The SMYD1 and skNAC transcription factors contribute to neurodegenerative diseases

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ABSTRACT
SMYD1 and the skNAC isoform of the NAC transcription factor have both previously been characterized as transcription factors in hematopoiesis and cardiac/skeletal muscle. Here we report that comparative analysis of genes deregulated by SMYD1 or skNAC knockdown in differentiating C2C12 myoblasts identified transcripts characteristic of neurodegenerative diseases, including Alzheimer’s, Parkinson’s and Huntington’s Diseases (AD, PD, and HD). This led us to determine whether SMYD1 and skNAC function together or independently within the brain. Based on meta-analyses and direct experimentation, we observed SMYD1 and skNAC expression within cortical striata of human brains, mouse brains and transgenic mouse models of these diseases. We observed some of these features in mouse myoblasts induced to differentiate into neurons. Finally, several defining features of Alzheimer’s pathology, including the brain-specific, axon-enriched microtubule-associated protein, Tau, are deregulated upon SMYD1 loss.

1. Introduction

SET and MYND Domain 1 (SMYD1) is a transcription factor characterized extensively in hematopoietic cells, cardiac/skeletal muscle (where it is essential for embryonic survival) (Yahalom et al., 2018; Tracy et al., 2018; Franklin et al., 2016; Marayama et al., 2015; Rasmussen et al., 2015; Rasmussen and Tucker, 2018; Fujii et al., 2016; Hsia and Zon, 2005; Tan et al., 2006; Jing and Zon, 2011; Paik and Zon, 2010) and in endothelial cells where it acts as a SRF-interacting partner required for angiogenesis (Ye et al., 2016). SMYD1 belongs to a family of three orthologous isoforms, SMYDIA (employed throughout this study and termed SMYD1), SMYD1B, and SMYD1C whose SET domains are split by a MYND protein-interaction domain (reviewed in Tracy et al., 2018). In addition to its role as a TF, SMYD1A and B possess histone methyltransferase activity by catalyzing methylation of histone H3 lysine K4 (H3K4m3), H3K9m1 and potentially other histone methylation marks during cardiac remodeling (Tracy et al., 2018). SMYD1 also monomethylates a single lysine within the hematopoietic stress response factor, Tribbles3/TRB3 (Nie et al., 2017).

SMYD1 interacts with the cardiac/skeletal and hematopoietic-specific TF skNAC, an alternatively spliced isoform of NACA (Yotov and St-Arnaud, 1996), which is required for transactivation of Myoglobin (MB) (Sims et al., 2002). Consistent with their physical interaction, the temporal and spatial expression patterns of skNAC are almost identical to those of SMYD1 (Sims et al., 2002; Raval et al., 2012; Park et al., 2010). Smyd1 and Sknac mutant embryos exhibit similar gene dysregulation (Park et al., 2010).

Here we employed global gene expression and integrated KEGG database pathway analyses (RanochaSato et al., 2016) to determine what targets are deregulated by SMYD1, skNAC or both in C2C12 myocytes following three days of differentiation to myoblasts. As anticipated, we observed numerous hematopoietic and cardiac/skeletal muscle targets, including MB. However, we observed numerous others that showed no obvious hematopoietic or cardioskeletal phenotypes. Unexpectedly, these included a number of highly deregulated transcripts characteristic of neurodegenerative diseases, including Alzheimer’s (AD), Parkinson’s (PD) and Huntington’s (HD) Diseases (readdressed in Results and Discussion). The cause of each of these diseases is death of neurons and other...
cells within the brain, and each can affect different regions of the brain (Smyth, Grosser et al., 2020; Lezi and Swerdlow, 2012; CanuSarasso et al., 2018; O'Brien et al., 2017; Bray, 2020; Elouzi, Vedam-Mai et al., 2019). In essence, Alzheimer’s destroys memory, while Parkinson’s and Huntington’s affect movement. Each of these diseases is progressive, debilitating and incurable (Smyth, Grosser et al., 2020; Lezi and Swerdlow, 2012; CanuSarasso et al., 2018; JT O'Brien et al., 2017; Bray, 2020; Elouzi, Vedam-Mai et al., 2019).

The above observations, coupled with the finding that mouse knockouts of SMYD1 paralogs SMYD4 and SMYD5 each resulted in significant behavioral phenotypes (GiauSenanarong et al., 2019), led us to hypothesize that SMYD1 and skNAC function together, not only in hematopoietic and cardiovascular/skeletal tissues, but in neuronal cells as regulators of genes disrupted in AD, PD and HD. This hypothesis was strengthened by our observation that SMYD1 and skNAC are expressed strongly within the cortical striata of human brains, weakly in the cortical striata of mouse brains and within the cortical striata of transgenic models of each of the inflammatory diseases noted above. We show that both SMYD1 and skNAC are expressed in C2C12 myocytes induced to differentiate to neurons, but only SMYD1 is essential for this process. Finally, either together or individually, SMYD1 and skNAC regulate several defining components of neurodegeneration, and particularly Alzheimer’s pathology, including the brain-specific, axon-enriched microtubule-associated protein, Tau.

These findings open heretofore unanticipated potential for SMYD1 and skNAC function in neurodegenerative disorders that in 2019 exceeded $290 billion in treatment and hospitalization (Alzheimer's Association Report, 2019).

2. Materials and methods

2.1. Mice and animal husbandry

Mice were bred and housed in the pathogen-free animal facility of the University of Texas. All experiments received approval from the Institutional Animal Care and Use Committees (Protocol ID AUP-2012-00169). Approximately 3 week old transgenic (TG) mouse models were purchased from the Jackson Laboratory. Further details are provided in S-Methods.

