Angiotensin II Induces Skeletal Muscle Atrophy by Activating TFEB-Mediated MuRF1 Expression

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Rationale: Skeletal muscle wasting with accompanying cachexia is a life threatening complication in congestive heart failure. The molecular mechanisms are imperfectly understood, although an activated renin–angiotensin aldosterone system has been implicated. Angiotensin (Ang) II induces skeletal muscle atrophy in part by increased muscle-enriched E3 ubiquitin ligase muscle RING-finger-1 (MuRF1) expression, which may involve protein kinase D1 (PKD1).

Objective: To elucidate the molecular mechanism of Ang II–induced skeletal muscle wasting.

Methods and Results: A cDNA expression screen identified the lysosomal hydrolase-coordinating transcription factor EB (TFEB) as novel regulator of the human MuRF1 promoter. TFEB played a key role in regulating Ang II–induced skeletal muscle atrophy by transcriptional control of MuRF1 via conserved E-box elements. Inhibiting TFEB with small interfering RNA prevented Ang II–induced MuRF1 expression and atrophy. The histone deacetylase-5 (HDAC5), which was directly bound to and colocalized with TFEB, inhibited TFEB-induced MuRF1 expression. The inhibition of TFEB by HDAC5 was reversed by PKD1, which was associated with HDAC5 and mediated its nuclear export. Mice lacking PKD1 in skeletal myocytes were resistant to Ang II–induced muscle wasting.

Conclusion: We propose that elevated Ang II serum concentrations, as occur in patients with congestive heart failure, could activate the PKD1/HDAC5/TFEB/MuRF1 pathway to induce skeletal muscle wasting. (Circ Res. 2015;117:424-436. DOI: 10.1161/CIRCRESAHA.114.305393.)

Key Words: angiotensin II • gene expression regulation • heart failure • histone deacetylase 5 • muscle RING-finger-1 • protein kinase D • transcription factor EB

Skeletal muscle plasticity assures functional adaptation to physiological and pathological conditions by regulating muscle mass and fiber type.1–3 Muscle mass is regulated by a well-controlled balance between protein synthesis and degradation.4 Increased protein degradation and decreased protein synthesis result in skeletal muscle atrophy. Muscle disuse, neurological disorders, and aging belong to the best-studied conditions leading to atrophy. However, muscle atrophy and wasting also accompany diseases, such as cancer5 and end-stage congestive heart failure (CHF),6 where cachexia is the major constituent.5,7 Although, the detailed mechanism of CHF-induced muscle atrophy is unknown, angiotensin II (Ang II) has been implicated in this process. First, the renin–angiotensin aldosterone system is activated and Ang II serum levels are increased in patients with CHF.8,9 Second, reduced renin–angiotensin aldosterone system activity by angiotensin-converting enzyme inhibition reduced cachexia in patients with CHF.6 Third, Ang II leads to decreased muscle mass by increased ubiquitin proteasome system (UPS)-dependent muscular protein degradation.10 Ang II increases UPS-mediated protein degradation by inducing muscle RING-finger-1 (MuRF1) expression.11–13 This muscle-enriched E3 ubiquitin ligase is a key mediator of muscle atrophy.1 However, the Ang II–activated signaling pathway increasing MuRF1 expression in muscle is not well understood. To search for novel transcription factors involved in Ang II–induced MuRF1 expression, we performed a cDNA expression screen. The basic helix-loop-helix (bHLH) transcription factor EB (TFEB) was identified as potent MuRF1 inducer. TFEB activity was regulated via the Ang II/protein kinase D1 (PKD1)/histone deacetylase-5 (HDAC5) signal transduction pathway. Inhibiting TFEB abolished Ang II–induced atrophy in vitro. We suggest that Ang II–induced skeletal muscle wasting could be mediated at least in part by the PKD1/HDAC5/TFEB/MuRF1 pathway.

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Methods

An expanded Materials and Methods section is included in the Online Data Supplement.

Results

To discover novel regulators of MuRF1 expression, we performed a cDNA expression screen using a luciferase reporter controlled by the human MuRF1 promoter (−5002 bp upstream of the transcription start site, Hs_MuRF1-Luc) and a human skeletal muscle cDNA library according to our previous work. The screening procedure is described (Online Figure I). We expressed pools of clones from the cDNA expression library in COS-7 cells and quantified activation of Hs_MuRF1-Luc by luciferase assays. After sib selection, a cDNA encoding TFEB was identified as strong inducer of Hs_MuRF1-Luc (Online Figure II). TFEB belongs to the MITF/TFE family of bHLH-leucine zipper transcription factors. Recently, TFEB was shown to bind to specific E-box motifs in the promoter of lysosomal genes, so called coordinated lysosomal expression and regulation (CLEAR) elements. We analyzed the sequence of the −543 bp promoter of MuRF1 and found 4 (Figure 2B). E-box 1 (−41 to −46 bp), E-box 2 (−63 to −68 bp), and E-box 3 (−139 to −144 bp) showed a high degree of sequence conservation across species. Because E-box 4 (−299 to −304 bp) was less conserved, and not present in all species, we focused on E-boxes 1, 2, and 3 for further analyses (Figure 2B). To investigate the importance of E-boxes 1 to 3 for TFEB-induced MuRF1 expression, site-directed mutagenesis was used to mutate these E-box motifs from CANNNTG to ATINNTG, known to inhibit E-box functionality, in the −543 bp Hs_MuRF1-Luc construct (Figure 2B). Mutation of E-box 1 and 3 abolished TFEB-induced MuRF1 expression, whereas mutation of E-box 2 had only minor effects (Figure 2C).

To uncover cis-regulatory elements in the MuRF1 promoter mediating responsiveness to TFEB, we generated Hs_MuRF1-Luc deletion mutants. Deletion of nucleotides from position −5002 bp down to −300 bp relative to the transcription start site of the MuRF1 promoter had no effect on TFEB-induced Hs_MuRF1-Luc activity in HEK-293 cells (Figure 2A; Online Figure III). However, further size reduction of the MuRF1 promoter led to a decline in TFEB responsiveness. More specifically, MuRF1 promoter fragments shorter than 300 bp showed a decreased TFEB responsiveness (Figure 2A). These data implicated TFEB binding sites between 300 bp and the transcription start site of the MuRF1 promoter. We used the −543 bp MuRF1 promoter fragment for further analysis. Because TFEB is known to bind to specific E-box motifs in the promoter of lysosomal genes, we performed ChIP-PCR to elucidate if TFEB binds to the conserved E-box motifs E-box 1, 2, and 3 in the endogenous MuRF1 promoter. We next used chromatin immunoprecipitation (ChIP) followed by qRT-PCR (ChIP-qPCR) to elucidate if TFEB binds to the conserved E-box motifs E-box 1, 2, and 3 in the endogenous MuRF1 promoter. ChIP-qPCR experiments were performed using an anti-TFEB antibody to test if endogenous TFEB was bound to the endogenous MuRF1 promoter. These experiments confirmed direct binding of endogenous TFEB to E-box 1, 2, and 3 of the endogenous MuRF1 promoter (Figure 2D). In addition, we tested whether overexpressed TFEB binds to the endogenous MuRF1 promoter. Chromatin of C2C12 myoblasts transfected with vector control, FLAG-TFEB or TFEB-Myc(His)6, was immunoprecipitated with anti-FLAG and Ni²⁺-NTA agarose.
respectively. An enrichment of the MuRF1 promoter sequence surrounding E-boxes 1, 2, and 3 in the immunoprecipitated DNA from FLAG-TFEB and TFEB-Myc(His)₆, respectively, indicated binding of overexpressed TFEB to the endogenous MuRF1 promoter (Online Figure IV A and IVB). Because MuRF1 is known to mediate Ang II–induced skeletal muscle atrophy,11–13 we tested if Ang II regulates TFEB binding to the MuRF1 promoter. ChIP-PCR assays were performed using chromatin from Ang II–treated C2C12 cells. This experiment showed that Ang II increased TFEB binding to E-boxes 1, 2, and 3 in the endogenous MuRF1 promoter (Figure 2E). Because starvation was shown to increase TFEB activity in other cell types,23,24 we tested if starvation increases binding of TFEB to the endogenous MuRF1 promoter. Indeed, starvation increased binding of endogenous TFEB to the endogenous MuRF1 promoter in C2C12 myoblasts (Figure 2F). In summary, our data show that TFEB constitutively binds to conserved E-box elements in the MuRF1 promoter in myocytes and that Ang II signaling, as well as starvation, increases the amount of TFEB bound to E-boxes 1, 2, and 3 in the MuRF1 promoter.

