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

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ABSTRACT

**Rationale:** Skeletal-muscle wasting with accompanying cachexia is a life threatening complication in congestive heart failure (CHF). The molecular mechanisms are imperfectly understood, although an activated renin-angiotensin aldosterone system (RAAS) 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-D (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 siRNA prevented Ang II induced MuRF1 expression and atrophy. The histone deactylase-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 CHF patients, could activate the PKD1/HDAC5/TFEB/MuRF1 pathway to induce skeletal muscle wasting.

**Keywords:** TFEB, MuRF1, cardiac cachexia, skeletal muscle atrophy, Angiotensin II, transcription factor EB, histone deacetylase 5, protein kinase D1, congestive heart failure, gene expression/regulation, transcriptional regulation, skeletal myopathy exercise.

**Nonstandard Abbreviations and Acronyms:**
- ACE: angiotensin converting enzyme
- Ang II: angiotensin II
- bHLH-LZ: basic helix-loop-helix leucine zipper
- CHF: congestive heart failure
- ChiP: chromatin immunoprecipitation
- CLEAR: coordinated lysosomal expression and regulation
- GAPDH: glyceraldehyde 3-phosphate dehydrogenase
- HDAC5: histone deacetylase 5
- HRP: horseradish peroxidase
- Luc: luciferase
- MuRF: muscle RING-finger
- PKD1: protein kinase D1
- qRT-PCR: quantitative real-time PCR
- RAAS: renin-angiotensin aldosterone system
- siRNA: small interfering RNA
- TFEB: transcription factor EB
- UPS: ubiquitin proteasome system
INTRODUCTION

Skeletal muscle plasticity assures functional adaptation to physiological and pathological conditions by regulating muscle mass and fiber type.\(^1\) Muscle mass is regulated by a well-controlled balance between protein synthesis and degradation.\(^2\) Increased protein degradation and/or 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 cancer\(^1\) or end-stage congestive heart failure (CHF)\(^6\), where cachexia is the major constituent.\(^6\), \(^7\) 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 (RAAS) is activated and Ang II serum levels are increased in CHF patients.\(^8\), \(^9\) Second, reduced RAAS activity by angiotensin converting enzyme (ACE) inhibition reduced cachexia in CHF patients.\(^6\) 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.\(^11\) This muscle-enriched E3 ubiquitin ligase is a key mediator of muscle atrophy.\(^14\) 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.

METHODS

An expanded Materials and Methods section is included in the Online 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 (-5,002bp upstream of the transcription start site, Hs_MuRF1-Luc) and a human skeletal muscle cDNA library according to our previous work.\(^15\), \(^16\) 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. Following sib selection, a cDNA encoding the transcription factor EB (TFEB) was identified as strong inducer of Hs_MuRF1-Luc (Online Figure II). TFEB belongs to the MITF/TFE family of basic helix-loop-helix (bHLH) transcription factor EB (TFEB) has been shown to regulate lysosomal biogenesis, autophagy and lipid metabolism.\(^17\), \(^18\) However, regulation of MuRF1 expression by TFEB has not been reported.

To confirm the results from the cDNA expression screen we generated cDNA expression constructs of TFEB and tested if overexpression of TFEB activates Hs_MuRF1-Luc activity. TFEB increased Hs_MuRF1-Luc activity in a dose dependent manner indicating that TFEB activates MuRF1 expression (Figure 1A). Because MuRF1 is primarily contained in skeletal muscle and heart\(^14\) whereas TFEB is ubiquitously expressed,\(^21\), \(^22\) quantitative real-time PCR (qRT-PCR) was used to test if TFEB was also expressed in striated muscle. To investigate whether TFEB is expressed in a fiber-type specific manner we quantitated its expression in muscle primarily containing fast twitch/type II fibers (tibialis anterior, extensor digitorum longus), and both fast twitch/type II and slow twitch/type-I fibers (soleus, gastrocnemius/plantar). To compare muscular TFEB expression with expression in liver and spleen, both organs were included into the analysis. Our data showed that TFEB expression in skeletal muscle and the heart is similar with its expression in liver where the function is well described.\(^18\) No evidence was found for fibre type related differences in TFEB expression. However, because TFEB was contained...
in all skeletal muscle and all parts of the heart analyzed, TFEB could contribute to transcriptional regulation of MuRF1 in muscle (Figure 1B). To test if TFEB increases endogenous MuRF1 mRNA expression and protein content in myocytes, we used qRT-PCR and Western blot analysis of lysates from C2C12 myoblasts transfected with cDNA expression plasmids encoding TFEB. Overexpressed TFEB increased endogenous MuRF1 mRNA expression (Figure 1C) and protein content (Figure 1D) in these cells. In addition to MuRF1, we analyzed the effect of TFEB on MuRF2 and MuRF3 expression, homologous MuRF family members that are also restricted to striated muscles. In contrast to MuRF1, TFEB did not elevate MuRF2 and MuRF3 expression (Figure 1C). Loss-of-function experiments were performed to investigate if TFEB is required for basal MuRF1 expression in C2C12 myoblasts. The siRNA mediated TFEB knockdown led to reduced MuRF1 mRNA expression and protein content in C2C12 myoblasts in vitro (Figure 1E and F).

