

Ski Regulates Muscle Terminal Differentiation by Transcriptional Activation of *Myog* in a Complex with Six1 and Eya3^{*[5]}

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Overexpression of the *Ski* pro-oncogene has been shown to induce myogenesis in non-muscle cells, to promote muscle hypertrophy in postnatal mice, and to activate transcription of muscle-specific genes. However, the precise role of *Ski* in muscle cell differentiation and its underlying molecular mechanism are not fully understood. To elucidate the involvement of *Ski* in muscle terminal differentiation, two retroviral systems were used to achieve conditional overexpression or knockdown of *Ski* in satellite cell-derived C2C12 myoblasts. We found that enforced expression of *Ski* promoted differentiation, whereas loss of *Ski* severely impaired it. Compromised terminal differentiation in the absence of *Ski* was likely because of the failure to induce myogenin (*Myog*) and p21 despite normal expression of *MyoD*. Chromatin immunoprecipitation and transcriptional reporter experiments showed that *Ski* occupied the endogenous *Myog* regulatory region and activated transcription from the *Myog* regulatory region upon differentiation. Transactivation of *Myog* was largely dependent on a MEF3 site bound by Six1, not on the binding site of *MyoD* or MEF2. Activation of the MEF3 site required direct interaction of *Ski* with Six1 and Eya3 mediated by the evolutionarily conserved Dachshund homology domain of *Ski*. Our results indicate that *Ski* is necessary for muscle terminal differentiation and that it exerts this role, at least in part, through its association with Six1 and Eya3 to regulate the *Myog* transcription.

Originally identified as a transduced avian retroviral oncogene (1–3), *Ski* is an evolutionarily conserved gene in species ranging from flies to humans (4). The retroviral *v-ski* protein corresponds to residues 21–441 of chicken *c-Ski*, which is a nuclear protein of 750 residues (5). The amino-terminal half of *c-Ski* is the most highly conserved segment, containing two distinct domains that mediate protein-protein interactions (6). The more conserved of these, the Dachshund homology domain (DHD),² defines the *Ski* gene family, which includes

Ski, *SnoN*, *Dach*, *Fussel-15*, *Fussel-18*, and *Corl* (7–14). The DHD has been implicated in the interactions of *Ski* with Smad2/3, nuclear receptor co-repressor, Skip, and retinoic acid receptor α (15–23). The second conserved region comprises the SAND domain (Sp100, AIRE-1, NucP41/75, and DEAF-1 domain) that mediates the interactions of *Ski* with Smad4, FHL2, and MeCP2 (24–26). The C-terminal region of *c-ski* is missing in *v-ski* and contains a tandem repeat/leucine zipper motif that mediates both homodimerization and heterodimerization with *SnoN* (6, 27).

Ski does not bind DNA directly (28) but interacts with several different transcription factors to modulate transcription as either a co-activator or a co-repressor depending on its DNA binding partner. Both *v-ski* and *c-ski* cause oncogenic transformation and induce myogenic differentiation in non-muscle avian embryo fibroblasts (2, 3, 29). The latter activity involves activation of muscle-specific genes, including the myogenic regulatory factor (MRF) genes, *MyoD* and myogenin (*Myog*) (29–31). Transgenic mice expressing *v-ski* or *c-ski* cDNAs develop selective hypertrophy of type IIb fast skeletal muscle fibers (32). Moreover expression of *Ski* increases in skeletal muscle at midgestation of mouse development (33). The requirement of the endogenous gene for normal muscle development was demonstrated by the observation that *Ski*-null mice show a marked decrease in skeletal muscle mass (34).

Further studies indicated that regulation of myogenesis by *Ski* might be mediated by its ability to activate transcription driven by the regulatory elements of muscle-specific genes, such as myosin light chain 1/3 (*MLC1/3*), the muscle creatine kinase (*MCK*), and most importantly *Myog* (35–37). The *Ski*-responsive cis element of *Myog* resides in a 184-bp regulatory region immediately upstream of the *Myog* promoter. This region has been shown to be sufficient for the complete recapitulation of the temporal and spatial expression pattern of *Myog* during embryogenesis (36, 38–40). DNA binding sites for *MyoD* and myocyte enhancer binding factor 2 (MEF2) in this regulatory region were found to be necessary for *Myog* transcription (40–42). Transient reporter assays have shown that *Ski* cooperates with *MyoD* and MEF2 to activate transcrip-

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² The abbreviations used are: DHD, Dachshund homology domain; MRF, myogenic regulatory factor; *Myog*, myogenin; MEF, myocyte enhancer factor;

MHC, myosin heavy chain; CHIP, chromatin immunoprecipitation; shRNA, short hairpin RNA; GM, growth medium; DM, differentiation medium; Dox, doxycycline; TFIIE, transcription factor IIE; DAPI, 4',6'-diamidino-2-phenylindole; TRE, tetracycline response element; CMV, cytomegalovirus; miR30, microRNA-30; *Dach*, Dachshund; MLC, myosin light chain; MCK, muscle creatine kinase; tTA, tetracycline-controlled transactivator; TMP, SIN-TREmiR30-PIG.

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tion from the 184-bp *Myog* regulatory region (36). However, no direct interactions between Ski and these muscle-specific transcription factors have been reported.

In vitro studies have revealed that terminal differentiation of myoblasts proceeds through a highly ordered sequence of events. These cells express MyoD while proliferating, but when growth stimuli are removed, they initiate expression of Myog followed by the induction of the cyclin-dependent kinase inhibitor p21 and irreversible withdrawal from the cell cycle. Subsequently these postmitotic myocytes express muscle-specific contractile proteins such as myosin heavy chain (MHC) and finally fuse into multinucleated myotubes (43). This process is governed mainly by two families of transcription factors, the MRFs and MEF2 (44–46). The MRF gene family includes *MyoD*, *Myf5*, *Myog*, and *MRF4* (44, 47, 48). All MRF family members share a highly conserved basic region and adjacent helix-loop-helix motif (bHLH) that mediates binding to a consensus DNA sequence, CANNTG, known as the E box, that is present in the regulatory regions of many muscle-specific genes. Forced expression of any MRF gene is capable of inducing expression of muscle-specific genes and activation of myogenic differentiation even in non-muscle cells. The MEF2 proteins belong to the superfamily of MADS (MCM1-aga-mous-deficient serum response factor) box transcription factors and directly bind an A+T-rich element found in the promoters and enhancers of many muscle specific genes (49). Genetic analysis reveals that members of the MEF2 family are also essential for terminal muscle differentiation (50).

Another cis element, the MEF3 site (consensus sequence, TCAGGTT), is also present in the 133-bp *Myog* regulatory region. Studies of transgenic mice demonstrated that mutation of this MEF3 site abolishes correct expression of a *Myog-LacZ* transgene during embryogenesis (51). Two skeletal muscle-specific members of the Six family (*sine oculis* homeodomain-containing transcription factors), Six1 and Six4, bind to the MEF3 element and transactivate *Myog* transcription (51). *Drosophila sine oculis* (*so*) has been shown to act synergistically with *eyes absent* (*eya*) and *dachshund* (*dac*) by direct protein-protein interactions. Similar interactions underlie the synergism of their mammalian homologues Six, Eya, and Dach (10, 52–59). This evolutionarily conserved regulatory network of Eya/Six/Dach has been shown to regulate myogenesis in chicken somite culture and in the chick limb and to activate transcription of reporters containing the *Myog* MEF3 site (52, 53). Interaction of mammalian Dach with Six protein is mediated by the evolutionarily conserved DHD motif (60). This has led to the suggestion that by virtue of its possession of these conserved domains (7, 10, 12) Ski might also interact with Six and Eya proteins to regulate *Myog* expression and thereby control commitment of myogenic cells to terminal differentiation (52).

In this study, we addressed this possibility by exploiting the well characterized mouse muscle satellite cell line, C2C12, as a model system. Using retroviral vectors to achieve tetracycline-regulated overexpression or knockdown of Ski, we asked whether Ski might not only stimulate but also be required for terminal differentiation of C2C12 cells. To probe the mechanism underlying the transcriptional regulation of *Myog* by Ski,

the E box, MEF2, and MEF3 sites in the *Myog* regulatory region were investigated as the possible cis response elements. Using co-immunoprecipitation, we asked whether direct binding of Ski to MyoD, MEF2c, Six1, and/or Eya3 mediates its transcriptional activity. Finally chromatin immunoprecipitation (ChIP) assays were performed to determine whether Ski resides at the endogenous *Myog* regulatory region prior to or concomitant with the initiation of terminal differentiation.

EXPERIMENTAL PROCEDURES

Construction of Retroviral Vectors—The replication-defective retroviral vector LNTX was kindly provided by Dr. Fage and was described earlier (61). A DNA fragment containing the entire coding region of the human *SKI* cDNA was excised from the plasmid pSHH*SKIN1D* using BamHI and ClaI, blunt-ended with T4 DNA polymerase, and inserted into the LINTX vector at the PmeI site to produce LNTX-huSKI.

