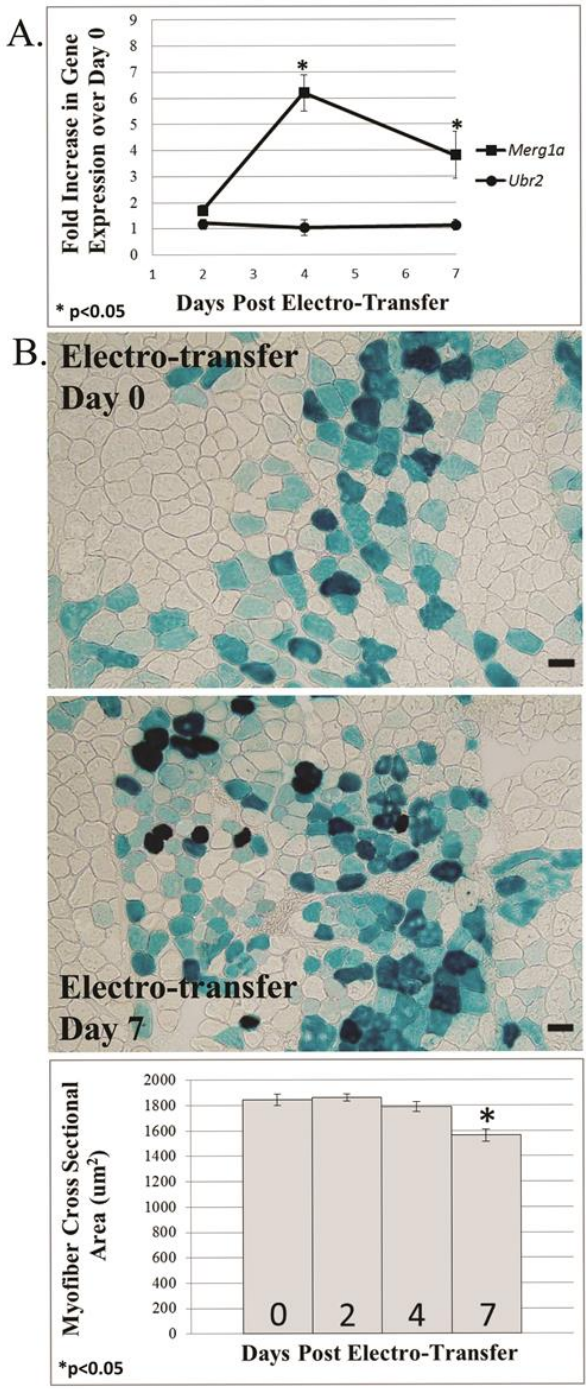

Figure 2. Ectopic expression of the Merg1a K+ channel gene induces atrophy, but not expression of the gene encoding the Ubr2 E3 ligase.

Mafbx/ATROGIN1, although it does indeed increase the levels of both MURF1 transcript and protein.[23] This separation of MURF1 and *Mafbx*/ATROGIN1 modulation at the level of a membrane protein is most interesting. Because the literature reports that *Murf1* transcription, and not that of *Mafbx*, is increased by NF-κB factors[26] and that NF-κB is basically required for disuse muscle atrophy[26-28] this separation of E3 ligase modulation suggests that MERG1a may be modulating *Murf1* expression through NF-κB activity. Indeed, a study by Wang et al. (2002), reveals that cells expressing HERG have higher endogenous levels of the active form of NF-κB than those cell lines with little to non-detectable levels of endogenous HERG.[29] Certainly these findings beg for further exploration into the potential effect of MERG1a on the NF-κB family of transcription factors (likely through the IKK-β/IκB-α/NF-κB pathway) as well as the potential modulation of FOXO1 and 4 transcription factors (potentially through the PI3K/AKT/FOXO pathway).[1,4,26,30] Obviously, the MERG1a K^+ channel may also interact with other known or unknown pathways.

Our data show that hind limb suspension decreases mouse GM CSA by 25.6% while ectopic *Merg1a* expression alone induces an approximately 10% lower (15.3%) decline in GM CSA. This demonstrates that *Merg1a* expression and the consequences of this channel up-regulation are not solely responsible for SKM atrophy. Indeed, we have already shown that *Merg1a* expression does not induce expression of *Mafbx*/Atrogin1,[23] an E3 ligase known to be responsible for a large percentage of SKM atrophy induced by immobilization,[30] although it does induce expression of *MuRF1*,[23] another E3 ligase important to immobilization induced SKM atrophy.[30] To further explore the potential effects of *Merg1a* expression on SKM and its contributions to the atrophic process, we decided to determine if a member of the UBR family of ligases, known to be involved in SKM atrophy, is modulated by MERG1a. The UBR1 and UBR2 E3 ligases are two members of the UBR family (recently described as having 7 constituents) which are known to recognize substrate proteins having a destabilizing N-terminal residue and an internal lysine residue for ubiquitin conjugation.[31] Expression of these E3 ligases is induced in skeletal muscle undergoing atrophy as a result of cancer cachexia, although *Ubr2* is up-regulated earlier in the atrophic time course and also more dramatically and more specifically than *Ubr1*.[20] In fact, *Ubr2* up-regulation in the GMs of mice bearing malignant tumors is reported to occur within the time frame during which *Merg1a* expression is highest in both suspended mice and in those expressing *Merg1a* as a result of electro-transfer (during the first 7 days). The reported *Ubr1* expression time frame for cachectic mice (second week) does not correlate well with the *Merg1a* expression time courses we have reported for either hind limb suspended or mice ectopically expressing *Merg1a*.[23] The existing data suggest that *Merg1a* levels would be low when *Ubr1* levels are demonstrated to begin to increase, so that the increase in *Ubr1* transcript levels would not likely be a direct result of *Merg1a* expression.

