Estrogen-related receptor α regulates skeletal myocyte differentiation via modulation of the ERK MAP kinase pathway

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Murray J, Huss JM. Estrogen-related receptor α regulates skeletal myocyte differentiation via modulation of the ERK MAP kinase pathway. Am J Physiol Cell Physiol 301: C630–C645, 2011. First published May 11, 2011; doi:10.1152/ajpcell.00033.2011.—Myocyte differentiation involves complex interactions between signal transduction pathways and transcription factors. The estrogen-related receptors (ERRs) regulate energy substrate uptake, mitochondrial respiration, and biogenesis and may target structural gene programs in striated muscle. However, ERRα’s role in regulating myocyte differentiation is not known. ERRα and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) are coordinately upregulated with metabolic and skeletal muscle-specific genes early in myogenesis. We analyzed effects of ERRα overexpression and loss of function in myogenic models. In C2C12 myocytes ERRα overexpression accelerated differentiation, whereas XCT790 treatment delayed myogenesis and resulted in myotubes with fewer mitochondria and disorganized sarcomeres. ERRα−/− primary myocytes showed delayed myogenesis, resulting in structurally immature myotubes with reduced sarcosome assembly and mitochondrial function. However, sarcomeric and metabolic gene expression was unaffected or upregulated in ERRα−/− cells. Instead, ERRα−/− myocytes exhibited aberrant ERK activation early in myogenesis, consistent with delayed myotube formation. XCT790 treatment also increased ERK phosphorylation in C2C12, whereas ERRα overexpression decreased early ERK activation, consistent with the opposing effects of these treatments on differentiation. The transient induction of MAP kinase phosphatase-1 (MKP-1), which mediates ERK dephosphorylation at the onset of myogenics, was lost in ERRα−/− myocytes and in XCT790-treated C2C12. The ERRα-PGC-1α complex activates the Dusp1 gene, which encodes MKP-1, and ERRα occupies the proximal 5′ regulatory region during early differentiation in C2C12 myocytes. Finally, treatment of ERRα−/− myocytes with MEK inhibitors rescued normal ERK signaling and myogenesis. Collectively, these data demonstrate that ERRα is required for normal skeletal myocyte differentiation via modulation of MAP kinase signaling.

orphan nuclear receptors; mitogen-activated protein kinases; gene regulation; skeletal muscle

THE ESTROGEN-RELATED RECEPTORS (ERRs) are a family of nuclear receptor transcription factors comprising three related isoforms, ERRα (NR3B1), ERRβ (NR3B2), and ERRγ (NR3B3), encoded by distinct genes (15). Although structurally related to the estrogen receptors, ERRs are not responsive to estradiol and have no endogenous ligand identified to date. We previously showed (22) that ERRα and ERRγ overexpression increases fatty acid (FA) uptake and β-oxidation in primary cardiac myocytes. In cell-based analyses ERRα is required for induction of electron transport/oxidative phosphorylation genes and mitochondrial biogenesis in conjunction with its coactivator, peroxisome proliferator-activated receptor-γ (PPARγ) coactivator (PGC)-1α (37, 53, 68). The in vivo role for ERRα in regulating energy metabolism has been most intensively studied in whole body ERRα knockout models (31), which have revealed its importance in mitochondrial energy generation in heart and brown adipose during metabolic stress (20, 63). ERRα is also involved in regulating mitochondria-related processes such as antioxidant defense in skeletal muscle and cytokine-induced reactive oxygen species production in macrophages (47, 58). Recent genomic studies employing transcript expression profiling and chromatin immunoprecipitation (ChIP) in heart showed that ERRα and ERRγ regulate genes involved at all steps of oxidative energy metabolism from substrate uptake through mitochondrial ATP synthesis and export (12). ERRα displays tissue-dependent transcriptional effects, activating mitochondrial enzyme genes in highly oxidative tissues that express PGC-1α and PGC-1β, while in white adipose and in glycolytic skeletal muscle ERRα is complexed with corepressors, such as RIP140, to repress oxidative metabolism (7, 21, 23, 43, 54, 55). ERRα expression is enriched in adult oxidative type I skeletal muscle and heart and is required for maximal expression of genes involved in oxidative energy metabolism in terminal myotubes in culture (37, 67, 68). However, whether the ERRα pathway plays a fundamental role in regulating myogenesis has not been investigated.

Myocyte differentiation is a complex process, involving a highly coordinated cascade of regulatory events that activate functionally related gene programs to transition proliferative myoblasts (MB) to terminally differentiated myotubes (MT) with enhanced energetic and contractile functions (51). Muscle-specific transcription factors regulate the MB cell cycle exit and subsequent fusion to form multinucleate MT and coordinate induction of muscle-specific gene expression (41, 60). The basic helix-loop-helix myogenic regulatory factors (MRFs) (MyoD, Myf5, myogenin, and MRF4) heterodimerize with ubiquitous E proteins at consensus E-box DNA elements in regulatory regions of the skeletal muscle-specific genes, such as Tnni1 and Clcn4 (34, 39, 41). MRFs are expressed only in skeletal muscle and exhibit specific temporal expression patterns during myocyte differentiation, reflecting their relative importance at distinct stages. Myf5 and MyoD are involved in myocyte lineage determination, cell cycle withdrawal, and regulation of myogenin expression in adult satellite cells. In postmitotic myocytes myogenin, MRF4, and MyoD regulate processes including cell fusion and contractile protein expression and assembly (36, 41, 48, 50, 51). The MADS-box binding myocyte enhancer factor-2 (MEF2) transcription factors are coordinately up-
regulated with myogenin during differentiation and cooperate with MRFs to regulate transcription of skeletal muscle genes and contribute to fiber type specification (4).

Myogenesis is also controlled by signal transduction pathways, including the extracellular signal-regulated kinase (ERK) MAP kinase pathways (2, 10). The activation profile of ERK during myogenesis is biphasic. ERK activation in MB promotes proliferation and is inhibitory to differentiation. To initiate myogenesis ERK must be repressed, but at later differentiation stages ERK activity is necessary for terminal differentiation and to promote MT formation (3, 52, 61, 70). The inactivation of ERK at the onset of myogenesis is mediated by MAP kinase phosphatase-1 (MKP-1), a member of the dual-specificity phosphatase (DUSP) family. MKP-1 expression peaks at day 1 (d1) of differentiation, then declines rapidly as differentiation proceeds. MKP-1 overexpression causes precocious contractile protein induction in MB, while introduction after the onset of myogenesis inhibits MT formation (3, 27). Therefore, proper temporal regulation of ERK pathway signaling is necessary for myogenesis to proceed normally.

