Original Article

Reduced expression of *Sytl 1* and *Ccdc21* and impaired induction of *Mt I* by oxidative stress in *SII-K1* knockout mice

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ABSTRACT: SII-K1 is a member of the transcription elongation factor S-II family. In the mouse, SII-K1 is expressed exclusively in the liver, kidney, heart, and skeletal muscle. Here, we report that deletion of the *SII-K1* gene in mice resulted in the downregulation of the synaptotagmin-like 1 (*Sytl 1*) gene in liver and of the coiled-coil domain-containing 21 (*Ccdc21*) gene in liver and kidney. Moreover, the induction of the metallothionein I (*Mt I*) gene in *SII-K1*-deficient mice liver was impaired in diethyl maleate-induced oxidative stress conditions. Our results suggest that SII-K1 regulates these genes *in vivo*.

Keywords: Transcription, elongation, knockout mice, oxidative stress

1. Introduction

The transcription elongation process is highly regulated by RNA polymerase II (RNAPII) and various elongation factors. During elongation of primary transcripts, RNAPII can encounter the DNA sequence, which interferes with the transcription and results in transcriptional arrest (*1*). Cleavage of the nascent RNA *via* the endonucleolytic activity of RNAPII is required to relieve the arrested state. S-II, also designated as TFIIS, is a transcription elongation factor that facilitates the elongation process by promoting RNAPII-mediated cleavage of the nascent RNA, which leads to the resumption of elongation *in vitro* (*2*). Recent studies have revealed that S-II regulates the expression of multiple genes *in vivo* (*3*). For example, mouse S-II, also referred to as general S-II, was involved in the transcription elongation of the *Bcl-*x_L gene in fetal liver *via* its activity of transcription-arrest relief (*4,5*). Yeast S-II is involved in the transcription of the *SSM1* and *IMD2* genes (*6,7*). The requirement of S-II function in mouse development (*4*) and oxidative stress resistance in yeast (*8*) clearly indicate the physiological relevance of S-II *in vivo*.

Mammalian tissues express several S-II-related genes. Among these, general S-II is ubiquitously expressed, whereas the others are expressed in a tissue-specific manner. SII-K1 is one of the tissue-specific S-IIs expressed exclusively in mouse liver, kidney, heart, and skeletal muscle (*9*). There are significant similarities in the N-terminal and C-terminal conserved amino acid regions between general S-II and SII-K1. Purified recombinant SII-K1 stimulates RNAPII *in vitro*, as does general S-II, which suggests that SII-K1 acts as a transcription elongation factor (*9*). The expression of general S-II is detected in throughout mouse development, whereas SII-K1 mRNA is barely detectable before 15- and 17-day-old embryos (*9*). It was demonstrated that *Xenopus* SII-K1 participates in the induction of the mesoderm marker genes and in the development of mesoderm-derived tissues (*10*). These findings raise the possibility that SII-K1 plays roles in the regulation of developmental process and gene expression that are distinct from those of general S-II. To understand the role of SII-K1 in the regulation of mouse development and gene expression, we generated *SII-K1*-deficient mice and assessed the extent of development and gene expression defects.

2. Materials and Methods

2.1. *Generation of SII-K1-deficient mice*

Genomic DNA fragments were cloned from a 129/Sv genomic DNA library (STRATAGENE, La Jolla, CA, USA) using the *SII-K1* cDNA as a probe. The 4.7 kb *XbaI-XhoI* genomic DNA fragment (5' arm) located in the 5' upstream region of exon 1 of the *SII-K1* gene and the 5.2 kb *SacI* genomic DNA fragment

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(3' arm) containing exons 2 and 3 of the gene were used to construct the targeting vector (Figure 1A). Electroporation of the targeting vector into E14 embryonic stem (ES) cells, selection of neomycin-resistant clones, and injection of the correctly targeted ES cells into blastocysts were performed as described previously (*11*,*12*). The resulting female chimeric mouse was bred with C57BL/6J male mice (CLEA Japan Inc., Tokyo, Japan) to obtain F_1 mice that were heterozygous for the mutation. Heterozygotes were then backcrossed to C57BL/6J mice.