2.2. Cell culture and production of stable cell lines

We employed C2C12 (purchased from ATCC). Phoenix A cells were the kind gift of Dr. Gary Nolan. Production of recombinant retroviral constructs and infection of cell lines was performed as detailed in S-Methods and as described at http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html. Briefly, Phoenix A cells were plated and then transfected with retroviral construct DNA using Fugene 6 reagent (Roche). Approximately 48 h post-transfection, supernatants were selected with 3 μg/ml puromycin and split at 80% confluency.

2.3. Mammalian expression

Vectors pBK-CMV-SMYD1A, pBK-CMV-SMYD1B, pBKCMV-SMYD1C and pBK-CMV-SMYD1B-YND-mutant were described previously (Rasmussen et al., 2015; Rasmussen and Tucker, 2018). pSilencer5.1-U6-SMYD1 (shRNA), pSilencer5.1-U6-skNAC (shRNA) and pSilencer5.1-U6-Scrable (shRNA) were generated with software (shRNA selector) available at http://hydra1.wistar.upenn.edu/Projects/shRNA/shRNAindex.htm). Transient transfections were performed with FuGENE 6 reagent according to the manufacturer’s instructions.

2.4. Antibodies, western blotting and immunohistochemistry (IHC)

Anti-SMYD1 3h2a monoclonal antibody (mAb) was described previously (Sims et al., 2002). Rabbit anti-skNAC polyclonal antibody (UT143) was generated as detailed in S-Methods by Cocalico Biologicals. Anti-FLAG M2 mAb is from Sigma (cat #F3165); anti-Acetylcholinesterase (CAT) biotinylated monoclonal (HR2) and anti-Neuron-Specific beta-III Tubulin (NST) biotinylated monoclonal Ab (Catalog # BAM1195) were each purchased from Invitrogen (Catalog # MA3-042) Filamentous actin (F-actin) was stained with FITC-conjugated phalloidin (Sigma-Aldrich; St. Louis, MO). Nuclei were labeled with 4’-6-diamidino-2-phenylindole (DAPI). For immunoprecipitation, we employed protein-A immobilized on Sepharose CL-4B (cat #P3391) as detailed in S-Methods.

Our Western blotting procedure, described previously (Rasmussen et al., 2015; Rasmussen and Tucker, 2018), was performed on 12.5% SDS-PAGE with the above mentioned commercial and home-generated Abs as detailed in S-Methods. For IHC, mice were anesthetized, perfused intracardially with ice cold HBSS and drop fixed overnight in 4% paraformaldehyde (PFA). Brain sections (25μm) were collected by cryostat, mounted and developed with antibodies noted above as detailed in S-Methods.

2.5. Retroviral shRNA silencing

pSilencer5.1-U6-SMYD1 (shRNA), pSilencer5.1-U6-skNAC (shRNA) and pSilencer5.1-U6-Scrable (shRNA) were generated as follows: The shRNA target sequences were selected by employing siRNA selector (http://hydra1.wistar.upenn.edu/Projects/shRNA/shRNAindex.htm). Design Tool (Ambion) was used to convert the target sequences into hairpin shRNA-encoding DNA oligonucleotide sequences. These oligonucleotide sequences were annealed and ligated into pSilencer 5.1 Retrovector (Ambion).

2.6. Microarray analysis

C2C12 myoblasts or skNAC-shRNA KD-transduced C2C12 myoblasts were induced for 3 days in culture via serum withdrawal to myoblasts as described previously (Sims et al., 2002). Total RNA was purified with TRizol reagent (Life Technologies, Inc. Rockville, MD) and hybridized to DNA-spotted arrays (printed in house as described in: Jing and Zon, 2011; Paik and Zon, 2010). Details of microarray, hybridization, scanning, creation of image files and analyses are provided in S-Methods. Differentially expressed genes (nominal p < 0.05) of SMYD1 or skNAC vs. their respective WTs were determined and their datasets were analyzed using the Ingenuity Pathway Analysis (IPA) ‘Core Analysis’ utility (Chen et al., 2013; Kuleshov et al., 2016; Meeson et al., 2001) and displayed as volcano plots as detailed in S-Methods.

2.7. Conversion of myoblasts to neurons

C2C12 cells were converted to neurons via a method described in detail in S-Methods. Briefly C2C12 myocytes were converted to myoblasts as previously described (Yotov and St-Arnaud, 1996), incubated for 3 days in a media containing 3 mm Neuodazine (Nz; LGC Standards, IRC80247). The media was replaced every 2 days with 4 mm Nz prior to their harvest and analysis at day 6.

2.8. Purification of mouse subcortical regions

Mice were anesthetized, perfused intracardially with HBSS and drop-fixed overnight in 4% paraformaldehyde (PFA). Brain sections (25μm) were collected using a cryostat, mounted onto charged slides and then stored at −80°C until use. Brain sections were rehydrated in PBS, blocked for 1 h at RT in 10% calf serum, 0.2% Triton X-100, in 1 × PBS prior to further analyses.