The replication-defective retroviral vector TMP-tTA was modified from the SIN-TREMiR30-PIG (TMP) vector (kindly provided by Dr. Scott Lowe) (62–64) by replacing its *GFP* gene with the tetracycline transactivator (65) gene (66). The sequences of shRNAs targeting mouse *Ski* were chosen using RNAi Codex and are designated by their positions in the mouse *Ski* cDNA sequence (GenBankTM accession number AF435852): m*Ski*1145 (bases 1145–1163), m*Ski*1819 (bases 1819–1837), and m*Ski*977 (bases 977–995). DNA forms of these shRNA inserts were generated by PCR amplification of 97-base synthetic oligonucleotides using *Pfu* DNA polymerase (Invitrogen) and a common set of primers (5'-cagaggctcagaag-gtatattgctgttgacagtgcg-3' and 5'-cgcggaattccgaggcagtag-gca-3'). The PCR products were subsequently digested with XhoI and EcoRI and inserted between these sites within TMP-tTA vector to generate TMP-tTA-m*Ski*1145, TMP-tTA-m*Ski*1819, and TMP-tTA-m*Ski*977. Clones containing these shRNA-encoding inserts were sequence-verified.

Tissue Culture and Transfection—Proliferating mouse C2C12 myoblasts were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 20% fetal bovine serum (Atlanta Biologicals), 100 μ g/ml penicillin, 100 units/ml streptomycin, and 0.002% Fungizone (Invitrogen). To avoid spontaneous differentiation, cells were always kept in subconfluent (60–70%) conditions. Terminal differentiation was induced by switching subconfluent cells (80%) to differentiation medium (DM) consisting of Dulbecco's modified Eagle's medium, 2% heat-inactivated horse serum, and antibiotics as in GM. Morphological differentiation, judged by myotube formation, was documented by digital photography of phase-contrast microscopic images.

Retroviral Packaging and Infection—The retrovirus packaging cell line PA317 (ATCC number SD3443) was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 μ g/ml penicillin, and 100 units/ml streptomycin. They were transfected with retroviral constructs by using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. 24 h after transfection, the medium was harvested, and live cells were removed by centrifugation at 1000 \times *g* for 10 min. The retrovirus-containing supernatant

was used to infect exponentially growing C2C12 cells at 40% confluence in the presence of 10 ng/ml Polybrene (Sigma). The infection was repeated with freshly harvested virus 12 h later. After an additional 12 h of culture, C2C12 cells were switched to GM plus 2 μ g/ml doxycycline (Dox; an analog of tetracycline) and antibiotics (G418 at 750 μ g/ml for LNITX-based constructs and puromycin at 2 ng/ml for TMP-tTA-based constructs). Two weeks later the resulting individual colonies were isolated using cloning discs according to the manufacturer's protocol (PGC Scientifics, 62-6151-14) and expanded in GM with Dox and G418 or puromycin. After culture in GM minus Dox, clones were screened by Western blot analysis for tetracycline-regulated Ski overexpression or knockdown.

Western Blotting—Whole-cell extracts were prepared from confluent C2C12 cells on 100-mm culture dishes as follows: cells were scraped into 400 μ l of lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, 0.1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM NaF, and Complete protease inhibitor mixture (Roche Applied Science)). After 10-min incubation on ice, the suspension was subjected to three freeze-thaw cycles, and cell debris were removed by centrifugation at 12,500 rpm for 15 min at 4 °C. Protein concentrations were determined by Bradford assay (Bio-Rad), and equal amounts of proteins were boiled in protein loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, and 2% β -mercaptoethanol), separated by 6% SDS-PAGE, and transferred to Immobilon-P membranes (0.45 μ m, Millipore).

For most antibodies, blots were preblocked for 1 h at room temperature in blocking buffer (20 mM Tris, pH 7.6, 125 mM NaCl, 0.1% Tween 20, and 5% (w/v) nonfat dry milk) and incubated with primary antibodies in the same solution overnight at 4 °C. The membranes were then washed with TBST (20 mM Tris, pH 7.6, 125 mM NaCl, and 0.1% Tween 20) and incubated for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase or alkaline phosphatase followed by washes with TBST. Signals were detected using either enhanced chemiluminescent horseradish peroxidase substrate (Pierce) or CDP-Star[®] alkaline phosphatase chemiluminescent substrate (1:50; Tropix) and exposure to HyBlot CL autoradiography film (Denville Scientific).

When using anti-Ski G8 monoclonal antibody, the blots were preblocked overnight at 4 °C in blocking buffer (PBS (7.7 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, and 150 mM NaCl, pH 7.2), 0.4% casein, 1% polyvinylpyrrolidone (40,000), 10 mM EDTA, and 0.2% Tween, pH 7.2) and then incubated with primary antibody in the same solution for 30 min at room temperature. The membranes were washed with blocking buffer and further incubated with alkaline phosphatase-conjugated secondary antibodies (Sigma) for 30 min at room temperature. Subsequently the blots were washed with blocking buffer and then with assay buffer (0.1 M EDTA, 1 mM MgCl₂, and 0.02% azide, pH 10.0), and the signal was detected with CDP-Star alkaline phosphatase chemiluminescent substrate as above.

The following primary antibodies were used for immunoblotting: Ski (monoclonal antibody (1:2000; G8, Learner Research Institute Hybridoma Core Facility) or rabbit polyclonal antibody (1:1000; H329, Santa Cruz Biotechnology,

Inc.), Myog (1:1000; F5D, Santa Cruz Biotechnology, Inc.), TFIIIE- α (1:3000; C-17, Santa Cruz Biotechnology, Inc.), MHC (1:1000; MF-20, Developmental Studies Hybridoma Bank), MyoD (1:1000; 5.8A, Santa Cruz Biotechnology, Inc.), and anti-FLAG (1:5000; M2, Sigma). The secondary antibodies used were: alkaline phosphatase-conjugated anti-mouse IgG (Fc-specific) and anti-rabbit IgG (whole molecule) (1:30,000; Sigma) and horseradish peroxidase-conjugated Mouse IgG TrueBlot[™] and Rabbit IgG TrueBlot (1:1000; eBioscience).

Immunofluorescence—C2C12 myoblasts (2×10^4) were seeded into each well of LAB-TEK[®] 8-well chamber slides (Nunc) coated with 1 μ g/cm² laminin (Invitrogen) in GM and switched to DM for 3 days. Cells were fixed in 3.7% paraformaldehyde, PBS for 30 min at room temperature; permeabilized with 10% goat serum, 1% Triton X-100, and PBS for 10 min; and incubated with blocking buffer (PBS, 10% goat serum, and 0.1% Tween 20) for 1 h at room temperature and then with primary antibodies overnight at 4 °C. Chambers were washed with PBST (PBS and 0.1% Tween 20), incubated with secondary antibodies for 1 h at room temperature, washed with PBS, and mounted in Vectashield aqueous mounting medium with DAPI (Vector Laboratories). Images were obtained using an Olympus BX50 upright fluorescence microscope equipped with Polaroid digital camera PDMC2, Polaroid PDMC2 software, and fluorescent illumination. Images were assembled using Photoshop CS (Adobe).

The following primary antibodies were used for immunofluorescence: MyoD (1:100; 5.8A, Santa Cruz Biotechnology, Inc.), Myog (1:100; F5D, Santa Cruz Biotechnology, Inc.), MHC (1:200; MF20, Developmental Studies Hybridoma Bank), and p21 (1:100; SX118, BD Pharmingen). Secondary antibodies were Alexa 488- or Alexa594-conjugated goat anti-mouse IgG antibody (1:300; Molecular Probes). Control experiments performed with normal IgG as the primary antibody yielded no signal above the background.

Quantification was performed by counting at least 1000 DAPI-stained nuclei in more than 10 random fields per culture plate. For MHC, the differentiation index = nuclei within MHC-stained multinucleate myotubes/total number of DAPI-stained nuclei, and the fusion index = the average number of nuclei per MHC-stained myotube. For nuclear proteins, the differentiation index = number of antibody-stained nuclei/total number of DAPI-stained nuclei. All experiments were performed in triplicate on three independent cultures, and the standard deviation was calculated.

Real Time PCR—RNA was isolated using the RNeasy kit (Qiagen) with DNase I treatment according to the manufacturer's protocol, and cDNA was generated using reverse transcriptase SuperScript[™] III (Invitrogen) with random hexamer primers according to the manufacturer's instructions. Quantitative real time PCR (iCycler iQ[™], Bio-Rad) was performed using SYBR[®] Green PCR Core Reagents (Applied Bioscience) according to the manufacturer's protocols. PCR was performed for 40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s followed by a single 72 °C extension step for 5 min. Primer sequences used for real time PCR will be provided upon request. Analyses were performed in triplicate on RNA samples from three independent experiments. Threshold cycles (Ct) of

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target genes were normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and relative transcript levels were calculated from the Ct values as $Y = 2^{-\Delta Ct}$ where Y is -fold difference in amount of target gene versus *Gapdh* and $\Delta Ct = Ct_x - Ct_{Gapdh}$.