For genotyping, genomic DNA isolated from mouse tail was analyzed by PCR assay using the following primer pairs (forward primer/reverse primer): wild-type allele, 5'-GGTGCACGAAAGGAGAACTG-3'/5'-CCTGGAATGTCCTGGCAATG-3'; targeted-allele, 5'-GTTATTAGTGGAGAGGCCCCA-3'/5'-AACTGTCAGAGCATGTGCGTCATGC-3'. This four-primer multiplex PCR resulted in a 320-bp product for the wild-type allele and a 430-bp product for the targeted allele. All animal experiments were approved by the institutional committee on animal experimentation and were performed in compliance with the corresponding animal welfare laws.

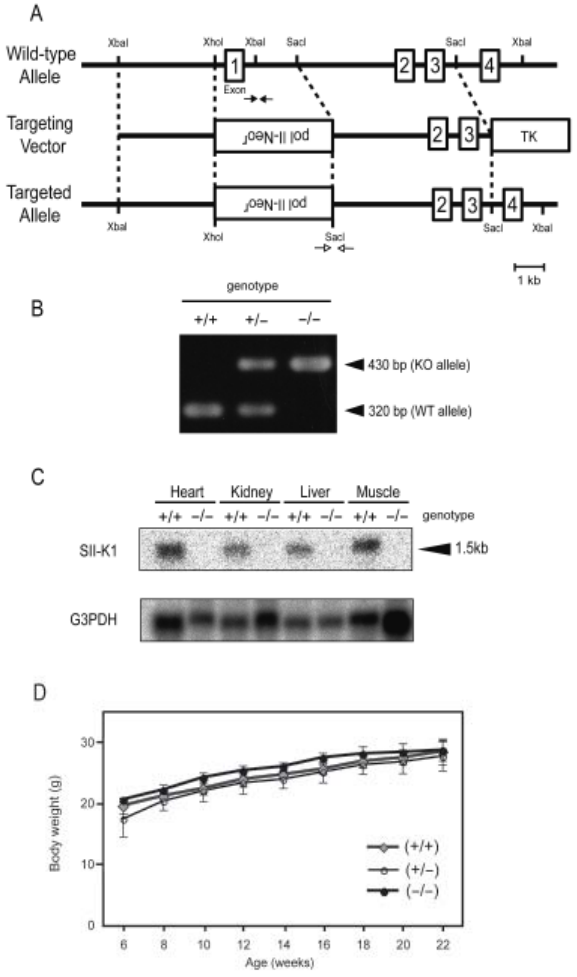

Figure 1. Generation of *SII-K1*-deficient mice. (**A**) Targeted disruption of the *SII-K1* gene. Diagrams of the wild-type allele of the mouse *SII-K1* gene, the targeting vector, and the targeted allele are shown. Numbered boxes indicate exons. Broken lines indicate the regions of homology used for homologous recombination. *Pol-II Neo*^{r} and *TK* represent the neomycin resistant gene and the herpes simplex virus thymidine kinase gene, respectively. (**B**) Genotyping of progeny from the heterozygous intercross using PCR with the indicated primer pairs in Figure 1. (**A**) (arrows). +/+, wild-type; +/–, heterozygous mutant; –/–, homozygous mutant. (**C**) Northern blot analysis of *SII-K1* mRNA from mouse heart, kidney, liver, and skeletal muscle. (**D**) Body weight was measured once every two weeks, from 6 to 22 weeks of age. Results are expressed as the mean ± S.D. (n = 4 in each group).

2.2. *Northern blot analysis of SII-K1*

Northern blot hybridization of SII-K1 mRNA was carried out using a probe that consisted of a PCR product corresponding to nucleotides +34 to +350 of the *SII-K1* cDNA. We used GAPDH mRNA as a control, which was detected with a GAPDH cDNA probe that was amplified using the following primers: 5'-ACCACAGTCCATGCCATCAC-3'/5'-TCCACCACCCTGTTGCTGTA-3'.

2.3. *Total RNA extraction and DNA microarray analysis*

Total RNA was extracted from five livers and four kidneys from *SII-K1*$^{-/-}$ and wild-type mice using the QIAGEN RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan) in accordance with the manufacturer's instructions. To verify the quality of this RNA, the yield and purity were determined spectrophotometrically and visually confirmed using formaldehyde-agarose gel electrophoresis. A pool of RNA samples from each genotype was used for microarray analysis.