Differentiating myocytes also undergo extensive metabolic reprogramming, switching from glycolytic to oxidative ATP production and dramatically increasing mitochondrial number to support the high rate of ATP turnover required in contracting muscle (32, 38). Regulation of the mitochondrial oxidative gene program during skeletal muscle differentiation has not been characterized as thoroughly as the contractile gene program given that oxidative gene expression is not exclusive to the skeletal muscle. Muscle-specific transcription factors are known to directly regulate targets of the energetic program, including cytochrome oxidase subunits and muscle-specific isoforms of soluble (Ckm) and mitochondrial (Ckm2t) creatine kinases (9, 30, 39, 44, 64). However, transcription factors that target genes involved in mitochondrial energy metabolism in adult muscle may also be recruited during myogenesis to drive the metabolic program required for proper terminal differentiation of MT (11, 28). How the metabolic transcription factors, such as the ERR pathway, are temporally regulated during differentiation and integrated with the known myogenic program is not fully understood.

The present studies demonstrate a role for ERRα in regulating myocyte differentiation in primary skeletal myocytes and in the C2C12 myogenesis model. Modulation of the ERRα pathway with the use of complementary approaches revealed a prodifferentiation role for ERRα. Overexpression of ERRα in C2C12 myocytes promotes MT formation and increases expression of metabolic and contractile genes, while loss of ERRα in primary myocytes results in delayed differentiation, impaired MT formation, and decreases in oxidative substrate metabolism. The effects of ERRα on myogenesis are mediated, in part, by alterations in the ERK1/2 MAP kinase signaling pathway at distinct stages of myogenesis. ERRα overexpression in C2C12 reduces ERK phosphorylation at early stages of myogenesis. Loss of ERRα in primary myocytes results in an aberrant increase in ERK phosphorylation early in myogenesis and inhibition of MT formation, and this differentiation delay can be rescued by treatment with MEK inhibitors. Our results also suggest that ERRα may regulate the ERK pathway through direct regulation of the Dusp1 gene, encoding MKP-1. Our data demonstrate that ERRα is necessary for myogenesis to proceed normally and expand the role of ERRα to include regulation of ERK signaling during differentiation.

MATERIALS AND METHODS

Cell culture and reagents. C2C12 were obtained from American Type Culture Collection (cell line CRL-1772, Manassas, VA) and were originally prepared from mouse skeletal muscle (72). MB were cultured in growth medium (DMEM; Mediatech, Manassas, VA) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), and all experiments were performed in cells below passage 35. To induce differentiation to MT, medium was changed to DMEM containing 2% horse serum (HS; Atlanta Biologicals) when MB reached confluence. All other cell culture reagents were purchased from Mediatech unless otherwise stated and are certified endotoxin free. Cells were not assessed for mycoplasma contamination, but multiple preparations and laboratory sources of cells produced similar results. Adenovirus infection to express green fluorescent protein (GFP) or ERRα was initiated in C2C12 myocytes at the time of differentiation (d0 MT), or for MB overexpression trials subconfluent cultures (4 × 10^3 cells) were infected and RNA or protein was collected at 48 h after treatment. Cloning and preparation of adenovirus expressing GFP (Ad-GFP) or ERRα (Ad-ERRα) has been described previously (22). C2C12 myocytes were treated with 10 μM XCT790 (Sigma-Aldrich, St. Louis, MO) or DMSO in growth medium or differentiation medium prepared with charcoal-stripped FBS or HS, respectively, and medium was changed daily.

The protocols for primary skeletal MB isolation and culture were modified from previously described methods (33, 46). All animal protocols were approved by the Institutional Animal Care and Use Committee at the City of Hope. ERRα−/− mice are of a mixed strain background (C57BL6J/svJ29) and have been characterized previously (6, 20, 31, 63). Hindlimb muscles were dissected from one litter of 5-day-old wild-type (WT) or ERRα−/− mice (5–8 mice) and finely minced in a small volume of 1× PBS containing 200 μl penicillin, 200 μg/ml streptomycin, and 0.1% Fungizone. Ca^{2+}- and bicarbonate-free HBSS with HEPES (CBFHH) digestion medium (in mM: 137 NaCl, 5.35 KCl, 0.81 MgSO4, 5.55 dextrose, 0.44 KH2PO4, 0.34 Na2HPO4, 20 HEPES, and 0.125 CaCl2, with 0.5 mg/ml collagenase, pH 7.4) containing 20 μg/ml gentamycin, 2 mM1-glutamine, 20 μM penicillin, 0.5 μg/ml streptomycin, and 0.5 μg/ml Fungizone was then added to muscle fragments. After incubation, muscle fragments were incubated at 37°C for 15 min. Then, after trituration, muscles were incubated at 37°C for 5 min, and liberated myocytes were removed and pelleted. Trituration and incubation steps were performed three times. Myocytes were resuspended in Ham’s F-10 medium containing 20% FBS, 100 μM penicillin, 100 μg/ml streptomycin, and 2.5 mg/ml basic FGF (bFGF) (Promega, Madison, WI) and passed through a cell strainer. Myocytes were plated on collagen-coated dishes for 20 min at 37°C and then removed to fresh collagen-coated dishes for culturing. After 2 wk of culture, cells were maintained in medium consisting of 40% Ham’s F-10, 40% DMEM, 20% FBS containing 100 μM penicillin, 100 μg/ml streptomycin, and 2.5 mg/ml bFGF. Primary myocyte differentiation medium consisted of DMEM containing 5% HS, 100 μM penicillin, and 100 μg/ml streptomycin. MT diameter was assessed with brightfield pictures of WT and ERRα−/− primary MT at d3 of differentiation. Fifty cells each of WT or ERRα−/− MT were analyzed with ImagePro Plus 6.3 (Media Cybernetics, Bethesda, MD) software. Where indicated, primary MB were pretreated with 5 μM U0126 (Cell Signaling, Danvers, MA), 10 μM PD98059 (Enzo Life Sciences, Plymouth Meeting, PA), or DMSO for 5 h in growth medium, and then total protein was harvested. The remaining MB were switched into differentiation medium containing 5 μM U0126, 10 μM PD98059, or DMSO, and total protein from MT was harvested at the indicated time points. Differentiation medium containing U0126, PD98059, or DMSO was changed daily.
**Metabolic assays.** Glucose oxidation rates were measured as previously described (67). Briefly, cells were cultured in T25 tissue culture flasks until grown to the appropriate stage of differentiation. After incubation in low-glucose medium (1 g/l) for 6 h, [U-14C]-glucose (MP Biomedical, Solon, OH) was added to medium at 1 μCi/ml, and flasks were sealed with airtight plugs and incubated at 37°C for 1 h. Cells were lysed by media acidification with injection of TCA through the airtight port. Oxidized glucose was measured by detecting 14CO2 released and collected in KOH-saturated filter paper suspended from center wells inside the flasks. FA oxidation was performed essentially as described previously (22). Primary myocytes plated in 24-well plates were grown to confluence or induced to differentiate for 5 days. Plating of MT and MB was staggered to allow assay on the day of harvest. Cells were washed with Ca2+/Mg2+-free PBS followed by incubation in PBS plus Ca2+/Mg2+ containing 125 μM [1H-H9-10]palmitic acid (Perkin Elmer NEN, Waltham, MA) and 1 mM carmine. Incubations were performed for 2 h at 37°C. Oxidized palmitate in the aqueous fraction was purified on Dowex 1 resin (Sigma-Aldrich) and quantitated by scintillation counting. Data were normalized for background and for total cellular protein quantitated with the Micro BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Rates were calculated as nanomoles of [3H-palmitate oxidized per hour per milligram of protein.