ChIP—Chromatin immunoprecipitation experiments were performed essentially as described before (67). Briefly C2C12 cells cultured in GM or DM for 2 days were cross-linked with 1% formaldehyde, PBS for 10 min at room temperature. Fixed cells were scraped and resuspended at 2×10^7 cells/ml in lysis buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% deoxycholate, and 0.1% SDS). Suspensions were sonicated to yield chromatin with an average DNA length of 200–500 bp. Equal amounts of chromatin from each sample (2×10^6 cells/assay) were preabsorbed at 4 °C for 1 h with 40 μ l of a 50% slurry of preblocked protein A beads (Repligen; previously incubated with 1 mg/ml salmon testes DNA, 10 mg/ml bovine serum albumin, and 0.05% sodium azide in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA)). After pelleting the beads, supernates were incubated overnight at 4 °C with either 2 μ g of rabbit polyclonal Ski antibody (H329, Santa Cruz Biotechnology, Inc.) or normal rabbit IgG. Antibody-chromatin complexes were then captured by incubation with 40 μ l of a 50% slurry of preblocked protein A beads at 4 °C for 1 h. The beads were washed sequentially in lysis buffer, high salt buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton-100, 0.1% deoxycholate, and 0.1% SDS), lithium salt buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, and 0.5% deoxycholate), and TE buffer. Complexes were eluted from the beads in 150 μ l of elution buffer (10 mM Tris, pH 8.0, 5 mM EDTA, and 1% SDS), and formaldehyde cross-linking was reversed by overnight incubation at 65 °C. After treatment with RNase A and proteinase K, DNA was isolated by phenol extraction and ethanol precipitation. The optimal PCR cycle numbers were determined by real time PCR, and 5% of purified DNA was analyzed by regular PCR using HotStart-IT Taq Master Mix (USB). 25% of each reaction mixture was resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. The following PCR primer sets were used: the mouse *Myog* regulatory region (–169 to +39, GenBank accession number M95800), 5′-gggcaaaaggagagggaag-3′ and 5′-agtggcaggaa-caagcctt-3′; the non-promoter region downstream of *Myog* gene (+1943 to +2185 downstream of the *Myog* gene, GenBank accession number NW_001030662.1) serving as a negative control, 5′-gtcaagaactgacttaaggcc-3′ and 5′-gacactaggagaagggtg-gag-3′; and the mouse *Smad7* regulatory region (–274 to –142, GenBank accession number NW_001030635.1), 5-tagaaac-cgatctgtgtttgcg-3 and 5-cctctgctcggtggttccactgc-3. For input control, 10% of cross-linked chromatin was purified as described above and assessed for PCR by using the same sets of primers.

Reporter Assays—A 202-bp DNA fragment (–184 to +18, GenBank accession number M95800) containing the *Myog* regulatory region was amplified by PCR using C57BL/6 genomic DNA as template. *Myog184*-luciferase reporter carrying the luciferase gene downstream of this *Myog* regulatory region was generated by inserting HindIII-StuI-digested PCR product into the HindIII-SmaI site of the pGL3-Basic vector (Promega).

Myog-luciferase constructs with E box, MEF2, and MEF3 mutations (*Myog184*-E1E2m, *Myog184*-MEF2m, and *Myog184*-MEF3m) were generated from *Myog184*-luciferase using the QuikChange® site-directed mutagenesis kit (Stratagene). SKI expression vector pCDNA-huSKI was described previously (23).

Cells (1×10^5) were seeded in 12-well plates and transfected at 80% confluence using Lipofectamine 2000 (Roche Applied Science) with a combined total of 4 μ g of expression vector and reporter plasmid DNAs. 18 h after transfection, cells were switched to DM for 48 h prior to harvest and lysis in $1 \times$ Reporter lysis buffer (Promega) with one round of freeze-thaw followed by incubation at room temperature for 20 min. Cell debris were removed by centrifugation, and luciferase activity in the supernatant was determined by a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol using a MAXline microplate luminometer (Molecular Devices). The relative light units were generated by normalizing firefly luciferase units to *Renilla* luciferase units of the co-transfected pTK-*Renilla*-luc vector. The experiments were done in duplicate, and the reported results represent at least three independent experiments.

Co-immunoprecipitation—C2C12 cells (~50% confluent, 100-mm dishes) were transfected with expression plasmids for FLAG-tagged *Six1*, FLAG-tagged *Eya3*, FLAG-tagged *Mef2c*, *MyoD*, and full-length *SKI* or its mutants using Lipofectamine 2000. 18 h after transfection, cells were refed GM or switched to DM and harvested 2 days later in 1 ml of NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and Complete protease inhibitors mixtures). After brief sonication, cell debris were removed by centrifuge at 10,000 rpm for 15 min at 4 °C. The lysates were preabsorbed with protein A beads, and protein concentrations were determined by the Bradford protein assay. Immunoprecipitations using equal amounts of proteins with either rabbit polyclonal anti-Ski or normal rabbit IgG were collected by overnight incubation with protein A beads (Repligen) at 4 °C. Precipitates were washed five times in NETN buffer, resuspended and boiled in protein loading buffer, separated by 6% SDS-PAGE, and analyzed by Western blotting. If precipitating and primary Western blotting antibodies were from the same species, either horseradish peroxidase-conjugated Mouse IgG TrueBlot or Rabbit IgG TrueBlot was used as the secondary antibody accordingly.

RESULTS

Forced Expression of SKI Stimulates Myogenic Differentiation in C2C12 Cells—Overexpression of Ski has been reported to induce non-muscle fibroblasts to differentiate into myotubes (30, 31), suggesting a role of *Ski* in myogenic lineage determination and terminal differentiation. To assess the role of *Ski* in terminal differentiation independent of its possible role in myogenic lineage determination, C2C12 cells that have already committed to myogenic fate and require only serum deprivation to undergo terminal differentiation were used in this study. To regulate Ski expression, C2C12 cells were infected with LNIT-huSKI, a retroviral vector that allowed both G418 selection and Dox regulation of SKI expression (Fig. 1A). Several

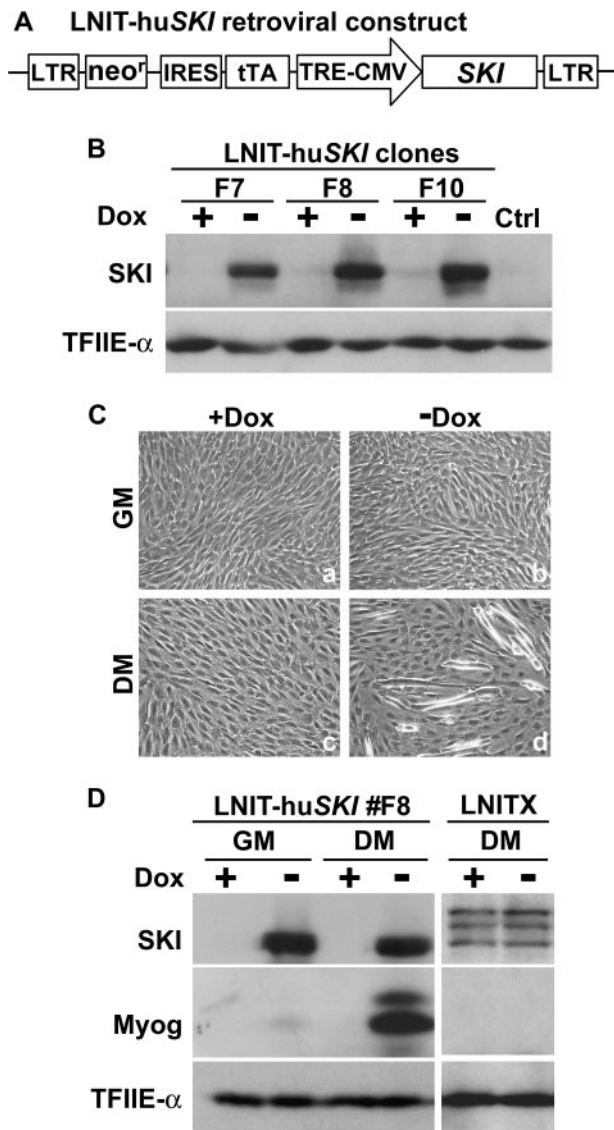


FIGURE 1. *Ski* stimulates differentiation of C2C12 myoblasts. *A*, schematic representation of retroviral vector LNIT-huSKI showing the 5' and 3' long terminal repeats (*LTR*), neomycin resistance gene (*neo*^r), internal ribosomal entry site (*IRES*), *tTA* (65), the minimal CMV promoter regulated by tetracycline response elements (*TRE-CMV*), and human *Ski* coding region (*Ski*). *B*, Dox-regulated *Ski* expression in LNIT-huSKI clones. Western analysis of *Ski* expression in representative C2C12 LNIT-huSKI clones cultured under induced condition (–Dox) or suppressed condition (+Dox) for 3 days. A vector-only clone (LNITX) was used as a negative control (Ctrl). TFIIIE- α was a loading control. *C*, a representative C2C12 LNIT-huSKI clone (F8) was cultured in GM or DM for 1 day with or without Dox, and phase-contrast microscopy shows myotube formation only in the absence of Dox. *D*, enhanced Myog expression by overexpression of *Ski*. LNIT-huSKI F8 clone or a vector-only clone (LNITX) was cultured in GM or DM for 1 day with or without Dox, and Western blot analysis revealed the expression of Myog and *Ski*. TFIIIE- α was a loading control. Note that endogenous *Ski* expression in the right panel was revealed only after much longer exposure than the left panel where overexpression of *Ski* was detected.

