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LETTER TO THE EDITOR

The role of ZKSCAN3 in the transcriptional regulation of autophagy

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The transcriptional repressor ZKSCAN3 has been reported to be an important regulator of genes involved in autophagosome formation and lysosomal biogenesis. These results have been based on acute knockdown studies in cultured cells. Here, we have generated and analyzed a mouse model lacking Zkscan3 expression. In vivo, we do not find evidence that this factor is an important regulator of autophagic or lysosomal gene expression.

Because enucleated cells appear to be fully capable of activating macroautophagy/autophagy,[1] the transcriptional regulation of this process has often been ignored or underappreciated.[2] Nonetheless, a growing number of transcription factors appear to modulate the expression of various autophagic components.[2] Most notably, the transcription factor TFEB activates a broad range of genes associated with autophagosome formation and lysosomal biology.[3] In contrast, it has been suggested that ZKSCAN3 can act in an opposing fashion to repress the expression of an overlapping set of genes involved in autophagy and lysosomal biogenesis.[4] ZKSCAN3 belongs to a family of transcriptional regulators, which contain zinc finger motifs (ZNFs), a Krüppel-associated box (KRAB) and a SCAN domain.[5,6] The KRAB domain is a very potent transcriptional repression module and it is thought to bind to corepressors and/or transcription factors via protein-protein interaction. Overexpression of ZKSCAN3 has been associated with augmenting tumor cell growth both in vitro and in vivo.[7-9] In contrast, a previous report found that knockdown of ZKSCAN3 induced growth arrest and augmented senescence, and that these biological effects could be attributed, at least in part, to the increased expression of a host of genes involved in autophagy and lysosomal biology.[4] These experimental results were obtained in several established transformed human tumor cell lines after acute knockdown of ZKSCAN3 using lentiviral shRNA constructs. Our preliminary observations using similar strategies largely confirmed these observations (data not shown).

Intrigued by the possibility that ZKSCAN3 could potentially function in vivo as a negative regulator of autophagic flux, we sought to generate zkscan3 knockout (KO) mice using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 approach. We generated several independent lines. All lines gave similar results and thus for the sake of clarity, we provide an in-depth analysis of one line that contained a large 8.1-kb deletion spanning from exon 2–6 of the Zkscan3 locus (Fig. 1A and B). The loss of ZKSCAN3 protein was confirmed using lysates obtained from wild-type (WT) or zkscan3 knockout animals (Fig. 1C). Although acute knockdown of ZKSCAN3 has dramatic effects on the growth of transformed cells,[4] mice lacking Zkscan3 were born in the expected Mendelian ratio (Fig. 1D). These mice did not manifest any discernable phenotypic abnormalities as WT and KO mice were indistinguishable by weight, size and appearance (Fig. 1E).