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 -5,002 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 -543bp MuRF1 promoter fragment for further analysis. Because TFEB is known to bind to specific E-box motifs in the promoter of lysosomal genes, so called CLEAR elements, the sequence of the -543bp MuRF1 promoter fragment was analyzed. Sequence analysis for conserved E-box motifs found four (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-box 1, 2 and 3 for further analyses (Figure 2B). To investigate the importance of E-box 1 to 3 for TFEB induced MuRF1 expression, site-directed mutagenesis was used to mutate these E-box motifs from CANNTG to ATNNTG, known to inhibit E-box functionality. The -543 bp 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). These data indicate that E-box 1 and 3 in the human MuRF1 promoter mediate TFEB induced MuRF1 expression. We next used chromatin-immunoprecipitation followed by qRT-PCR (ChIP-PCR) to elucidate if TFEB binds to the conserved E-box motifs E-box 1, 2, and 3 in the endogenous MuRF1-promoter. ChIP-PCR 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 to the endogenous MuRF1-promoter (Figure 2D). In addition, we tested if 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-box 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 IVA, B). Because MuRF1 is known to mediate Ang II-induced skeletal muscle atrophy, 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-box 1, 2, and 3 in the endogenous MuRF1-promoter (Figure 2E). Since starvation was shown to increase TFEB activity in other cell types, 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-box 1, 2, and 3 in the MuRF1-promoter.

Although the function and regulation of TFEB in non-muscle cells is well described, 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 or mutant TFEB (Figure 3A) and
transfected into C2C12 cells. Overexpressed wild type TFEB was localized in the nucleus, cytosol and vesicular structures (Figure 3B, Figure 4C). Deletion of amino acids (AA) 1-127 (128-C), containing the glycine-rich (GR) domain, or AA129-237, containing regulatory serine residues in TFEB, had no effect on TFEBs subcellular localization (Figure 3B). In contrast, deletion of AA238-400, encoding the bHLH and leucine zipper (LZ) domain, and AA299-352, encoding only the bHLH domain, abolished nuclear localization of TFEB in C2C12 myocytes (Figure 3B). These data 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 increase MuRF1 expression (Figure 3C). In contrast, TFEB deletion mutants AA128-C and AA129-237 increased MuRF1 expression but not to the same extent as wild type TFEB (Figure 3C). We used western blot analysis to test if deletion mutant TFEB AA299-352 would also fail to increase the endogenous MuRF1 protein content in C2C12 cells. No increase in endogenous MuRF1 protein content was detected after transfection of this deletion mutant (Figure 3D). These data indicate that in myocytes TFEB is localized to the cytoplasm and to the nucleus, and that its nuclear localization is important for its effects on MuRF1 expression.