Although the function and regulation of TFEB in non-muscle cells is well described,18–20,25 its function in myocytes is not well understood. We next performed immunocytochemistry and immunofluorescence microscopy to investigate subcellular localization of TFEB in C2C12 myoblasts. We generated cDNA expression plasmids encoding wild-type FLAG-TFEB (TFEB) or empty (control) expression plasmid for 24 hours. Immunoblotting (IB) with anti-FLAG, anti-MuRF1, or anti-GAPDH antibody was performed. E and F, C2C12 myoblasts were transfected with scrambled siRNA (siScr, −) or siRNA targeting TFEB (siTFEB, +, 100 nmol/L each) for 24 hours. E, qRT-PCR analysis was performed to measure TFEB and MuRF1 expression. GAPDH expression was used as reference. Data are represented as mean±SD. *P<0.05, ***P<0.001. n=3 each. F, Western blot analysis was performed with anti-TFEB, anti-MuRF1, or anti-GAPDH antibody. EDL indicates extensor digitorum longus; GP, gastrocnemius/plantar Lis, LA, left atrium; LV, left ventricle; n.s., not significant; RA, right atrium; RV, right ventricle; Sol, soleus; and TA, tibialis anterior.
indicate that the bHLH domain mediates nuclear localization of TFEB. Luciferase assays were used to test if these changes in subcellular localization also affected the ability of TFEB to increase MuRF1 expression. As expected TFEB deletion mutants AA299-352 and AA238-400, which were not found in the nucleus, failed to initiate individual E-boxes (mutated nucleotides are shown in bold). Asterisks indicate homology. C, COS-7 cells were transfected with a TFEB expression plasmid and the indicated MuRF1 promoter constructs (-543 bp) harboring E-box mutations as shown in B. Data are represented as mean±SD. *P<0.05, **P<0.01, ***P<0.005. D, Chromatin immunoprecipitation (ChIP) assay performed on chromatin from C2C12 myoblasts using antibodies against TFEB. Primers flanking E-boxes 1, 2, and 3 of the MuRF1 promoter were used. Values indicate the fold-enrichment over chromatin immunoprecipitated with antibodies against IgG. n=5. E and F, ChIP assay performed on chromatin from angiotensin II (Ang II, +) and vehicle-treated (−) C2C12 myoblasts (E). ChIP assay performed on chromatin from serum starved (+) and untreated (−) C2C12 myoblasts (F). Chromatin was immunoprecipitated with antibodies against TFEB. Antibodies against IgG were used as control. n=3. MuRF1 indicates muscle RING-finger-1.

Figure 2. Transcription factor EB (TFEB) regulates MuRF1 expression via conserved E-box elements. A, COS-7 cells were transfected with wild-type FLAG-TFEB expression plasmid or empty vector control (control), along with MuRF1 promoter constructs, as indicated. Data are represented as mean±SD. *P<0.05, **P<0.01, ***P<0.005. B, Schematic diagram of the human MuRF1 promoter. Positions of conserved E-box motifs (CANNTG) in the MuRF1 promoter relative to the transcription start (ATG) are indicated. Alignment shows genomic homology of individual E-box motifs between indicated species. Homo sapiens (mut.) indicates mutated nucleotides to inactivate individual E-boxes (mutated nucleotides are shown in bold). Asterisks indicate homology. C, COS-7 cells were transfected with a TFEB expression plasmid and the indicated MuRF1 promoter constructs (-543 bp) harboring E-box mutations as shown in B. Data are represented as mean±SD. *P<0.05, **P<0.01, ***P<0.005. D, Chromatin immunoprecipitation (ChIP) assay performed on chromatin from C2C12 myoblasts using antibodies against TFEB. Primers flanking E-boxes 1, 2, and 3 of the MuRF1 promoter were used. Values indicate the fold-enrichment over chromatin immunoprecipitated with antibodies against IgG. n=5. E and F, ChIP assay performed on chromatin from angiotensin II (Ang II, +) and vehicle-treated (−) C2C12 myoblasts (E). ChIP assay performed on chromatin from serum starved (+) and untreated (−) C2C12 myoblasts (F). Chromatin was immunoprecipitated with antibodies against TFEB. Antibodies against IgG were used as control. n=3. MuRF1 indicates muscle RING-finger-1.
regulation of its nuclear-to-cytoplasmic export.27 Using luciferase assays, we found that HDAC5 inhibited TFEB-induced MuRF1 expression (Figure 4A). To elucidate the nature of this inhibitory effect, we performed immunocytochemistry and coimmunoprecipitation experiments to test whether TFEB and HDAC5 colocalized and physically interacted with each other, respectively. As shown by immunocytochemistry staining, TFEB and HDAC5 colocalized in the nucleus and in cytosolic vesicular structures when coexpressed in C2C12 myoblasts (Figure 4B). Coimmunoprecipitation experiments showed that TFEB and HDAC5 physically interacted with each other (Figure 4C). Further coimmunoprecipitation experiments were performed to map the interacting region in TFEB and HDAC5 using expression plasmids encoding wild-type and deletion mutants of TFEB and HDAC5 (Figure 4D and 4E). Deletion of amino acids 1 to 127 of TFEB (TFEB 128-C) abolished interaction with wild-type HDAC5. Deletion of AA129-237 in TFEB resulted in decreased binding between TFEB and HDAC5. Removal of the bHLH domain (TFEB Δ299 to 352) or the bHLH-leucine zipper domain in TFEB (TFEB Δ238-400) had no effect on interaction between TFEB and HDAC5 (Figure 4D). These data indicate that direct interaction between TFEB and HDAC5 is mediated by AA1-237 at the amino terminus of TFEB. Moreover, using truncated variants of HDAC5 in coimmunoprecipitation experiments followed by Western blotting, we found that the HDAC5 deletion mutants AA100-C and AA175-C failed to interact with wild-type TFEB (Figure 4E). Luciferase assays were performed to elucidate if interaction between TFEB and HDAC5 is required for the repressive effect of HDAC5 on TFEB activity. As anticipated, activity of those TFEB mutants that did not interact with HDAC5 were not repressed (Figure 4F). Likewise, HDAC5 deletion mutants AA100-C and AA175-C that did not interact with TFEB, or AA1 to 664 that lacks the deacetylase domain of HDAC5, did not repress TFEB-induced MuRF1 expression. Whereas, the HDAC5 deletion mutant AA51-C that still interacts with TFEB, inhibited TFEB-induced MuRF1 expression to similar extend as wild-type HDAC5 (Figure 4G). These data indicate that direct interaction between TFEB and HDAC5 is required for HDAC5-mediated inhibition of TFEB.