We next sought to understand whether the absence of Zkscan3 resulted in an induction of autophagy/lysosomal gene expression consistent with its putative function as a transcriptional repressor. We harvested the brain, heart and liver from 5 WT and KO mice. These organs were chosen based on previous observations that ZKSCAN3 is highly expressed in these tissues.[10] Surprisingly, Zkscan3 deletion had no marked effect on the expression of a host of either autophagy (Map1lc3b, Ulk1, Wipi2, Diras2 and Rela) or lysosomal genes (Ubqln2, Bloc1s1, Rilp, Sgsh and Hexa) previously reported to be direct and indirect targets of ZKSCAN3 (Fig. 1F-H).[4] While we did note that a few genes were modestly reduced in some tissues, this reduction was actually the opposite from what was expected following deletion of a repressor. These expression results were obtained under fasting conditions because previous results have suggested that the effects of ZKSCAN3 inhibition are more marked under conditions of nutrient depletion.[4] Nonetheless, we also found that the expression of these target genes were also not induced in randomly fed mice (Fig. S1), or when we analyzed other lines of zkscan3 KO mice generated from independent CRISPR/Cas9 targeting events (data not shown). Consistent with this lack of transcriptional change, we saw no evidence of increased steady-state levels of autophagy proteins such as LC3B in tissues obtained from zkscan3 KO animals (Fig. 1I). Furthermore, using WT or KO mouse embryonic fibroblasts (MEFs), we observed that the absence of Zkscan3 did not alter the induction of autophagy following nutrient withdrawal, as both WT and KO cells had a similar fall in SQSTM1/p62
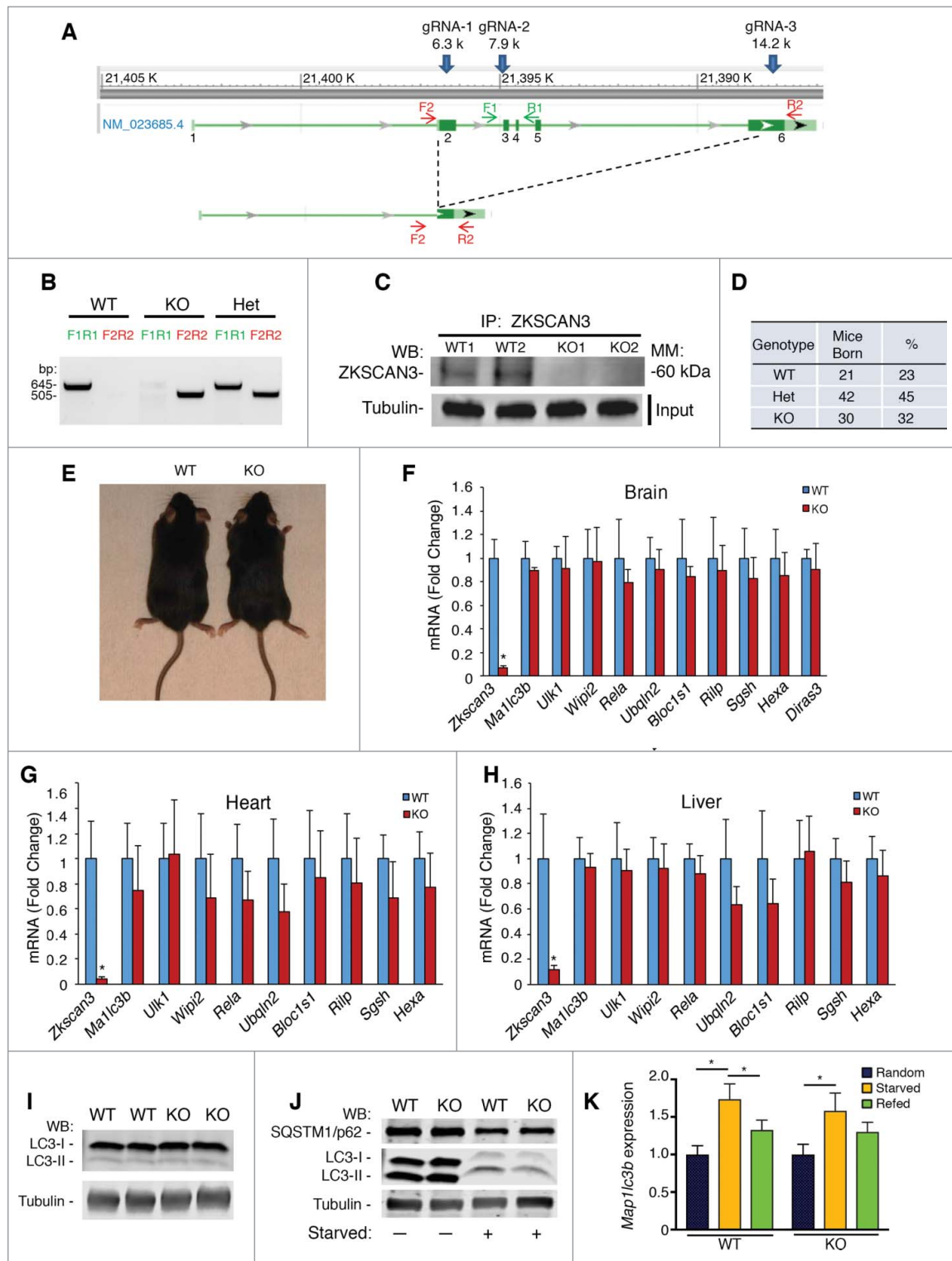
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Figure 1. The role of ZKSCAN3 in transcriptional regulation of autophagy. (A) CRISPR-mediated deletions in the *zkscan3* knockout mouse line. The genomic sequence and intron/exon sequence of *Zkscan3* are shown. Given putative alternative transcripts, the diagram shows the strategy to use 3 gRNA targeting 3 different locations in exon 2, exon 3 and exon 6 of the *Zkscan3* gene. This strategy leads to generating this knockout line with a deletion of 8083 base pairs (bp), with complete loss of exon 2–5 and partial loss of the 6th exon (14,050 to 14,957). Primers F1 and R1 are for the WT allele, while F2 and R2 can only amplify the KO allele. (B) Genotyping result demonstrates the product of primer F1 and R1 (645 bp in WT alleles) and that of F2 and R2 (505 bp in KO alleles). Heterozygous mice have both alleles. (C) Representative result of immunoprecipitation (IP) followed by western blot (WB) demonstrating loss of the 63 k_D ZKSCAN3 protein in KO heart tissue. Analysis is from 2 WT (WT1, WT2) and 2 knockout (KO1, KO2) mice. Loading control for tubulin expression represents a WB of the input lysates used for immunoprecipitation. (D) Analysis of approximately 100 live consecutive births from *Zkscan3*$^{+/-}$ heterozygous (Het) crosses demonstrating no evidence for embryonic lethality in mice lacking *Zkscan3*. (E) Representative appearance of male WT and KO mice at 8 wk of age. (F-H) qPCR analysis for selected genes involved in autophagy and lysosomal biogenesis in the brain (F), heart (G) and liver (H) of WT and KO mice. n = 5 mice per group, *p < 0.05, error bars represent standard deviation (SD). (I) Representative western blot of brain lysate of 2 WT and 2 *zkscan3*$^{-/-}$ mice demonstrating no difference in LC3B expression. (J) Representative western blot of SQSTM1/p62 and LC3 from WT and KO MEFs under fed (-) and starved (+) conditions. (K) Analysis of *Map1lc3b* expression in hearts of WT or KO mice under randomly fed conditions (n = 8 WT, n = 7 KO), starved conditions (n = 5 WT, n = 7 KO) and 8 h after refeeding (n = 4 WT, n = 4 KO). Values were normalized to random fed conditions for each genotype. *p < 0.05.