Although, it is possible that the time course for *Merg1a* expression is more drawn out in mice experiencing muscle atrophy as a result of cancer cachexia, we nonetheless designed *Ubr2* specific primers and monitored the time course of *Ubr2* expression in response to both hind limb suspension and electro-transfer of *Merg1a* into the GMs of mice. We discovered that, although hind limb suspension does induce transcription of both *Merg1a* and *Ubr2* within seven days of suspension, *Merg1a* expression alone is not sufficient to yield increased levels of *Ubr2* transcript within this time frame. One might speculate that the electro-transfer of *Merg1a* into GMs needs additional time for insertion of this K^+ channel into the plasma membrane and physiological activation. However, our earlier work,[22,23] demonstrated that the time frame applied (7 days post electro-transfer of *Merg1a*) does indeed produce both transcription (rtPCR) and translation (western blotting) of the *Merg1a* construct and a statistically significant 16% ($p<0.05$) decrease in GM CSA. We also have shown that this decrease in CSA can be inhibited by co-electro-transfer and co-expression of *Merg1a* and a dominant negative (i.e., dysfunctional pore mutant) form of the *Merg1a* channel 7 days after electro-transfer into skm.[22] The inhibition of *Merg1a*-induced atrophy by this dysfunctional pore mutant demonstrates that the electro-transferred *Merg1a* K^+ channel is functional. Further, we have shown that both pharmacological treatment of mice with an ERG channel pore blocker (oral gavage with astemizole) and electro-transfer of the dominant negative *Merg1a* into GM of hind limb suspended mice inhibited atrophy (i.e., inhibited the decrease in muscle fiber CSA,[22]). This evidence demonstrates (albeit indirectly) that the MERG1a channel is functional (located in the membrane and conducting current) within 7 days of electro-transfer. Therefore, it appears that *Merg1a* expression alone does not affect expression of *Ubr2* within 7 days; nonetheless, it is still feasible that an even longer post electro-transfer time frame may be necessary for adequate activation of the MERG1a channel to induce *Ubr2* transcription. Further, this work does not rule out the possibility that MERG1a may affect or interact with some other factor produced during hind limb suspension which induces or participates in the induction of *Ubr2* expression. For example, HERG protein and current expression is known to be regulated by kinases such as stress-responsive serum- and glucocorticoid-inducible kinase isoforms SGK1 and 3 in HEK cells,[32] protein kinase

A[33] and AMP-dependent protein kinase (AMPK; 1). It is possible that such a factor(s) is necessary to modulate channel activity and produce atrophy and, although present during hindlimb suspension, is not present when *Merg1a* is electro-transferred into muscle. We also must consider that the electro-transfer itself could interfere with the presence or production of some factor necessary for MERG1a-induced *Ubr2* transcription, although we have not produced or read any evidence of this. Of course, even though we have shown that our system of *Merg1a* electro-transfer into mouse GMs will induce transcription of significant levels of *Merg1a* and the *MuRF1* E3 ligase,[22,23] the simplest issue may be that it may not have produced the levels of MERG1a necessary to induce *Ubr2* transcription.

Finally, our work does not rule out the possibility that MERG1a may affect *Ubr2* expression in muscle atrophying as a result of cancer cachexia. Previous studies support the existence of potential cellular connections between UBR2 and ERG. For example, *Ubr2* expression is reported to activate the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α), which is involved in the onset of cachexia;[11,29] and, interestingly, TNF-α stimulation of TNF receptor 1 (TNFR1) has been shown to impair human ERG (HERG)/IKr current density (and prolong action potential duration) by stimulation of reactive oxygen species in canine cardiomyocytes.[33] Is it possible that TNF-α binds its receptor and affects ERG in such a way as to affect *Ubr2* expression? Work by Wang et al., (2002) demonstrates that TNF-α affects cell growth and apoptosis more stringently in cells expressing ERG than those that do not, suggesting that TNFR1 may be more abundant in the ERG-expressing cells.[34]Further, it is reported that cells positive for endogenous ERG protein also contained more abundant TNFR1 than cells that did not express high levels of endogenous ERG, suggesting that perhaps ERG channels somehow recruit TNFR1 to the cell membrane. Indeed, the same study showed that HERG and TNFR1 co-immunoprecipitate from cell lysates.[34] Therefore, it does seem reasonable to suggest that, although *Merg1a* expression alone may not induce *Ubr2* expression, it may do so as a result of some missing factor that may be more significantly abundant under physiological atrophy producing conditions.

To our knowledge, this is the first report of an increase in *Ubr2* expression in SKM as a response to disuse atrophy and also the first exploration of possible modulation of *Ubr2* E3 ligase expression by MERG1a. We conclude that, although hind limb suspension of mice induces an increased expression of genes encoding *Merg1a* and *Ubr2* in GMs, the electro-transfer (and subsequent expression) of *Merg1a* alone into GMs of mice does not affect expression of *Ubr2* within 7 days of electro-transfer. The data suggest that either *Merg1a* does not affect *Ubr2* expression or that, if it does, then it either needs more than 7 days to induce this expression or it must have access to another factor, one induced by conditions produced during skeletal muscle atrophy resulting from disuse and/or cancer cachexia.

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