**PKD1 Inhibits HDAC5-Mediated Repression of TFEB**

We previously reported that the stress responsive serine/threonine kinase protein kinase D1 (PKD1) plays a regulatory role in muscle remodeling via phosphorylation and nuclear export of HDAC5.28,29 Therefore, we reasoned that PKD1 might play a role in regulation of the HDAC5/TFEB/MuRF1 axis. PKD1 phosphorylates HDAC5 enabling binding of 14-3-3 chaperon proteins and mediating its nuclear export in a CRM1 (chromosomal maintenance 1)-dependent manner.28 Phosphoserines 259 and 498 in HDAC5 serve as binding sites for the chaperone protein 14-3-3.30 To confirm if binding between PKD1 and
HDAC5 leads to PKD1-mediated phosphorylation of the 14-3-3 consensus sites in HDAC5, we performed a UAS-luciferase assay as published recently. In this assay, the N terminus of HDAC5 is fused with the GAL4 DNA-binding domain, and 14-3-3 is fused with the VP16 transactivation domain. Under normal growth conditions, HDAC5 is not phosphorylated in COS-7 cells. Thus, GAL4-HDAC5 cannot interact with 14-3-3-VP16 and the GAL4-dependent luciferase reporter (UAS-luciferase) cannot be activated. Expression plasmids encoding these fusion proteins, together with UAS-luciferase, were transfected into COS-7 cells together with wild-type, constitutive-active, or inactive (kinase dead) PKD1 and increasing amounts of constitutive-active PKD1 expression plasmids, respectively. Wild-type PKD1 and constitutive-active PKD1 increased UAS-luciferase activity (Figure 5A). This activation was dependent on the amount of PKD1 transfected (Figure 5B). These data suggest that HDAC5 interacts avidly with wild-type HDAC5 (Figure 5C). Further mapping experiments showed that AA360-601 in HDAC5 were responsible for interaction between HDAC5 and PKD1 (Figure 5D; Online Figure V). In addition, we identified the cysteine-rich region 1a (C1a) in PKD1 to be responsible for the interaction between PKD1 and HDAC5 (Figure 5E and 5F). We hypothesized that PKD1 inhibits HDAC5-mediated repression of TFEB-induced MuRF1 expression by facilitating nuclear export of HDAC5. Immunocytochemistry showed that colocalization of TFEB and HDAC5 in the nucleus was abolished when PKD1 was coexpressed in C2C12 myoblasts (Figure 5G), indicating that PKD1 controls TFEB activity via regulation of its physical interaction with its repressor HDAC5. Luciferase assays were performed to elucidate if PKD1 inhibits HDAC5-mediated repression of TFEB-induced MuRF1 expression. Expression plasmids encoding HDAC5, TFEB, and Hs_MuRF1-Luc reporter construct (∼543 bp) and expression plasmids encoding WT TFEB, WT HDAC5, and HDAC5 deletion mutants data are represented as mean±SD. **P<0.01, ***P<0.005. n=3. MuRF1 indicates muscle RING-finger-1; and n.s., not significant.
Figure 5. Protein kinase D1 (PKD1) relieves histone deacetylase-5 (HDAC5)-mediated transcription factor EB (TFEB) repression. 

A, HEK293 cells were transfected with UAS-luciferase and expression plasmids encoding GAL4 fused with the wild-type (WT) HDAC5 N-terminal extension together with 14-3-3-VP16 and expression plasmids of WT, constitutive active (CA), or kinase inactive (kinase dead, KD) PKD1 as indicated. Values were normalized to expression of CMV-LacZ and calculated as fold-increase. Data are represented as mean±SD. *P<0.05; **P<0.005. n=3. B, HEK293 cells were transfected with UAS-luciferase and expression plasmids encoding GAL4 fused with the WT HDAC5 N-terminal extension together with 14-3-3-VP16 and increasing amounts of expression plasmids of PKD1 CA (from 6.25 to 400 ng), as indicated. Values were normalized to expression of CMV-LacZ and calculated as fold-increase. Data are represented as mean±SD. *P<0.05, **P<0.01, ***P<0.005. n=3. C, Coimmunoprecipitation (Co-IP) assay with lysates from COS-7 cells expressing Myc-PKD1 and FLAG-HDAC5, as indicated. HDAC5 fusion proteins were immunoprecipitated (IP) with anti-FLAG antibody and PKD1 fusion proteins were detected with an antibody directed against Myc. Input proteins were detected by Western blot (immunoblot, IB) with antibodies directed against the FLAG or Myc tag. n=3. D, Based on Co-IP data, amino acids 360 to 601 of HDAC5 were identified to be required for physical interaction with PKD1 and, therefore, define a PKD1 binding site. E, Co-IP assay of FLAG-PKD1 deletion mutants coexpressed with Myc-HDAC5 to identify the HDAC5 binding domain of PKD1. PKD1 fusion proteins were IP with an anti-FLAG antibody and HDAC5 fusion proteins were detected with an antibody directed against Myc. Input proteins were detected by Western blot (IB) with antibodies directed against the FLAG or Myc tag. n=3. F, Based on the Co-IP data shown in (E), amino acids 1 to 201 of PKD1 were identified to be required for physical interaction with HDAC5 and, therefore, define a HDAC5 docking site. Positions of cysteine-rich region 1a (C1a, yellow), C1b (orange), pleckstrin homology domain (PH; green), and kinase domain (red) of PKD1 are depicted. G, COS-7 cells were transfected with GFP (green fluorescent protein)-HDAC5 and FLAG-TFEB together with an empty vector (pCDNA_3.1) or constitutive active PKD1. Immunostaining was performed with anti-FLAG and anti-GFP antibody. H, HEK293 cells were transfected with expression plasmids encoding WT FLAG-TFEB, HDAC5-Myc, or PKD1 CA proteins, as indicated, together with the Hs_MuRF1_Luc reporter construct (−543 bp). Values were normalized to expression of CMV-LacZ and calculated as the fold-increase in luciferase/CMV-LacZ ratio compared with the reporter alone. Data are represented as mean±SD. *P<0.05; **P<0.005. n=5. MuRF1 indicates muscle RING-finger-1.
Ang II–Induced Atrophy Is Attenuated by Knockdown of TFEB in C2C12 Myotubes

Because Ang II activates PKD1 activity27 and induces muscle atrophy by increasing MuRF1 expression,11–13 we reasoned that TFEB mediates Ang II–induced MuRF1 expression. To test this hypothesis, we used siRNA targeting TFEB in C2C12 myotubes and treated these cells either with Ang II or vehicle for 24 hours. C2C12 myotubes transfected with scrambled siRNA and treated with Ang II or vehicle for 24 hours served as control. A qRT-PCR showed that knockdown of TFEB decreased Ang II–induced MuRF1 mRNA expression (Figure 6A). To test whether Ang II–induced MuRF1 expression was mediated by HDAC5, we transfected a signal-resistant FLAG-HDAC5 S259/498A, harboring alanines in place of serines 259 and 498, which are required for nuclear export of HDAC5, into C2C12 myoblasts for 24 hours and treated these cells either with Ang II or vehicle for 24 hours. C2C12 myoblasts transfected with vector control and treated with Ang II or vehicle for 24 hours served as control. qRT-PCR showed that overexpression of signal-resistant HDAC5 reduced Ang II–induced MuRF1 mRNA expression (Figure 6B). In addition, we tested whether or not TFEB mediates Ang II–induced myofiber atrophy. To test this hypothesis, we used siRNA targeting TFEB in differentiated C2C12 myotubes and treated these cells either with Ang II or vehicle for 48 hours. C2C12 myotubes transfected with scrambled siRNA and treated with Ang II or vehicle for 48 hours served as control. Myotube diameters were measured using ImageJ software. As expected, Ang II treatment induced atrophy of C2C12 myotubes. Knockdown of TFEB signficantly reduced Ang II–induced atrophy of C2C12 myotubes (Figure 6C and 6D). These results indicate that TFEB mediates Ang II–induced MuRF1 expression and myocyte atrophy.

PKD1 Ablation in Skeletal Myocytes Attenuates Ang II–Induced Muscular Atrophy In Vivo

Given that Ang II is a strong activator of the PKD1/HDAC5/TFEB/MuRF1 axis, we hypothesized that absence of PKD1 inhibits Ang II–induced muscle atrophy in vivo. We generated mice with a skeletal muscle–specific deletion of PKD1 (PKD1loxP/loxP; MCK-CRE; conditional PKD1 knockout, PKD1 cKO) to investigate the importance of muscular PKD1 for Ang II–mediated muscle atrophy in vivo as recently described.28,31