2.9. Dendritic tree reconstruction

Briefly, C2C12 neurons were sliced into 10 mm thick blocks and stained with a Fd Rapid GolgiStain™ kit (FD NeuroTechnologies, Ellicott City, MD, USA). The blocks were sectioned using a cryomicrotome.
transcripts for SMYD1 loss and 1209 significant replicates yielded 1672 significantly robust. Differential expression (DE) analysis performed in two independent (summary statistics and transcripts listed in S-Tables 1 and 2). DE genes

2.10. RT-qPCR and endpoint PCR

RT-qPCR was conducted as previously described (Hsia and Zon, 2005) and detailed in S-Methods. Briefly, total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen, Santa Clara, CA), cDNA synthesized with qScript cDNA supermix (Quanta), and RT-qPCRs were performed using PerfeCTa SYBR Green FastMix (Quanta) with 1 μl of 20X-diluted cDNA generated from 500 ng of total RNA. RT-qPCR primers, designed to amplify the junction between two exons, are listed in S-Fig. 2. CT values were normalized against Gapdh. The normalized level of mRNA was determined as 2−Δ(ΔCt) where Ct is the threshold cycle, ΔCt is the input of interest, and CT is the housekeeping control (assuming that Ct is inversely proportional to the initial concentration of mRNA and that the amount of product doubles with every cycle). PCR products were analyzed via electrophoresis over agarose or SDS-PAGE. Primers designed using Primer 3 software for SMYD1: 5′-GTAAGAACGGGACAGGACGCT-3′; 5′-CTCCTTCACCACCTTCGTGAG-3′ and for skNAC: 5′-ATTCCACG-CAGGCAAACACACA-3′; 5′-TGTAACCTGCAGGAGACCCAGT.

3. Results

3.1. Determination of SMYD1 and skNAC transcriptional targets in myoblasts

We employed global gene expression analyses to determine SMYD1 and skNAC transcriptional targets in C2C12 myocytes following their differentiation to myoblasts. We employed shRNA-mediated knockdown (KD) of SMYD1 or skNAC. Their KD efficiencies, as shown in S-Fig. 1, were robust. Differential expression (DE) analysis performed in two independent replicates yielded 1672 significantly (nominal p < 0.05) altered transcripts for SMYD1 loss and 1209 significantly (nominal p < 0.05) altered transcripts for skNAC KD with respect to wildtype (WT) controls (summary statistics and transcripts listed in S-Tables 1 and 2). DE genes were submitted to Ingenuity Pathway Analysis (IPA) (Chen et al., 2015) to identify molecular pathways. To identify network, we overlaid and merged SMYD1 and skNAC KOs via the “fold-change” (FC) algorithm as logFC expression values (Chen et al., 2013; Kuleshov et al., 2016).

Using the Ingenuity Knowledge Base algorithm (Chen et al., 2013), association between SMYD1 and skNAC networks were identified with the IPA “Grow” tool (Chen et al., 2013), in which a maximum of 20 target molecules for each factor were connected to targets within their respective networks. We employed default settings (Chen et al., 2013; Kuleshov et al., 2016) for all IPA pathway functions. We then performed KEGG pathway analyses (KanehisaSato et al., 2016) employing Enrichr™ (Kuleshov et al., 2016)—a comprehensive resource for curated gene sets and a search engine that accumulates biological knowledge for further discovery.

3.2. SMYD1 and skNAC loss deregulates factors involved in heart and skeletal muscle development, cardiac muscle contraction, inflammatory responses and TCA cycle

We first analyzed differential gene expression and as represented in volcano plots (Fig. 1). Genes deregulated by SMYD1 KD (left panel) are shown with nominal p values < 0.004 (blue dots) and log2-fold changes >1.3 (green dots). Genes deregulated by skNAC KD (right panel) are shown with nominal p values < 0.003 (blue dots) and log2-fold changes >1.4 (green dots). Genes that pass both the cutoff for log2FC and p value are shown as red dots, whereas genes that did not reach these criteria are indicated in gray. S-Fig. 2B, displays the IPA network association between SMYD1, MB, and skNAC (in blue). The input data included nominally significant genes (p < 0.05) for SMYD1 or skNAC KD vs. C2C12 myoblast WT.

3.3. SMYD1 and skNAC regulate neurogenic factors implicated in neurodegenerative diseases

We observed numerous KEGG pathways significantly deregulated, including heart/skeletal muscle development and cardiac muscle contraction (Table 1, S-Fig. 2 and data not shown). This was not unexpected, given that SMYD1 regulates skNAC, which, in turn, regulates MB (C. Li et al., manuscript under submission); MB, in turn, supplies ferrous iron (haem) to numerous pathways within cardiac and skeletal muscles of all vertebrates (Meeson et al., 2001).

However, totally unexpected was our finding that loss of SMYD and skNAC led to significant deregulation of genes typically associated with neurodegenerative diseases. Foremost of the SMYD1 GO pathways was Alzheimer’s Disease (AD), the most common cause of dementia (p < 1.48E-11, Table 1) (Smyth, Grosser et al., 2020; Elkouzi, Vedam-Mai et al., 2019). Loss of SMYD1 also significantly deregulated a number of genes typically expressed in Parkinson’s (p < 1.37E-9) and in Huntington’s (p < 1.41E-7). Diseases. Loss of skNAC also deregulated each of these pathways, but to a more modest extent than SMYD1: Alzheimer’s (p = 2.93E-6), Huntington’s (p ≤ 3.29–4) and Parkinson’s (p ≤ 1.37E-3) (Table 1).

As shown in Table 1 and the Volcano plot of Fig. 2, transcripts common to each of the neurodegenerative diseases included Cytochrome c oxidase (COX) isoforms, involved in the conversion of arachidonic acid to the prostaglandin precursor of prostaglandins (Smyth, Grosser et al., 2020); NDUFA isoforms, which function in a complex with the mitochondrial electron transport chain downstream of COX, which is deregulated in AD, PD, and HD (Lezi and Swerdlow, 2012); and ATPase isoforms which drive the electron transport chain and have been associated with Aβ plaque burden in Alzheimer’s (Holper et al., 2019).