G418-resistant clones were isolated and propagated in GM containing Dox to suppress *Ski* overexpression. They were then subdivided and tested for *Ski* expression after 3-day cultures in either the presence or absence of Dox. Western blot analysis of cell extracts revealed obvious induction of *Ski* expression in several LNIT-huSKI clones after Dox withdrawal (Fig. 1*B*), whereas a clone infected with the LNITX empty vec-

tor did not overexpress *Ski* regardless of Dox treatment (Fig. 1*D*, right panel).

Using these clones, we examined the effect of *Ski* expression on myotube formation. LNIT-huSKI cells were cultured in GM with or without Dox for 4 days. Upon reaching 80% confluence, the cells were induced to differentiate by switching to DM while maintaining the presence or absence of Dox. 1 day after initiating differentiation, LNIT-huSKI cells that expressed exogenous *Ski* had prematurely formed myotubes, but no myotubes were observed in the same clone cultured in the presence of Dox to suppress *Ski* overexpression (Fig. 1*C*). Western analysis of these cells revealed expression of Myog only in cells that expressed exogenous *Ski* (Fig. 1*D*, left panel). These results were not due to Dox treatment because the vector-only controls showed no obvious difference in Myog or endogenous *Ski* expression in the presence or absence of Dox (Fig. 1*D*, right panel). These data indicate that the increased *Ski* expression stimulates the commitment of C2C12 myoblasts to terminal muscle differentiation.

Generation of C2C12 Clones with Inducible Knockdown of *Ski* Expression—The gain-of-function study above indicated that, as in avian cells, overexpression of *Ski* induces muscle terminal differentiation of C2C12 cells. However, those results do not necessarily implicate endogenous *Ski* in this process. To address this issue we asked whether knocking down endogenous mouse *Ski* expression would affect the differentiation of C2C12 cells. To accomplish this and avoid potential deleterious effects on the long term cell viability due to the loss of endogenous *Ski*, we used tetracycline-regulated expression of shRNAs to knock down *Ski* in C2C12 cells. The TMP-tTA vector used for this purpose was modified from the TMP retroviral vector (62–64) by substituting the *tTA* gene for the *GFP* gene so that this single retrovirus carries both the tetracycline-controlled transactivator (*tTA*) and its tetracycline response element (*TRE*) (Fig. 2*A*). DNA sequences that encoded shRNAs targeting mouse *Ski* were inserted within the framework of microRNA-30 (*miR30*) downstream of the *TRE-CMV* promoter (Fig. 2*A*). Transcripts of this cassette resembling natural microRNA-30 could be generated upon removal of Dox, resulting in the knockdown of *Ski*. Three different shRNA inserts targeting mouse *Ski* gene were designed, and retroviral constructs, designated as TMP-tTA-m*Ski*977, -1145, and -1819, were generated.

PA317 packaging cells were transfected with each of the three TMP-tTA-m*Ski* vectors (or the empty vector), and viruses were harvested to infect C2C12 myoblasts. Puromycin-resistant clones were isolated and propagated in GM plus Dox to prevent shRNA expression. These clones were then subdivided and cultured with or without Dox for 6 days prior to testing for conditional knockdown of *Ski*. Western analysis of several TMP-tTA-m*Ski*1819 clones revealed a highly efficient Dox-dependent knockdown of *Ski* without significant leakiness (Fig. 2*B*). TMP-tTA-m*Ski* cells grown in the absence of Dox produced nearly undetectable *Ski* levels, whereas the same clones grown in the presence of Dox expressed *Ski* at levels similar to that of the vector-only control. Of ~40 clones tested with each of the three TMP-tTA-m*Ski* shRNAs, 70–80%

Regulation of Muscle Differentiation by Ski-Six1-Eya3

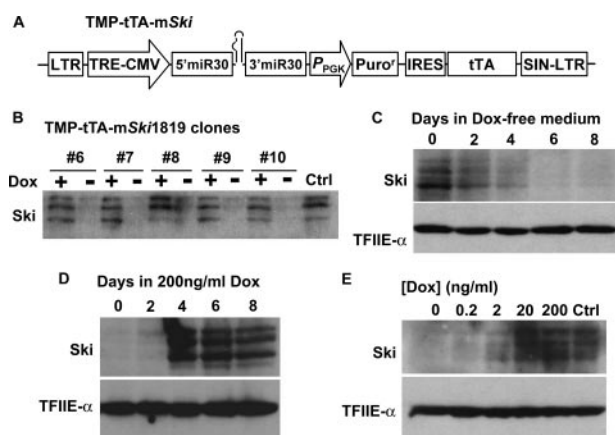


FIGURE 2. Dox-regulated knockdown of Ski in C2C12 cells via shRNAs in the context of miR30. *A*, schematic representation of TMP-tTA-mSki retroviral vector showing the 5' long terminal repeat (*LTR*) and 3' self-inactivating long terminal repeat (*SIN-LTR*), puromycin resistance gene (*Puro'*) driven by the phosphoglycerate kinase (*PGK*) promoter, internal ribosomal entry site (*IRES*), *tTA*, and a microRNA cassette downstream of TRE-CMV promoter. The shRNAs targeting mouse *Ski* were inserted into the microRNA cassette between the 5' and 3' flanking sequences derived from the miR30 primary transcript (5' *miR30* and 3' *miR30*). *B*, Dox-regulated knockdown of Ski in TMP-tTA-mSki1819 clones. Western analysis of Ski expression was performed on representative C2C12 TMP-tTA-mSki1819 clones or the vector-only clone (*Ctrl*) cultured with (+Dox) or without Dox (–Dox) for 1 week. *C*, Western blot analysis revealed decreased Ski expression in C2C12 TMP-tTA-mSki1145 clone in response to removal of Dox. Cells were cultured in the presence of 200 ng/ml Dox for 8 days, switched into Dox-free medium, and analyzed after culture for the indicated number of days. *D*, Western blot analysis revealed restoration of Ski expression in C2C12 TMP-tTA-mSki1145 clone in response to addition of Dox. Cells were cultured in the absence of Dox for 8 days to achieve the maximal knockdown of Ski and then switched into medium containing 200 ng/ml Dox for the indicated number of days. *E*, Western blot analysis revealed dose response of Ski knockdown to Dox in a C2C12 TMP-tTA-mSki1145 clone. Cells were cultured in the absence of Dox for 6 days and switched into medium containing the indicated concentration of Dox for another 6 days. TFIIIE- α was used as a loading control, and a vector-only clone (*Ctrl*) shows the endogenous Ski level.

exhibited Dox-dependent knockdown of Ski, indicating the high efficiency of this system (data not shown).

Ski Knockdown Is Reversible and Dox Dose-dependent—To determine the kinetics of Ski knockdown, a C2C12 TMP-tTA-mSki1145 clone was propagated in Dox-containing medium, transferred to Dox-free medium, and monitored for Ski expression over an 8-day period. Western analysis showed that knockdown of Ski was apparent within 4 days after Dox removal and was virtually complete after 6 days (Fig. 2*C*). This knockdown was completely reversible; readdition of Dox to these cells restored normal Ski expression within 4 days (Fig. 2*D*). The extent of Ski knockdown was also doxycycline dose-dependent; expression of Ski was barely detectable at 2 ng/ml Dox or less and was comparable to the vector-only control at 20 ng/ml Dox or more (Fig. 2*E*). Taken together, these data demonstrated that this single vector system allows tightly regulated and reversible knockdown of endogenous Ski.

Impaired Myotube Formation in the Absence of Ski—Using this Dox-regulated knockdown system, we next evaluated the consequences of the loss of Ski on terminal differentiation. A C2C12 TMP-tTA-mSki1145 clone was either kept in GM plus Dox to maintain Ski expression or switched to GM minus Dox for 7 days to achieve the maximal knockdown of Ski. Subsequently upon reaching 80% confluence, these cells were switched from GM to DM while continuing the maintenance or