Figure 6. Transcription factor EB (TFEB) knockdown reduces endogenous muscle RING-finger-1 (MuRF1) expression and inhibits angiotensin (Ang II)–induced atrophy of C2C12 myocytes. A, Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of MuRF1 expression in C2C12 myotubes following transfection with scrambled control siRNA (siScr) and siRNA targeting TFEB (siTFEB; 100 nmol/L each) for 24 hours and following treatment with 500 nmol/L Ang II or vehicle (−) for 24 hours. Glyceraldehyde 3-phosphate dehydrogenase expression was used as reference. Data are represented as mean±SEM. **P<0.01. n=3. B, qRT-PCR analysis of MuRF1 expression in C2C12 myoblasts following transfection with signal-resistant FLAG-HDAC5 S259/498A (harboring alanines in place of serines 259 and 498 which are required for nuclear export of histone deacetylase-5 [HDAC5]). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as reference. Data are represented as mean±SEM. *P<0.05. n=3. C, C2C12 myoblasts were transfected with siScr or siTFEB (100 nmol/L each), differentiated for 5 days, and treated with 500 nmol/L Ang II or vehicle for 48 hours. Myotubes were photographed and myotube width was measured using ImageJ software. Number of myotubes belonging to a given range of myotube diameters is shown in a size distribution diagram. D, Changes in mean myotube width following siRNA-mediated knockdown of TFEB and Ang II or vehicle treatment is shown. *P<0.05, **P<0.01, ***P<0.005. n=3 each. n.s. indicates not significant; and siRNA, small interfering RNA.
PKD1 WT/WT; MCK-CRE mice were used to control for unspecific MCK-CRE–mediated effects on the skeletal muscle. PKD1 cKO mice and PKD1 WT/WT; MCK-CRE controls were treated with chronic infusion of Ang II (1.5 µg/kg per minute)12,13,32 via osmotic minipumps for 24 hours and 7 days. Ang II treatment led to a significant decrease in gastrocnemius/plantaris weight in PKD1 WT/WT; MCK-CRE mice, but not PKD1 cKO mice after 7 days of treatment (Figure 7A). Accordingly, myocytes cross-sectional area was reduced in Ang II treated PKD1 WT/WT; MCK-CRE mice, but not in PKD1 cKO animals after 7 days of Ang II treatment (Figure 7B). ChIP-PCR assays were performed using chromatin from gastrocnemius/plantaris of 24-hour Ang II– and vehicle-treated PKD1 WT/WT; MCK-CRE and PKD1 cKO mice. This experiment showed that Ang II increased TFEB binding to E-box 1 on the endogenous MuRF1 promoter of PKD1 WT/WT; MCK-CRE, but not PKD1 cKO mice (Figure 7C). A qRT-PCR and Western blot analysis showed that 24-hour Ang II treatment increased MuRF1 mRNA and protein expression in gastrocnemius/plantaris of PKD1 WT/WT; MCK-CRE mice, but not PKD1 cKO (Figure 7D and 7E). In contrast, MuRF1 mRNA and protein expression remained unchanged after 7 days of Ang II treatment (Figure 7D and 7E). These data show that Ang II–induced skeletal muscle atrophy is attenuated in conditional PKD1 knockout (PKD1 cKO) mice.
muscular atrophy is at least partially regulated via PKD1-dependent regulation of TFEB-mediated MuRF1 expression.

Discussion
We identified TFEB as a novel transcriptional regulator of MuRF1. Our study shows that nuclear TFEB specifically binds to conserved E-box motifs in the MuRF1 promoter localized close to its transcription start site. In myocytes, binding of TFEB to the MuRF1 promoter was increased by Ang II treatment and by starvation in vitro. We demonstrated that PKD1, together with HDAC5, controls TFEB activity at the MuRF1 promoter. Our data imply that the PKD1/HDAC5/TFEB/MuRF1 axis mediates Ang II–induced skeletal muscle atrophy (Figure 8). Inhibition of this signaling pathway could be important in combating Ang II–associated muscle wasting disorders, such as CHF-induced cachexia.

We searched for novel regulators of MuRF1 expression. To this end, we used a cDNA library generated from human skeletal muscle and skeletal muscle cells to perform a mechanistic analysis. This approach differs from prior studies investigating the function of TFEB, which have primarily been performed in HeLa cells and in liver.23,24 These studies showed that TFEB is ubiquitously expressed.33 However, expression of TFEB in the skeletal muscle and the heart and transcriptional regulation of the muscle enriched E3 ubiquitin ligase MuRF1 clearly argue for its functional importance in muscle cells. TFEB was reported to promote expression of genes involved in early and late lysosomal biogenesis by direct binding to specific E-box motifs at their promoters.23,24 TFEB also regulates biogenesis of autophagosomes, the fusion of autophagosomes with lysosomes, and autophagic flux.18 Based on its function in lysosomal biogenesis, the gene network regulated by TFEB was clearly clustered in close proximity to the transcription start site of MuRF1. Importantly, these E-box motifs were also clustered. In contrast, E-box 4 that is located further upstream of the transcription start site of MuRF1 and that is not conserved throughout species was not involved in TFEB-mediated MuRF1 expression. In summary, our data indicate that TFEB increases MuRF1 expression via conserved and clustered E-box elements located within the proximal promoter region of MuRF1.

Previous studies showed that TFEB is predominantly localized in the cytoplasm of HEK-293T cells and that its phosphorylation status and cytosolic-to-nuclear shuttling regulates TFEB activity.20 The kinases mTORC1 (mammalian target of rapamycin complex 1) and ERK were shown to be important in that regard.20,34,35 In the presence of nutrients, mTORC1 phospho-lation of TFEB thereby facilitating its binding to 14-3-3 proteins and mediating its retention in the cytoplasm. Conversely, reduced mTORC1 activity increases TFEB shuttling into the
nucleus. Whether or not mTORC1 and ERK regulate TFEB activity in myocytes is unknown. However, we report that TFEB is localized to the cytoplasm and the nucleus of myocytes. Our data are supported by others showing that 20% to 30% of TFEB is contained in the nucleus of several cell lines, such as patient-derived fibroblasts, HeLa cells, ARPE-19 cells, and mouse embryonic fibroblasts. Our observation that TFEB knockdown reduced baseline MuRF1 expression in C2C12 cells indicates that TFEB plays a role in regulation of baseline gene expression and supports the notion that nuclear TFEB is functionally active. We also describe the fact that nuclear TFEB was bound to the MuRF1 promoter already at baseline and that this binding was increased by Ang II treatment or starvation. TFEB mutants that do not bind to E-box motifs are unable to translocate to the nucleus did not increase MuRF1 expression. Importantly, when localized in the nucleus TFEB and HDAC5 interacted with each other. HDAC5, which is primarily localized to the nucleus, directly interacted with TFEB in the nucleus and inhibited its activity. HDAC5 deletion mutants not interacting with TFEB did not repress TFEB-induced MuRF1 expression. Our data indicate that the activity of TFEB is regulated at least at 2 levels, first by regulation of the subcellular localization of TFEB and second by repression of its activity by HDAC5 once it is localized to the nucleus.

Recently, we reported that myogenin, another HLH transcription factor, is involved in neurogenic atrophy and that its activity is regulated by HDAC4 and HDAC5. Our data implicated additional targets of these HDACs promoting neurogenic atrophy. We also showed that myogenin was necessary, but not sufficient, to regulate the genetic program for muscle atrophy and proposed additional important signals for this process. We report that TFEB could at least partially account for these differences. We define TFEB as additional target of these HDACs promoting neurogenic atrophy and that TFEB's activity is regulated via its interaction with HDAC5. However, the importance of this pathway in neurogenic atrophy is unknown.

MuRF1 is a key enzyme in UPS-dependent skeletal muscle atrophy and mediates UPS-dependent degradation of myofibrillar proteins, such as myosin heavy chain and troponin I. MuRF1 expression and protein content are increased in various animal models of muscle atrophy, and germ-line MuRF1 deletion prevents muscle atrophy in mice. MuRF1 is also increased in skeletal muscle of CHF patients with wasting. Importantly, MuRF1 expression was increased in Ang II–induced muscle atrophy indicating that Ang II increases muscle protein breakdown via MuRF1. However, the signaling pathway mediating Ang II–induced MuRF1 expression in muscle was unknown. Recently, investigators reported that Ang II regulates MuRF1 expression in an Akt-Foxo–independent pathway, leaving the question about the role of Ang II–induced MuRF1 expression still open. We show that Ang II induces skeletal muscle atrophy by activation of TFEB. This process involves HDAC5-mediated repression of TFEB-induced MuRF1 expression and negative regulation of HDAC5 by PKD1. We hypothesize that Ang II induces MuRF1 expression via the PKD1/HDAC5/TFEB axis. Based on our data, we speculate that elevated Ang II serum concentrations, as occur in patients with CHF, could activate this pathway. Because cardiac cachexia is a life threatening complication, therapeutic interventions counteracting muscle atrophy and its accompanying weakness could be useful to prevent immobilization and increase quality of life. However, further studies are needed to elucidate the importance of this pathway in vivo.