Microarray analysis was performed as previously reported (*13*) using a whole mouse genome 4x 44K oligo microarray kit (G4122F, Agilent Technologies, Palo Alto, CA, USA). Total RNA (800 ng) was labeled with either Cy3 or Cy5 dye using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescently labeled targets of wild-type, as well as *SII-K1*$^{-/-}$ samples were hybridized to the same microarray slide with 60-mer probes. A flip labeling (dye-swap or reverse labeling with Cy3 and Cy5 dyes) procedure was followed to nullify the dye bias associated with unequal incorporation of the two Cy dyes into cDNA. The use of a dye-swap approach provides a more stringent selection condition for changed gene expression profiling than use of a simple single/two-color approach. Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner G2565BA. For the detection of significantly differentially expressed genes between *SII-K1*$^{-/-}$ and wild-type samples each slide image was processed by Agilent Feature Extraction software (version

9.5.3.1). This program measures Cy3 and Cy5 signal intensities of whole probes. Dye-bias tends to be signal intensity dependent, therefore the software selected probes using a set by rank consistency filter for dye-normalization. Said normalization was performed by LOWESS (locally weighted linear regression) which calculates the log ratio of dye-normalized Cy3- and Cy5-signals, as well as the final error of log ratio. The significance (*p*) value based on the propagate error and universal error models. In this analysis, the threshold of significant differentially expressed genes was < 0.01 (for the confidence that the feature was not differentially expressed). In addition, erroneous data generated due to artifacts were eliminated before data analysis using the software. The differentially expressed gene lists (up- and down-regulated genes) were generated (data not shown) and annotated using the GeneSpring version GX 7.3.1 (Agilent).

2.4. *Reverse transcription and real-time quantitative PCR analysis*

Total RNA (1 μg) was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). A real-time PCR assay was performed on the Thermal Cycler Dice Real-Time System TP850 (TaKaRa Bio, Shiga, Japan) using SYBR Premix Ex Taq II (TaKaRa) and the following primer sets: *Sytl 1*, 5'-CCAGGGTCTCCATAGAGGTG-3'/5'-TGAGGGTACATATGGTGAGAACA-3'; *Ccdc21*, 5'-AGATGGAACAGCTGCACTCC-3'/5'-CCCTCTTCTTGTGCCAGTTC-3'; *Mt I*, 5'-CACCAGATCTCGGAATGGAC-3'/5'-AGGAGCAGCAGCTCTTCTTG-3'; *Mt II*, 5'-CATGGACCCCAACTGCTC-3'/5'-GCAGCAGCTTTTCTTGCAG-3'; NAD(P)H quinone oxidoreductase 1 (*Nqo 1*), 5'-AGCGTTCGGTATTACGATCC-3'/5-AGTACAATCAGGGCTCTTCTCG-3'; β-actin, 5'-CTAAGGCCAACCGTGAAAAG-3'/5'-ACCAGAGGCATACAGGGACA-3'. Real-time PCR was performed in duplicate for each sample and was normalized to β-actin expression levels.

2.5. *Diethyl maleate treatment*

Ten- to fourteen-week-old male wild-type and *SII-K1*$^{-/-}$ mice were used (n = 4 in each group). Groups of mice were injected subcutaneously with 10 mmol/kg of diethyl maleate (DEM) (Wako, Osaka, Japan) or with corn oil vehicle at a volume of 10 mL/kg.

3. Results

3.1. *Generation of SII-K1-deficient mice*

To disrupt the *SII-K1* gene, we constructed a targeting vector to replace exon 1, which contains the initiation codon and part of the 5' sequence of exon 2 of the *SII-K1* gene, with a neomycin resistance cassette (Figure 1A). We then introduced this targeting vector into ES cells and established ES cell lines in which the *SII-K1* gene was targeted. Disruption of the *SII-K1* gene by homologous recombination was confirmed by Southern blot hybridization analysis (data not shown). We obtained a chimeric mouse by injecting the heterozygous mutant (*SII-K1*$^{+/-}$) ES cells into C57BL/6J blastocysts. This mouse was crossed with C57BL/6J mice to establish the *SII-K1*$^{+/-}$ mouse line. To determine whether homozygous mutant (*SII-K1*$^{-/-}$) mice were viable, *SII-K1*$^{+/-}$ mice were intercrossed and the genotypes of the progeny were determined (Figure 1B). The distribution of genotypes in the progeny was consistent with a Mendelian distribution, which signifies that *SII-K1*$^{-/-}$ mice are viable (Table 1). Northern blot analysis confirmed the complete loss of SII-K1 mRNA expression in the organs of adult *SII-K1*$^{-/-}$ mice (Figure 1C). We observed little difference in body weight between wild-type and SII-K1 mutant mice up to four months (from six weeks of age after birth) (Figure 1D). *SII-K1*$^{-/-}$ mice were fertile and had no abnormalities in terms of overt appearance and health up to over one year of age (data not shown). These results indicate that SII-K1 is not essential for mouse development.

