Identification of Novel Genetic Regulatory Region for Proprotein Convertase FURIN and Interferon Gamma in T Cells

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The proprotein convertase enzyme FURIN promotes the proteolytic maturation of pro-proteins and thereby it serves as an important factor for maintaining cellular homeostasis. In T cells, FURIN is critical for maintaining the T regulatory cell dependent peripheral immune tolerance and intact T helper cell polarization. The enzymatic activity of FURIN is directly associated with its expression levels, but genetic determinants for cell-type specific Furin gene regulation have remained elusive. By exploring the histone acetyltransferase p300 binding patterns in T helper cells, a putative regulatory region at ca. 20kB upstream of Furin gene was identified. When this region was deleted with CRISPR/Cas9 the production of Furin mRNA was significantly reduced in activated mouse T cells. Genome-wide RNA profiling by sequencing revealed that the novel Furin regulator region also impacted the expression of several genes that have previously been associated with the Th1 type hallmark cytokine IFNγ regulation or function. Finally, Furin genetic regulatory region was found to specifically promote the secretion of IFNγ by activated T cells. In sum, our data unravels the presence of Furin expression regulatory region in T cells that has characteristics of a super-enhancer for Th1 cell fate.

Keywords: Furin regulation, super-enhancer, interferon gamma, p300, proprotein convertase

INTRODUCTION

The enzymes of the proprotein convertase subtilisin/kexin (PCSK) family are characterized by their common domain structure and unique cleavage target sequence (R/K)X(R/K) where X represents a 0, 2, 4, or 6 amino acid long spacer (1). PCSKs control the bodily homeostasis by regulating the bioavailability of several hormones, growth factors, other enzymes, and adhesion molecules. Proprotein convertases are also important factors in infections as they proteolytically modify various pathogenic structures, such as viral proteins, for example in HIV and SARS-Cov-2, or bacterial toxins (2). Although PCSKs show partly overlapping target specificity in vitro, their expression pattern and subcellular localization are divergent, and studies with knock-out animals and human mutations have unequivocally revealed distinct physiological roles for each convertase (3, 4).

The PCSK prototype enzyme, FURIN, is ubiquitously expressed and critical for embryonic development (5). In T cells, Furin (Fur) gene is upregulated by T cell receptor engagement and
IL-12 cytokine and its expression is essential for maintaining intact peripheral immune tolerance, normal T helper cell polarization and activation (6–9). Furin is also upregulated via Toll like receptor signaling in myeloid cells wherein it exerts an anti-inflammatory function (10). Changes in Furin expression level are associated with a wide variety of pathological processes ranging from tumor development and progression (11), infectious diseases (12, 13) to autoimmune disorders such as rheumatoid arthritis (14) or Sjögren’s syndrome (15).

The gene encoding FURIN is located on chromosome 15 in human and chromosome seven in mouse. Furin mRNAs arise from three different promoters, P1, P1A, and P1B leading to transcripts, which vary in 5′ untranslated region but translate into identical protein products (16). P1A and P1B promoters resemble those of housekeeping genes while the P1 promoter is inducible by cytokines [e.g., IL-12, TGFβ-1 (7, 17)] or by other external factors [e.g., thyroid hormone, hypoxia (18, 19)] that activate transcription factors including C/EBP-β, GATA-1, Smads, STAT-4 or HIF-1 (16, 19–21).

Super-enhancers or stretch enhancers (SE) are clusters of putative enhancers, binding transcriptional coactivators on an average 10-fold higher density than typical enhancers (TE) (22–24). They can be identified using the Rank Ordering of Super-enhancer (ROUSE) algorithm on the basis of active enhancer marks such as histone three acetylation on lysine 27 residue (H3K27ac), mediator complex subunit 1 (MED1), and binding marks such as histone three acetylation on lysine 27 residue (H3K27ac) enhancer (ROSE) algorithm on the basis of active enhancer (St. Louis, MO, USA) and maintained in growth medium: Murine thymoma cell line EL-4 was purchased from Sigma.

Cell Culture and Activation

Murine thymoma cell line EL-4 was purchased from Sigma (St. Louis, MO, USA) and maintained in growth medium:

- RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Gibco, ThermoFisher Scientific, Waltham, MA, USA), 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 U/ml penicillin (Lonza), 100 U/ml streptomycin (Lonza), 1 mM sodium pyruvate (Lonza), 4.5 g/l glucose (Lonza), 0.05 mM 2-mercaptoethanol (Sigma).