We speculate that the Ang II/PKD1/HDAC5/TFEB/MuRF1 pathway described here is involved in skeletal muscle wasting of CHF patients. However, we have not proved such an association. To test whether this pathway is involved in skeletal muscle atrophy caused by CHF, the atrophic response of PKD1 cKO mice subjected to standard heart failure models, such as coronary ligation to induce myocardial infarction, transverse aortic constriction, genetic heart failure models, such as deletion of the muscle limb protein, and toxic doxorubicine-induced cardiomyopathy could be investigated. However, CHF is a complex syndrome that in addition to the renin–angiotensin aldosterone system involves many different neuroendocrine pathways, such as the sympathetic nervous system, the endothelin system, and inflammatory mediators. In addition, CHF leads to immobility, which also causes muscle atrophy. All these factors add to the complexity of the aforementioned experiment. We specifically focused on Ang II–mediated muscle atrophy and investigated its downstream signal transduction pathway. Ang II serum levels are increased in heart failure and its role in muscle wasting is well established. However, our mouse model spans only 1 week and we did not show heart failure in our model. Further studies are needed to elucidate whether or not the PKD1/HDAC5/TFEB/MuRF1 axis can also be used and activated by other signaling pathways involved in CHF-associated muscle wasting. Finally, the amount of Ang II infused into mice to induce muscle atrophy in this study surely resulted in higher Ang II plasma levels than those observed in patients with CHF. We used this Ang II dose because it induces atrogene gene expression after 24 hours and muscle atrophy after 7 days. We cannot directly compare Ang II plasma levels between patients and our contrived mouse model. Nonetheless, our data show that deletion of PKD1 prevents Ang II–induced muscle atrophy, when Ang II was infused at a rate of 1.5 μg/kg per minute.

Acknowledgments

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Disclosures

None.
References


Novelty and Significance

**What Is Known?**

- Renin–angiotensin aldosterone system activation in congestive heart failure is associated with skeletal muscle wasting.
- Angiotensin II (Ang II) induces skeletal muscle atrophy via ubiquitin proteasome system induction via the E3 ubiquitin ligase muscle RING-finger-1 (MuRF1).

**What New Information Does This Article Contribute?**

- We show that Ang II increases MuRF1 expression and mediates skeletal muscle wasting.
- The novel pathway regulates the lysosomal hydrolase-coordinating transcription factor TFEB via histone deacetylase-5 (HDAC5) and protein kinase D1 (PKD1).
- TFEB influences not only lysosomal and autophagosomal but also ubiquitin proteasome system–dependent protein degradation.

Muscle wasting often occurs in patients with advanced stages of heart failure and worsens prognosis. Renin–angiotensin aldosterone system activation with increased Ang II is implicated. Ang II increases muscular protein degradation through increased MuRF1 expression. This muscle-enriched E3 ubiquitin ligase is a key mediator of muscle atrophy. However, the Ang II–dependent signaling pathway was unknown. We report that TFEB regulates MuRF1 expression and thereby ubiquitin proteasome system–dependent protein degradation. Recentely, TFEB was shown to regulate biogenesis of lysosomes and autophagosomes in nonmuscle tissue. We show that TFEB directly binds to the MuRF1 promoter. Ang II/PKD1/HDAC5 signal transduction pathway controls TFEB activity. TFEB inhibition abolished Ang II–induced atrophy in vitro. Likewise, myocyte-specific PKD1 deletion attenuated Ang II–induced atrophy in mice in vivo. Our study provides evidence for novel signaling downstream of Ang II that mediates Ang II–induced skeletal muscle wasting. We conclude that TFEB serves as a nodal transcription factor not only regulating lysosomal and autophagosal but also ubiquitin proteasome system–dependent protein degradation. The PKD1/HDAC5/TFEB/MuRF1 axis could constitute a potential therapeutic avenue to attenuate Ang II–mediated muscle wasting disorders.
Angiotensin II Induces Skeletal Muscle Atrophy by Activating TFEB-Mediated MuRF1 Expression

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Supplemental Material.

Animal models.

Animal procedures were performed in accordance with the guidelines of the Max-Delbrück Center for Molecular Medicine and the Charité Universitätsmedizin Berlin, and were approved by the Landesamt für Gesundheit und Soziales (LaGeSo, Berlin, Germany) for the use of laboratory animals (permit number G 0229/12) and followed the “Principles of Laboratory Animal Care” (NIH publication No. 86-23, revised 1985), as well as the current version of German Law on the Protection of Animals.

Angiotensin II (AngII; American Peptide) dissolved in 150 mM NaCl and 1 mM acetic acid was delivered chronically, at a rate of 1.5 µg per kilogram of body weight per day to 8- to 10-wk-old male PKD1 wild type (PKD1WT/WT, MCK-CRE::TFEB, 24h: n = 6, 7 days: n = 6) and skeletal muscle loss of function PKD1loxP/loxP; MCK-CRE (cKO; 24h: n = 5, 7 days: n = 7) mice by using implanted osmotic minipumps (ALZET® model 2001) for 24h and seven days as described1. WT (24h: n = 5, 7 days: n = 5) and cKO (24h: n = 5, 7 days: n = 5) control mice underwent the same procedure except that osmotic pumps were only loaded with vehicle (150 mM NaCl, 1 mM acetic acid). Pair feeding of AngII and vehicle treated mice was performed to correct for AngII induced anorexia. 24h and seven days after implantation of AngII-loaded pumps *gastrocnemius/plantaris* was harvested, and muscle weight, body weight, and tibia length were measured. Muscle specimens were immediately snap frozen in liquid nitrogen and stored at -80°C until further analyses, and embedded for histological analyses.

Cell culture experiments. COS-7 cells (ATCC, CRL1651) and C2C12 cells (ATCC, CRL1772) were cultured in low glucose DME-Medium (PAA, D6046) HEK293 in high glucose DME-Medium (PAA, D5796) both supplemented with 10% FBS (PAA, A15-104) and penicillin and streptomycin (PAA, P11-010) in a humidified 5% CO2 atmosphere at 37°C. For differentiation of C2C12 myoblasts 2x10⁵ cells per well of a 6well-plate were seeded in DME-medium containing 10% FBS which was replaced 24 h later by low glucose DME-Medium containing 2% FBS with penicillin and streptomycin (PAA, P11-010). Differentiation was performed for indicated time points with daily medium exchange. Transfection of C2C12 cells was performed using Lipofectamine® and PLUS™ reagent (both Invitrogen). COS-7 cells were transiently transfected using FuGENE-6 (Roche) following manufacturers’ instructions, HEK293 cells were transiently transfected using polyethyleneimine (PEI, Polyscience Inc., #24313 linear MW2500) with a DNA to PEI ratio of 1 to 3.

Luciferase reporter assays. COS-7 cells were maintained in DME-medium (4.5 g/l glucose) supplemented with 2 mM L-glutamine, 10 % FBS, Penicillin and Streptomycin. COS-7 cells were transfected with cDNA expression plasmids encoding full length or mutant TFEB protein (pcDNA3.1_TFEB_C-myc-(His)₆ or pcDNA3.1_TFEB_N-FLAG), or empty vector control (pcDNA3.1_C-myc-(His)₆ or pcDNA3.1_N-FLAG), as indicated, and the Hs_MuRF1_Luc reporter construct (100 ng), using 2.4 µg/ml Polyethylenimine (Polysciences, linear MW 2.500) for 24h. To control for transfection efficacy pCMV lacZ (Clontech, 50 ng) was used in each sample. Transfected COS-7 cells were washed with ice cold PBS (PAA H15-002) and lysed in 200µL reporter lysis buffer (Promega, E3971). The supernatant was used for quantification of luciferase activity and β-Galactosidase in a Fluostar Optima instrument (BMG-Labtech). Luciferase activity was determined using Dual-Luciferase® Reporter Assay System (Promega) and normalized to fluorescence determined using FluoReporter® lacZ/Galactosidase Quantitation Kit (Invitrogen). Luciferase-to fluorescence ratios were further normalized to pGL3 basic activity.

cDNA library screening. A human male skeletal muscle cDNA library (Invitrogen, #11327-012) was titrated to achieve ~100 cDNA expression plasmids per well in a 96-well-plate. cDNA pools were transfected together with the human 5,002 bp (first screen) and 1,500 bp (second screen) Hs_MuRF1-Luc reporter construct, respectively, and CMV-lacZ construct in COS-7 cells. 24h after transfection luciferase expression was quantified. Sib-selection was performed to obtain single cDNAs capable of inducing the Hs_MuRF1-luciferase construct. For each well (96-well-format) 15,000 COS-7 cells were transfected with 130 ng of pooled cDNA together with 50ng of 5,002 bp or 1,000 bp
Hs_MuRF1-Luc reporter construct and 10 ng of CMV-LacZ (transfection control) using FuGENE6 transfection reagent (Roche) according to the manufacturer’s instructions. Luciferase activity was analyzed 20-24 h post-transfection. Pools of cDNA inducing MuRF1-Luc more than the plates double SD mean value were selected for sib-selection and further analysis. Single cDNAs activating the Hs_MuRF1-Luc construct were identified by sequencing.

