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Research Article

skNAC and Smyd1 in transcriptional control

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ABSTRACT

Skeletal and heart muscle-specific variant of the alpha subunit of nascent polypeptide associated complex (skNAC) is exclusively found in striated muscle cells. Its function, however, is largely unknown. Previous reports could demonstrate that skNAC binds to Smyd1 (SET and MYND domain containing protein 1). The facts that (a) SET domains have histone methyltransferase activity, and (b) MYND domains are known recruiters of histone deacetylases (HDACs), implicate the skNAC–Smyd1 complex in transcriptional control. To study potential target genes, we carried out cDNA microarray analysis on differentiating C2C12 myoblasts in which expression of the *skNAC* gene had been knocked down. We found and confirmed a series of targets, specifically genes encoding regulators of inflammation, cellular metabolism, and cell migration. Mechanistically, as shown by Western blot, ELISA, and ChIP analysis at selected promoter regions, transcriptional control by skNAC–Smyd1 appears to be exerted at least in part by affecting a series of histone modifications, specifically H3K4 di- and trimethylation and potentially also histone acetylation. Taken together, our data suggest that the skNAC–Smyd1 complex is involved in transcriptional regulation both via the control of histone methylation and histone (de)acetylation.

1. Introduction

Skeletal and heart muscle-specific variant of the alpha subunit of nascent polypeptide associated complex (skNAC) is encoded by a splice variant of the alpha subunit of nascent polypeptide associated complex, a ubiquitous protein which plays a role in the targeting of newly synthesized polypeptide chains within the cell [28].

In contrast to αNAC, the skNAC protein is exclusively found in skeletal and heart muscle tissue. Remarkably, the *skNAC* gene is not expressed in proliferating myoblasts, but becomes induced only after the induction of myogenic differentiation. In addition, we could previously demonstrate *skNAC* induction in muscle regeneration [18], and skNAC-deficient animals are characterized by impaired cardiac development, as well as skeletal muscle hypotrophy and regeneration defects [21], implicating the protein in the control of striated muscle development and regeneration.

In 1996, Yotov and St.-Arnaud could show that skNAC enhances transcription of the myoglobin gene, suggesting that the protein might act as a transcriptional (co)activator for this gene.

Its mechanism of action, however, is still largely enigmatic. Nevertheless, the facts that skNAC binds to the Smyd1 (mBop) protein, a SET-domain-containing histone methyltransferase (HMT) which also contains a MYND domain known to be involved in the recruitment of histone deacetylases (HDACs) [25], and that Smyd1 interacts with class I (HDAC 1–3), and class II (HDAC 4,5) HDACs [9], suggests that the skNAC/Smyd1 complex might control transcription at the epigenetic level, via influencing histone modification.

However, to date, the respective histone modification patterns and the associated target genes have only poorly been characterized: For human Smyd3, Hamamoto et al. [11], and Kim et al. [15], could detect di- and trimethylation activity, whereas for Smyd1, zebrafish and murine data exist, indicating that the protein induces methylation of H3K4, however, the authors did not discriminate between mono-, di- and trimethylation [26,27], whereas Smyd2 catalyzes H3K36 dimethylation [5].

Thus, to get insight into functional aspects of transcriptional control by skNAC and Smyd1, we inhibited expression of the respective genes in cultured myoblasts and analyzed target genes and histone modification patterns. The results demonstrate that skNAC influences the expression of genes encoding regulators of inflammation, cellular metabolism, and cell migration. In addition, we present data suggesting a potential mechanism of transcriptional regulation by the skNAC–Smyd1 complex via histone modification.

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2. Materials and methods

2.1. Tissue culture

Murine C2C12 and SOL8 cells were cultured in Dulbecco's modified Eagle's medium containing 20% or 10% fetal bovine serum (growth medium) at 37 °C and 5% CO_2. To induce differentiation, cells were grown to 80–90% confluence and then switched to differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum; DM).

2.2. Transfection with expression vectors

For transient transfection of expression vectors into C2C12 cells, the "TurboFect" reagent (Fermentas) was employed according to the manufacturer's instructions. Transfection efficiency was controlled with a GFP expression vector and was 40–50% in all experiments. pBK-CMV-Smyd1 and pBK-CMV-Smyd1$_{\text{HMTase mutant}}$ were gifts from Haley Tucker, Austin, Texas.

2.3. Transfection with specific siRNAs

siRNA transfection was carried out using predesigned, specific siRNAs purchased from Sigma. For inhibition of *Smyd1* and *skNAC* expression, the following siRNAs were used: Smyd1 siRNA: sense: 5′-CACAUCUUUGGUGUGAUCA-3′, antisense: 5′-UGAUCACACCAAAGAUGUG-3′; skNAC siRNA: sense: 5′-GACAGUUCCUGUUGAGAAAUU-3′, antisense: 5′-UUUCUCAACAGGAACUGUCUU-3′. As a negative control, a non-gene-specific, "scrambled" siRNA was employed: scrambled siRNA: sense: 5′-CGUACGCGGAAUACUUCGAUU-3′, antisense: 5′-UCGAAGUAUUCCGCGUACGUU-3′. C2C12 cells were transfected using the transfection reagent Interferin (Polyplus Transfection) according to the manufacturer's instructions.