Cell clones created from EL-4 cell line were maintained in the same growth medium. Prior activation, cells were first starved in 1% FBS containing culture medium for 1 h then left unstimulated or stimulated with 10 ng/ml PMA (Sigma) and 100 ng/ml ionomycin (Sigma) for the time period mentioned. For evaluating the cytokine production from cell culture supernatants with ELISA, cells were washed once with PBS and resuspended in serum-free X-Vivo medium (Lonza) and activated as above. Thereafter cells were pelleted, and supernatants were collected and frozen until cytokine measurements with ELISA.

sgRNAs, Plasmids, and Cloning

For designing single guide RNA (sgRNA) sequences, 150 bp sequences both up- and downstream the highest p300 binding peaks (according to GEO datasets GSM994508, GSM994516, GSM1480824) in the mouse Furin genomic region (chr7: 87,533,000-87,570,000 mm9) were inputed to an on-line CRISPR design tool. The generated sgRNA sequences were ranked according to predicted cleavage success efficiency. Chosen gRNA sequences were further analyzed using CHOPCHOP (30). Three sgRNAs were chosen both upstream (L) and downstream (R) of the p300 binding sites either in the Furin promoter region (PRO) or ca. 20kb upstream Furin transcription starting site (putative super-enhancer site, SE) taking into account the predicted cleavage efficiency as well as possible off-target sequences. Table 1 summarizes the target sequences of chosen sgRNAs.

sgRNA sequences were cloned one by one into Cas9 expression plasmid px330 (a kind gift from Dr. Rafael Casellas, senior investigator at NIAMS, National Institutes of Health, MD, USA) following previously published protocol (31) to generate the px330-mFurin-SE-R/L and px330-mFurin-PRO-R/L constructs (Figure 2A shows the schematic representation of the genome editing strategy). Functionality and efficiency of these constructs were tested with T7 assay in NIH 3T3 (ATCC) cells. Briefly, genomic DNA was isolated using DNeasy kit (Qiagen, Hilden, Germany) from cells transfected with each construct individually by using TurboFect reagent (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Targeted genomic area was amplified with PCR using DreamTaq DNA polymerase (ThermoFisher Scientific) and primer pairs upstream and downstream of the targeted deletion (for SE: SE-L-For: 5′-TGTTTTTTCGCTTTTGAAAATGT-3′ and SE-R-Rev: 5′-CTGGGACTCTCACACTTGCT-3′; for PRO: PRO-L-For: 5′-GGGCTCAAATCCTTGCGGTA-3′ and PRO-R-Rev: 5′-CAGCTGAGCTTCTGAGGAC-3′). PCR products were annealed in a reaction containing 1x NEBuffer 2 (New England Biolabs, MA, USA) and run on 10% polyacrylamide gels.
TABLE 1 | Summary of sgRNA target sites for p300 binding area deletion.

| Name                | Sequence                                    | Genomic location (mm9) | Predicted cleavage efficiency | Off-target |
|---------------------|---------------------------------------------|------------------------|-------------------------------|------------|
| mFurin-SE-sgRNA-L1  | caccgGAAGGCGATGTCCTCCG                         | Chr7: 87,561,784-87,561,801 | 65.62                        | MM0 = 0, MM1 = 0, MM2 = 1, MM3 = 11 |
| mFurin-SE-sgRNA-L5  | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,561,743-87,561,760 | 35.83                        | MM0 = 0, MM1 = 1, MM2 = 6, MM3 = 116 |
| mFurin-SE-sgRNA-L11 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,561,781-87,561,790 | 45.86                        | MM0 = 0, MM1 = 0, MM2 = 8, MM3 = 27 |
| mFurin-SE-sgRNA-R1  | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,562,511-87,562,528 | 54.66                        | MM0 = 0, MM1 = 0, MM2 = 2, MM3 = 46 |
| mFurin-SE-sgRNA-R3  | caccgGCGGTTGTTGTAGGCT                         | Chr7: 87,562,529-87,562,546 | 32.67                        | MM0 = 0, MM1 = 0, MM2 = 7 |
| mFurin-SE-sgRNA-R12 | caccgGTATGCTCCACGTTTTT                       | Chr7: 87,562,423-87,562,439 | 54.48                        | MM0 = 0, MM1 = 1, MM2 = 9, MM3 = 115 |
| mFurin-PRO-sgRNA-L1 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,548,976-87,548,995 | 51.23                        | MM0 = 0, MM1 = 0, MM2 = 4 |
| mFurin-PRO-sgRNA-L2 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,548,994-87,549,011 | 41.95                        | MM0 = 0, MM1 = 0, MM2 = 3, MM3 = 10 |
| mFurin-PRO-sgRNA-L3 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,548,945-87,548,962 | 41.95                        | MM0 = 0, MM1 = 0, MM2 = 3, MM3 = 10 |
| mFurin-PRO-sgRNA-R1 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,549,484-87,549,500 | 71.91                        | MM0 = 0, MM1 = 0, MM2 = 3, MM3 = 6 |
| mFurin-PRO-sgRNA-L5 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,549,974-87,549,990 | 57.24                        | MM0 = 0, MM1 = 0, MM2 = 2, MM3 = 43 |
| mFurin-PRO-sgRNA-R3 | caccgGTGAGGGAGGTCTGCTG Music                    | Chr7: 87,550,585-87,550,602 | 45.35                        | MM0 = 0, MM1 = 0, MM2 = 8 |