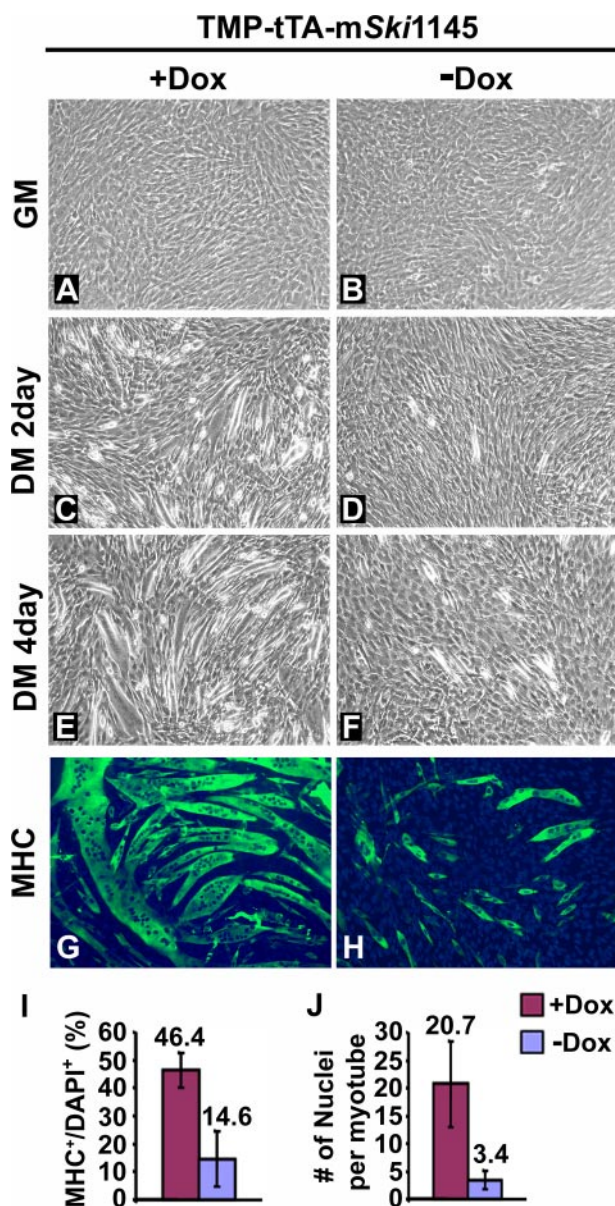


FIGURE 3. Loss of Ski prevents terminal differentiation of C2C12 myoblasts. Prior to the following assays, cells were cultured for 7 days in GM either with Dox as a control or without Dox to achieve maximal knockdown of Ski. *A–F*, a C2C12 TMP-tTA-mSki1145 clone was analyzed for the effect of Ski knockdown on myotube formation by phase-contrast microscopy (left panel). Cells were cultured to 80% confluence in GM with (+Dox) or without Dox (–Dox), switched to DM with continued presence or absence of Dox, and cultured for 2 and 4 days. Representative fields are shown at 25 \times magnification. *G* and *H*, TMP-tTA-mSki1145 cells were cultured as described above and kept in DM for 3 days. Myotubes were labeled by indirect immunofluorescence with antibody against MHC (green), and nuclei were counterstained with DAPI (blue). Representative fields are shown at 100 \times magnification. *I* and *J*, quantitative analysis of the immunofluorescence assays in *G* and *H*. Red bars represent culture in the presence of Dox (+Dox), and blue bars represent culture in the absence of Dox (–Dox). The percentage of DAPI-stained nuclei in MHC-positive cells (*I*) and the average number of nuclei per MHC-positive myotube (*J*) were calculated as described under “Experimental Procedures.” Data above the bar represent means of three independent experiments. Error bars show S.D.

suppression of Ski expression. Phase microscopy of the cultures prior to switching to DM revealed that the morphology of TMP-tTA-mSki and TMP-tTA cells in GM was similar and not affected by the loss of Ski expression (Fig. 3, *A* and *B*, and sup-

plemental Fig. S1, A and B). This was not true for cells that were switched into DM to induce differentiation. The TMP-tTA-mSki cells in which Ski expression was maintained exhibited extensive myotube formation (Fig. 3, C and E). However, when Ski expression was knocked down in the TMP-tTA-mSki clone, no obvious myotube formation was visible within 2 days (Fig. 3D), and only a few small myotubes had formed after 4 days (Fig. 3F). The lack of myotube formation was not due to delayed differentiation kinetics because in the absence of Dox TMP-tTA-mSki cells did not show significant myotube formation even after 6 days in DM (data not shown). Furthermore the differentiation defect of TMP-tTA-mSki cells in the absence of Dox was not due to the withdrawal of Dox because myotube formation of the TMP-tTA control was independent of Dox (supplemental Fig. S1, C and D). Thus the results indicate that Ski expression is required for terminal muscle differentiation of C2C12 cells.

Decreased myotube formation in the absence of Ski could be due to a block at any stage in the differentiation pathway or a failure of fully differentiated myocytes to fuse. To distinguish between these possibilities, we performed immunofluorescence for MHC, which is a terminal differentiation marker expressed in both unfused myocytes and multinucleated myotubes. Consistent with the morphological observations above, TMP-tTA-mSki cells exhibited extensive formation of MHC-positive multinucleated myotubes when expressing Ski, whereas the vast majority of cells from the same clonal line were MHC-negative when Ski expression was knocked down (Fig. 3, G and H). The effects of loss of Ski on terminal differentiation and fusion were further quantified by measuring both the percentage of nuclei in MHC-positive cells (differentiation index) and the average number of nuclei per MHC-positive myotube (fusion index). We found that the loss of Ski caused significant ($p < 0.05$) drops in both the differentiation index (from 46.4 ± 6.2 to $14.6 \pm 9.8\%$) and fusion index (20.7 ± 7.7 to 3.4 ± 1.7 nuclei per myotube) (Fig. 3, I and J), respectively. The results indicate that in the absence of Ski, both differentiation (~3-fold fewer MHC-positive cells) and myotube formation (~6-fold fewer nuclei per myotube) were severely impaired (Fig. 3, I and J). Thus the loss of Ski resulted in a differentiation block upstream of myocyte fusion.

Loss of Ski Inhibits Commitment to Terminal Muscle Differentiation—To determine the stage at which terminal differentiation was blocked by the absence of Ski, we further examined the expression of an early differentiation marker, Myog, and a late differentiation marker, MHC. As shown in Fig. 4A, when maintained in GM, neither the C2C12 TMP-tTA-mSki1145 cells nor the TMP-tTA cells expressed Myog or MHC at detectable levels regardless of Dox treatment. However, after 3 days of culture in DM, TMP-tTA-mSki cells in which Ski expression was maintained by Dox treatment expressed both Myog and MHC at high levels. This was also the case when TMP-tTA cells were cultured in DM regardless of Dox treatment. In contrast, when the same TMP-tTA-mSki clone with Ski expression knocked down were cultured in DM, Myog and MHC were expressed at very low levels (Fig. 4A). Similar results were obtained using TMP-tTA-mSki clones expressing the other two shRNAs, indicating that the differen-

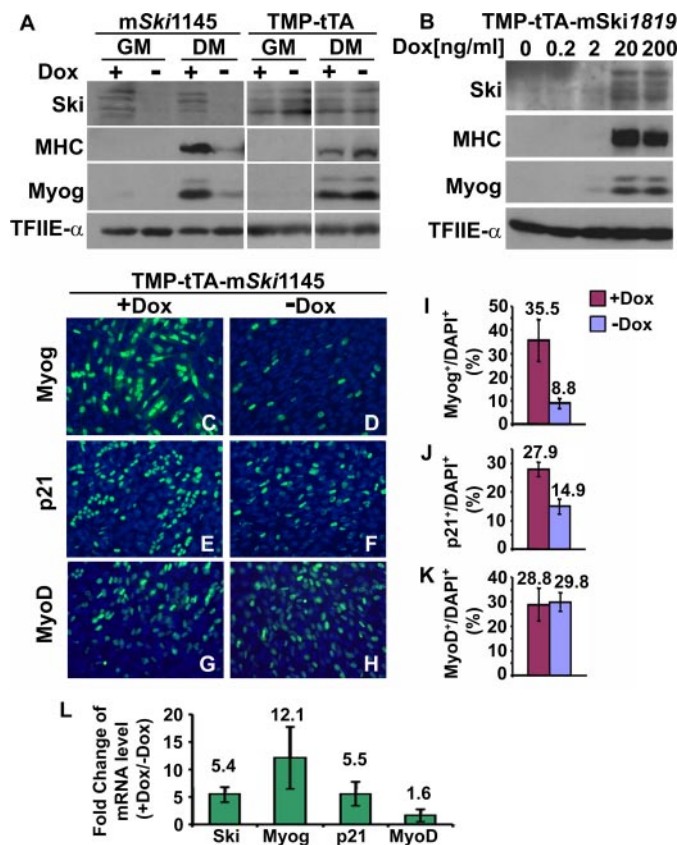


FIGURE 4. Loss of Ski reduces the expression of muscle-specific genes at both mRNA and protein levels. A, a representative C2C12 TMP-tTA-mSki1145 clone was cultured as described in the legend to Fig. 3 and kept in DM for 3 days prior to Western analysis for Ski, MHC, and Myog expression. TFIIE- α was used as a loading control. B, Western blotting revealed expression of Ski, MHC, and Myog in a C2C12 TMP-tTA-mSki1819 clone cultured in DM for 3 days with the indicated concentrations of Dox (0–200 ng/ml). C–H, indirect immunofluorescence was performed on a C2C12 TMP-tTA-mSki1145 clone cultured in DM with or without Dox for 3 days. Differentiating cells were detected with antibodies against Myog, p21, or MyoD (green), and total nuclei were counterstained with DAPI (blue). Representative fields are shown at 400 \times magnification. I–K, quantitative analysis of the immunofluorescence assays in C–H. Red bars represent culture in the presence of Dox (+Dox), and blue bars represent culture in the absence of Dox (–Dox). The fraction of nuclei positively stained for Myog (I), p21 (J), and MyoD (K) were calculated as described under “Experimental Procedures.” Data above the bar represent means of three independent experiments. Error bars show S.D. L, quantitative real time PCR analysis of Ski, Myog, p21, and MyoD mRNA levels in a C2C12 TMP-tTA-mSki1145 clone cultured in DM with or without Dox for 3 days. –Fold change of the transcript level in culture in the presence of Dox over that in the absence of Dox was calculated as described under “Experimental Procedures.” Data above the bars represent means from three independent experiments performed in triplicate. Error bars show S.D.

tiation block was not due to an off-target effect of the shRNA (supplemental Fig. S2). In addition, a Dox dose-response experiment with a C2C12 TMP-tTA-mSki clone expressing a different shRNA (mSki1189) revealed that the expression of Myog and MHC dropped in parallel with the decrease in endogenous Ski expression (Fig. 4B). The loss of Myog expression indicated that differentiation was blocked at a very early stage in the absence of Ski expression.