RNA isolation, cDNA synthesis and quantitative real-time PCR. Total RNA was isolated from skeletal muscle or cultured cells using TRizol® Reagent (Invitrogen) according to the manufacturer’s protocol. 14. RNAs from C2C12 myotubes following TFEB siRNA treatment were isolated with RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. cDNA synthesis of 1 μg of RNA per sample was carried out by using the SuperScript® First-Strand Synthesis System (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) and self-designed primers (for primer sequences see Online Table I). PCR reactions were performed in a Step-One™ Plus thermocycler (Applied Biosystems) as described recently using a cDNA standard curve. 1, 2, 5, 6. To correct for potential variances between samples regarding differences in mRNA extraction and reverse transcription efficiency, gene expression was normalized to the expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1, 2, 5, 6.

Generation of cDNA expression plasmids and reporter gene assays. The human -5,002 bp MuRF1 Promoter was amplified from genomic DNA by PCR using primer pairs containing restriction enzyme consensus sequences (primer sequences are shown in Online Table II). The PCR product and the vector pGL3 basic (Promega E1751) were digested using respective restriction enzymes (New England Biolabs GmbH) and ligated using T4 ligase (Promega). The resulting reporter plasmid (Hs_MuRF1-Luc) was sequence verified and used for transfection of COS-7 or C2C12 cells. Deletion mutants of the human MuRF1 promoter were generated using Hs_MuRF1_Luc as template (primer sequences are shown in Online Table II). The E-box mutants of the MuRF1 promoter were generated using the Phusion® Site-Directed Mutagenesis Kit. To mutate the consensus E-box motif from CANNNTG to ATNNNTG primers were designed according to the manufacturer’s protocol (primers are shown in Online Table II). All reporter constructs were verified by sequencing. For generation of the cDNA expression vectors pcDNA3.1_TFEB_C-Myc-(His)6, pcDNA3.1_TFEB_N-FLAG and pZSGreen1N1_TFEB expressing murine TFEB with an C-terminal Myc-(His)6 tag, an N-terminal FLAG tag and an C-terminal GFP tag, respectively, mouse TFEB cDNA was purchased from imaGenes (IRAVp968G09140D) and subcloned into pcDNA_3.1myc-(His)6 (-A) (Invitrogen), pcDNA_3.1 FLAG (Invitrogen) and vectors pZSGreen1N1 (Clontech) (primers are shown in Online Table III). TFEB deletion mutants were generated using Phusion® Site-Directed Mutagenesis Kit and the full length pcDNA3.1_TFEB_N-FLAG expression plasmid as template (primers are shown in Online Table III). Expression plasmids of HDAC5, deletion mutants of PKD1, HDAC5, HDAC5 S259/498A, GAL4-HDAC5, UAS-GAL4, 14-3-3-TAD were recently published. 1, 4, 7. The expression plasmid pcDNA3.1_PKD1_N-FLAG encoding wild type human PKD1 was used as template to generate PKD1 deletion mutants by PCR (primers are shown in Online Table IV).

Immunostaining of myoblasts and myotubes in vitro. For immunofluorescence microscopy cells were cultured on glass cover slips, fixed with 4% Para-Formaldehyde (20 min, 4°C), permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature, blocked with 5% serum corresponding to the secondary antibodies host and incubated with specific primary antibody at 4°C over night in a water saturated atmosphere as recently published. After washing with PBS for three times, histological sections as well as cells were incubated with fluorophore coupled secondary antibody. Stained cells were embedded in ProLong Gold® Antifade Reagent that contained DAPI for nuclei staining (Invitrogen). Pictures were taken with the Leica CTR 6500HS microscope and the Leica DFC 360 FX digital camera, and analyzed with Leica LASAF 2.3.5 build 5379 Version 2010 software. Confocal microscopy was performed with a Zeiss LSM 700 microscope and analyzed with Zeiss ZEN 2009 software.
Protein extraction and immunoblotting. Protein analyses were performed as recently published2,9. Shortly, cells were lysed in ice-cold extraction buffer (1:3 wt/vol; 10 mM Tris HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 25 % glycerol, 0.5 % sodium dodecyl sulfate (SDS), 0.5 % Nonident P-40, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng/ml protease inhibitor cocktail) using FastPrep-24 instrument (MP Bio) according to the manufacturers instructions (30 s, 2,000 rpm). Lysates were cleared by centrifugation (4°C, 10 min, 12,000 x g). The supernatant was assayed for protein concentration using Bio-Rad Protein Assay, frozen and stored at -80°C until usage. Protein (20 µg) was separated by 10 % SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto PVDF membranes (Amersham Pharmacia Biotech). Membranes were incubated with specific primary and secondary antibody and the signal was visualized with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific).

Antibodies. Following antibodies were used for western blot, coimmunoprecipitation and chromatin immunoprecipitation analyses: goat anti-TFEB (Abcam, ab2636 and ab122910), anti-Goat-HRP (Abcam, ab6741), mouse anti-GAPDH (Millipore, MAB 374), mouse anti-MuRF1 (Abcam, ab57865), mouse anti-Myc (Millipore, 05-419), rabbit anti-Myc (Upstate, 06-549), rabbit anti-FLAG (Cell Signaling, 2368), anti-Mouse HRP (Cell Signaling, 7076), anti-rabbit HRP (Cell Signaling, 7074), anti-mouse AlexaFluor488 (Invitrogen, A11001) and anti-rabbit AlexaFluor488 (Invitrogen, A11008) antibody.

Coimmunoprecipitation. Transfected cells were washed with ice cold PBS and resuspended in lysis buffer (50 mM potassium phosphate, pH 7.4; 150 mM NaCl, 0.5 % Triton X-100). Lysates were cleared by centrifugation at 10,000 x g for 10 min at 4°C. For coimmunoprecipitation, supernatants were incubated with 30 µl of prewashed anti-FLAG M2 agarose (Sigma, A2220) for 2 h at 4°C. Immunoprecipitated proteins were eluted from agarose by 5 min boiling at 95°C in Laemmli sample buffer (125 mM Tris-HCl, 10% glycerol, 10% SDS, 130 mM DTT). Proteins were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose. Western blot analysis was performed as described above.