One hundred fifty base pair sequences from both up- and downstream of the most abundant p300 binding regions in Furin promoter area (PRO) and 20 kb upstream Furin transcription site (Super-enhancer area, SE) were analyzed for targeting with Cas9 endonuclease activity. Twenty sequences of each region were evaluated according to predicted Cas9 cleavage efficiency and off-target effect, and three sequences/region were chosen for experimental procedures. Upper case letters show the sequences in mouse genome, lower case letters refer to additional nucleotides for restriction enzyme digestion during cloning to the px330 vector. Predicted cleavage efficiency and off-target prediction are assessed using ChopChop (33). Off-target values show the number of off-targets with 0 (MM0), 1 (MM1), 2 (MM2), 3 or more (MM3) mismatches.

Generation of EL-4 Cell Clones With Targeted Deletion of Putative Super-Enhancer (SE) or Promoter (PRO) Region

Stable EL-4 cell clones with deleted SE and PRO regions were generated according to a previously published protocol (32). Briefly, 24 h before transfection, EL-4 cells were adjusted to a concentration of 0.5 × 10⁶ cells/ml in complete growing medium. A total of 6 × 10⁶ cells were transfected with the mixture of 6 µg px330-mFurin-sgRNA-R and 6 µg px330-mFurin-sgRNA-L (for PRO or SE deletion) or as control 12 µg px330 vector (“empty”) constructs together with 0.6 µg pMax-FlpF-GFP plasmid by using the Amaxa SF Cell Line 4D-Nucleofector TM X Kit L (Lonza). Various different pairings of sgRNA R and L constructs were used: two different pairs of PRO deleting constructs (px330-PROR1 + px330-PROL3 and px330-PROR1 + px330-PROL5) or three for the SE constructs (px330-SEL1 + px330-SER1, px330-SEL1 + px330-SEL2, and px330-SEL5 + px330-SEL2). The transfected cells were resuspended in complete growth medium in a concentration of 0.5 × 10⁶ cells/ml.

Seventy two hours after the transfection GFP + cells were sorted by using FACs Aria Fusion (BD, Franklin Lakes, NJ, USA) and seeded individually (1 cell/well) into 96 well-plates (Nunc, Roskilde, Denmark). Cell clones were expanded and screened for deletion of the targeted genomic areas by PCR. For this purpose, whole genomic DNA was isolated from the cell clones using QuickExtract DNA Extraction Solution (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer’s protocol. Deletion efficiency was investigated using PCR. PCR products were run on 2% agarose gels and EL-4 cell clones showing a clear deletion at the targeted region were retained for further experiments. In case of SE region, successful deletion produced a 360 bp product instead of the 1,108 bp wild type band while PRO region deletion led to a 320 bp product and the undeleted region was 843 bp long.

qRT-PCR Analysis of Furin mRNA Expression

A total of 4 × 10⁶ cells of three individual SE or PRO deleted “empty” px330-transfected EL-4 clones were either left unstimulated or stimulated with PMA/ionomycin for 6 h. Thereafter cells were collected, washed once in PBS and RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer’s protocol. RNA from each cell clone was reverse transcribed using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Gene expression was investigated with real-time quantitative PCR using the CFX96 Real-Time PCR Detection System (Bio-Rad). Primer sequences used...
Cell Growth and Viability Assays
Three individual SE or PRO deleted, or “empty” EL-4 cell clones were seeded into complete growth medium at a concentration of 3 x 10^5 cells/ml, and the numbers of alive and dead cells were determined by staining with 0.4% Trypan Blue solution (Sigma). Additionally, cytotoxicity was investigated using the Cytotoxicity Detection LDH Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, samples were taken from each growing cell clones daily, and split to two. Half of the samples were supplemented with 1% Triton X-100 (Sigma). One hour later supernatants were collected from Triton X-100 treated and untreated cells, and the absorbance at 490 nm (reference absorbance 680 nm) was measured. The lactate dehydrogenase activity (which is proportional to the absorbance of its colorimetric product) of the Triton X-100 treated cells was considered as 100% and the activity measured from the medium without any cells as 0%.