**Plasmids, transient transfection, and chromatin immunoprecipitation.**

The 5.0.Dusp1.Luc promoter-reporter contains the region of the Dusp1 gene encompassing 5004 to +59 bp, relative to the predicted transcription start site (tss) cloned into pGL3-Basic vector (kindly provided by Dr. Eisuke Nishida, Kyoto University). The pcDNA3.1-Flag-ERRα and pcDNA-myc-his/PGC-1α have been described previously (21). Transient transfection in C2C12 myocytes by the calcium phosphate method has been described previously (67). Briefly, subconfluent C2C12 myoblasts were plated in 24-well plates at a density of 13,000 cells/well 1 day before transfection. Plasmids were used at the following final concentrations in 0.5 ml of growth medium: 5.0- Dusp1-Luc, 2 μg/ml; pcDNA3.1(-), pcDNA3.1-Flag-ERRα, or pcDNA-myc-his/PGC-1α, 0.5 mg/ml; and pRL-TK-Renilla, 0.25 μg/ml. Luciferase activity was assayed in MB 48 h after transfection and in d1 MT 24 h after change of confluent cells to 2% HS-DMEM. Luciferase activity was assayed with Dual-Glo reagents (Promega) on the DTX 880 Multimode Detector (Beckman-Coulter, Brea, CA). Firefly luciferase activity was normalized to that of Renilla luciferase, which was expressed downstream of the minimal thymidine kinase promoter from the pRL-TK-Renilla plasmid.

ChIP using cross-linked chromatin isolated from subconfluent C2C12 MB and d1 MT was performed in Ad-ERRα-expressing myocytes, essentially as described previously (67). The chromatin was sheared with a microtip sonicator (Thermo-Fischer Sonic disembrator 100) set at 40% power for 8 rounds of 25 pulses each. The resulting sheared chromatin was immunoprecipitated overnight at 4°C with 5 μg of antibody against either ERRα (Abcam, Cambridge, MA; ab-16363) or normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA; sc-2027) with Immobilized Protein G Plus (Thermo Scientific). The resin was washed the following day and eluted with Tris-EDTA (pH 7.5) incubated at 65°C for 10 min. The eluted chromatin was purified with the QIAquick PCR purification kit per manufacturer’s instructions (Qiagen, Valencia, CA). The purified genomic DNA was used in PCR reactions (15 μl) performed in 96-well format in triplicate containing 1× SYBR Green reagent (iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA). Primers were used to amplify a 309-bp region of the Dusp1 gene promoter corresponding to -1092 to -1112 (5′-GGACTCTACTGGCTAC-CCTGT-3′) and -804 to -822 (5′-CCCCCCAACACAATTTTCT-3′) relative to the predicted tss. The Tbp gene was amplified in parallel reactions to normalize for nonspecific binding of the antibodies as previously described (22).

**Quantitative real-time PCR.** Real-time PCR was performed to quantify relative transcript levels in RNA collected from MB or successive d1 through d4 of MT formation with RNAzol B (TelTest, Friendswood, TX). One microgram of RNA was reverse transcribed in 20-μl reactions with the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad Laboratories) with a 1:1 mixture of oligo(dT) and random hexamers for 30 min at 42°C. The resulting cDNA was used in PCR reactions (15 μl) performed in 96-well format in triplicate containing 1× SYBR Green reagent (Bio-Rad iQ SYBR Green Supermix), 0.4 μM gene-specific primers, and 0.5 μl of first-strand reaction product (diluted 1:2) as previously described (20). Primer and probe sequences are shown in Supplemental Table S3. Cycling and detection were performed with the Bio-Rad IQ5 Real Time PCR system. Experimental transcript levels were normalized to 36B4 ribosomal RNA analyzed in separate reactions.

**Immunofluorescence.** WT or ERRα−/− primary myocytes were plated at a density of 8 × 104 cells/well of a two-well chamber slide (Thermo Scientific). 45 MT were fixed in 4% formaldehyde for 15 min and blocked for 30 min in 1% BSA, 0.3% Triton X-100, and 3% normal goat serum in PBS. C2C12 MB were fixed 2 days after adenoviral infection, and immunofluorescence was performed as described for primary myocytes. Cells were incubated with the following primary antibodies at 4°C overnight: α-actinin (Sigma-Aldrich), cytokerin e (BD Biosciences, Franklin Lakes, NJ), MF20 (Developmental Studies Hybridoma Bank, Iowa City, IA), or troponin I (Tnl; Santa Cruz Biotechnology). Alexa Fluor 568 and Alexa Fluor 488 (Invitrogen, Carlsbad, CA) were used as secondary antibodies, and nuclei were stained with Hoechst 33258 (Sigma-Aldrich). MT were analyzed with the Olympus AX70 Automated Upright microscope at ×600 magnification or the Zeiss Inverted LSM510 META 2-Photon microscope at ×630 magnification. Brightfield images were taken on the Leica DM IL microscope at ×100 magnification. Mitochondria were visualized with MitoTracker Red (Invitrogen) as described previously (29). Images were taken in monochrome and pseudocolored in Image Pro Plus 6.3.