levels and a similar rise in the LC3-II:LC3-I ratio under starved conditions (Fig. 1J). Similarly, in vivo, while expression of *Map1lc3b*, which encodes LC3B, was dynamically regulated by nutrient availability, the overall response to starvation and re-feeding was similar between WT and KO mice (Fig. 1K).

Taken together, our results do not support an important in vivo role for ZKSCAN3 in the transcriptional regulation of autophagy. There are several potential explanations of these observations and why they conflict with a previous in vitro analysis.[4] First, there may be important differences between the role of ZKSCAN3 in human and mouse tissues. It should be noted that at the protein level, there is only an approximate 80% level of amino acid conservation between mouse and human ZKSCAN3. Another possibility is that there are often important differences in the cellular response between acute shRNA knockdown and stable germline knockouts. In that regard, ZKSCAN4 is highly related to ZKSCAN3 and might be able to compensate for the loss of the latter in our stable knockout mice. Finally, and perhaps most likely, there may be substantial differences in the regulation of autophagy between normal tissues and tumor cell lines. These and other explanations are likely to account for the differences in these sets of observations. Nonetheless, given the fact that inhibiting ZKSCAN3 has been viewed as an attractive target to augment autophagy therapeutically,[11] we think our in vivo results may be of interest to the community.

Materials and methods

Generation of zkscan3 knockout mice by the CRISPR/Cas9 method

zkscan3 knockout mice were generated using the CRISPR/Cas9 method as reported previously.[12] Briefly, 3 single guide RNAs (sgRNAs) were designed to target the *Zkscan3* gene, targeting exon 2, exon 3 and exon 6 separately. The nucleotide sequences for these sgRNAs were as follows: *Zkscan3*-Ex2:GGAGCTACTGGTCATAAAGG; *Zkscan3*-Ex3: AAGTAGCCAGATGGAGCCCG and *Zkscan3*-Ex6: ATAGAACCGCCTGGTTCCTG. These sequences were cloned into a sgRNA vector using OriGene sgRNA cloning services and were then used as templates to synthesize sgRNAs using the MEGAshortscript T7 kit (ThermoFisher, AM1354). *Cas9* mRNA was *in vitro*-transcribed from plasmid MLM3613 (Addgene, 42251; deposited by Dr. Keith Joung) using the mMESSAGEmMACHINE T7 Ultra kit (ThermoFisher, AM1345). For microinjection, *Cas9* mRNA (100 ng/μl) was mixed with all 3 sgRNAs (20 ng/μl of each sgRNA) and then microinjected into the cytoplasm of fertilized eggs collected from C57BL/6N inbred mice. The injected zygotes were cultured overnight in M16 medium at 37°C in 5% CO_2. The next morning, embryos that had reached the 2-cell stage of development were implanted into the oviducts of pseudopregnant foster mothers (Swiss Webster, Taconic Farm). From the first generation, we identified 5 individual lines with long genomic DNA deletion, ranging from 7 kb to 8 kb. We named the lines 1295, 1297, 1298, 1440, and 1444. The majority of our experiments were performed using Line 1444.