2.4. RNA isolation, Northern blot analysis, and qPCR

RNA isolation and Northern blot analysis were carried out as previously described [19]. Semi-quantitative real time PCR analysis was carried out using the Rotor-Gene 2000 system (Corbett Research, Mortlake, Australia). Gene expression was analyzed using the GoTaq qPCR Master Mix (Promega). For detection of different transcripts, self- and pre-designed primers (Qiagen QuantiTect Primer Assays) were used. Primer sequences are listed in Table 1. In each experiment, melting curve analysis was performed to verify that a single transcript was produced. RT-qPCR relative gene expression was calculated using the comparative CT ($2^{-\Delta\Delta C_T}$) method, where expression was normalized to GAPDH. Non-RT- and non-template controls were run for all reactions. Unless otherwise specified, data from at least three independent experiments were expressed as mean ± SD, $n=3$–5. Significance was accepted at $p < 0.05$.

2.5. cDNA microarray analysis

Genome-wide expression analysis was performed using SurePrint G3 Mouse GE 8x60K Microarrays (Design ID 028005) (Agilent Technology). skNAC-specific siRNA- and scrambled siRNA-transfected cells were differentiated for 72 h followed by RNA extraction. A quality check of each RNA sample as well as each cDNA sample was carried out by analysis on an Agilent 2100 Bioanalyzer and by photometric analysis. Data annotation analysis was performed using Agilent GeneSpring software. For microarray analysis, three samples from three different transfections were

Table 1
Summary of all qPCR primers used in this study.

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)
Accn3	CAGGTGGCTGAGCGGGTTCG	AGTCCATTGCCAGCACCGCC
Calpain 1	CACCAAGGAAGCCAGCCCCAG	GTTTTCATGGCGGCCCAAGCC
Calpain 3	TCAGAGACTCAGACCTGGACCCCA	TCCGCAGCCGCACCAACTTC
CIITA	GAGGCGACTCCAGGCAGCAGG	CCAGGGCATCACCATCAGGCTC
Cyp2s1	GGGGCTCTGTACTCGGGGCT	GCCAATGTTCCACGCCCGCT
Ffar2	GGACGGACGCGGGTTAGGGA	GGGCAGCCCATGTCTTCACGG
Foxo1	CCTGTCGTACGCCGACCTCATCAC	GTCCATGGACGCAGCTCTTCTCCG
Foxr1	GACCTTCACCACGACACACT	CCACATCCACAGATTGGGCT
GM 14316	CGGGATCCCTCTGCGAAAGCGGGCACCTT	CGGAATTCTGGCGCTGCTCCTTGCATCCTT
IL4	GGGACGCCATGCACGGAGATG	TGCGAAGCACCTTGGAAGCCC
IL6	GGTGACAACCACGGCCTTCCC	AAGCCTCCGACTTGTGAAGTGGT
IL17B	CTGACTTGGTGGGATGGACT	CTTCCATTCGAGCGTAGGGC
INFγ	TGGAGCTTTGACGAGCACCGA	CCAGGGGGCCCGACCTTTCC
Mef2c	GAGCTGAGCGTGCTGTGCGA	GCTCTCGTGCGGCTCGTTGT
Myogenin	TGGGTGTGCATGTGAGCCCC	CGCTGGGCTGGGTGTTAGCC
Myoglobin	CCCTGGAGGGTTGAGCACGGT	AGGCCACCTGGTCCTGAAGGG
Ppargc-1α	GTGGTGCCACCGCCAACCAA	AGCCGGAGACTGGGCCGTTTA
Rasgrf2	CCAGCAGACCGCTTACCGCA	CCGCCATGTGTGACCCGCTT
SIRT1	TTGGCACCGATCCTCGAAC	CCCAGCTCCAGTCAGAACTAT
Slc30a3	AGCGGGGCGTCCGAGTGGTC	CAGCATGGCACCCGCCTCAA
SRF	CCCGGCCATAGGGGCAGGAA	GCTTCTCGGTTCCACCCGGC
UCP3	AACCCAGGGGCTCAGAGCGT	GTCCGCTCCCTTGGGGGTGT
Capn1-338	GGGCGCACTACAAGAGTTTG	GCCATGCTGTGACCTCTATAAC
Capn1+1074	CCACAGCAGCAGAGCAGAG	GAGGAAAGGGGCAAATGAAC
Capn1+562	CTCCCTGGTCCTCCACCTAC	GGGACTGAAAGACACCCTAAG
Capn1+1032	ACCATTTGCCTGCCTGTAAC	TCAGTGCGTGGAGTTTTCAG
Capn3+24k	GCTGATGGGCGTGACTTAG	AGTTGGAAGGGGCAAACAC
Naca+1737	GCACCGAGCCTTTCATCAC	AAGCATTATCAACCCCTTTGAG
Naca+712	GATCTGGACGAGGAGGTTAAG	TCGCAGAGCAAGAAGAACAC
Srf+1646	CCCAGGTTGTAGGTGGTTTC	CAAGGATGGGAGTCGTCTTG
Srf-435	TTCTCCCACCACTTCCTGAG	GAGAGGGAAAGGCAAGAGTG
SRF+1290	TCCTTCAAAGCCCGTACAAC	AGGCAGGAGTTGGATGACAG
SRF+308	GGCAAGTCAGCGAGGAATC	CAGCAAGAGCGGCTTTAGTAAC

For detection of Accn3, Calpain 1, CIITA, GM14316, Rasgrf2 and skNAC novel and pre-designed primers (Qiagen QuantiTect Primer Assays) were used. For detection of the MyHC isoforms, Untr6, Actb and Gapdh only pre-designed primers were employed.