3.2. *Reduced expression levels of Sytl 1 in the liver and Ccdc21 in the liver and kidney of SII-K1*$^{-/-}$ *mice*

To investigate genes that are regulated by SII-K1, we screened for genes with decreased expression in *SII-K1*$^{-/-}$ mice. From a whole mouse genome 4× 44K oligo microarray analysis, candidate genes were filtered for transcripts that were decreased above 2-fold in *SII-K1*$^{-/-}$ mice compared with wild-type mice and there were 14 transcripts in the liver and 12 transcripts in the kidney that were passed this filtering criteria. To confirm the downregulation of these transcripts observed in microarray analysis above, expression levels of each gene were determined using real-time quantitative PCR analysis. This revealed a significant downregulation of the mRNA levels of the two genes, *Sytl 1* (approximately 20% of wild-type) and *Ccdc21* (approximately 30% of wild-type), in the liver of *SII-K1*$^{-/-}$ mice (Figures 2A and 2B). The Sytl1 protein is a Rab27 effector that is involved in secretion (*16*), whereas the function of Ccdc21 is uncertain. The

Table 1. Genotype of the offspring from the intercross of *SII-K1*$^{+/-}$ mice

Genotype			Total
+/+	+/–	–/–	
60	118	61	239

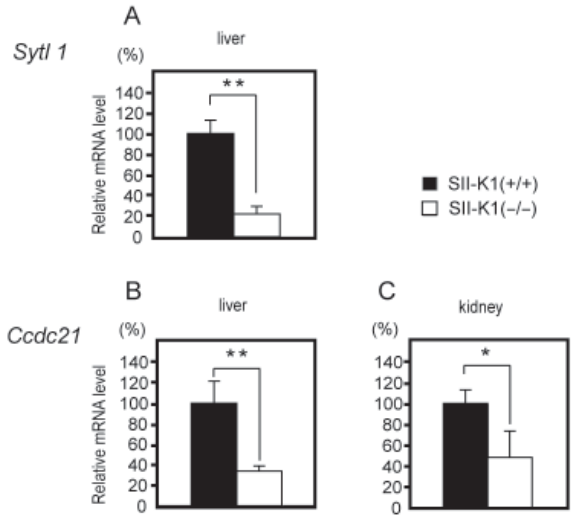

Figure 2. Gene expression analysis in *SII-K1*$^{-/-}$ mice. The expression levels of each gene were analyzed in liver (**A** and **B**) and kidney (**C**) using real-time PCR. The relative mRNA levels in *SII-K1*$^{-/-}$ mice are presented by defining the mean value for wild-type mice as 100% (n = 5 in each group). Error bars show the standard deviation. Asterisks indicate significant differences (Student's t-test, * $p < 0.05$, ** $p < 0.01$).

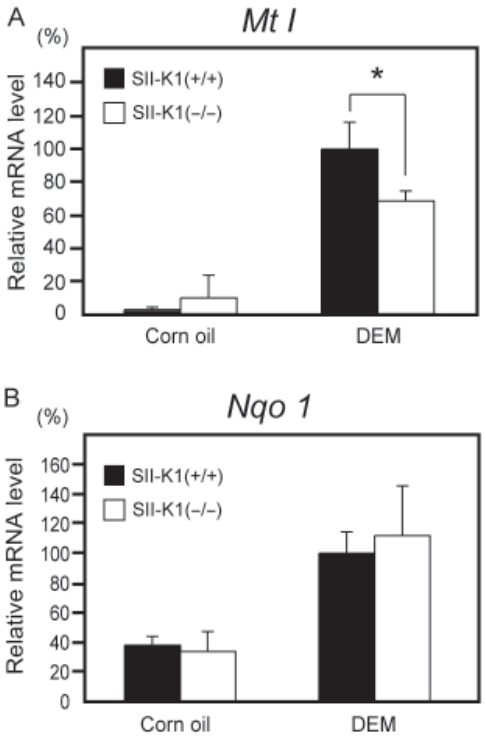

Figure 3. Gene expression analysis in the liver of *SII-K1*$^{-/-}$ mice under DEM-induced oxidative stress conditions. The expression levels of each gene were analyzed by real-time PCR. Relative mRNA levels in *SII-K1*$^{-/-}$ mice are presented by defining the mean value for wild-type mice exposed to DEM as 100% (n = 4 in each group). Error bars show the standard deviation. The asterisk indicates a significant difference (Student's t-test, * $p < 0.05$).