These and other deregulated neurodegenerative transcripts are readdressed in detail below.

3.4. SMYD1 and skNAC accumulate in human brains within a region implicated in neurodegenerative diseases

The above results were unsuspected since all previous publications had detected SMYD1 and skNAC exclusively in developing or mature cardiomyocytes, endothelial cells, skeletal muscle myoblasts and myocytes (Yahalom et al., 2018; Tracy et al., 2018; Franklin et al., 2016; Murayama et al., 2015; Rasmussen et al., 2015; Rasmussen and Tucker, 2018; Fujiji et al., 2016; Hsia and Zon, 2005; Tan et al., 2006; Jing and Zon, 2011; Faik and Zon, 2010; Ye et al., 2016), or in the case of SMYD1C, CD8 T cells (Nie et al., 2017).

We searched the literature, and while we found no evidence for SMYD1 and/or skNAC in the mouse brain, we did find evidence for both in the human brain (Holper et al., 2019; Lein et al., 2007; Oh et al., 2014; Miller et al., 2017; Hawrylycz et al., 2012). Fig. 2A and B displays our meta-analyses of samples of four normal human brains in which both SMYD1 and skNAC were identified by in situ RNA hybridization (in red) within the cerebral cortex. skNAC is expressed more nearly overlapping fashion, within the subcortex of normal human donors (Fig. 2A and B). The subcortex constitutes a group of diverse neural formations deep within the brain, which include the thalamus, the hypothalamus and the striatum (Uddin and Ghulam, 2018). As shown in
Transcripts highlighted in green are down-regulated and in red are up-regulated based on the overlaid logFC values carrying a negative value or a positive value, association between SMYD1, MB, and skNAC labeled in blue. The input data included nominally signi...and downstream relationships. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In early disease staging, detection of markers of AD extend randomly throughout the brain; ie, degeneration follows a predictable, nonrandom pattern (Hawrylycz et al., 2012; Arendt et al., 2018). The initial sites of degeneration are ill-defined. While controversial (Keller et al., 2018; Arendt et al., 2018), the prevailing concept is that the disease originates within subcortical regions such as locus coeruleus or nucleus basalis of Meynert (reviewed in (Arendt et al., 2018), (GuptaLee et al., 2019)). The hallmark of HD is massive loss/disruption of the medium spiny neurons of the striatum. Early and diffuse cortical and subcortical neurodegeneration underlies the pathology (reviewed in Myers and McGonigle, 2019). PD patients show distributed alteration of temporal variability. However, the variability showing the highest correlation with clinical score is associated with the subcortical network reviewed in Jankowsky et al., 2004.

3.5. Mouse models of neuroinflammatory diseases

The above transcriptional and localization data encouraged us to analyze SMYD1 and skNAC under conditions of ongoing neuroinflammatory disease. As a first step toward this end, we selected the best available transgenic (TG) mouse models for AD, HD and PD.

Several models of AD are available (reviewed in 46). Most incorporate mutations within amyloid precursor protein (APP) and/or presenilin (PSEN). We elected to assess APP/PS1 double Tg mice (Pickrell et al., 2015) (this and other mutant strains detailed in Materials and Methods) which express chimeric mouse/human APP protein (Mo/HuAPP695Swe) and a mutant human PSEN (PS1-dE9) (Konnova et al., 2018). Both genes are directed to CNS neurons. Both mutations are associated with early-onset Alzheimer’s disease.

There are several categories of TG mice that carry the distinguishing feature of HD—CAG repeats of varying length within the mouse Huntingtin (Htt) genomic locus (Goldberg et al., 2003). Preferred are Knock-In (KI) models, in which the HD mutation is replicated by directly engineering CAG repeats of varying length. Toward this end, we employed the xQ175 KI allele in which the human Htt exon 1 carrying an ~190 amino acid CAG repeat tract replaces the murine Htt exon 1 (Brüggemann et al., 2013). Numerous studies of HD disease pathogenesis and assessment of potential therapeutic interventions have employed these KI mice (reviewed in 46).

While an ultimate mouse model that address all Parkinson’s-related questions is yet to be developed, a number of existing models are useful in answering specific questions (reviewed in 46). The PARK loci, recently identified by GWAS, are highly favored, and we chose one—the homozygous Parkin (Park2tm1Shn) KO (Konnova et al., 2018). Parkin mice harbor mutation of the exon most commonly observed in human autosomal recessive juvenile Parkinsonism patients (Brüggemann et al., 2013) and have been employed broadly in neurobiological research (Maiti et al., 2018).

3.6. The SMYD1-skNAC complex accumulates within the subcortex of normal and neuroinflammatory transgenic mouse models

We obtained, bred and analyzed each of the TG mouse models discussed above. At ~6 weeks of age, we isolated the subcortical layers of TG and WT littermate control brains to determine if, as in the human, SMYD1 and skNAC accumulate. If so, do they form heterodimeric complexes as they do in heart and skeletal muscle (Sims et al., 2002; Ravat et al., 2012).