RNA Sequencing and Data Analysis
Three independent SE or “empty” EL-4 cell clones were left unstimulated or stimulated with PMA/ionomycin for 6 h as described above. Total RNA was isolated using RNeasy kit (Qiagen) according to the manufacturer’s protocol. RNA library (with RiboZero kit for rRNA removal) construction and RNA sequencing services (using Illumina PE150 platform) were provided by Novogene (Beijing, China). The quality of the sequenced reads was assessed using the FastQC software. Reads were aligned to the mouse genome (mm10/ GRCm38.p5) with the STAR aligner (33), and genes without transcripts in any of the conditions (“empty” or SE, unstimulated or stimulated) were filtered out of the analysis. Statistical analysis of differential gene expression was assessed by DESeq2 algorithms (34) using DESeq2 normalization based on the median ratios (35) and apeglm shrinkage (36). Experimental design for different comparisons included the genotype (SE vs. “empty”) and the status (stimulated or unstimulated) of the cells. We considered genes as differentially expressed when the Benjamini–Hochberg adjusted p-value [false discovery rate (FDR)] was < 0.05. Clustered heatmap was generated by using the heatmap package (Version 1.0.12.) (37) in the R software. For gene set enrichment analyses, genes were ordered on the basis of fold change values (log2FC) and gene set enrichment was analyzed using WebGestalt.

Cytokine Measurements
Cytokine (IL-2, IL-4, IL-17A, TNFα, TGFβ, IFNγ) productions of three individual SE or “empty” EL-4 cell clones were assessed from PMA/ionomycin-stimulated and unstimulated cells with commercial ELISA kits (eBioscience, San Diego, CA, USA) following the protocol provided by the manufacturer. Optical densities were analyzed with the drc package (39) in R, statistical differences between sample groups were examined by comparing the log(conc) values using unpaired two-tailed Student t-tests, p < 0.05 was considered a statistically significant difference.

RESULTS
p300 Binding Is Pronounced at 20 kb Upstream of Furin Gene in CD4+ T Helper Cells
p300 is a histone acetyltransferase responsible for acetylating the chromatin at histone H3K27 thus activating the gene expression machinery (40). Vahedi et al. (29) sought for the key genomic regulators of CD4+ T helper cell fate by identifying putative super-enhancer (SE) regions with exceptionally high p300 loading in mouse Th1, Th2, and Th17 cells. By ranking the genomic regions according to the magnitude of p300 load they identified an area at ca. 20kb upstream of the transcription starting site of Furin gene that is highly occupied by p300 in Th1 type cells. This region ranked as one of the genomic sites with the most abundant p300 binding (together with Bach2, Rgs1, Il2r, IFNγ, Il7r, Tbx21, Stat5 and others) and it was thus defined as a putative super-enhancer for Th1 cell fate. The evaluation of the genomic location of the p300 binding sites in different T helper cell subtypes at chr7: 87,500,000-87,600,000 of the mouse genome demonstrated the highest amount of p300 binding at putative SE site in Th1 type cells while the other investigated T cell types showed a lower amount of p300 binding (Figure 1A). In addition to the aforementioned putative super-enhancer region, high p300 binding was observed adjacent to promoter regions (PRO) of Furin gene in all Th subtypes. Upstream of the PRO and downstream of the SE region, two other genomic area showed enriched p300 binding in Th1 and Th2 but not in Th17 cells, whereas p300 binding was low or missing at the surrounding genomic locations.

Figure 1B shows the Furin region in the mouse genome (chr7:87,533,000-87,570,000; mm9 genome assembly). The two aforementioned genomic areas (SE and PRO) that showed high p300 binding in all type of T helper cells are highlighted in blue (SE) and green (PRO) color (Figures 1A, B). To further evaluate the role of p300 binding at the putative Furin super-enhancer site we studied the H3K27ac histone marks in several tissues from openly available data of the mouse ENCODE project (42). In tissue and cell line samples analyzed in the frame of the ENCODE project, the genomic region at the promoter area of Furin gene showed typically H3K27ac enrichment near Furin’s constitutively active promoters P1A and P1B (16). Moreover, bone marrow-derived macrophages (BMDM), erythroleukemia cell lines (CH12 and Mel), olfactory bulbs (olfact) and placenta, the H3K27ac peak at the promoter region overlapped with the
p300 binding peak occurring in Th cell subgroups although this p300 peak does not overlap with any of the known Furin promoters but localizes between the P1 and P1A promoters. However, a H3K27ac enrichment at the putative Furin super-enhancer region could only be seen in bone marrow, liver and Mel samples suggesting that this genomic area is active, and in an open state only in restricted cell and tissue types.