**Western blot analysis.** Cytoskeletal stabilization buffer (CSB) was used to extract soluble protein fraction from MB and MT (26). Cells were first washed with CSB (in mM: 150 KCl, 20 PIPES, 10 imidazole, 1 MgCl2, 1 EGTA, 0.2 DTT, and 200 mM sodium fluoride, with 50 mM stauroporine, 1× Sigmafast protease inhibitors, and 200 μM sodium orthovanadate) without detergent followed by 5-min room temperature incubation with CSB plus 0.05% Triton X-100 to gently lyse cells and leave cytoskeletal and sarcomeric structures intact. The remainder of the cellular protein was then solubilized with RIPA buffer containing 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 1% NP-40. Total protein was collected from primary myocytes with RIPA/HBSS (1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2.5 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 250 mM mannitol) (40) with the addition of 1× Sigmafast protease inhibitors, 1 mM DTT, 200 μM sodium orthovanadate, and 50 mM sodium fluoride. Proteins were resolved by SDS-PAGE followed by electroblotting onto nitrocellulose (Bio-Rad). Membranes were blocked with 3.5–5% nonfat milk, and primary antibody hybridization was performed overnight at 4°C in Tris-buffered saline containing 0.1% Tween and 1% BSA. The following antibodies for analysis of MAPK signaling pathways were purchased from Cell Signaling Technology: p44/p42 MAPK (Erk1/2), phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-eukaryotic elongation factor 2 (eEF2) (Thr56), ribosomal S6 kinase (RSK)1/RSK2/RSK3, phospho-p90RSK (Ser380), S6 ribosomal protein, and phospho-S6 ribosomal protein (Ser235/236). The PGC-1α and p21 antibodies were purchased from Santa Cruz Biotechnology. The β-tubulin antibody (developed by Dr. Michael Klymkowsky) and the MF-20 [myosin heavy chain (MHC)] antibody (developed by Dr. Donald Fishman) were provided by the Developmental Studies Hybridoma Bank (developed by Dr. Michael Klymkowsky) and the MF-20 [myosin heavy chain (MHC)] antibody (developed by Dr. Donald Fishman) were provided by the Developmental Studies Hybridoma Bank (developed by Dr. Michael Klymkowsky) and the MF-20 [myosin heavy chain (MHC)] antibody (developed by Dr. Donald Fishman).
ERRs promote skeletal myocyte differentiation

ERR regulatory axis is activated during myotube differentiation in skeletal myocyte cultures. Recent studies have emphasized the role of ERRα in direct transcriptional regulation of genes encoding enzymes involved in energy metabolism with its coactivator PGC-1α or through regulation of other metabolic transcription factors (20, 47, 63). Genomewide analyses in heart have identified additional classes of putative ERRα target genes, encoding muscle-specific structural and contractile proteins (12). Thus we sought to investigate whether the ERRα pathway may play a more central role in myocyte differentiation by coupling activation of the metabolic and contractile gene programs. To characterize this novel role for ERRα, we first employed C2C12 myocytes, which differentiate from proliferating MB to postmitotic fused MT upon culturing in low-serum conditions (Fig. 1A). The myogenic gene expression and signal transduction pathways influencing C2C12 myogenesis have been extensively studied (17, 51, 60). We compared relative expression of ERR isoforms and PGC-1α in d0 MB and d3 and d5 MT with that of myogenic regulatory factors and muscle-specific sarcomeric genes (Fig. 1B). ERRα and PGC-1α were readily detectable in proliferating MB but exhibited further stepwise induction in MT, although the relative magnitude of PGC-1α upregulation from...
MB to MT (>15-fold) was greater than that of ERRα (3-fold). ERRγ transcript levels were very low in MB but were upregulated by d3 MT and dramatically increased by d5 of myogenesis (28-fold), subsequent to the upregulation of ERRα and PGC-1α. A similar MB to MT increase was observed at the protein level for these factors (Fig. 1B). Our findings are in general agreement with recently reported expression profiles of ERRα and ERRγ transcripts in C2C12 (66). The onset of ERRα and PGC-1α induction coincided with that of the myogenic regulatory factor myogenin. The putative ERR target genes, TnI and mitochondrial creatine kinase (Ckmt2), and the established target pyruvate dehydrogenase kinase 4 (PDK4), were upregulated in parallel with ERRα and PGC-1α (Fig. 1C). These results suggest that ERRs may be involved in regulating gene programs important for skeletal myocyte differentiation.

**ERRα overexpression promotes differentiation in C2C12 skeletal myocytes.** Given that endogenous ERRα expression is induced during myogenesis, we wanted to assess how early ERRα overexpression would influence MT formation in mouse C2C12 skeletal myocytes. C2C12 MB transduced with Ad-ERRα in growth medium exhibited precocious expression of the sarcomeric protein TnI, while control MB did not express this differentiation marker (Fig. 2A). Simultaneous introduction of Ad-ERRα with the switch to low-serum differentiation medium advanced MT formation. Brightfield images as well as immunofluorescence detection of α-actinin revealed that ERRα-expressing MT were more advanced compared with GFP-expressing cells (Fig. 2A). Furthermore, consistent with the role of ERRα in regulating mitochondrial oxidative capacity (22, 53), we also observed increased mitochondrial staining by MitoTracker Red (Fig. 2A) and cytochrome c (data not shown).

Fig. 2. Overexpression of ERRα promotes C2C12 myocyte differentiation. A: subconfluent C2C12 MB were infected with adenovirus (Ad) encoding green fluorescent protein (GFP) (a, c) or ERRα (b, d). The differentiation marker TnI (a, b) was detected by IF, and MitoTracker Red was used to visualize mitochondria (c, d) in cells maintained in growth medium (×400 magnification; scale bar = 10 μm). Confluent MB were infected with GFP (e, g) or ERRα (f, h) upon switch to differentiation medium, and brightfield images (e, f) were taken in d3 MT (×100 magnification; scale bar = 100 μm). Sarcomeric structure was visualized by using IF to detect α-actinin as described in Fig. 1A (×630 magnification; scale bar = 10 μm). Representative images are shown. B: analysis of ERRγ (Esrrg), PGC-1α (Ppargc-1a), myogenin (Myog), PDK4 (Pdk4), mitochondrial creatine kinase (Ckmt2), muscle creatine kinase (Ckm), myosin heavy chain (MHC2a (Myh2)), and TnI (Tnni1) transcript expression by quantitative real-time PCR in C2C12 MB and d3 MT infected with the indicated adenoviral constructs. Expression levels were normalized to 36B4 mRNA levels. Triplicate samples were analyzed, and data are expressed as means ± SE. *Significant difference (P < 0.05) between Ad-GFP and Ad-ERRα conditions. GM, growth medium; DM, differentiation medium.
Real-time quantitative PCR analysis of transcript expression showed that myogenin was only modestly increased (38%) in the Ad-ERRα MT, while expression of other myogenic factors was not significantly changed (Fig. 2B, Supplemental Table S1). ERRα overexpression caused a downregulation of ERRγ transcript, whereas PGC-1α expression was moderately increased. During skeletal myocyte differentiation, changes in expression of genes encoding metabolic and contractile proteins have been well characterized (5, 16, 38, 39, 44, 45). We examined the transcript expression pattern of several markers in ERRα-expressing myocytes. Expression of the fast isoform of MHCa (Myh2), a sarcomeric protein and marker of differentiation, was induced in ERRα-overexpressing cells. Expression of several other sarcomeric genes showed equal differentiation-dependent induction in GFP- and ERRα-expressing MT. ERRα overexpression in d3 MT induced robust activation of the Pdk4 (4.5-fold) and Ckm2 (19-fold) genes, both encoding skeletal muscle-enriched isofoms of mitochondrial proteins. In contrast, only modest activation of other ERRα target genes, encoding MCAD, GLUT4 and hexokinase, was observed in ERRα-expressing MT at the same stage (12, 21, 57, 62, 67) (Supplemental Table S1). Adenoviral transduction alone had no effect on the differentiation-dependent induction trends of target gene classes represented in Fig. 2B (Supplemental Fig. S1). Although some differences in magnitude of induction between the MB and d3 MT stages were observed in uninfected versus Ad-GFP-expressing C2C12 myocytes, the patterns were the same, suggesting that differentiation is not altered with adenoviral transduction. These magnitude differences do not alter the conclusions regarding effects of ERRα overexpression on gene expression. Thus ERRα overexpression advances MT formation in parallel with induction of contractile and metabolic programs, supporting a role for ERRα in promoting the skeletal myocyte differentiation program.