Briefly, mice models of Alzheimer’s Disease (TG-AD), Huntington’s Disease (TG-HD) and Parkinson’s Disease (TG-PD) as well as WT controls were transcardially perfused using ice cold Hank’s balanced salt solution (HBSS) and the brains were minced. Subcortical enrichment was achieved using a validated protocol (Kim et al., 2016a) of subsequent enzymatic dissociation, density gradient separation, and magnetic bead sorting as...
| Term                        | Enrichr path analysis: KEGG SMYD1 KD | Term                        | Enrichr path analysis: KEGG skNAC KD |
|-----------------------------|----------------------------------------|-----------------------------|----------------------------------------|
| Alzheimer disease           | 38/175, 1.48E-11, 3.98E-09, -1.16, 28.92 | Parkinson disease           | 31/144, 1.37E-09, 9.23E-08, -1.32, 26.85 |
| Thermogenesis               | 44/231, 3.94E-11, 5.30E-09, -1.29, 30.89 | Huntington disease          | 33/192, 1.41E-07, 7.57E-06, -1.13, 17.83 |
| Oxidative phosphorylation   | 30/134, 9.34E-10, 8.38E-08, -1.23, 25.56 | Cardiac muscle contraction  | 19/78, 2.71E-07, 1.22E-05, -2.20, 33.22 |
| Non-alcoholic fatty liver   |                                        |                             |                                        |
| disease (NAFLD)             |                                        |                             |                                        |
| Citrate cycle (TCA cycle)   | 10/32, 1.77E-05, 5.94E-04, -2.46, 26.94 | Adrenergic signaling in      | 20/148, 1.06E-03, 2.95E-02, -1.44, 9.86 |
| cardiomycocytes             |                                        |                             |                                        |
| Ribosome                    | 22/170, 1.10E-03, 2.95E-02, -1.09, 7.42 |                             |                                        |

**Table 1**

KEGG pathway analyses identify the progression of Alzheimer disease and Parkinson disease. The pathway analysis showed that Alzheimer disease is significantly enriched in the pathway Arthritis and the pathway Oxidative phosphorylation. Parkinson disease is significantly enriched in the pathway Oxidative phosphorylation and the pathway Oxidative phosphorylation.

| Term                        | Enrichr path analysis: KEGG skNAC KD |
|-----------------------------|----------------------------------------|
| Alzheimer disease           | 24/175, 2.93E-06, 7.87E-04, -1.16, 14.78 |
| Insulin resistance          | 15/110, 2.18E-04, 2.21E-02, -1.74, 14.65 |
| Non-alcoholic fatty liver   | 18/151, 3.02E-04, 2.21E-02, -1.41, 11.40 |
| Huntington disease          | 21/192, 3.29E-04, 2.21E-02, -1.14, 9.15 |
| Cardiac muscle contraction  | 11/78, 1.10E-03, 3.65E-02, -2.17, 14.77 |
| Hedgehog signaling pathway  | 8/44, 9.72E-04, 3.65E-02, -2.05, 14.22 |
| Protein processing in        | 18/163, 7.61E-4, 3.65E-02, -1.51, 10.82 |
| endoplasmic reticulum        |                                        |                             |                                        |
| Ubiquitin mediated          | 16/138, 8.67E-04, 3.65E-02, -1.36, 9.59 |
| proteolysis                 |                                        |                             |                                        |
| Parkinson disease           | 16/144, 1.37E-03, 3.65E-02, -1.25, 8.24 |
| Ribosome                    | 18/170, 1.24E-03, 3.65E-02, -1.10, 7.38 |
| Human T-cell leukemia virus  | 23/245, 1.49E-03, 3.65E-02, -0.82, 5.36 |
| 1 infection                 |                                        |                             |                                        |

**Enrichr pathway analysis:**

- **KEGG SMYD1 KD**
  - Alzheimer disease
  - Thermogenesis
  - Oxidative phosphorylation
  - Huntington disease
  - Cardiac muscle contraction
  - Non-alcoholic fatty liver disease (NAFLD)
- **KEGG skNAC KD**
  - Alzheimer disease
  - Insulin resistance
  - Non-alcoholic fatty liver disease (NAFLD)
  - Huntington disease
  - Cardiac muscle contraction
  - Hedgehog signaling pathway
  - Protein processing in endoplasmic reticulum
  - Ubiquitin mediated proteolysis
  - Parkinson disease
  - Ribosome
  - Human T-cell leukemia virus 1 infection
detailed in Materials and Methods. Enriched protein fractions were fractionated on SDS-PAGE and subjected to anti-SMYD1 Western and anti-skNAC co-immunoprecipitation (co-IP) analyses.

As shown in Fig. 3A and B, we confirmed expression of SMYD1 and skNAC in the mouse cortex of each of the three TG subcortices (TG-AD, TG-PD and TG-HD). We also confirmed low, and previously undetected, cortical transcripts in WT littermates of each of the above (Fig. 3C). Co-IPs of SMYD1 from subcortical lysates, following by probing for interaction with skNAC (Fig. 3D) revealed that SMYD1 and skNAC interact to varying extents within each. Reverse Co-IPs, employing anti-skNAC pulldowns probed with anti-SMYD1 Westerns blotting confirmed the interaction was reciprocal (Fig. 3E).

These data provided confidence that pursuit of additional, long-term studies in these mice models would be informative.