The early stages of muscle differentiation are marked by a well characterized progression of protein expression including the myogenic regulatory factors (MyoD and Myog) and the cell cycle regulator (p21) (43, 44, 47, 48). To obtain a quantitative assessment of the early disruption of the myogenic differentia-

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tion program due to the loss of Ski expression, we investigated the percentage of cells expressing these proteins during the differentiation of C2C12 TMP-tTA-m*Ski* clones in the absence or presence of Dox. Myog and p21 are markers of commitment to terminal differentiation and withdrawal from the cell cycle, respectively. Their increased expression can be detected in both differentiating myocytes and fully differentiated multinucleated myotubes and is widely used to assess the early stage of differentiation. Within 3 days of switching to DM, 35.5% of TMP-tTA-m*Ski* cells were Myog-positive when cultured in the presence of Dox (Fig. 4, C and I), and 27.9% were p21-positive (Fig. 4, E and J). In sharp contrast, in the same TMP-tTA-m*Ski* clone with Ski expression knocked down, the percentages of Myog- and p21-positive cells decreased to 8.8% (Fig. 4, D and I) and 14.9% (Fig. 4, F and J), respectively. On the other hand, the expression of MyoD, a constitutive myogenic lineage marker of C2C12 cells, was comparable in the presence and absence of Ski expression (Fig. 4, G, H, and K). These results indicate that the loss of Ski blocked a step in the differentiation pathway downstream of *MyoD*. Results obtained with a representative TMP-tTA-m*Ski*1145 clone are shown in Fig. 4, and similar results were obtained with two other clonal lines (data not shown).

To determine whether the observed Ski-dependent changes in protein expression were due to reductions in the expression of their mRNAs, we performed real time PCR on RNA isolated from TMP-tTA-m*Ski* cells cultured for 3 days in DM plus or minus Dox. Concomitant with the 5.4-fold lower expression of *Ski* mRNA in TMP-tTA-m*Ski* cells in the absence of Dox, *Myog* and *p21* mRNA levels were reduced by 12- and 5.5-fold, respectively. On the other hand, the level of *MyoD* mRNA was not significantly affected by the loss of Ski (Fig. 4L). These results mirror those obtained in the analyses of protein expression and indicate that Ski is necessary for the transcription or accumulation of mRNAs that are important for initiating muscle terminal differentiation.

Ski Occupies Myog Regulatory Region in Differentiating Myoblasts—Because Ski is known to be a co-regulator of transcription it seemed likely that the changes we detected in the expression of muscle-specific mRNA might be due to direct effects of Ski on transcription. This possibility was especially appealing for *Myog* not only because its expression is required for the initiation of terminal differentiation (43, 44, 47, 48) but also because published reporter gene assays have demonstrated that transient overexpression of Ski can transcriptionally activate the *Myog* regulatory region (36, 37).

To investigate whether endogenous Ski might directly regulate *Myog* transcription, we performed ChIP assays on C2C12 cells cultured in either GM or DM. DNA was isolated from the chromatin immunoprecipitated with anti-Ski antibodies and was analyzed by PCR using primers that amplify the *Myog* regulatory region including the E1 box, a MEF2 site, and a MEF3 element (40–42, 51, 68) (Fig. 5A). The ChIP assays revealed that Ski was not bound to this endogenous *Myog* regulatory region in proliferating C2C12 cells cultured in GM. However, Ski became associated with this region when the cells were stimulated to differentiate by switching them into DM (Fig. 5B, upper panel). The specificity of this interaction was verified by negative results in control ChIP assays using either a nonspe-

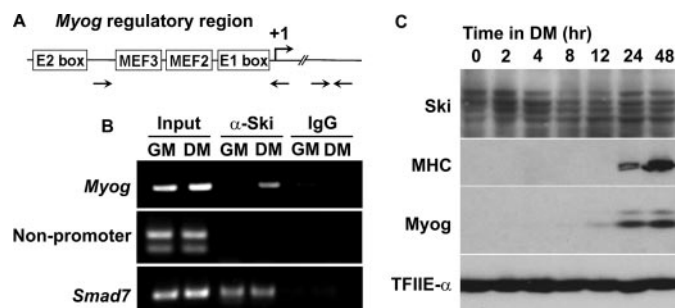


FIGURE 5. Ski binds the endogenous *Myog* regulatory region upon differentiation. A, schematic representation of the *Myog* regulatory region. E boxes (E1 and E2), the MEF2 binding site, and the MEF3 binding site are indicated as open boxes relative to the transcriptional start site (+1). Primer sets used to amplify sequences spanning the *Myog* regulatory region or a non-promoter region downstream of the *Myog* gene are represented as arrows. B, ChIP analysis of Ski occupancy of the endogenous *Myog* regulatory region in proliferating and differentiating cells. Equivalent amounts of cross-linked chromatin from C2C12 cells cultured in GM or in DM for 2 days were precipitated with an antibody against Ski (α -Ski) or a normal rabbit IgG. The DNA isolated from precipitated chromatin was analyzed by PCR using primers spanning *Myog* regulatory region (upper), non-promoter region (middle), or *Smad7* regulatory region (lower), and following electrophoresis, PCR products were visualized by ethidium bromide staining. C, Western analysis of Ski, MHC, and Myog expression in C2C12 cells cultured in GM or in DM for the indicated periods of time. TFIIIE- α was used as a loading control.

cific antibody (normal IgG) and the same primers amplifying the *Myog* regulatory region or the Ski antibody and primers amplifying a non-promoter region downstream of *Myog* gene (Fig. 5B, top and middle panels). Furthermore the observation that Ski was expressed at similar levels in cells cultured in GM and DM indicated that the increased interaction between Ski and the endogenous *Myog* regulatory region upon differentiation was not due to a parallel increase in Ski expression (Fig. 5C). Likewise the fact that the *Smad7* regulatory region, which is known to be bound by Ski (69), was occupied by Ski in both proliferating and differentiating cells (Fig. 5B, bottom panel) indicated that the chromatin binding ability of Ski did not depend merely on the change from GM to DM. Thus Ski selectively binds the *Myog* regulatory region in concert with a signal that initiates differentiation. These results suggest that the requirement for endogenous Ski in the initiation of terminal muscle differentiation might be due to its direct role in activating of *Myog* transcription.

MEF3 Binding Site Is Required for the Activation of Myog Regulatory Region by Ski—In light of the above results we sought to define the Ski-response cis element in the *Myog* regulatory region. A 184-bp *Myog* regulatory region sequence has been defined as a muscle-specific regulatory region and contains a number of transcriptional factor binding sites that are critical for activation of *Myog* transcription during differentiation (38, 40–42, 51, 68). Among others, they include two consensus MyoD-binding E boxes (proximal E1 box and distal E2 box), a MEF2 binding site, and a MEF3 site (Fig. 6A, upper panel). Ski does not bind DNA directly, but it has previously been shown to synergize with MyoD and MEF2 in activating transcription of *Myog* reporters containing this 184-bp sequence (36). This synergy requires the binding of these transcription factors to their response elements in the *Myog* regulatory region. To address whether Ski activation of *Myog* regulatory region was mediated only through these binding sites, we

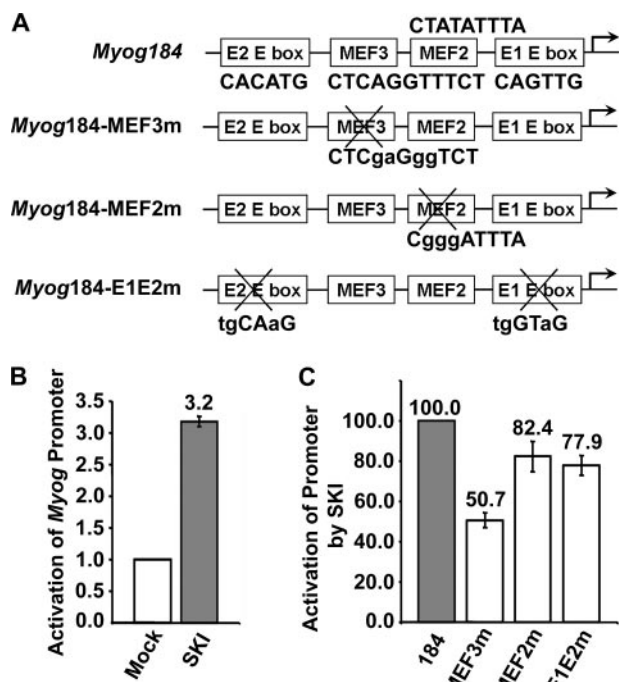


FIGURE 6. The MEF3 binding site is required for maximal activation of *Myog* promoter by SKI. A, schematic representation of the 184-bp proximal *Myog* regulatory region and its mutants. The intact E1 and E2 boxes, MEF2 site, and MEF3 site are indicated as open boxes; the mutated elements are marked by "X." The sequences of wild-type binding sites are indicated in uppercase, and the mutated bases in these binding sites are indicated in lowercase. B and C, C2C12 cells were co-transfected with the wild-type or mutated reporters described above along with a *SKI* expression vector or an empty vector (*Mock*) and cultured in DM for 2 days. Luciferase activity (relative light units) of each sample was calculated as described under "Experimental Procedures." B, activation of wild-type *Myog* regulatory region (*Myog184*) by SKI was calculated as the -fold change of relative light units in SKI-transfected cells over that in the empty vector-transfected cells, which was set to an arbitrary unit of 1. Data are expressed as the mean values from three independent experiments performed in triplicate. Error bars represent the S.D. C, the activation of wild-type and mutated *Myog* regulatory region by SKI were calculated as described in B. The activation of the mutated *Myog* regulatory regions (*Myog184-E1E2m*, *Myog184-MEF2m*, and *Myog184-MEF3m*) by SKI is expressed as the percentage of that with the wild type, which was set at 100%. Data are expressed as the mean data of each reporter from three independent experiments performed in triplicates. Error bars represent the S.D.