The Putative Super-Enhancer Regulates Furin mRNA Expression in Activated T Cells

To investigate how the identified p300 enrichment regions PRO and SE regulate Furin mRNA expression in T cells, we deleted these genomic areas with high p300 load in all investigated Th subtypes by employing CRISPR/Cas9 technology. To this end we
cloned sgRNA sequences targeting the genomic DNA upstream (L) or downstream (R) of either the SE or the PRO area to the Cas9 expression vector px330. EL-4 mouse thymoma cell line was then transfected with pairs of either PRO or SE deleting constructs using various px330-L and px330-R combinations (Figure 2A). Transient transfection of EL-4 cells for the targeted deletions resulted in a relatively low deletion efficiency (data not shown). To overcome this challenge, we generated several stable EL-4 cell lines with full deletion of SE (above) or PRO (below) region or transfected with without gRNA (“empty”) were kept for further investigations. A cell clone with heterozygous PRO region deletion was used for PCR control (control lane (L) or downstream (R) of either the SE or the PRO area to the genomic DNA upstream (L) or downstream (R) sequence of the p300 binding areas were designed, cloned to Cas9 expression vectors and transfected in pairs (SEL and SER or ORL and ORR) into EL-4 mouse thymoma cells. (B) Deletion efficiency in EL-4 cell clones was assessed with PCR using primer pairs outside of the targeted deletion area. Three independent EL-4 cell clones with full deletion of SE (above) or PRO (below) region or transfected without gRNA (“empty”) were kept for further investigations. A cell clone with heterozygous PRO region deletion was used for PCR control (control lane +) L = 1kb DNA ladder. (C) “Empty,” SE area deleted (SE) and PRO region deleted (PRO) EL-4 clones were left unstimulated or stimulated with PMA and ionomycin for 6 h and Furin mRNA expression was examined with qRT PCR. Normalized Furin expressions were compared using Student’s t-tests (**p < 0.01). (D–F) Viability and proliferation of EL-4 cell clones. “Empty,” SE, and PRO clones were cultured under identical conditions, and the number of alive (D) and dead (E) cells was determined manually for 96 h. Additionally, samples from each EL-4 clone culture were taken daily and cytotoxicity was determined using LDH assay (F). Data are shown as means ± SEM (n = 3/genotype). (D–F) The experiment was repeated three times with similar results.

The Deletion of the Super-Enhancer Area Does Not Affect the Splicing of Furin mRNA

To further evaluate how the SE region impacts Furin mRNA expression, we compared the RNA transcription profiles of resting and activated SE-deleted and “empty” EL-4 T cell clones using RNA sequencing (RNAseq). Read alignment to the entire Furin region led to similar results as observed with qRT PCR: in steady state, both wild type “empty” and SE-deleted EL-4 clones expressed Furin at comparable levels. Upon PMA/ ionomycin stimulation for 6 h, a 20-times increase in the number of aligned reads was observed in the “empty” clones while Furin expression was upregulated ca. three-fold in the SE EL-4 clones (Figure 3A).
Alternative splicing can affect the stability, localization, or translation of the mRNA, and as such, it can play an important role in gene regulation (51). There are ten alternative transcripts of mouse *Furin* in the RefSeq database (52), eight of which are predicted with computational methods while two transcripts are experimentally validated (5). To investigate how the deletion of the putative SE region affects the exon usage of *Furin* transcripts, we generated Sashimi plots with the built-in tool of the Integrative Genomics Viewer [IGV (53)]. Sashimi plots (54) are used to depict RNAseq reads aligning to the exons of a gene, loops representing bridges between separate exons where a read aligns to more exons thus visualizing possible alternative splicing events. We investigated the quality and the quantity of mRNA isoforms originating from the *Furin* region in wild type (“empty”) and SE-deleted cell clones (Figure 3B). In steady-state, we could not detect any marked differences in *Furin* transcription between the “empty” and SE EL-4 clones; the quantity of the reads aligning to any given genomic location (the heights of the bars on the diagrams) were similar, and the junction coverage, the number of
reads aligning to separate exons (represented by the thickness of the loops on Figure 3B), was also equal. The activation of T cells for 6 h resulted in a pronounced elevation (ca. 15 to 20 times) in all read counts aligning to the translating exons of Furin in the “empty” cell clones whereas the induction of Furin expression in SE-deleted cell clones was reduced globally (ca. three times more aligning reads across all encoding exons).

Gene expression can also be regulated by lncRNAs, especially those transcribing from the antisense strand of the protein-coding DNA (55). We observed the expression of a known lncRNA gene (Gm44851) overlapping the alternative first non-coding exon of Furin, with a low transcription level in steady-state (two to six reads both in “empty” and SE EL-4 clones). PMA/ionomycin stimulus led to a ca. 100-fold elevation in Gm44851 transcription, however, there was no statistically significant difference between the “empty” and SE-deleted EL-4 clones (data not shown).