3.7. Differentiation of C2C12 myocytes into neurons

Our knockdown/gene array results summarized in Table 1 strongly implicated SMYD1 and skNAC in neuroinflammatory diseases. This was particularly unexpected, as our results were obtained from 3 day-
stimulated C2C12 myoblasts—not neurons. However, C2C12 myocytes are competent to differentiate into a number of cell lineages, including chondrocytes and osteoblasts, via addition of BMP4 (Chen et al., 2003), and into tenocytes, via addition of Myostatin/Chlorpromazine (MZ) (Ker et al., 2011; Uemura et al., 2017; Watanabe et al., 2004; Riazi et al., 2005). Notably in the present context, MZ, a member of the imidazole-based small molecule family, also was shown to promote neurogenesis in pluripotent stem cells (Kim et al., 2014).

Of the various conditions optimized for C2C12 cell neural differentiation (Chen et al., 2003; Ker et al., 2011; Uemura et al., 2017; Watanabe et al., 2004; Riazi et al., 2005; Kim et al., 2014), we chose successive incubation with MZ followed by incubation with Neudozine (NZ), an antipsychotic medication primarily used to treat psychotic disorders such as schizophrenia (López-Munoz et al., 2005).

Briefly, C2C12 myocytes, following 3 days of differentiation to myoblasts by serum withdrawal, were initially treated with MZ, which binds to microtubules and inhibits their conversion to angiogenesis by myoblasts by serum withdrawal, were initially treated with MZ, which binds to microtubules and inhibits their conversion to other targets of Table 1 and Fig. 1. Regardless, the data of Fig. 4 promoting mononucleates (Uemura et al., 2017; Watanabe et al., 2004). Note that even at this late myoblast differentiation stage, we began to detect what appeared to be dendritic-like cell body extensions (arrows in Fig. 4C, D). C2C12 mononucleates were then incubated with NZ for 6 more days. As shown in Fig. 4E–H, the NZ-treated mononucleotides converted to a significant degree into neurogenic cells, as determined by phenotype and neuronal-specific staining (Materials and Methods). We observed that MZ/NZ treatment also converted satellite cells, obtained from mouse single muscle fibers, to neurons (data not shown).

We suspect that C2C12 myoblasts spontaneously underwent neural differentiation to a level sufficient to identify the neuroinflammatory-restricted targets of Table 1 and Fig. 1. Regardless, the data of Fig. 4

![Fig. 4. Neurogenesis of C2C12 skeletal muscle myocytes. C2C12 myocytes (top row) were programmed to myoblasts (center row) via serum withdrawal/ media change as previously described for 3 days (Sims et al., 2002). Myoblasts were treated with Myoxygen (MZ) for 1 day and then with Chlorpromazine (Neurazine, NZ) for 6 days as indicated by the schematic and detailed in Materials and Methods. Cell types were identified by morphology and by staining with FITS-phallolidin to identify F-Actin (F-Ac, green) or with neuronal antibodies (red) to identify neuron-specific tubulin (NST) and choline acetyltransferase (CAT) positive neurons. Scale bars = 50 mm (yellow). Shown are two representative images (Experiment 1 and Experiment 2) representative of 5 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image1)

3.8. SMYD1, but not skNAC is required for C2C12 neuron reprogramming

We employed SMYD1- and skNAC sh-RNAs to determine if either SMYD1, skNAC or both were essential for reprogramming C2C12 myoblasts to neurons. C2C12 myocytes were infected with either SMYD1- or skNAC-shRNA, cultured 3 days to form myoblasts, and then cultured with MZ and NZ under the neurogenesis protocol described above and in Materials and Methods.

As shown in Fig. 5A, we detected a clear reduction in acquisition of neuronal phenotype following SMYD1-shRNA KD, but no observable change was observed following skNAC KD relative to mock-transduced C2C12 controls.

To quantify the reduction in SMYD1- and skNAC-silenced cells, we employed three-dimensional reconstructions using Neurolucida software (Peng et al., 2015) and detailed in S-Methods) of each mouse reprogrammed neuron that displayed the parameters of somato-dendritic morphology. Shown in Fig. 5B are typical examples calculated from a minimum of 180 dendrites imaged. The majority of the control and skNAC-depleted spiny neurons (Fig. 5Da, c) had medium sized soma and signifi cant dendrites. In contrast, SMYD1 KD neurons (Fig. 5Bb) had signifi cantly smaller soma and dendrites (P = 0.01; indicated by **). Scale bars (yellow) = 50 mm. Results and statistics calculated from 5 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
spine-laden dendrites. Their density increased gradually towards distal segments, with some showing numerous collateral branches. However, in SMYD1 sh-RNA treated neurons (Fig. 5Db), the somato-dendritic pattern, significantly lower density in relation to their size of their cell bodies. A statistically significant difference (p ≤ 0.01) between scrambled control and SMYD KD was determined by one-way analysis of variance (Uylings et al., 1989).

These data indicated that SMYD1 is the rate-limiting factor—at least in this in culture format of myoblasts to neuronal differentiation.

3.9. Transcripts associated with neuroinflammatory diseases are deregulated by SMYD1 and/or skNAC loss in reprogrammed C2C12 neurons

We preformed RT-qPCR of RNA isolated from reprogrammed skNAC-shRNA or SMYD1-shRNA transduced C2C12 neurons in an attempt to confirm and quantify expression of selective neurogenerative transcripts identified by expression arrays (see Fig. 1). We analyzed the data according to either singular loss of SMYD1 or skNAC as well as dual loss of both. Scrambled sequences of SMYD1 or skNAC sh-RNAs served as controls (CNTR); oligonucleotide primers employed for RT-qPCR are listed in Table 2.