mRNA levels of the *Ccdc21* gene were also reduced in the kidney of *SII-K1*$^{-/-}$ mice (Figure 2C). These results suggest that SII-K1 is involved in the control of the expression levels of a specific subset of genes, *Sytl 1* and *Ccdc21*, *in vivo*.

3.3. *Impaired induction of the Mt I gene in the liver of SII-K1*$^{-/-}$ *mice under DEM-induced oxidative stress conditions*

S-II participates in the induction of certain genes as a response to environmental stimuli (*6,7*). Moreover, it was demonstrated that S-II (*DST1*) confers resistance against oxidative stress in yeast (*8*). It is also well known that certain oxidative stress-inducible genes are activated in response to oxidative stress in the mammalian liver, where SII-K1 is expressed. These findings led us to hypothesize that SII-K1 contributes to the induction of oxidative stress-inducible genes in the liver under oxidative stress conditions. To test this hypothesis, we examined the effect of diethyl maleate (DEM), which is an oxidative stress agent, on *SII-K1*$^{-/-}$ mice. Because *Mt* gene is activated by various oxidative stress agents to protect organs against oxidative stress, especially in the liver (*14*), we analyzed *Mt* gene induction. Real-time PCR assays revealed that the expression levels of the *Mt I* gene were reduced by about 30% in the liver of *SII-K1*$^{-/-}$ mice 7.5 h after DEM treatment when compared with wild-type mice (Figure 3A). In contrast, there was no significant difference in *Mt II* gene expression levels in the liver between *SII-K1*$^{-/-}$ and wild-type mice treated with DEM (data not shown). The level of induction of *Nqo1*, which is also induced by DEM (*15*), in the liver was not significantly different between *SII-K1*$^{-/-}$ and wild-type mice (Figure 3B). These results suggest that SII-K1 is specifically involved in the induction of the *Mt I* gene in the liver under DEM-induced oxidative stress conditions.

4. Discussion

In the present study, we found that the tissue-specific transcription elongation factor SII-K1 is involved in the expression of a specific subset of genes in tissues where SII-K1 is expressed. We also found that SII-K1 is not essential for mouse development.

Previously, we reported that the *Bcl-x*$_L$ gene, which is downregulated in the fetal liver of *S-II* knockout mice, has a transcription arrest site and that the arrest at this site is relieved by general S-II (*4,5*). As SII-K1 is a transcription elongation factor, *Sytl 1* and *Ccdc21*, which were downregulated in *SII-K1* knockout mice, may also have transcription arrest sites that can be relieved by SII-K1. Sytl1, also known as Slp 1, is a Rab27 effector protein and has been reported to be part of secretory machineries, *e.g.* amylase secretion by the exocrine pancreas (*16*). SII-K1 may affect the secretion of certain proteins *via* the transcriptional regulation of the *Sytl 1* gene in the liver.

We provided evidence that SII-K1 participates in

the induction of the *Mt I* gene in the liver under DEM-induced oxidative stress conditions. It was reported that hepatocytes from MT I and II double knockout (MT null) mice exhibited enhanced sensitivity to the oxidative stress and cytotoxicity induced by cadmium or *tert*-butylhydroperoxide (*17*), which suggests that MT is important for the protection of hepatic cells against oxidative stress. Our finding that deficiency of SII-K1 reduced the induction of the *Mt I* gene under DEM-induced oxidative stress conditions suggests that SII-K1 confers oxidative stress resistance through the induction of the *Mt I* gene in the liver. In addition, SII-K1 may be involved in the suppression of carcinogenesis in liver, because MT null mice showed high susceptibility to cisplatin-induced hepatocarcinogenicity (*18*).

Taken together, our results imply that mammals may have acquired SII-K1, in addition to general S-II, to achieve proper expression of certain genes, which include *Sytl 1* and *Ccdc21*. Moreover, the reduced induction of the *Mt I* gene in *SII-K1*$^{-/-}$ mouse liver suggests that SII-K1 participates in the protection of tissues that express SII-K1 against environmental stresses.

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