Recently, we reported that the bHLH transcription factor myogenin increases MuRF1 expression and that class II histone deacetylases (HDACs) inhibit the activity of myogenin at the MuRF1 promoter. Because TFEB was localized to both the nucleus and the cytoplasm of myocytes, we hypothesized that class IIa HDACs inhibit the activity of nuclear TFEB. According to our working hypothesis we focused on HDAC5, because Ang II was shown to repress HDAC5 activity in smooth muscle cells via regulation of its nuclear-to-cytoplasmic export. 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 if 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 full length and deletion mutants of TFEB and HDAC5 (Figure 4D, 4E). Deletion of amino acids 1-127 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 Δ299-352) or the bHLH-LZ 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 1-664 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. 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-dependent manner. Phosphoserines 259 and 498 in HDAC5 serve as binding sites for the chaperone protein 14-3-3. 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 to the GAL4 DNA-binding domain, and 14-3-3 is fused to 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 (CA), or inactive (kinase-dead) PKD1 and increasing amounts of PKD1 CA expression plasmids, respectively. Wild-type PKD1 and constitutive-active PKD1 increased UAS-luciferase activity (Figure 5A). This activation was dependent upon the amount of PKD1 transfected (Figure 5B). These data indicate that PKD1 creates phospho-14-3-3 recognition motifs in HDAC5, which recruit 14-3-3 chaperone proteins eventually leading to its nuclear export. Coimmunoprecipitation experiments showed that wild-type PKD1 interacted 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, F). 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 MuRF1-Luc were transfected together with PKD1 in COS-7 cells and their effect on MuRF1 promoter activity was investigated. Indeed, PKD1 abolished HDAC5-mediated inhibition of TFEB induced MuRF1 expression (Figure 5H).

Ang II-induced atrophy is attenuated by knockdown of TFEB in C2C12 myotubes.

Because Ang II activates PKD1 activity and induces muscle atrophy by increasing MuRF1 expression, 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 h. C2C12 myotubes transfected with scrambled siRNA and treated with Ang II or vehicle for 24 h 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 h and treated these cells either with Ang II or vehicle for 24 h. C2C12 myoblasts transfected with vector control and treated with Ang II or vehicle for 24 h served as control. qRT-PCR showed that overexpression of signal-resistant HDAC5 reduced Ang II induced MuRF1 mRNA expression (Figure 6B). Additionally, 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 h. C2C12 myotubes transfected with scrambled siRNA and treated with Ang II or vehicle for 48 h served as control. Myotube diameters were measured using ImageJ software. As expected, Ang II treatment induced atrophy of C2C12 myotubes. Knockdown of TFEB significantly reduced Ang II-induced atrophy of C2C12 myotubes (Figure 6C, D). 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 activates PKD1 activity and induces muscle atrophy by increasing MuRF1 expression, 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. PKD1WT/WT; MCK-CRE mice were used to control for unspecific MCK-CRE-mediated effects on the skeletal muscle. PKD1cKO mice and PKD1WT/WT; MCK-CRE controls were treated with chronic infusion of Ang II (1.5 µg/KG/min) via osmotic minipumps for 24 h and seven days. Ang II treatment led to a significant decrease in gastrocnemius/plantarism weight in PKD1WT/WT; MCK-CRE controls.
mice, but not PKD1 cKO mice after seven days’ treatment (Figure 7A). Accordingly, myocytes-cross-sectional area (MCSA) was reduced in Ang II treated PKD1WT/WT; MCK-CRE mice, but not in PKD1 cKO animals after seven days’ Ang II treatment (Figure 7B). ChIP-PCR assays were performed using chromatin from gastrocnemius/plantaris of 24 h Ang II- and vehicle-treated PKD1WT/WT; MCK-CRE and cKO mice. This experiment showed that Ang II increased TFEB binding to E-box 1 on the endogenous MuRF1-promoter of PKD1WT/WT; MCK-CRE, but not cKO mice (Figure 7C). A qRT-PCR and Western blot analysis showed that 24 h Ang II treatment increased MuRF1 mRNA and protein expression in gastrocnemius/plantaris of PKD1WT/WT; MCK-CRE mice, but not PKD1 cKO (Figure 7D, E). In contrast, MuRF1 mRNA and protein expression remained unchanged after seven days’ Ang II treatment (Figure 7D, E). These data show that Ang II-induced 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 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 named CLEAR (Coordinated Lysosomal Expression and Regulation) and the specific E-box motifs TFEB binds to CLEAR elements. The consensus sequence of the CLEAR element was resolved as CACGTG,23, 24 which is consistent with a specific E-box motif (CANNTG). The sequence of the conserved E-box motifs in the MuRF1-promoter to which TFEB binds clearly differs from the described CLEAR element consensus sequence. E-boxes 1 and 3, predominantly mediating TFEB-induced MuRF1 expression in myocytes, share the consensus site CATGTG, which was highly conserved throughout species. Nevertheless, since TFEB strongly increased MuRF1 expression via these E-box motifs and the fact that increased binding of TFEB to the MuRF1 promoter was found after Ang II treatment and starvation supports the idea that these E-box motifs are functional. The distinct differences in the sequence between the E-box motifs in the MuRF1 promoter and CLEAR elements imply that TFEB also regulates the expression of genes not involved in lysosomal or autophagosomal biogenesis. Although we would like to speculate that the PKD1/HDAC5/TFEB axis also regulates lysosomal and autophagosomal protein degradation in muscle, such experiments were beyond the scope of this work. We contribute the findings that TFEB additionally regulates expression of MuRF1, a muscle enriched E3 ubiquitin ligase, which plays a key role in UPS dependent protein degradation in muscle atrophy.14 Therefore, we believe that TFEB serves as a nodal transcription factor regulating not only lysosomal and autophagosomal, but also UPS dependent protein degradation in muscle cells.