As shown in Fig. 6A, SMYD1 KD of transcripts determined in Table 1 detected 13 unique targets with 9 of these confirmed by RT-qPCR (Fig. 6A). skNAC KD yielded 7 unique targets with 4 confirmed (Fig. 6B, left panel). Note that the magnitude of fold change mediated by skNAC KD was almost a log lower than that achieved by SMYD1 KD, with the exception of ADAM17 (6 B, right panel). These data support the observations of Fig. 5 and further suggested that the dominant factor in the system is SMYD1.

There were 7 transcripts with 5 statistically deregulated by both SMYD1 and skNAC (Fig. 6C). In Fig. 6D, we observed 5 deregulated transcripts which are particularly relevant to AD. As anticipated from the data of Table 1, NDUF, COX and ATPase transcripts dominated each of the C2C12 neuronal categories. The large number of transcripts deregulated by SMYD1 and skNAC suggest that, while SMYD1 may rate limiting, SMYD1 and skNAC might be functioning as heterodimers, at least in most of the cases. The results are discussed below in the context of neuroinflammation and are illuminated in the AD pathway of S-Fig. 4.

Table 2

| GENE | OLIGONUCLEOTIDE PRIMERS | ACCESSION # |
|------|-------------------------|-------------|
| SKMYD1 KD | | |
| NDUF56 | GGTGAGCTGGTGAATGAGTTGG | NM_001166102 |
| COX8A | GCCGAGGCCGGAGGCGGCGGTCG | NM_001038609 |
| NDUF52 | CCAATTCGCGCACAGTGGA | NM_001289726 |
| APAP1 | GCCGAGGCCGGAGGCGGCGGTCG | NM_001347092 |
| ADUFV1 | CTGTAGTGGACTGATGTTGGTGA | NM_00135998 |
| skNAC KD | | |
| NDUF56 | GGTGAGCTGGTGAATGAGTTGG | NM_00142556 |
| COX8A | GCCGAGGCCGGAGGCGGCGGTCG | NM_001081304 |
| NDUF52 | CCAATTCGCGCACAGTGGA | NM_001001975 |
| APAP1 | GCCGAGGCCGGAGGCGGCGGTCG | NM_001166102 |
| ADUFV1 | CTGTAGTGGACTGATGTTGGTGA | NM_001291005 |
| Neuroinflammation | | |
| ATPF | AGAGAAGGAGTGAGTGGTGG | NM_00142556 |
| COX7A2 | AGAGAAGGAGTGAGTGGTGG | NM_001347092 |
| ATP88PK | AGAGAAGGAGTGAGTGGTGG | NM_001166102 |
| KCHIP3 | AGAGAAGGAGTGAGTGGTGG | NM_001291005 |
| Controls | | |
| GAPDH | AGAGAAGGAGTGAGTGGTGG | NM_001038609 |
| ACTB | AGAGAAGGAGTGAGTGGTGG | NM_001289726 |
3.10. SMYD1 and/or skNAC regulate factors central to neuroinflammation including Tau

Foremost among this group is Microtubule-Associated Protein Tau (MAPT). Tau/MAPT transcripts undergo complex and highly regulated alternative splicing. This leads to their differential expression in the nervous system, depending on stage of neuronal maturation and neuron type (Strang et al., 2019; Caillot-Boudin et al., 2015). Mapt mutations are associated with several neurodegenerative disorders, particularly Alzheimer’s (Smyth, Grosser et al., 2020; Lezi and Swerdlow, 2012; Canu-Sarasso et al., 2018; JT O’Brien et al., 2017; Bray, 2020; Elkouzi, Vedam-Mai et al., 2019). While the normal function of Tau is to organize microtubules, in Alzheimer’s Tau collapses into tangled aggregates termed “Tau’s tangles”. Brain alterations result via a complex interplay among abnormal Tau, beta-amyloid proteins and several other factors (Smyth, Grosser et al., 2020; Lezi and Swerdlow, 2012; Canu-Sarasso et al., 2018; JT O’Brien et al., 2017; Bray, 2020; Elkouzi, Vedam-Mai et al., 2019).

The consequence of Tau neurofibrillary tangles leads to “senile plaques” composed of extracellular deposits of aggregated Amyloid-beta protein (A-beta)—the proposed causative agent of AD (Strang et al., 2019; Caillot-Boudin et al., 2015). A-beta is enzymatically processed to Amyloid Precursor Protein (APP) primarily via several alpha-secretases, which generates A-beta as well as gamma secretases also required to generate APP (Li et al., 2019). ATP6 encodes a transcription factor (repressed by SMYD1 and skNAC; Fig. 6D) that activates target genes required for the unfolded protein response during endoplasmic reticulum (ER) stress (López-Hurtado et al., 2018). Immunochemistry studies revealed that neurons in postmortem brain samples of AD patients display prominent expression of markers of ER stress (López-Hurtado et al., 2018). ATP6 also is deregulated in HD and PD (Colla, 2019; Talya et al., 2019). Dual SMYD1 + skNAC downregulation of KCHIP3 is particularly interesting in this regard. KCHIP3/DREAM is part of an endogenous neuroprotective mechanism that interacts with and accelerates ATP6 processing as well as neuronal survival in the striatum of R6/2 mice (Li et al., 2019)—a model of HD (Konnova et al., 2018).

3.11. Key aging factors underlying neurodegeneration are deregulated by SMYD1 and/or skNAC loss

Aging is the strongest risk factor for neurodegenerative diseases including (reviewed in Thies and Bleiler, 2011). Aging is accompanied by increased mitochondrial reactive oxygen species whose dysregulation damages mtDNA and/or mitochondrial components (Thies and Bleiler, 2011). Numerous mechanisms have implicated mitochondria in aging and in multiple neurodegenerative diseases, including AD, PD, and HD (Lezi and Swerdlow, 2012; Colla, 2019; Talya et al., 2019; Thies and Bleiler, 2011).