The transcriptional activity at the active enhancers results in the production of enhancer RNAs (eRNAs), which may regulate their target gene by altering the chromatin architecture and histone modification, or by recruiting specific proteins to the enhancers (56). Super-enhancers are reported to produce higher amounts of eRNAs than typical enhancers (22). To evaluate eRNA transcription at the putative Furin super-enhancer

![Figure 4](image-url)
Wild type “empty” and super-enhancer deleted SE EL-4 clones were stimulated (PMA/ionomycin, 6 h). Total RNA was isolated and submitted to RNA sequencing. Forty differentially expressed genes were found with the DESeq2 algorithms (34), which could be grouped to three categories based on similarities in genomic localization, holding FURIN target sequence or their common relation to IFNγ.

region we compared the RNA alignments of resting and PMA/ionomycin stimulated “empty” and SE-deleted cell clones. We also examined the read alignment to the M10 area, a genomic region (30 kb upstream Furin, 10 kb downstream Blm) with reported Furin enhancer activity (48) (Figure 3C). In “empty” EL-4 clones, T cell activation resulted in 20–30 eRNA reads aligning to the super-enhancer region indicative of activation-induced eRNA production. Logically, in resting or activated SE clones where the p300-binding region is deleted, no eRNA reads aligned to the super-enhancer region. In contrast, the eRNA production at M10 in EL-4 cells was not evidently dependent of the SE region or T cell activation (8–16 aligning eRNA reads/condition).

The Furin Super-Enhancer Region Regulates Gamma Interferon Gene Signature

Next, we compared the genome-wide RNA expression profiles of unstimulated and stimulated (6 h, PMA/ ionomycin) “empty” and SE-deleted EL-4 cell clones (Supplementary Figure 1, Supplementary Table 1). This analysis revealed differential expression of a total of 141 genes, among those only immune system process (GO:0002376) associated genes were significantly over-represented (FDR = 0.04, data not shown). As the impact of SE on the Furin expression was only significant in activated cells, we next compared directly the gene expression profiles of activated “empty” and SE clones (Figure 4A). This analysis revealed that the Furin SE region significantly regulated the expression of a total of 40 transcripts (p adj< 0.05), some of which have been reported to share common ontologies or other features (Figure 4, Table 2). For example, SE region impacted the expression of both Furin and Fes, the gene directly downstream and in a short genomic distance to Furin. Two genes encoding previously identified FURIN target molecules [NOTCH1 (62) and ADAM33 (57)] were among the SE-dependent genes as well. Additionally, FURIN cleavage could be predicted with the ProP software6 (77) in the protein product of six other SE-dependent genes, namely Arc, Gimap4, Il18bp, Mst1r, Sh2d3c, and S16a6 genes (Table 2).

A remarkable number of the SE-dependent genes have previously been associated with the Th1 signature cytokine IFNγ. Many of these exert GTPase activity [F830016B08Rik coding immunity-related GTPase 4 (IFGGA4) protein], or are IFNγ-inducible guanylate binding proteins (Gbp3, Gbp6, Gbp7, Gbp9, Gbp11), Gimap4, Gvin1 (also known as Vlig1 and Igtp). Also several genes that either regulate or are regulated by IFNγ were found to be SE-dependent. Adam33 is regulated in an IFNγ dependent manner in airway smooth muscle cells of asthma patients (58), Calhm6 (also known as Fam26f or Isam) both regulates IFNγ in NK cells (60) and is itself upregulated by IFNγ in lymphocytes (61), Il18bp is regulated by IFNγ (70) and it inhibits the levels of circulating IL-18 with a consequential IFNγ reduction (69). Irf8 is an IFNγ inducible transcription factor with a crucial role in myelopoiesis (71). Ly6c expression positively correlates with IFNγ production by effector T cells (72, 73), and endogenous IFNγ induces Ly6c expression (74). Ly75 (Dec205/CD205) is a surface molecule

6https://services.healthtech.dtu.dk/service.php?ProP-1.0
FIGURE 5 | Furin super-enhancer regulates IFNγ in activated T cells. “Empty” and SE-deleted EL-4 T cell clones (n = 3/genotype) were left unstimulated or activated with PMA and ionomycin for 24 and 48 h. The production of (A) IL-2, (B) IFNγ, (C) TNFα and (D) IL-17 were evaluated from the supernatants with ELISA. The cytokine levels were compared using Student’s t-test (*p < 0.05). Three independent “empty” and SE EL-4 clones were stimulated three times, ELISA measurements were repeated two to three times leading to similar results.

on a subset of dendritic cells (75). Slamf9 expression is also upregulated upon IFNγ stimulus in bone marrow-derived macrophages (76). Lastly, also Furin has been shown to control ifnγ expression, and both Furin and IFNγ expression are co-regulated via the IL-12/STAT4 pathway (7). As the number of significantly differentially expressed genes in activated “empty” and SE clones was limited, we further ranked all genes according to the difference in expression levels between stimulated SE and “empty” cells (log2foldchange) and evaluated enriched gene ontology terms and KEGG pathways. These analyses did not reveal any additional gene set enrichments between the activated “empty” and SE clones (data not shown).

In sum, RNAseq data indicate that the Furin SE region regulates the transcription of genes that are located at its vicinity (Furin, Fes), FURIN target molecules as well as several genes that are associated with the IFNγ cytokine regulation or function.