ERRα inverse agonist delays differentiation in C2C12 myocytes. We then evaluated whether inhibition of ERRα activity by ligand would modulate MT formation in the C2C12 model system. Treatment of C2C12 cells at the onset of differentiation with the ERRα inverse agonist XCT790 (68) caused a delay in MT formation. XCT790-treated myocytes formed fewer high-order, multinucleate fused MT by d3 in differentiation medium compared with vehicle controls (Fig. 3A). Immunofluorescence analysis of sarcomeric proteins demonstrated that XCT790-treated MT were thinner than control MT with impaired myofibril formation. Treatment with XCT790 inhibited mitochondrial redistribution along assembled sarcomeres as observed in mature MT, resulting in a perinuclear distribution. While ERRα and myogenin levels were unaffected in C2C12 d3 MT, expression of PGC-1α, ERRγ, and Gabpa were reduced in XCT790-treated d3 MT (Fig. 3B, Supplemental Table S1). Coincident with structural effects, XCT790 treatment decreased expression of genes encoding sarcomeric proteins, including MHC isoforms 1 and 2a (Myh7, -50%; Myh2, -60%; Fig. 3C), Tn (Tnn1, -24%), and titin-cap (Tcap1, -58%) (Supplemental Table S1). Furthermore, XCT790 treatment consistently downregulated mitochondrial oxidative enzyme genes, including MCAD (Acadm, -66%), PDK4 (Pdk4, -50%), and the muscle-specific creatine kinase mitochondrial CK2 (Ckm2, -58%) (Fig. 3C, Supplemental Table S1), suggesting that differentiation-dependent metabolic gene induction requires transcriptional activation by ERRα. Collectively, the results suggest that ERRα is involved in regulation of metabolic and sarcomeric gene expression during myogenesis and thus plays a critical role in the normal myogenic program.

Impaired differentiation and myotube maturation in ERRα−/−myocytes. Our acute gain- and loss-of-function studies suggested that ERRα can promote and is necessary for myocyte differentiation. Furthermore, ERRα−/−mice have reduced skeletal muscle mass relative to WT (Supplemental Fig. S2) but otherwise do not exhibit an overt muscle defect at baseline. In light of these observations we sought to investigate the capacity for primary myocytes isolated from ERRα whole body knockout mice to undergo differentiation in culture. During the first 48 h in differentiation medium, ERRα−/−cells formed fewer MT that were shorter with fewer nuclei per MT than those in the WT culture, although by d3 the number of MT in ERRα−/−cultures was typically equivalent to that in WT cultures (Fig. 4A). Quantitation of d3 MT thickness showed that ERRα−/−MT were consistently thinner than WT independent of the region measured for cell width (Fig. 4B). Immunofluorescence detection of sarcomeric structural proteins α-actinin and TnI revealed impaired sarcomeric assembly in ERRα−/− d5 MT compared with the well-defined Z-banding pattern clearly seen in WT tubes at the same stage, suggesting an additional late-stage growth defect (Fig. 4C). Finally, coincident with the structural defects observed, the ERRα−/−MT also had fewer mitochondria, as determined by staining for cytochrome c. Thus the absence of ERRα in skeletal myocytes results in delayed myogenesis and impaired maturation in the resulting MT.