Most of the ATP of a cell is produced through oxidative phosphorylation (OXPHOS) in mitochondria driven by the electron transport chain composed of 4 respiratory complexes (CX1, CX2, CX3, and CX4) (reviewed in Berg et al., 2002). SMYD1 and skNAC loss deregulate primarily component polypeptides within CX-I (NDUFA11, NDUFA10 and
ATPase) and CX-IV (COX7B, COX5A, ATPD5 and COX7A2) (Fig. 6A–D, S-Fig. 4). Deregulation of both of these enzyme complexes have been implicated in AD, PD and HD (Canu-Sarasso et al., 2018; JT O’Brien et al., 2017; Bray, 2020; Elkozi-Vedam-Mai et al., 2019; GiauSenanarong et al., 2019; Berg et al., 2002). Previous studies also have correlated disruption of CX-I and IV with Aβ plaque burden in the hippocampi of AD TF mice (Goldberg et al., 2003; Pickrell et al., 2015).

Mitochondrial dysfunction, because of its critical role in energy production and cellular metabolism, is a strongly implicated susceptibility factor for AD, PD and HD (Lezi and Swerdlow, 2012; Canu, Sarasso et al., 2018). SMYD1 and skNAC modulated expression of a family of Cyclo-oxygenase (COX) isoforms (Fig. 6C, S-Fig. 4), which are required both for the formation of prostanooids (including prostaglandins) (Smyth, Grosser et al., 2028) as well as for modulation of NDUFA isoforms—a complex within the mitochondrial electron transfer chain (Lezi and Swerdlow, 2012). UQCRCC2/CX3, also repressed by both SMYD1 and skNAC, localizes to the mitochondrion, where as part of the ubiquinol-cytochrome c reductase complex (CX-III), it is required for mitochondrial OXPHOS (Berg et al., 2002). UQCRCC2 and additional components of CX-III are down-regulated in early onset AD as well as in HD and PD (Adav et al., 2019).

Finally, oxidative phosphorylation and glycolysis are metabolically linked, as pyruvate is transferred to the mitochondria to produce energy (Calsolaro and Edison, 2016). Several molecular markers of glycometabolism, including the SMYD1 and skNAC target, ATP5D (Fig. 6C, S-Fig. 4), are downregulated in AD and other neuroinflammatory diseases (Ding et al., 2014; Spremkle et al., 2017).

4. Discussion

Synaptic dysfunction in Alzheimer’s, Parkinson’s and Huntington’s disease is largely caused by failed protein homeostasis, because defective, unfolded proteins accumulate pathogenic protein aggregates at synapses (Herm and Dorostkar, 2016). Gene deregulation underlying several transcriptional consequences have been observed in the present analyses of two, previously unanticipated players—SMYD1 and skNAC. While the data presented here must be considered preliminary, they strongly suggest that the use of reprogrammed C2C12 neurons can be informative in deducing/confirming proteins downstream of SMYD1 and skNAC involved in neuroinflammation. We currently are utilizing both the neuroinflammatory mouse models of Fig. 3, as well as diseased human brain tissues from the Alzheimer’s Disease Center at University of Texas Southwestern Medical Center to carry out direct tests of the hypotheses generated in this report.

Histone methylation generally is associated with transcriptional repression (Tracy et al., 2018). SMYD1 has been shown to catalyze tri-methylation of histones H3K9m3, H3K9m1 and potentially additional, but as yet uncharacterized, histone methylation marks (Tracy et al., 2018; Paik and Zon, 2010). There are two exceptions: Tribbles3 and skNAC. When directly methylated by SMYD1, Tribbles3 is activated to act as a co-repressor of SMYD1-mediated transcription during oxidative stress (Nie et al., 2017). We also showed recently that SMYD1 methylation of skNAC is required for its full transcriptional activation of Myoglobin (C. Li et al. submitted). In this report, we observed that only 5 of 28 SMYD1 or SMYD1/skNAC dual targets were activated. This strongly indicated that the repressive role of histone or non-histone modification, as well as the more conventional transcriptional repression documented for both SMYD1 and skNAC (Yahalom et al., 2018; Tracy et al., 2018; Franklin et al., 2016; Murayama et al., 2015; Rasmussen et al., 2015; Rasmussen and Tucker, 2018; Fujii et al., 2016; Hsia and Zon, 2005), are essential features for future exploration. To comprehensively address this issue, we plan to carry out global transcriptional analyses, including Chip-seq of both human and mouse genomes following SMYD1 and skNAC loss, using approaches we have performed previously for a number of TFs (Dekker et al., 2016, 2019; Kim et al., 2016b; Ippolito et al., 2014). We also plan to determine the methylation status catalyzed by SMYD1 HMTase activity that may supplement the transcriptional regulation determined here using methods we have employed previously (Rhee et al., 2014; An et al., 2010). Results of these experiments will be critical in providing a full mechanistic account of the role of SMYD1 and skNAC in neuroinflammatory disease.

Finally, we submit that ultimately the data here, coupled with the above approaches, may render SMYD1, or a small molecule mimic, an excellent clinical biomarker of neurodegenerative diseases.

Author contributions

RDM and HOT designed research; LZ, TAS, and GRT performed research; RDM, LZ, RDM, GRT and HOT analyzed data; RDM and HOT wrote the manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

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