mutated the MEF2 binding site or both E boxes (E1 and E2) to eliminate binding by their corresponding transcriptional factors (Fig. 6A). In addition, we mutated the MEF3 site, although it had not previously been implicated in activation by Ski. C2C12 myoblasts were transfected with the wild-type *Myog* reporter (*Myog184*) or its mutated derivatives (*Myog184-MEF3m*, *Myog184-MEF2m*, and *Myog184-E1E2m*; see Fig. 6A) along with a *SKI* expression vector or an empty vector, and luciferase activity was measured 2 days after switching the cells to DM. SKI expression potentiated the activity of the wild-type *Myog* reporter (Fig. 6B) and surprisingly activated the transcription of the reporters carrying mutations of either the MEF2 site or both E boxes to ~80% of the level observed with the wild-type reporter (Fig. 6C). These results suggested that activation of *Myog* regulatory region by SKI was not mediated exclusively by these two regulatory elements. However, activation of the reporter bearing a mutated MEF3 site by SKI was only 50% of that of the wild type (Fig. 6C), indicating that this site may be the major SKI-responsive cis element within the *Myog* regulatory region.

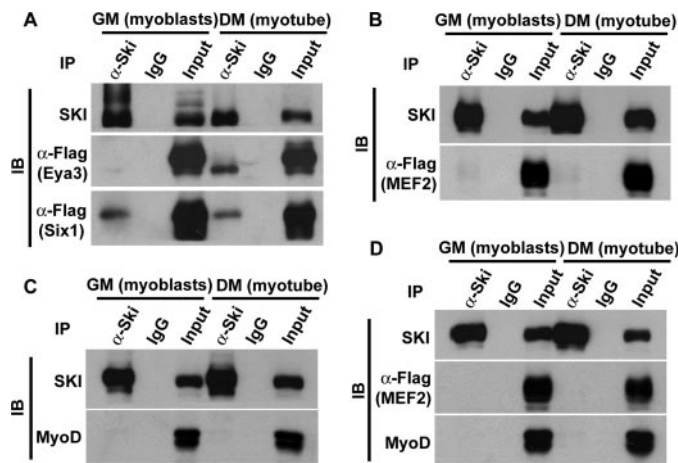


FIGURE 7. SKI interacts with Six1 and Eya3 but not with MyoD or MEF2. A–D, C2C12 cells were co-transfected with expression plasmids for SKI and FLAG-tagged Six1 and Eya3 (A), MEF2-FLAG (B), MyoD (C), or MEF2-FLAG and MyoD (D) and cultured in GM or DM for 2 days. Lysates were immunoprecipitated with a rabbit antibody against Ski (α -Ski lanes), and precipitates were analyzed by Western blotting using anti-Ski, anti-FLAG, or anti-MyoD antibodies. 10% of the input for immunoprecipitation (*Input* lanes) confirms that comparable amounts of these proteins were used in the immunoprecipitation assays. Immunoprecipitation (*IP*) with a normal rabbit IgG (*IgG* lanes) was used as a negative control.

SKI Associates with Eya3 and Six1 in Differentiating Muscle Cells—We have shown that activation of *Myog* transcription by SKI is mediated mainly through the MEF3 site in the regulatory region. Because Ski does not bind DNA directly, it seemed likely that its association with the endogenous *Myog* regulatory region and its activation of *Myog* transcription are mediated by its association with transcription factors that bind to the MEF3 element. Six1 has been shown to bind to the MEF3 site of the *Myog* regulatory region (51) and to synergize with Eya to positively regulate the transcription driven by this cis element (53). In addition, because Dach, a Ski family member, forms a trimeric complex with Six1 and Eya3 to regulate muscle-specific gene expression (53), it seemed possible that Ski may be tethered to *Myog* regulatory region via an association with Six1 and Eya3 in a similar manner. We therefore investigated whether Ski interacts with Six1 and Eya3 in muscle cells undergoing terminal differentiation. C2C12 cells were co-transfected with SKI and FLAG-tagged Six1 and Eya3 expression vectors, and cells were either cultured in GM or induced to differentiate in DM for 48 h. Extracts of these cells were immunoprecipitated with either rabbit anti-Ski or normal rabbit IgG and analyzed for co-precipitation of Six1 and Eya3 by Western blotting with anti-FLAG. As seen in Fig. 7A, neither Six1 nor Eya3 was precipitated by normal rabbit IgG, whereas Six1 was co-precipitated with SKI at comparable levels in proliferating (GM) and differentiating (DM) cells. In contrast, co-precipitation of Eya3 with SKI was barely above background in proliferating cells but clearly detectable in differentiating cells. Thus in muscle cells Ski is constitutively associated with Six1 but interacts with Eya3 only upon differentiation.

Because a previous study suggested that Ski activated transcription of *Myog* regulatory region in cooperation with MyoD and MEF2 (36), we performed similar co-precipitation assays to examine the possible interactions of Ski with these proteins. Surprisingly neither MyoD nor MEF2C co-precipitated with

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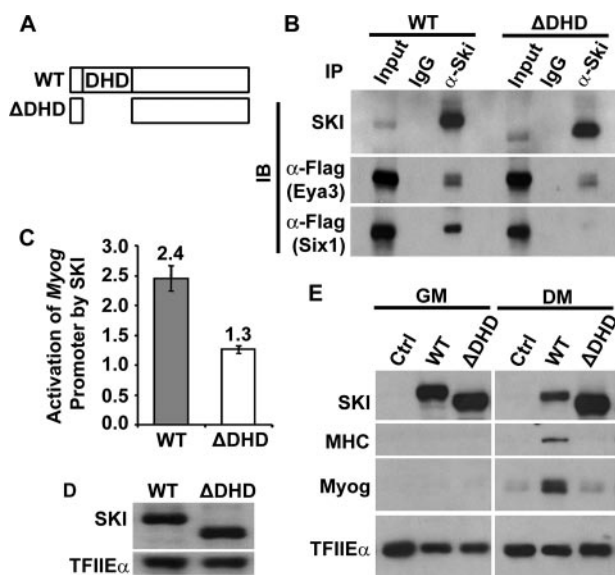


FIGURE 8. The DHD of SKI is required for its association with Six1 and its activation of *Myog* transcription. *A*, schematic diagrams represent SKI and the DHD deletion mutant (SKI Δ DHD). *B*, interaction of SKI with Six1 was mediated by its DHD. C2C12 cells were co-transfected with expression plasmids for FLAG-tagged Six1 and Eya3 and full-length SKI (wild type (WT)) or SKI Δ DHD (described in *A*) and cultured in DM for 2 days. Immunoprecipitation (IP) assays were performed as described in the legend to Fig. 7. *C*, C2C12 cells were co-transfected with the wild-type *Myog* reporter (*Myog*184) and expression vectors for wild-type SKI or SKI Δ DHD and cultured in DM for 2 days. Luciferase activity of each sample was calculated as described under "Experimental Procedures," and the -fold activation of *Myog* reporter by wild-type SKI and SKI Δ DHD were calculated as described in the legend to Fig. 6*B*. Data are expressed as the mean values from three independent experiments performed in triplicate. Error bars represent the S.D. *D*, Western blotting of the same lysates used for the luciferase assays in *C* revealed similar expression of wild-type SKI and SKI Δ DHD. TFIIIE- α was used as a loading control. *E*, cells were cultured in GM in the absence of Dox for 6 days to achieve maximal knockdown of Ski and then transfected with expression vectors for wild-type human SKI or SKI Δ DHD. Cells were then switched to DM and cultured for 2 day prior to harvest. Western blotting revealed expression of SKI, MHC, and Myog. TFIIIE- α was used as a loading control (Ctrl).

SKI in either proliferative or differentiating C2C12 cells (Fig. 7, *B* and *C*). Considering that MyoD and MEF2 activate the transcription of some muscle-specific genes in a cooperative manner (44), it seemed possible that their interactions with SKI might require the presence of both proteins. To test this possibility, similar co-immunoprecipitation experiments were performed with extracts of C2C12 cells that were co-transfected with SKI and both MyoD and MEF2 expression vectors. Once again both proteins were expressed at high levels, but neither of them co-precipitated with SKI in C2C12 cells cultured in GM or DM (Fig. 7*D*). Although negative results are inconclusive, these observations and the results of the reporter assays suggest that transcriptional cooperation of Ski with MyoD and MEF2 may be mediated by an indirect interaction with a DNA-bound Ski-Six1-Eya3 complex.