Furin Super-Enhancer Promotes the Production of Th1 Cytokine IFNγ in Activated T Cells

Data from the RNA sequencing experiments imply that Furin SE may have a role in regulating Th1 type responses, such as the production of IFNγ. To test this hypothesis directly, we evaluated the production of T cell cytokines in “empty” and SE EL-4 T cell clones. To this end, EL-4 cell clones were stimulated with PMA/ionomycin for 24 h and 48 h, or left untreated, and cytokine levels were measured in the cell culture supernatants using commercial ELISA kits. Remarkably, deleting the Furin SE region significantly reduced the production of IFNγ by activated T cells at both 24 and 48 h (Figure 5) whereas the production of IL-2, TNFα, and IL-17 were unaffected by SE deletion. Th2 type hallmark cytokines were below the level of detection in both “empty” and SE EL-4 cell clones at all tested time points (data not shown). These data indicate that the p300 binding enrichment site ca. 20 kb upstream of Furin gene has a potential to serve as a genuine super-enhancer region for IFNγ production and T helper 1 type cell fate.

DISCUSSION

Although the structure of Furin gene and its promoters have been described already in the 1990’s, its genetic regulation is still not well-understood. The development of DNA sequencing technologies now allows for further deciphering regulatory sequences that control the spatial and temporal expression of
genes. ChIP-seq using antibody against the acetyltransferase and transcriptional coactivator p300 protein predicts the presence of active enhancers with high accuracy, more p300 binding referring to higher transcriptional activity of a target gene (40). p300 has a dual role in the regulation of gene expression: as a chromatin-modification enzyme it can regulate chromatin accessibility, and by recruiting transcription factors it can help in the assembling of the transcriptional machinery. Further, genes determining cell identity are associated with super-enhancers, which are marked by exceptionally high p300 binding. This feature is useful for identifying genes that play key roles in defining cell fate. The exceptionally high p300 histone acetylase binding upstream of Furf in gene in mouse Th1 cells (29) referring to the presence of a super-enhancer (SE) at this genomic area appoints Furf in as a critical element of Th1 fate. Indeed, FURIN has previously been reported to regulate IFNy (7) and to be essential for generating pathogen-specific Th1 lymphocytes after Toxoplasma gondii infection (8). However, a lower level of p300 binding was also found in the promoter region of other investigated T helper cell types (Th1, Th2, and Th17) as well as in the SE region of Th2 and Th17 cells. The quantity of p300 binding can be decisive in defining the rate of RNA transcription, and two other binding sites with lower p300 load were found upstream of the PRO and downstream of the SE area in Th1 and Th2 cells. Understanding the cell-specific regulation of Furf in expression would benefit from the functional evaluation of the other two putative enhancer sites in the future.

In this work, we examined the regulatory role of the genomic regions appointed by high levels of p300 binding in mouse Th cells by generating cell clones with complete deletion of these sequences in Furf in promoter region (EL-4 PRO) or ca. 20 kb upstream Furf in (EL-4 SE) and comparing Furf in expression to that of the wild type (EL-4 “empty”) cells. Surprisingly, deletion of the p300 binding sequence in the promoter region has not caused any changes of Furf in expression in either steady-state or following stimulation although analysis of this sequence revealed putative binding sites for different transcription factors (such as EVI-1, HAND-1, PAIX4, and c-REL, data not shown). Notably, the p300 peak present in the PRO region in primary mouse Th cells does not overlap with the reported Furf in promoter sequences but it is rather situated between the inducible P1 and the housekeeping-like P1A and P1B promoters.

On the other hand, deletion of the SE region led to significant reduction in stimulation-dependent upregulation of Furf in expression while, in steady-state, Furf in expression levels were similarly low in all three types of EL-4 cell clones. The importance of the SE region in EL-4 cells was underlined by the fact that this genomic region could be deleted referring to an open—thus accessible—chromatin state in this cell type. In contrast, targeting the same sequence in NIH 3T3 cells proved to be unsuccessful (data not shown) suggesting that in fibroblast cells the SE area can be closed, and as such, not accessible for the activity of Cas9 endonuclease. In line with the ubiquitous expression of Furf in, the PRO region could be deleted in both EL-4 and NIH 3T3 cells (data not shown). The deletion of the SE sequence caused a significant decrease but not complete lack of Furf in mRNA in stimulated cells, allowing the analysis of cellular processes which are dependent on dose-dependent Furf in expression. For example, although Furf in deficiency in mouse T cells was associated with increased T cell numbers (6), decrease in FURIN level seems not to influence cell proliferation and survival. However, it is important to note that the EL-4 cell line model might not fully reflect the true role of the SE region and it will be thus important to replicate our findings in more physiological settings, ideally by generating an SE targeted mouse strain.