Time course analysis of mRNA expression during myogenesis (MB through d4) in the ERRα−/− model (Fig. 5A, Supplemental Table S2) revealed an increase in d1 MyoD expression but slightly lower myogenin in d2 ERRα−/− MT. Interestingly, ERRγ was induced in d1 and d2 MT only, coinciding with impaired tube formation, whereas the greatest increase in PGC-1α transcript was observed in the late-stage ERRα−/− MT. In ERRα−/− mice, upregulation of ERRγ and PGC-1α has been observed in adult tissues and is thought to be a compensatory response to loss of ERRα expression (20, 63). Analysis of structural and metabolic transcript levels at the same time points revealed, unexpectedly, either no change or slightly increased expression in ERRα−/− myocytes (Fig. 5B, Supplemental Table S2). Genes encoding sarcomeric proteins, such as Tn (Tnnl1), α-skeletal actin (Acta1), and MHC (Myh6), were upregulated in ERRα−/− myocytes beginning at various time points, and all were elevated in terminal d4 ERRα−/− MT compared with WT. Western blot analysis for MHC, Tn, and α-actinin (Fig. 5C) revealed that the steady-state protein levels reflected the transcript pattern, despite impaired sarcomere assembly in d5 ERRα−/− MT (Fig. 4C). A similar moderate induction was observed for many metabolic genes encoding substrate transporters (Cd36), β-oxidation (Acadm), and mitochondrial ATP synthesis (Atp2a2, Ckm2) enzymes at various time points in ERRα−/− myocytes (Fig. 6A, Supplemental Table S2). Pdk4 displayed no change while Ckm expression was decreased after d2, consistent with impaired myogenesis. Importantly, regulators of mitochondrial biogenesis, Gabpa/NRF-2a and Tfam, which are
Fig. 3. XCT790 treatment inhibits differentiation in C2C12 myocytes. A: mitochondria were visualized by MitoTracker Red (MT Red) staining in C2C12 MT treated with vehicle or 10 μM XCT790 in differentiation medium for 3 days (×400 magnification; scale bar = 10 μm). Brightfield images of MB and d3 MT were taken to assess tube formation, and representative images are shown (×100 magnification; scale bar = 50 μm). IF was performed to visualize the sarcomeric proteins α-actinin, MHC, and TnI (×630 magnification; scale bar = 10 μm). B: expression levels of ERRα (Esrra), myogenin (Myog), PGC-1α (Ppargc-1a), and ERRγ (Esrrg) were assessed by quantitative real-time PCR in C2C12 MB or d3 MT treated with vehicle or 10 μM XCT790. Expression levels were normalized to 36B4 mRNA levels. Triplicate samples were analyzed, and results are expressed as means ± SE. *Significant difference in Ad-GFP vehicle-treated control vs. XCT790 treated (P < 0.05).
also direct ERRα target genes, were upregulated in the ERRα+/− MB as well as in d1–d4 MT (Supplemental Table S2). These changes are consistent with the pattern of ERRγ and PGC-1α expression in the ERRα+/− myocytes at various differentiation stages. However, despite these expression patterns, diminished mitochondrial capacity was observed in terminal ERRα−/− MT, evidenced by dramatic reduction in glucose and FA oxidation rates (Fig. 6B). Importantly, metabolic capacity was similar in WT and ERRα+/− MB; thus the early delay in ERRα−/− myogenesis is dissociated from metabolic deficiency. Fulco et al. (13) demonstrated that AMP-activated protein kinase (AMPK) activation in response to nutrient deprivation inhibits differentiation in skeletal myocytes. Therefore, we investigated AMPK activation in response to nutrient deprivation in ERRα+/− primary myocytes. By Western blot analysis WT myocytes showed the expected pattern of reduced phospho-ERK (p-ERK) through d2 MT that were smaller with poorly organized sarcomeres. Given this pattern, we sought to investigate the activation status of signaling pathways with distinct roles during early- and late-phase myogenesis. The MEK/ERK pathway has a complex regulatory role in skeletal myogenesis (59, 70). In cell culture models of myogenesis, MEK/ERK activity is reduced through d2, followed by MEK/ERK activation at d3 of differentiation and beyond, which stimulates protein synthesis and MT fusion (59). By Western blot analysis WT myocytes showed the expected pattern of reduced phospho-ERK (p-ERK) through d2 MT followed by ERK activation by d3 of differentiation (Fig. 7A, left). In contrast, ERRα+/− myocytes exhibited an aberrant increase in p-ERK at d1 and d2 after induction of myogenesis compared with WT myocytes. This early activation is consistent with delayed MT formation in ERRα+/− myocytes. By d3 of differentiation, however, p-ERK levels had declined in ERRα−/− MT to a level slightly lower than that observed in WT d3 MT. Downstream of ERK, the phosphorylation of p90RSK in ERRα+/− myocytes was increased in MB and d1 and d2 MT but decreased by d3 of differentiation compared with WT d3 MT. 

ERRα−/− myocytes exhibit altered ERK/MAPK signaling during early myogenesis. The observed defects in ERRα+/− myocyte differentiation appeared biphasic, with a delay in early myogenesis followed by differentiation to form terminal MT that were smaller with poorly organized sarcomeres. Given this pattern, we sought to investigate the activation status of signaling pathways with distinct roles during early- and late-phase myogenesis. The MEK/ERK pathway has a complex regulatory role in skeletal myogenesis (59, 70). In cell culture models of myogenesis, MEK/ERK activity is reduced through d2, followed by MEK/ERK activation at d3 of differentiation and beyond, which stimulates protein synthesis and MT fusion (59). By Western blot analysis WT myocytes showed the expected pattern of reduced phospho-ERK (p-ERK) through d2 MT followed by ERK activation by d3 of differentiation (Fig. 7A, left). In contrast, ERRα+/− myocytes exhibited an aberrant increase in p-ERK at d1 and d2 after induction of myogenesis compared with WT myocytes. This early activation is consistent with delayed MT formation in ERRα+/− myocytes. By d3 of differentiation, however, p-ERK levels had declined in ERRα−/− MT to a level slightly lower than that observed in WT d3 MT. Downstream of ERK, the phosphorylation of p90RSK in ERRα−/− myocytes was increased in MB and d1 and d2 MT but decreased by d3 of differentiation compared...
levels may be inhibited through additional effects of acute ERR/MEK/ERK response to XCT790 treatment, indicating that protein synthesis was decreased in early differentiation stages and increased in d3 MT by ERR/α−/− primary myocytes (Fig. 7A, right), in complete opposition to the pattern observed in ERR+/− primary myocytes (Fig. 7A). Phosphorylation of p90RSK was also reduced, while eEF2 phosphorylation was increased, consistent with release of p90RSK-mediated inhibition of EF2 kinase. Thus promotion of myogenesis in ERR/α-overexpressing myocytes correlates with prodifferentiation signaling by the MEK/ERK pathway.

**ERRα directly targets the Dusp1 gene and regulates MKP-1 expression during myogenesis.** We sought to explore a potential mechanism for the altered MEK/ERK signaling in ERRα−/− myocytes. A transient spike in expression and activity of MKP-1 at the onset of myogenesis drives the d1 MEK/ERK inactivation during myogenesis (3). Whereas WT cells displayed a greater than twofold increase at d1 followed by downregulation from d2 to d5, the ERRα−/− myocytes exhibited an inverse pattern of MKP-1 expression (Fig. 8A). MKP-1 was significantly lower at d1 of differentiation and higher in subsequent days of myogenesis compared with WT, correlating with the perturbed activation state of MEK/ERK in the ERRα−/− cells during myogenesis. Interestingly, expression of MKP-1 is reduced in the skeletal muscle but not the heart of ERRα−/− mice (Supplemental Fig. S3). These results suggest that ERRα may regulate MKP-1 expression. Additionally, treatment of C2C12 myocytes with the ERRα inhibitor XCT790 inhibited the d1 MKP-1 upregulation at the transcript and protein levels.
while overexpression of ERRα modestly enhanced d1 induction of MKP-1 transcript (Fig. 8B).

To investigate the potential for direct regulation of MKP-1 expression by ERRα, we analyzed the Dusp1 gene 5′ regulatory region. TRANSFAC (35) analysis of the Dusp1 gene upstream 5-kb promoter region identified several sequences corresponding to ERRα response element (ERRE) consensus sites (Fig. 8C). Several ERREs cluster with E-boxes throughout the promoter and with Sp1 consensus sites in the proximal region (<1 kb) of the gene. We performed transient cotransfection experiments with a 5.0.Dusp1.Luc promoter-reporter construct in C2C12 MB and d1 MT (Fig. 8C). A 3.5-fold increase in luciferase expression was observed between MB and d1 MT, suggesting that the cloned region of the Dusp1 gene contained response elements supporting transcriptional induction that paralleled the endogenous transcript pattern. We tested the responsiveness to ERRα transactivation in MB in order to minimize the regulatory effects of endogenous factors. Cotransfection of ERRα or PGC-1α alone modestly induced the Dusp1 promoter-reporter construct, while transfection of ERRα and PGC-1α together caused over fivefold activation.