Recent data suggest that only the subset of TFEB binding sites associated with proximal promoter regions in lysosomal genes is functional. Most TFEB binding sites were localized in close proximity to the transcription start sites, with most sites lying between positions -300 bp to +100bp of the 5’ end of genes. Genes highly responsive to TFEB often display clustered TFEB binding sites.24 We
found E-box 1, 2, and 3 in the MuRF1 promoter, the sites at which TFEB binds, are located close 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 regulate TFEB activity. The kinases mTORC1 and ERK were shown to be important in that regard. 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 associated with HDAC5. 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 two 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 HDAC5 and describe 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. Thus, 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 regarding 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 CHF patients, 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/TFEB/HDAC5/MuRF1 pathway described here is involved in skeletal muscle wasting of EFH patients. However, we have not proved such an association. To test if this pathway is involved in skeletal muscle atrophy caused by CHF the atrophic response of PKD1 KO 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 RAAS, 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 model spans only one week and we did not show heart failure in our model. Further studies are needed to elucidate whether or not the PKD1/TFEB/HDAC5/MuRF1 axis can also be utilized and activated by other signaling pathways involved in CHF-associated muscle wasting. Finally, the amount of Ang II infused into mice to induced 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 h and muscle atrophy after seven 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/min.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

P.D.-B., C.P.-T., D.L., M.K., F.S., K.S., S.S., R.B.-D., E.N.O. and J.F. designed and analyzed experiments and prepared the manuscript. All authors read and approved the manuscript.
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FIGURE LEGENDS

Figure 1. A cDNA expression screen identified the transcription factor EB (TFEB) as activator of the human MuRF1 promoter. (A) Luciferase assays performed on cell extracts of COS-7 cells transfected with Hs_MuRF1-Luc (-543 bp) with increasing amounts of FLAG-TFEB (TFEB) or empty (-) plasmid. Luciferase activity was normalized to expression of CMV-LacZ and expressed as fold-increase. Data are represented as mean ± SD. **P<0.005, ***P<0.001. (B) qRT-PCR analysis of TFEB expression in skeletal muscle, heart, liver and spleen of C57Bl6/N mice (n = 4-5). GAPDH expression was used as reference. Data are represented as mean ± SD. GP indicates gastrocnemius/plantarlis, Sol, soleus; TA, tibialis anterior, EDL, extensor digitorum longus; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle. (C) qRT-PCR of MuRF1, MuRF2 and MuRF3 expression following transfection of cDNA expression plasmids encoding for FLAG-TFEB (+, TFEB) or empty (-) expression plasmid in C2C12 myoblasts for 24 h. GAPDH expression was used as reference. Data are represented as mean ± SD. **P<0.005, ***P<0.001. n = 3 each. (D) C2C12 cells were transfected with expression plasmids encoding wild type FLAG-TFEB (TFEB) or empty (control) expression plasmid for 24h, as indicated. Immunoblotting (IB) with anti-FLAG, anti-MuRF1 or anti-GAPDH antibody was performed. (E, F) C2C12 myoblasts were transfected with scrambled control siRNA (siScr, -) or siRNA targeting TFEB expression in luciferase assays, or their interaction with HDAC5 in transfected cells. Luciferase activity was 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 each. (D) Western blot analysis was performed with anti-FLAG, anti-MuRF1 or anti-GAPDH antibody.

Figure 2. 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). * indicates 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<0.005. (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. Positions of conserved E-box elements are indicated. (E, F) Chromatin immunoprecipitation (ChIP) assay performed on chromatin from C2C12 myoblasts using antibodies against IgG. n=5. (E, F) ChIP assay performed on chromatin from angiotensin II (Ang II, +) and vehicle treated (-) C2C12 myoblasts. 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.