Chromatin immunoprecipitation. For chromatin immunoprecipitation (ChIP) experiments, 750,000 C2C12 cells were seeded into 10 cm dishes and after 24 h a transient transfection or indicated treatments were performed. Cells were cross-linked with 1% formaldehyde for 8 min at room temperature. Fixation was stopped by adding 2.5 M glycine to a final concentration of 100 mM and incubation for 10 min at room temperature. Cells were harvested and washed three times with ice cold PBS containing protease inhibitors (Roche) and centrifuged at 4,000 x g for 5 min at 4°C. For tissue ChIP gastrocnemius/plantaris muscles were pulverized on dry ice to powder according to a recently published protocol 10. Tissue powder was suspended in PBS at room temperature containing protease inhibitor cocktail (Roche) and cross-linked with 1% formaldehyde for 15 min at room temperature. Fixation was stopped by adding 0.125 M glycine for 5 min at room temperature. Cross-linked tissue powder was washed three times with ice cold PBS containing protease inhibitors (Roche) and centrifuged at 4,000 x g for 5 min at 4°C. Pellets were suspended in SDS ChIP lysis buffer (10 mM EDTA, 50 mM Tris pH 8.1, 1% SDS). For cell lysis cell suspension was sonicated (Hielscher, UP50H) yielding a DNA fragment sizes of 100-500 bp. Following centrifugation (13,000 x g, 10 min, 4°C) 50 µl of clarified chromatin was used as input control and 100 µl for immunoprecipitation. Clarified chromatin was diluted 1:10 with ChIP RIPA buffer containing 0.1% SDS. Chromatin was immunoprecipitated with antibodies against TFEB (Abcam, ab2636) and immunoglobulin G (Sigma Aldrich, I5006), and anti-FLAG (Sigma) or Ni2+-NTA agarose (Life Technologies) for 2 h at 4°C. Coupled protein-DNA complexes were washed twice in low salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), twice in high salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl) and twice in TE-buffer (10 mM Tris HCl pH 8.8, 1 mM EDTA). Elution was performed by using 55 µl of TE-buffer with 1% SDS at 65°C and shaking at 1200 rpm for 10 min. 50 µl of eluate or input were supplemented with...
150 µl TE-buffer, 5µl 10 % SDS and 1µl RNase (Fermentas, EN0541) and incubated for 30 min at 37°C. Afterwards, proteins were digested by Proteinase K (20 µg/µl, VWR) and lysates were incubated for 6 h at 37°C and 6 h at 65°C. DNA was isolated using DNA purification columns (Macherey-Nagel, 740609, NT buffer 740595) as described by the manufacturer. Input DNA was eluted in 200 µl H2O and immunoprecipitated DNA was eluted in 50 µl H2O. From input and immunoprecipitated DNA 5 µl each were used for qRT-PCR analysis with primers listed in Online Table V.

**siRNA transfection.** C2C12 myoblast or myotubes were transfected with ON-TARGET plus SMART pool (Dharmacon, L-050607-01-0020) siRNA to knock down TFEB or ON-TARGETplus Non-targeting siRNA Control Pool (Dharmacon, D-001810-10-05) (both Thermo Scientific), respectively, using Dharmafect3 reagent (Dharmacon, T-2003, Thermo Scientific) according to the manufacturer’s protocol for 48 h. TFEB expression was analyzed by qRT-PCR.

**Statistics.** Values are presented as mean ± standard deviation (SD), unless otherwise stated. Gene expression was normalized to stably expressed GAPDH mRNA and calculated as relative change. Statistical comparisons were made using two-sided Student’s t tests and Mann-Whitney-U-Test as appropriate. A p-value of less than 0.05 was considered as statistically significant. Statistical tests were computed using SPSS (version 19.0.0.1) and Sigma Plot software (version 11.0). Plots were prepared by using the GraphPad Prism® 6 program (GraphPad Software, La Jolla, CA, USA), Adobe Illustrator CS6, version 16.0.0, and Photoshop CS6, version 13.0.

**References**


Supplemental Figure Legends

**Online Figure I. Schematic diagram of the cDNA expression screening strategy.** COS-7 cells were transfected with the human MuRF1-promoter fragment (5,002 bp) along with pools of cDNAs derived from a human skeletal muscle library and a final concentration of ~100 cDNAs per well, and CMV-LacZ as control. Results from a transfection assay in a representative 96-well plate are shown. The threshold was put to the 2-fold standard deviation (dashed lines) above the plate mean value (straight line). Several rounds of sib selection resulted in a single cDNA contained in each well, which was sequence analyzed.

**Online Figure II. MuRF1 promoter activation by TFEB.** COS-7 cells were transfected with the human MuRF1-promoter reporter gene construct (~5,002 bp) along with pools of cDNAs derived from a human skeletal muscle library, and CMV-LacZ as control. Results from a transfection assay in a representative 24-well plate are shown. Activating cDNAs, marked by arrows, were identified as TFEB. Black lines represent mean plate value (straight line) and mean value plus double standard deviation (dashed line), respectively.

**Online Figure III. TFEB induced MuRF1 expression occurs close to the transcription start site.** COS-7 cells were transfected with wild type FLAG-TFEB expression plasmid or empty vector control (control), along with MuRF1-promoter constructs, as indicated. Luciferase activity was normalized to the expression of CMV-LacZ and calculated as fold-increase. Data are represented as mean ± SD. *P<0.05, **P<0.01, ***P<0.005.

**Online Figure IV. Overexpressed TFEB binds to E-box 1, 2 and 3 in the endogenous MuRF1 promoter.** (A + B) Chromatin immunoprecipitation (ChIP) assay performed in C2C12 myoblasts showing binding of overexpressed wild type TFEB (FLAG-TFEB (A), TFEB-Myc(His)6 (B)), to E-box 1, 2 and 3 of the MuRF1 promoter. C2C12 myoblasts were transfected with expression plasmids encoding wild type FLAG-TFEB, TFEB-Myc(His)6 or empty control. Chromatin was immunoprecipitated with anti-FLAG and Ni2+-NTA agarose, respectively. Primers flanking the E-boxes of the MuRF1 promoter (as depicted above graph) were used to amplify DNA by qRT-PCR. Values indicate the fold-enrichment over chromatin immunoprecipitated with anti-FLAG and Ni2+-NTA agarose, respectively, from empty vector transfected cells. n = 3.

**Online Figure V. Physical interaction mapping between PKD1 and HDAC5.** Coimmunoprecipitation assay with lysates from COS-7 cells expressing wild type FLAG-PKD1, wild type Myc-HDAC5 and Myc-HDAC5 deletion mutants, as indicated. HDAC5-fusion proteins were immunoprecipitated (IP) with anti-Myc antibody and PKD1-fusion proteins were detected with an antibody directed against FLAG. Input proteins were detected by Western blot (IB) with antibodies directed against the FLAG or Myc tag. n = 3.
Online Figure I

Plasmids:

- cDNA library
  - CMV-sport6
  - human skeletal muscle cDNA library

- MuRF1 promoter
  - pGBl3 Basic
  - human MuRF1-promoter

- CMV-beta-Galactosidase
  - pCMV-LacZ

Cell culture:

**first round:**
- ~100 cDNAs/well
- COS-7 cells
  - Cell lysis and quantification of luciferase and fluorescence
  - cDNA pools used for sib selection

**second round:**
- ~10 cDNAs/well
- COS-7 cells
  - Cell lysis and quantification of luciferase and fluorescence
  - cDNA pools used for sib selection

**third round:**
- single cDNA/well
- COS-7 cells
  - Cell lysis and quantification of luciferase and fluorescence

Identification of single MuRF1 activating cDNAs by sequencing

Online Figure I
Online Figure II

$MuRF1$-promoter

fold activation

TFEB
Online Figure III

**MuRF1-promoter**

- 5002 bp
- 3935 bp
- 3500 bp
- 2960 bp
- 1565 bp
- 1006 bp
- 543 bp
- pGL3 basic

**fold-activation**

- **FLAG-TFEB**
- **control**

* Indicates statistical significance.
Online Figure VI

A

B

fold enrichment

fold enrichment

empty Myc(His)_6

empty FLAG

FLAG-TFEB

empty Myc(His)_6

TFEB-Myc(His)_6

E-box 1 E-box 2 E-box 3

E-box 1 E-box 2 E-box 3
Online Figure V

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**Input PKD1**
IB: FLAG

**Input HDAC5**
IB: Myc

**IP: Myc**
IB: FLAG
Online Table I. Primers used for qRT-PCR

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<td>Mm_MuRF2_forward</td>
<td>5’-AGC ACT TCT CTG AAT TAC AAG-3’</td>
</tr>
<tr>
<td>Mm_MuRF2_reverse</td>
<td>5’-TTC ATT TAG GGA ATT CAA CCA G-3’</td>
</tr>
<tr>
<td>Mm_MuRF3_forward</td>
<td>5’-AAC TTC ACG GTG GTT TTC AAG C-3’</td>
</tr>
<tr>
<td>Mm_MuRF3_reverse</td>
<td>5’-GTG CAG GCC TGA GCC TTC TGG C-3’</td>
</tr>
<tr>
<td>Mm_TFEB_forward</td>
<td>5’-GAG CTG GGA ATG CTG ATC C-3’</td>
</tr>
<tr>
<td>Mm_TFEB_reverse</td>
<td>5’-CTT GAG GAT GGT GCC TTT GT-3’</td>
</tr>
</tbody>
</table>