ChIP was then performed to demonstrate ERRα binding to the endogenous Dusp1 gene locus at critical time points during differentiation (Fig. 8D). Chromatin was cross-linked and purified with antibody against ERRα or IgG. ERRα binding was observed at the Dusp1 gene with the use of primers specific for the proximal promoter region (804 to 1112). Interestingly, ERRα occupation increased nearly fivefold between MB and d1 MT, coincident with peak expression of MKP-1 transcript. No enrichment in ERRα binding was observed at the independent genomic region corresponding to the Tbp gene. Taken together, these results suggest that aberrant ERK activation inhibits differentiation in ERRα−/− myocytes and that the key ERK phosphatase, Dusp1, is a direct ERRα target.

Inhibition of early ERK/MAPK activation in ERRα−/− myocytes rescues the differentiation delay. The response to various concentrations of U0126 (5, 10, and 25 μM) and PD98059 (10, 25, and 50 μM) was assessed in ERRα−/− and
WT myocytes. The higher concentrations of either compound caused rounding of a significant number of cells in both WT and ERRα−/− cultures, whereas the lower concentrations of U0126 and PD98059 caused no apparent toxicity. Five micromolar U0126 or 10 μM PD98059 was added to the growth medium of WT and ERRα−/− myocytes 5 h before the switch to differentiation medium, and treatments were maintained in differentiation medium. Both MEK inhibitors resulted in rescue of delayed myogenesis in ERRα−/− myocytes (Fig. 9A). Significant advancement of ERRα−/− MT formation was observed with MEK inhibitor treatment at both d1 and d2. The most dramatic results were observed at d1, at which point the number of ERRα−/− MT was increased over vehicle-treated ERRα−/− myocytes and nearly equivalent to wild-type MT treated with 5 μM U0126 or 10 μM PD98059 (Fig. 9A, top). Since results with both inhibitors were similar, only U0126 treatment was used in subsequent experiments. Treatment with 5 μM U0126 caused a significant reduction in ERK activation in ERRα−/− MB and d1 MT (Fig. 9B), suggesting that the differentiation delay in ERRα−/− myocytes is rescued by restoring the normal ERK activation pattern. Additionally, U0126 treatment did not significantly affect MKP-1 protein levels in ERRα−/− myocytes, which were downregulated in d1 and more dramatically in d2 ERRα−/− MT (Fig. 9C), consistent with the transcript data (Fig. 8A). Thus MEK inhibitor rescue of myogenesis in ERRα−/− myocytes was not due to changes in MKP-1 expression (Fig. 9C). Collectively, these results suggest that the disruption of the normal MKP-1 expression pattern at the onset of myogenesis in ERRα−/− myocytes results in aberrant ERK activation and delayed differentiation.

**DISCUSSION**

The ERRα transcriptional pathway has been shown in recent years to play a central role in the regulation of mitochondrial energy metabolism in many cell and tissue types, including striated muscle (20, 63). In the present study, we explored the hypothesis that ERRα may function more broadly as an essential regulatory component of myogenesis. Myocyte differentiation requires precise regulation of multiple gene programs, consisting of genes encoding contractile and sarcoplasmic reticulum proteins, along with ubiquitously expressed proteins involved in energy metabolism. Such coordination may be mediated by transcriptional regulators of energy metabolism genes, including the ERR isoforms and their PGC-1 coactivators, that are temporally induced as part of the myogenic program (Refs. 28, 66; present study). Our findings suggest that ERRα does promote differentiation when overexpressed and is required for normal myogenesis. A surprising finding...
was that the broader regulatory function for ERRα in myocyte differentiation and growth involves modulation of the MAPK/ERK pathway to promote differentiation in skeletal myocytes. These novel findings establish the importance of the ERR pathway to promote differentiation in skeletal myocytes.

Recent studies have implicated potential regulatory roles for ERRs beyond targeting genes involved in energy metabolism. ChIP and gene expression microarrays in heart showed that ERRs beyond targeting genes involved in energy metabolism, growth and energetics. pathway in integrating fundamental processes of muscle. These novel findings establish the importance of the ERR pathway to promote differentiation in skeletal myocytes.

Fig. 8. MAP kinase phosphatase-1 (MKP-1) is a target of ERRα. A: expression of MKP-1 transcript during myogenesis in ERRα−/− and WT myocytes was analyzed by quantitative real-time PCR and normalized to 36B4 mRNA levels. Samples were analyzed in triplicate, and results are expressed as means ± SE. *P < 0.05.

B, left: MKP-1 transcript expression was analyzed by quantitative real-time PCR in C2C12 MB and d1 MT overexpressing ERRα or treated with 10 μM XCT790. Samples were analyzed in triplicate, and results are expressed as means ± SE. *Significant difference from GFP-infected MT (P < 0.05). Right: Western blot analysis for MKP-1 transcript was performed in C2C12 confluent MB (CMB) and d1 and d2 MT treated with vehicle or 10 μM XCT790. β-Tubulin was included as a loading control.

C, top: schematic diagram of the -5.0.Dusp1.pGL3 luciferase reporter plasmid. The relative locations of ERR response elements (ERRE), E-boxes, and Sp1 sites in the Dusp1 promoter are shown. Arrows indicate the region amplified by PCR in chromatin immunoprecipitation (ChIP) experiments. Bottom left: relative luciferase expression of the Dusp1 promoter-reporter transiently transfected alone into C2C12 MB. Activity was assessed at 24 h after transfection in subconfluent MB or in MT after 1 day in low-serum differentiation medium. Bottom right: activity of the Dusp1 promoter-reporter transiently cotransfected with empty expression vector, pcDNA3.1, ERRα, and/or PGC-1α in C2C12 myoblasts. Luciferase expression was normalized to Renilla luciferase activity from cotransfected pRL.TK.Luc. Data represent means ± SE from 6 trials. *Significant difference from baseline, either MB or vector control; **significant difference compared with activation by ERRα or PGC-1α alone.

D, ChIP analysis for ERRα binding to Dusp1 gene in C2C12 myocytes. Immunoprecipitation was performed with chromatin isolated from Ad-ERRα infected myoblasts and d1 MT. The proximal promoter of the Dusp1 gene was amplified with primers recognizing the indicated regions (C). Top: immunoprecipitation of chromatin corresponding to the Dusp1 and Tbp genes by ERRα antibody compared with IgG is shown in a representative trial on agarose gels. As a control for chromatin differences between MB and MT, 10% input is shown. Bottom: enrichment was quantitated by real-time PCR and normalized to chromatin input and to nonspecific amplification of the Tbp gene locus. Data are reported as ERRα/IgG after normalization to chromatin input. *P < 0.05.
myogenesis in ERRα−/− myocytes may be sufficient to maintain target gene expression. The potential contribution of ERRγ in the ERRα−/− phenotype was not evaluated in the present study. Prior studies have shown that the ERRβ/γ agonist GSK4716 upregulates known inhibitors of myogenesis through a mechanism involving glucocorticoid receptor activation independent of any effects on energy metabolism (66). Regardless of whether compensatory upregulation of ERRγ and PGC-1α maintains metabolic and sarcomeric gene expression in ERRα−/− myocytes, this upregulation is not sufficient for normal myogenesis to proceed.