Figure 3. Induction of MuRF1 expression is mediated through TFEB’s bHLH domain. (A) Schemes of wild type TFEB and its deletion mutants used in this study. The presence of wild type and mutant TFEB protein in the nucleus (N) or cytoplasm (C), as detected by immunostaining, their ability to induce MuRF1 expression in luciferase assays, or their interaction with HDAC5 in coimmunoprecipitation assays, respectively, are indicated. GR indicates glycine-rich domain; bHLH, basic Helix-Loop-Helix domain; LZ, Leucine Zipper domain; PR, Proline-rich domain. (B) The subcellular distribution of wild type FLAG-TFEB and its deletion mutants in transfected C2C12 myoblasts was detected by immunofluorescence using anti-TFEB antibody together with A488-coupled secondary antibody. Nuclei were stained with DAPI (blue); scale bar 20 μm. (C) HEK293 cells were transfected with the Hs_MuRF1_Luc reporter construct (-543 bp) and expression plasmids encoding wild type or mutant TFEB, as indicated. Luciferase activity was normalized to expression of CMV-LacZ and calculated as fold-increase. Data are represented as mean ± SD.*,P<0.05; **P<0.001. n = 3. (D) C2C12 cells were transfected with expression plasmids encoding wild type (WT) FLAG-TFEB, TFEB deletion mutant Δ299-352 or empty expression plasmid (control) for 24 h, as indicated. Immunoblotting (IB) with anti-FLAG, anti-MuRF1 or anti-GAPDH antibody was performed.
Figure 4. TFEB induced MuRF1 expression is inhibited by HDAC5. (A) HEK293 cells were transfected with MuRF1-luciferase reporter (-543 bp) and expression plasmids encoding wild type TFEB, histone deacetylase (HDAC) 5 or empty vector control as indicated. Data are represented as mean ± SD. ***P<0.001. n = 3. (B) The subcellular distribution of wild type TFEB-GFP and HDAC5-Myc in transfected C2C12 myoblasts was detected by immunofluorescence. (a+b) indicate augmentations. neg. = negative control. (C) Co-IP with lysates from HEK293 cells expressing Myc-HDAC5 and FLAG-TFEB (top panel), or TFEB-Myc(His), and FLAG-HDAC5 (bottom panel), as indicated. Extracts were immunoprecipitated (IP) with anti-FLAG antibody and detected with an antibody directed against Myc. Input proteins were detected by Western blot (IB) with anti-FLAG and anti-Myc antibodies, respectively. n = 5. (D) Co-IP assay with lysates from HEK293 cells expressing wild type FLAG-TFEB or TFEB deletion mutants along with wild type Myc-HDAC5, as indicated. IP of TFEB-fusion proteins were using anti-FLAG antibody and detection by anti-Myc antibody. Input proteins were detected by Western blot (IB) with antibodies directed against FLAG and Myc. n = 3. (E) Co-IP assay with lysates from HEK293 cells expressing wild type Myc-HDAC5 or HDAC5 deletion mutants along with wild type FLAG-TFEB, as indicated. TFEB-fusion proteins were immunoprecipitated (IP) with anti-FLAG antibody and detected with an antibody directed against Myc. Input proteins were detected by immunoblot (IB) with antibodies directed against FLAG and Myc. n = 3. (F) HEK293 cells were transfected with the Hs_MuRF1Luc reporter construct (-543 bp) and expression plasmids encoding wild type TFEB, TFEB deletion mutants and wild type HDAC5 as indicated. Data are represented as mean ± SD. **P<0.01, ***P<0.005. n = 3. (G) HEK293 cells were transfected with the Hs_MuRF1Luc reporter construct (-543 bp) and expression plasmids encoding wild type TFEB, wild type HDAC5 and HDAC5 deletion mutants. Data are represented as mean ± SD. **P<0.01, ***P<0.005. n = 3.