Mm indicates Mus musculus, GAPDH, Glyceraldehyde-3 phosphate dehydrogenase; MuRF, Muscle RING-finger protein; TFEB, transcription factor EB.
Online Table II. Primers used for site directed mutagenesis of the MuRF1 promoter.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs_E-Box_1_mut_forward</td>
<td>5'-CCT CCT GGG GCT ATT GTG ACC AAG ATC-3’</td>
</tr>
<tr>
<td>Hs_E-Box_1_mut_reverse</td>
<td>5'-GAT CTT GGT CAC AAT AGC CCC AGG AGG-3’</td>
</tr>
<tr>
<td>Hs_E-Box_2_mut_forward</td>
<td>5'-CAT CGG AAT GCT ATG CTG GTC CCC TC-3’</td>
</tr>
<tr>
<td>Hs_E-Box_2_mut_reverse</td>
<td>5'-CCA AGC GGC TGG TGG GGC TTG AG-3’</td>
</tr>
<tr>
<td>Hs_E-Box_3_mut_forward</td>
<td>5'-GAT TGC TCA TCC CTG ATT GTG ATT TGA GAG-3’</td>
</tr>
<tr>
<td>Hs_E-Box_3_mut_reverse</td>
<td>5'-CTC TCA AAT CAC AAT CAG GGA TGA GCA ATC-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-5,002_for (NheI)</td>
<td>5'-CTA GCT AGC AAC AGG GCC ATG TGA ATG GC-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-3,938_for (NheI)</td>
<td>5'-GTG CTA GCC CTG GAG TTA TAG GTG TGA GC-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-3,500_for (NheI)</td>
<td>5'-GTG CTA GCC AGA ATC ATC AGC ATA TGG-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-2,960_for (NheI)</td>
<td>5'-GTG CTA GCC TGA GGT CCC ATG AGC AAG GAA G-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-1,565_for (NheI)</td>
<td>5'-GTG CTA GCC AGG CGT GAG TCA CTG TGC CC-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-1,006_for (NheI)</td>
<td>5'-GTG CTA GCC CAT TTC AAA ATC TGT AAG ATG-3’</td>
</tr>
<tr>
<td>Hs_MuRF1_Luc_-543_for (NheI)</td>
<td>5'-GTG CTA GCC TAC TCA GAA AAA TGT CTG ATG-3’</td>
</tr>
<tr>
<td>pGL3 basic reverse (XhoI)</td>
<td>5'-CCG CTC GAG CAT TCT GTG GGA AGG AAT GA-3’</td>
</tr>
</tbody>
</table>
## Online Table III. Primers used for generation of TFEB cDNA expression plasmids and its deletion mutants.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFEB pcDNA™3.1myc-(His)_6 for (EcoRI)</td>
<td>5'-TGA ATT CAT GGC TCA GCT CGC TCA G-3'</td>
</tr>
<tr>
<td>TFEB pcDNA™3.1myc-(His)_6 rev (KpnI)</td>
<td>5'-CAG GTA CCC AGA ACA TCA CCC TCC TCC ATG CT-3'</td>
</tr>
<tr>
<td>TFEB pcDNA™3.1 FLAG for (EcoRI)</td>
<td>5'-GGA ATT CGC TCA GCT CGC TCA GTG TGC T-3'</td>
</tr>
<tr>
<td>TFEB pcDNA™3.1 FLAG rev (KpnI)</td>
<td>5'-GGG TAC CTC ACA GAA CAT AAC CCT CCA TGC TGC T-3'</td>
</tr>
<tr>
<td>TFEB pZsGreen1-N1 for (NheI)</td>
<td>5'-CTA GCT AGC ATG GCT CAG CTC GCT CAG TGG-3'</td>
</tr>
<tr>
<td>TFEB pZsGreen1-N1 rev (Xhol)</td>
<td>5'-CCG CTC GAG CAG AAT ATG ACC CTC CTC CTC CA-3'</td>
</tr>
<tr>
<td>TFEB Δ1-128 forward</td>
<td>5'-AAG GTG CAG TCC TAC CTG GAG AAC-3'</td>
</tr>
<tr>
<td>TFEB Δ1-128 reverse</td>
<td>5'-ATG CCT AAC ACG CTG CCC CTG-3'</td>
</tr>
<tr>
<td>TFEB Δ129-237 forward</td>
<td>5'-ATG CCT AAC ACG CTG CCC CTG-3'</td>
</tr>
<tr>
<td>TFEB Δ129-237 reverse</td>
<td>5'-CAG CTC CCC GGG CAC A-3'</td>
</tr>
<tr>
<td>TFEB Δ238-400 forward</td>
<td>5'-CAG CAG GTG GTG AAG CAA GAG TTG-3'</td>
</tr>
<tr>
<td>TFEB Δ238-400 reverse</td>
<td>5'-CTG CAT CTC AGG GTT GAT GTA GCC CA-3'</td>
</tr>
<tr>
<td>TFEB Δ299-352 forward</td>
<td>5'-TCC CGG CGC CTG GAG ATG ACT-3'</td>
</tr>
<tr>
<td>TFEB Δ299-352 reverse</td>
<td>5'-GTG ATT GTC TTT CTT CTG CCG CTC CT-3'</td>
</tr>
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</table>
**Online Table IV. Primers used to generate deletion mutants of PKD1.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs_PKD1_1-201_forward (ClaI)</td>
<td>5’-ATC GAT AGC GCC CCT CCG GTC CTG CG-3’</td>
</tr>
<tr>
<td>Hs_PKD1_1-201_reverse (EcoRI)</td>
<td>5’-GAA TTC GGG ACG AAT CTG AAA GTC TTC-3’</td>
</tr>
<tr>
<td>Hs_PKD1_1-327_reverse (EcoRI)</td>
<td>5’-GAA TTC CGG CAC TTT AAT TTT AGA CAT C-3’</td>
</tr>
<tr>
<td>Hs_PKD1_1-574_reverse (EcoRI)</td>
<td>5’-GAA TTC GAC TGT GCT GCT TTT CCT CTT C-3’</td>
</tr>
<tr>
<td>Hs_PKD1_201-C_forward (EcoRI)</td>
<td>5’-GAA TTC CTT GGC GAA GTG ACC ATT AAT G-3’</td>
</tr>
<tr>
<td>Hs_PKD1_201-C_reverse (XhoI)</td>
<td>5’-CTC GAG TCA GAG GAT GCT GAC ACG CTC A-3’</td>
</tr>
</tbody>
</table>

PKD1 indicates protein kinase D1; Hs, Homo sapiens.
Online Table V. Primers used for ChIP-PCR of E-box 1, 2 and 3 of the \textit{MuRF1} promoter.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm\textsubscript{_MuRF1} E-box 1 forward</td>
<td>5’-TCC TGG GGC TCA TGT GAC-3’</td>
</tr>
<tr>
<td>Mm\textsubscript{_MuRF1} E-box 1 reverse</td>
<td>5’-CCC TCT GAT ATT TAT AGC TGC AC-3’</td>
</tr>
<tr>
<td>Mm\textsubscript{_MuRF1} E-box 2 forward</td>
<td>5’-CTC AAG CCC TGC CAG CAG-3’</td>
</tr>
<tr>
<td>Mm\textsubscript{_MuRF1} E-box 2 reverse</td>
<td>5’-CAG GAG GGG ACC AGC TG-3’</td>
</tr>
<tr>
<td>Mm\textsubscript{_MuRF1} E-box 3 forward</td>
<td>5’-CAA CAG CGA TTG CTC ATC CC-3’</td>
</tr>
<tr>
<td>Mm\textsubscript{_MuRF1} E-box 3 reverse</td>
<td>5’-AAG ATT TGG CCC TCT CAG ATC-3’</td>
</tr>
</tbody>
</table>