Indeed, the data presented here indicate that ERRα regulation of myocyte differentiation is not simply driven by metabolic modulation. In both ERRα loss-of-function models we observed inappropriate activation of ERK/MAPK coincident with MKP-1 downregulation, yet the two conditions have divergent effects on expression of metabolic and sarcomeric transcripts. These transcripts were unchanged or increased in ERRα−/− myocytes during early differentiation, associated with the noted upregulation in ERRγ expression. Consistently, mitochondrial DNA and substrate oxidation rates are equivalent in WT and ERRα−/− MB and early-stage MT (Fig. 6B; J. Murray, unpublished observation). Diminished mitochondrial function is observed only in the late-stage ERRα−/− MT. In contrast, acute inhibition of ERRα by XCT790 caused a uniform decrease in metabolic and sarcomeric transcripts in addition to its effects on MKP-1 expression. Thus the delay in myogenesis in both ERRα−/− primary myocytes and XCT790-treated C2C12 likely involves overlapping but distinct mechanisms with early delays associated with aberrant ERK activation.

The MEK/ERK signaling pathway has a biphasic role during myogenesis (1, 61). Activation of MEK/ERK early in myogenesis by growth factors maintains the proliferative state and inhibits myogenesis, while in later stages of differentiation MEK/ERK activation increases MT fusion and sarcomeric protein expression and assembly. The importance of this precise temporal signaling pattern by MEK/ERK is supported by studies demonstrating that treatment of myocytes with MEK inhibitors advances myogenesis early and blocks growth or causes cell death in fused late-stage MT (59). Thus downregulation of MEK/ERK activity is necessary for the onset of differentiation. The altered ERK signaling observed in ERRα−/− primary myocytes is sufficient to cause the differentiation delay, since treatment of these myocytes with either of the well-characterized MEK inhibitors advances myogenesis early and blocks growth or causes cell death in fused late-stage MT (59). Thus downregulation of MEK/ERK activity is necessary for the onset of differentiation. The altered ERK signaling observed in ERRα−/− primary myocytes is sufficient to cause the differentiation delay, since treatment of these myocytes with either of the well-characterized MEK inhibitors advances myogenesis early and blocks growth or causes cell death in fused late-stage MT (59). Thus downregulation of MEK/ERK activity is necessary for the onset of differentiation. The altered ERK signaling observed in ERRα−/− primary myocytes is sufficient to cause the differentiation delay, since treatment of these myocytes with either of the well-characterized MEK inhibitors advances myogenesis early and blocks growth or causes cell death in fused late-stage MT (59). Thus downregulation of MEK/ERK activity is necessary for the onset of differentiation. The altered ERK signaling observed in ERRα−/− primary myocytes is sufficient to cause the differentiation delay, since treatment of these myocytes with either of the well-characterized MEK inhibitors advances myogenesis early and blocks growth or causes cell death in fused late-stage MT (59). Thus downregulation of MEK/ERK activity is necessary for the onset of differentiation.

Fig. 9. MEK inhibitor treatment rescues delayed differentiation in ERRα−/− primary myocytes. A: WT and ERRα−/− primary myocytes were treated 5 h before initiation of differentiation with DMSO, 5 μM U0126, or 10 μM PD98059, and this treatment was maintained during differentiation. Brightfield pictures of WT and ERRα−/− primary myocytes were taken in MB and d1 and d2 MT to assess MT formation, and representative images are shown (×100 magnification; scale bars = 100 μm). B: WT and ERRα−/− primary myocytes were treated with 5 μM U0126 as in A. Total protein was harvested at the indicated time points, as described in MATERIALS AND METHODS. Western blot analysis of ERK activation was performed as in Fig. 7A. β-Tubulin was included as a loading control. C: MKP-1 expression in WT and ERRα−/− primary myocytes treated as in A was assessed by Western blot analysis at the indicated time points. EFTUD2 was included as a loading control.
Thus the perturbation in MKP-1 expression may mediate the changes in MEK/ERK activation associated with impaired differentiation and growth in response to ERRα modulation. The important role of MKP-1 in transient MEK/ERK inactivation during myogenesis has been established by studies showing that ectopic MKP-1 expression is sufficient to induce C2C12 MB differentiation (3, 27). However, sustained MKP-1 expression caused by Notch activation of the Dusp1 gene is sufficient to disrupt myogenesis (27). Consistent with cell-based data, skeletal muscle regeneration is impaired in MKP-1−/− mice, supporting the physiological relevance of MKP-1 in regulating myogenesis in vivo (56). These findings imply that the reduction of MKP-1 expression in skeletal muscle from ERRα−/− mice may affect muscle regeneration. We also demonstrated that ERRα directly regulates expression of the Dusp1 gene 5′ flanking region, which contains an ERRα-responsive site and is occupied by ERRα in a differentiation-dependent manner. This is consistent with results from previous studies demonstrating that Dusp1 is regulated by other nuclear receptors, including glucocorticoid and retinoic acid receptors (24, 71). Furthermore, a subsequent search using the Ensembl mouse regulatory build (version 4) revealed ERRα binding to the proximal Dusp1 promoter in embryonic stem cells within the same region we identified (8, 19). Several Dusp genes are transcriptionally induced in response to cell stress and growth factors, while Dusp1 is also responsive to FAs (42, 49). Induction of MKP-1 by FAs has been implicated in diabetes-associated downregulation of mitochondrial enzyme genes and associated loss of type I oxidative myofibers in high-fat-fed mice (49). Recent studies demonstrating MKP-1 effects on whole body metabolism through tissue-specific modulation of MAP kinase activity implicate an additional indirect mechanism through which ERRαs may regulate metabolism (69). Thus the direct regulation of MKP-1 by ERRα and PGC-1α and the downstream effects on muscle regeneration as well as on oxidative metabolism in adult skeletal muscle is an intriguing area for further study.

Collectively, our results demonstrate a novel role for ERRα as an essential regulatory component of the myogenic pathway. ERRα activation promotes differentiation and growth, while ERRα ablation inhibits differentiation in skeletal myocytes. These data expand the role of ERRαs to include the potential regulation of fundamental signal transduction pathways during myogenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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