Figure 5. PKD1 relieves HDAC5-mediated TFEB repression. (A) HEK293 cells were transfected with UAS-luciferase and expression plasmids encoding GAL4 fused to the wild type HDAC5 N-terminal extension together with 14-3-3-VP16 and expression plasmids of wild type (WT), constitutive active (CA) or kinase inactive (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 to the wild type HDAC5 N-terminal extension together with 14-3-3-VP16 and increasing amounts of expression plasmids of PKD1 CA (from 6.25 ng 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 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 (IB) with antibodies directed against FLAG and Myc tag. n = 3. (D) Based on the coimmunoprecipitation 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) Coimmunoprecipitation assay of FLAG-PKD1 deletion mutants coexpressed with Myc-HDAC5 to identify the HDAC5 binding domain of PKD1. PKD1-fusion proteins were immunoprecipitated (IP) with 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 coimmunoprecipitation data, 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-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 wild type FLAG-TFEB, HDAC5-Myc, or PKD1 CA proteins, as indicated, together with the Hs_MuRF1Luc reporter construct (-543 bp). Values were normalized to expression of CMV-LacZ and calculated as the fold-increase in luciferase to CMV-LacZ ratio compared to the reporter alone. Data are represented as mean ± SD. *P<0.05; **P<0.005. n = 5.
Figure 6. TFEB knockdown reduces endogenous MuRF1 expression and inhibits Ang II induced atrophy of C2C12 myocytes. (A) qRT-PCR analysis of MuRF1 expression in C2C12 myotubes following transfection with scrambled control siRNA (siScr) and siRNA targeting TFEB (siTFEB; 100 nM each) for 24h and following treatment with 500 nM Ang II or vehicle (-) for 24 h. GAPDH 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 HDAC5). GAPDH expression was used as reference. Data are represented as mean ± SEM. *P<0.05. n = 3. (C) C2C12 myoblasts were transfected with scrambled control siRNA (siScr) or siRNA targeting TFEB (siTFEB; 100 nM each), differentiated for 5 days, and treated with 500 nM Ang II or vehicle for 48h. 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.

Figure 7. Ang II induced skeletal muscle atrophy is attenuated in PKD1 cKO mice. Ang II (1.5 µg/KG/min) was delivered chronically to 8- to 10-wk-old male PKD1 wild type (WT; PKD1WT/WT; MCK-CRE) and skeletal muscle loss of function PKD1loxP/loxP; MCK-CRE (cKO) mice for 24h and 7 days, respectively, by using an implanted osmotic minipump. Vehicle treated wild type (PKD1WT/WT; MCK-CRE) and cKO mice were used as controls. Numbers of animals used are indicated. (A) Gastrocnemius/plantaris to tibia length ratios are shown. **P<0.01. n.s. not significant. (B) Myocyte cross sectional area (MCSA) of histological sections from gastrocnemius/plantaris (+SEM) of WT and cKO mice after 7 days of Ang II or vehicle treatment. Number of myotubes belonging to the given range of MCSA are shown in a size distribution diagram. (C) Chromatin immunoprecipitation (ChIP) assay performed on chromatin from gastrocnemius/plantaris of 24h Ang II and vehicle treated WT and cKO mice using anti-TFEB antibody. Primers flanking E-box 1 of the MuRF1 promoter were used. Values indicate the fold-enrichment over chromatin immunoprecipitated with anti-IgG antibody. n=3. *P<0.05, **P<0.01, n.s. not significant. (D) MuRF1 expression in gastrocnemius/plantaris (+SEM) of WT and cKO mice after 24h and 7 days of Ang II or vehicle treatment. GAPDH was used as a reference. Data are presented as mean±SEM. *P<0.05. (E) Western blot analysis on protein lysates from gastrocnemius/plantaris of 24h Ang II and vehicle treated WT and cKO mice using anti-MuRF1 and anti-GAPDH antibody. n.s., no specific signal.

Figure 8. The Ang II/PKD1/HDAC5 signaling pathway regulates TFEB-induced MuRF1 expression. The PKD1/HDAC5/TFEB/MuRF1 axis mediates Ang II induced skeletal muscle atrophy. Nuclear TFEB specifically binds to conserved E-box motifs in the MuRF1 promoter localized close to its transcription start site. PKD1 together with HDAC5 controls TFEB activity at the MuRF1 promoter. Inhibition of this signaling pathway could be important to combat Ang II associated muscle wasting disorders such as cardiac cachexia.
Novelty and Significance

What Is Known?