Mice

The *Zkscan3* mice were maintained in a C57BL/6N or C57BL/6J F1 background, and experimental mice were obtained by breeding the mice using heterozygous crosses. Genotyping for *Zkscan3* was performed using the following primers: 5′-GCTACTGGTTTCACTCACTCT-3′ (F1) and 5′-CACTCTAGC ATCAGCCTTCTTG-3′ (R1) for the WT allele, 5′-ATGCCACCCACAGTCATTAG -3′ (F2) and 5′-GAA-GATGTG AGCTACGCCTAAA-3′ (R2) for the KO allele. All animal studies were done in accordance with and approval of the National Heart, Lung, and Blood Institute (NHLBI) Animal Care and Use Committee.

Cell culture

MEFs were prepared from *Zkscan3* heterozygous breeders using embryos harvested on embryonic d 12–14. MEFs were cultured in growth medium consisting of DMEM (ThermoFisher, 11965–092) supplemented with 10% fetal bovine serum (ThermoFisher, 16000044). For starved conditions, the growth medium was replaced with Hank's balanced salt solution (ThermoFisher, 14025092) for 6 h before harvesting the cells.

Protein detection of ZKSCAN3

Hearts from WT and KO mice were lysed with RIPA buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1% Triton X-100 [Sigma-Aldrich, T9284], 0.1% SDS [Sigma-Aldrich, L3771], 150 mM NaCl) supplemented with protease inhibitor (Roche, 11873580001) and phosphatase inhibitor (Roche, 04906837001) cocktails. After homogenization, protein

Table 1. Primers used in the study for RT-PCR.

Name	Sequence
mDiras2F	GGTTGGGCTAATCTCTCTTTC
mDiras2R	CAGGATGGCAGGCAAATTA
mRelaF	GCATGCGATTCCGCTATAA
mRelaR	CTTGGTGGTATCTGTGCTTC
mWipi2F	CAGGCCTGTGTGGTTATG
mWipi2R	GGGAACCTCAGCAGATTTAG
mStx5aF	TGGAGGTGGTCCCATAAT
mStx5aR	CTAGGGTCCATCATGTCAATAG
mSec22bF	CACCTTCCTCTTCGTTTACTC
mSec22bR	GAGACCAAACAGTCCCAAATA
mUbqln2F	ACACCTACCACCACGAATA
mUbqln2R	CAGGTTACTAAGGCTGGAAAG
mBloc1s1F	GACCATGAGGTGAAGACTCTA
mBloc1s1R	GTTCTCCACCATTCCAATCC
mRilp-F	CTCCAACGACAAGGAAGATG
mRilp-R	CCCGATACCATAAGCCAAAG
mZkscan3-F	CTG GAG TAC TTG GAC AGG CAG
mZkscan3-R	ATC ATC TGG AAC CTG TGG AGG
mUlk1-F	CAC ACT CTG TGG TTC TCC TAT G
mUlk1-R	CAT CGT AGT GCT GGG ACA TAA T
mMaplc3b-F	ACG AGA GTG AGA GAG ATG AAG A
mMaplc3b-R	GTC TCC TGC GAG GCA TAA A
mRps21-F	AAA CCT ATG GCA TCT GCG GG
mRps21-R	ATC ATC TGA CTC GCC CAT CC

concentrations were measured and immunoprecipitation procedures were performed as described previously.[13] ZKSCAN3 antibody (ProteinTech, 20800–1-AP) was incubated with the protein lysate overnight (1 mg of protein) in a 4°C cold room, at a concentration of 2 μg of Ab/1 mg lysate. Protein A magnetic beads (Bio-rad, 1614013) were used according to the manufacturer's instructions. Immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The same antibody for ZKSCAN3 immunoprecipitation was used in the subsequent western blot (1: 1000), along with a True blot secondary antibody (Rockland, 18–4516–32). Other antibodies used include SQSTM1/p62 (American Research Products, 03-GP62-C), tubulin (Millipore, 05–829) and LC3 (Novus, NB100–2220).

Quantitative RT-PCR

The procedures for mRNA expression were as described previously.[14] The primers used are listed in Table 1. The expression of individual genes was normalized to the expression of the ribosomal gene *Rps21*. Three pairs of WT and KO mice that had been fasted for 12 h were used for each tissue analyzed. The data were normalized to the expression observed in WT samples.

Statistical analysis

Statistical significance for gene expression was by a Tukey-Kramer Multiple Comparison Test. A value of $p < 0.05$ was considered significant.

Abbreviations

CAS9	CRISPR associated protein 9
CRISPR	clustered regularly interspaced short palindromic repeats
KO	knockout
MEFs	mouse embryonic fibroblasts
shRNA	short hairpin RNA
WT	wild type

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No potential conflicts of interest were disclosed.

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