The DHD of Ski Is Required for Its Association with Six1 and Its Activation of Myog Transcription—It has been shown that Dach interacts with Six1 through its DHD (52, 53). It was therefore of interest to determine whether this conserved domain in Ski also mediates its interaction with Six1. The ability of a SKI mutant lacking the DHD to interact with Six1 was examined by coimmunoprecipitation (Fig. 8, *A* and *B*). Deletion of the DHD

from SKI (SKI Δ DHD) greatly reduced its ability to interact with Six1, although it did not affect interaction with Eya3.

Having identified the DHD as the Six1 binding domain in SKI, we sought to determine the effect of its deletion on transcriptional activation of *Myog*. Cotransfection experiments showed that SKI Δ DHD failed to activate transcription of the wild-type *Myog* reporter (*Myog*184) significantly compared with the wild-type SKI (Fig. 8*C*). Western blots of the transfected cells demonstrated that the lack of reporter activation by SKI Δ DHD was not due to a failure to express this protein at a level similar to that of the wild-type SKI (Fig. 8*D*). To confirm these results, we next asked whether the DHD of Ski was also required for activation of endogenous *Myog* expression upon differentiation. To answer this question we assessed whether reintroducing SKI or SKI Δ DHD into C2C12 TMP-tTA-*mSki* cells could overcome the loss of Myog and MHC expression due to the knockdown of Ski in these cells. C2C12 TMP-tTA-*mSki* cells were grown in GM minus Dox to achieve the maximal knockdown of endogenous mouse Ski and transfected with vectors expressing human SKI or human SKI Δ DHD, which are not subject to knockdown by the mouse *Ski*-targeted shRNA. These cells were analyzed for Myog and MHC expression after 2 days of culture in DM minus Dox. We found that wild-type SKI restored Myog and MHC expression. However, SKI Δ DHD failed to do so, and this inability was not attributable to poor expression of this mutant (Fig. 8*E*), indicating that the DHD is needed for the activation of *Myog* transcription.

DISCUSSION

The role of *Ski* in terminal differentiation has been evidenced by its ability to induce myogenesis in non-muscle cells *in vitro* and hypertrophy of type II fast muscle of adult mice when it was overexpressed (29–32). It remained of great interest to determine whether *Ski* also regulates terminal differentiation of muscle satellite cells, which are the committed myogenic cells and responsible for the skeletal muscle regeneration (70, 71). However, because *Ski*^{-/-} mice die at birth, we were not able to address this issue using this mouse genetic model (34). We therefore used a well established *in vitro* model, satellite cell-derived C2C12 myoblasts, to investigate the role of *Ski* in terminal differentiation and its underlying molecular mechanism. Our findings that differentiation was enhanced by overexpression of SKI and severely impaired in the absence of endogenous Ski indicate that Ski not only induces but also is necessary for differentiation of determined myoblasts. We further established a tight linkage between the myogenic activity of Ski and the expression of *Myog* at both mRNA and protein levels. Additional results suggest that direct regulation of *Myog* underlies the myogenic activity of Ski by demonstrating transcriptional activation of *Myog* by SKI in reporter assays and the association of Ski with the endogenous *Myog* regulatory region upon differentiation. Surprisingly transcriptional activation of *Myog* by SKI was largely mediated by its cooperation with Six1 and Eya3 through a MEF3 binding site, not with MyoD or MEF2 through the E boxes or the MEF2 binding site. The necessity of an evolutionarily conserved DHD for the interaction of SKI with Six1 is consistent with published data on the interaction between Dach and Six proteins (52). Our findings support a model in

which Ski substitutes for Dach in the standard Dach-Six1-Eya3 complex to regulate myogenesis.

Our earlier attempts to perform these studies using C2C12 myoblasts were frustrated by our inability to maintain cells in which Ski is constitutively overexpressed or knocked down. Here we surmounted this problem using inducible vectors, which allowed cloning and propagation of transduced cells expressing endogenous Ski at normal levels prior to acute induction or knockdown of Ski expression. The retroviral vector we constructed for regulated knockdown (TMP-tTA) was modified from the TMP retroviral vector of Lowe and co-workers (62) and others (63, 64). Because the TMP vector only carries the TRE-driven microRNA cassette whereas a second vector provides the *tTA* gene, two rounds of infection and antibiotic selection are required. This approach works well for cells whose activity is not compromised by prolonged passage, but it is not optimal for C2C12 myoblasts, which gradually lose their differentiation potential. Our new vector, TMP-tTA, carries the TRE-driven microRNA cassette and the *tTA* gene in a single retroviral vector so only one round of infection/antibiotic selection is needed. This vector has been proven as effective as the original TMP vector with regard to the efficiency, reversibility, dose dependence, and insignificant leakiness of knockdown. Given its simplicity and effectiveness, this knockdown vector is suitable to study any gene required for cell survival and is especially useful in cells whose activities of interest are sensitive to prolonged cell culture passage.

Earlier work showed that Ski stimulated myogenesis in non-muscle primary avian cells by inducing both *MyoD* and *Myog*, two genes controlling myogenic lineage determination and terminal differentiation, respectively (30). We therefore assumed that loss of Ski would lead to down-regulation of both of these genes and result not only in impaired differentiation but also in loss of myogenic identity. However, in the present report, we observed that loss of Ski only affected the expression level of *Myog* but not *MyoD* in determined C2C12 myoblasts, indicating that Ski is not necessary for maintaining myogenic identity. Given the pivotal role of *Myog* in the initiation of differentiation (43, 44, 47, 48), it is likely that regulation of *Myog* expression is the key mediator of the effect of *Ski* on terminal differentiation. Furthermore our data revealed that the regulation of *Myog* expression by Ski was not only at the protein level but also at the transcript level. This result along with the observed transactivation of *Myog* reporter by SKI and the occupancy of Ski on the endogenous *Myog* regulatory region places *Myog* as a direct transcriptional target of Ski.

As a non-DNA-binding transcription factor, Ski has to be brought into contact with promoters/enhancers by interaction with transcription factors that bind to specific DNA cis regulatory elements (28). Cis elements essential for transcriptional regulation of *Myog* include two E boxes and a MEF2 site that are bound by MyoD and MEF2, respectively (40–42, 51, 68). Previous studies have shown that MyoD and MEF2 cooperatively activate the *Myog* transcription through binding to these elements at the onset of myogenesis (44). This cooperative interaction has also been seen in the upstream regulatory sequences of other muscle-specific genes, including *MLC1/3* and *MCK* (35, 72–74). Interestingly Ski has been implicated in activation

of these promoters in cooperation with MyoD, and a previous report based solely on reporter assays suggested the same mechanism for the transactivation of *Myog* regulatory region by Ski (36). However, our results, using the same *Myog* regulatory region, appear to conflict with this report by showing that the destruction of E boxes or the MEF2 site had only a marginal effect on the ability of SKI to activate the transcription of *Myog* reporters. We cannot explain this discrepancy, but we believe that although reporter assays can be useful for defining cis response elements their biological significance requires confirmation by additional experiments. Our inability to detect direct interactions of SKI with MyoD or MEF2 and the absence of any previous data showing these interactions suggest an indirect mechanism for the cooperation between Ski and MyoD on *Myog* activation.

Recent studies demonstrating the presence of functional Pbx1 and MEF3 binding sites have revealed that the *Myog* regulatory region is more complex than previously believed (51, 53, 68, 75, 76). Our data underscore this complexity by showing that the MEF3 site instead of the previously implicated E boxes and MEF2 site mediated transactivation of *Myog* by SKI. These sites reside in close proximity within the *Myog* regulatory region suggesting that their combined occupancy by Six1, MyoD, and MEF2 may be the basis for the cooperation between Ski and these proteins. Our finding that activation of *Myog* transcription by Ski requires interaction with the MEF3-binding Six1 protein provides the mechanism for recruitment of Ski to the *Myog* regulatory region. This interaction and the observation that *Ski*^{-/-} mice exhibited a muscle defect similar to that of the *Six1*^{-/-} mice suggest a common mechanism by which both Ski and Six1 regulate myogenesis (34, 51, 59, 77–79).

The differentiation-dependent interaction between Ski, Six1, and Eya3 correlates well with the observation that the association of Ski with the endogenous *Myog* regulatory region occurs only in differentiating cells. Because growth factor signaling can block the ability of Eya to interact with Six proteins (80, 81), it is possible that the withdrawal of serum growth factors initiates terminal differentiation by freeing Eya to form the Ski-Six-Eya trimeric complex on the *Myog* regulatory region to activate its transcription. Dachshund has also been reported to transactivate the *Myog* reporter through the interaction with Six and Eya (53, 54). However, because activation of *Myog* expression and subsequent differentiation was almost abolished in the absence of Ski, we believe that in C2C12 myoblasts it is Ski, not Dach, that is the essential member of this trimeric complex. Our findings shed new light on the mechanism of the myogenic activity of Ski and the importance of the Ski-Six-Eya trimeric complex in muscle terminal differentiation.

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