- Renin-angiotensin aldosterone system (RAAS) activation in congestive heart failure (CHF) is associated with skeletal muscle wasting.
- RAAS inhibition reduces cachexia in CHF patients.
- Angiotensin II (Ang II) induces skeletal muscle atrophy via ubiquitin proteasome system (UPS) induction via the E3 ubiquitin ligase muscle RING-finger (MuRF) 1.

What New Information Does This Article Contribute?

- We show that Ang II increases \textit{MuRF1} expression and mediates skeletal muscle wasting.
- The novel pathway regulates the lysosomal hydrolase-coordinating transcription factor EB (TFEB) via histone deactylase-5 (HDAC5) and protein kinase D1 (PKD1).
- TFEB influences not only lysosomal and autophagosomal, but also UPS-dependent protein degradation.

Muscle wasting often occurs in patients with advanced stages of heart failure and worsens prognosis. RAAS activation with increased Ang II is implicated. Ang II increases muscular protein degradation through increased \textit{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 \textit{MuRF1} expression and thereby UPS-dependent protein degradation. Recently, TFEB was shown to regulate biogenesis of lysosomes and autophagosomes in non-muscle tissue. We show that TFEB directly binds to the \textit{MuRF1} promoter. Ang II/PKD1/HDAC5 signal transduction pathway controls TFEB activity. TFEB inhibition abolished Ang II-induced atrophy in vitro. Likewise, myocyte-specific \textit{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 autophagosomal, but also UPS-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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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 (PKD1<sup>WT/WT</sup>, MCK<sup>Cre</sup>) mice (<sup>n</sup> = 6, 7 days: <sup>n</sup> = 6) and skeletal muscle loss of function PKD1<sup>loxP/loxP, MCK<sup>Cre</sup></sup> (cKO; 24h: <sup>n</sup> = 5, 7 days: <sup>n</sup> = 7) mice by using implanted osmotic minipumps (ALZET<sup>®</sup> model 2001) for 24h and seven days as described<sup>1</sup>. WT (24h: <sup>n</sup> = 5, 7 days: <sup>n</sup> = 5) and cKO (24h: <sup>n</sup> = 5, 7 days: <sup>n</sup> = 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 <i>gastrocnemius/plantaris</i> 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% CO<sub>2</sub> atmosphere at 37°C. For differentiation of C2C12 myoblasts 2x10<sup>5</sup> 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<sup>®</sup> 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 MW 2500) 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_<i>TFEB</i>-C-myc -(His)<sub>6</sub>) or pcDNA3.1_<i>TFEB</i>-N-FLAG), or empty vector control (pcDNA3.1_C-myc -(His)<sub>6</sub> or pcDNA3.1_N-FLAG), as indicated, and the Hs_<i>MuRF1</i> _Luc reporter construct (100 ng), using 2.4 µg/ml Polyethylenimine (Polysciences, linear MW 2500) 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_<i>MuRF1</i>-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_<i>MuRF1</i>-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 RNAeasy Mini Kit (Quiagen) 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-OnePlus™ 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 ATNNTG 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 pcDNA3.1myc-(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 published\textsuperscript{2,9}. 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 (50 mM Tris HCl pH 6.8, 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/planitaris muscles were pulverized on dry ice to powder according to a recently published protocol \textsuperscript{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 (Hielischer, 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 Ni\textsuperscript{2+}-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
- MuRF1 promoter-pGL3 Basic
- pCMV-LacZ

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

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

- **third round:**
  - COS-7 cells
  - Cell lysis and quantification of luciferase and fluorescence
  - single cDNA/well
  - Identification of single MuRF1 activating cDNAs by sequencing
Online Figure II

*MuRF1*-promoter

![Graph showing fold activation of MuRF1 promoter with TFEB as x-axis.](image-url)
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

* * *
Online Figure VI

A

B

![Bar chart A](image1.png)

![Bar chart B](image2.png)
Online Figure V

<table>
<thead>
<tr>
<th>FLAG-PKD1</th>
<th>Myc-HDAC5</th>
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<tr>
<td>-</td>
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</tr>
<tr>
<td>WT</td>
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<td>540C</td>
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<tr>
<td>661C</td>
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MW [kDa]

130
170
130
70
55

Input PKD1
IB: FLAG

Input HDAC5
IB: Myc

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Mm_GAPDH_forward</td>
<td>5’-ATG GTG AAG GTC GGT GTG A-3’</td>
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<tr>
<td>Mm_GAPDH_reverse</td>
<td>5’-AAT CTC CAC TTT GCC ACT GC-3’</td>
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<tr>
<td>Mm_MuRF1_forward</td>
<td>5’-GAT TAT AAA TCT AGC CTG ATT C-3’</td>
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<tr>
<td>Mm_MuRF1_reverse</td>
<td>5’-TTG GTG TTC TTC TTT ACC CTC-3’</td>
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<tr>
<td>Mm_MuRF2_forward</td>
<td>5’-AGC ACT TCT CTG AAT TAC AAG-3’</td>
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<tr>
<td>Mm_MuRF2_reverse</td>
<td>5’-TTC ATT TAG GGA ATT CAA CCA G-3’</td>
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<tr>
<td>Mm_MuRF3_forward</td>
<td>5’-AAC TTC ACG GTG GGT TTC AAG C-3’</td>
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<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>
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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>
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<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>
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<tr>
<td>Hs_E-Box_2_mut_forward</td>
<td>5’-CAT CGG AAT GCT ATG CTG GTC CCC TC-3’</td>
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<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>
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<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 (Nhel)</td>
<td>5’-CTA GCT AGC AAC AGG GCC ATG TGA ATG GC-3’</td>
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<tr>
<td>Hs_MuRF1_Luc_3,938_for (Nhel)</td>
<td>5’-GTG CTA GCG CTG GGA TTA TAG GTG TGA GC-3’</td>
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<tr>
<td>Hs_MuRF1_Luc_3,500_for (Nhel)</td>
<td>5’-GTG CTA GCG AGA ATC ATC AGC ATA TGG-3’</td>
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<td>Hs_MuRF1_Luc_2,960_for (Nhel)</td>
<td>5’-GTG CTA GCC TGA GGT CCC ATG AGC AAG GAA G-3’</td>
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<td>Hs_MuRF1_Luc_1,565_for (Nhel)</td>
<td>5’-GTG CTA GCC AGG CGT GAG TCA CTG TGC CC-3’</td>
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<td>Hs_MuRF1_Luc_1,006_for (Nhel)</td>
<td>5’-GTG CTA GCC CAT TTC AAA ATC TGT AAG ATG-3’</td>
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<td>Hs_MuRF1_Luc_543_for (Nhel)</td>
<td>5’-GTG CTA GCC TCA GAA AAA TGT CTG ATG-3’</td>
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<td>pGL3 basic reverse (Xhol)</td>
<td>5’-CCG CTC GAG CAT TCT GTG GGA AGG AAT GA-3’</td>
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Online Table III. Primers used for generation of TFEB cDNA expression plasmids and its deletion mutants.

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

<table>
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<th>Name</th>
<th>Sequence</th>
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<tr>
<td>Hs_PKD1_1-201_forward (ClaI)</td>
<td>5’-ATC GAT AGC GCC CCT CCG GTC CTG CG-3’</td>
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<tr>
<td>Hs_PKD1_1-201_reverse (EcoRI)</td>
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<tr>
<td>Hs_PKD1_1-327_reverse (EcoRI)</td>
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<td>Hs_PKD1_1-574_reverse (EcoRI)</td>
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<tr>
<td>Hs_PKD1_201-C_forward (EcoRI)</td>
<td>5’-GAA TTC CTT GGC GAA GTG ACC ATT AAT G-3’</td>
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<tr>
<td>Hs_PKD1_201-C_reverse (XhoI)</td>
<td>5’-CTC GAG TCA GAG GAT GCT GAC ACG CTC A-3’</td>
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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 *MuRF1* promoter.

<table>
<thead>
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<td>Mm_MuRF1 E-box 1 reverse</td>
<td>5’-CCC TCT GAT ATT TAT AGC TGC AC-3’</td>
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<tr>
<td>Mm_MuRF1 E-box 2 forward</td>
<td>5’-CTC AAG CCC TGC CAG CAG-3’</td>
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<tr>
<td>Mm_MuRF1 E-box 2 reverse</td>
<td>5’-CAG GAG GGG ACC AGC TG-3’</td>
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<tr>
<td>Mm_MuRF1 E-box 3 forward</td>
<td>5’-CAA CAG CGA TTG CTC ATC CC-3’</td>
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<tr>
<td>Mm_MuRF1 E-box 3 reverse</td>
<td>5’-AAG ATT TGG CCC TCT CAG ATC-3’</td>
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