While in T cells complete lack of FURIN results in differential expressions of several genes (9), the deletion of the putative SE area affected only a restricted set of genes. These included genes that are in close genomic distance to the putative SE, reported and predicted FURIN targets, and IFNy signature genes. The regulatory effect of SE on Fes can be explained by the close genomic distance of Furf in and Fes rendering these two genes to the same genomic regulatory domain. However, Blm, upstream of Furf in, and in a similar (shorter) distance from the SE region as Fes (ca. 26 vs. 29 kb), was not differently expressed in stimulated SE and “empty” EL-4 clones although enhancers are able to activate gene expression independently of their orientation (78). The fact that FURIN target proteins are among the differentially expressed genes is in keeping with the observation that the expression of FURIN and other proroprotein convertase enzyme genes are often co-regulated with their cognate target molecules (79).

The putative super-enhancer influenced the expression of several genes, which are known to be regulators of or being regulated by IFNy. ELISA experiments demonstrated that IFNy was produced at lower levels by SE-deleted EL-4 clones compared to the wild type “empty” EL-4 cells. This finding is in accordance with the presence of the investigated super-enhancer in Th1 cells: super-enhancers are thought to regulate genes which are decisive for cell identity. Interestingly, complete deletion of Furf in T cells seems to have wider effects on cytokine production than SE-deletion in EL-4 cells. For example, while Furf in KO T cells produce elevated levels of IL-2 in steady-state but have impaired IL-2 responses upon T cell stimulus (9), SE-dependent reduction in Furf in expression does not influence IL-2 production in EL-4 cells indicating a dose-dependent role for Furf in in IL-2 regulation. TNFα and IL-17 productions remained unaffected by FURIN levels which is in accordance with earlier findings in FURIN cKO mice (6). Collectively, these data indicate that Furf in may have an expression level dependent mode of action on T cell proliferation, activation and T helper cell polarization.

To understand the mechanism by which the SE region regulates Furf in expression, we investigated the possibility of gene regulation via alternative RNA splicing. T cell activation by PMA/ionomycin stimulation caused more RNA fragments aligning to Furf in gene. Similarly to this finding, phorbol esters were reported to enhance Furf in promoter activity in neuronal cells (80). However, Furf in mRNA splicing profiles were intact in SE-deleted EL-4 clones, which suggests that RNA splicing is independent of the SE region. In addition, we could not detect significant difference in the transcription of the lncRNA gene Gm44851 either in steady-state or following stimulation. We also noticed eRNA transcription at the putative super-enhancer
site in “empty” EL-4 clones proving the enhancer activity of this genomic region. Upon stimulation, the amount of RNA reads aligning to this area increased suggesting elevated enhancer activity.

However, deletion of the SE sequence or PMA/ionomycin stimulation did not have a clear effect on the transcription of the earlier reported Furin enhancer, M10. The SE region includes target sequences for a different set of transcription factors (e.g., OCT1, PAX-6, NKK2-5, and HNF-1). The regulatory role of these transcription factors on Furin expression or in Th cell fate remains yet to be established. Notably, the SE region also includes a putative binding site for T-bet (GSM949921), a key regulator of Th1 polarization. Deletion of SE region can thus potentially disturb Th polarization (81) also by diminishing the T-bet dependent Th1 polarization events.

Our data demonstrate that the predicted super-enhancer indeed regulates Furin expression in stimulated mouse T cells. There are also reported super-enhancers upstream Furin gene in human cells, cell lines (CD4+CD25-Il17+ Th17, K562, Ly4, HeLa) and organs (liver, tonsils, small intestine, pancreatic islets and adipose nuclei) according to the super-enhancer database, dbSuper (82). Single nucleotide polymorphisms (SNPs) in the reported genomic regions are associated with stroke [rs4932370 (83)], blood pressure variation [rs4932371 (84)], rs8029440 (85)], and asthma [rs8029440 (86), rs74874915 (87)]. Super-enhancers are emerging targets of therapies where cell-type specific gene regulation is impaired, such as in cancers (23, 88) or autoimmune diseases (28, 89, 90). Targeting the super-enhancer would enable to influence Furin expression only in selected conditions when the super-enhancer is in an active state.

FURIN has recently emerged as a potential target in infectious diseases and cancer. Especially, as the critical determinant of SARS-CoV-2 infectivity—the spike protein, is now known to be processed by FURIN, inhibitors may have clinical value in harnessing this global epidemic (91). Upregulated FURIN level/activity can have both beneficial and pathological effects in malignant diseases. FURIN level is elevated in various tumor cell lines and primary tumors (11), and FURIN activity promotes many cancer-related processes, such as cell proliferation, migration or vascularization, for example (11). It is thus not surprising that FURIN inhibitors are suggested in the treatment of various cancers and to prevent infections (92). On the other hand, Furin overexpression is not a common feature of diseases associated with Furin: for example, Furin is downregulated in prostate cancer (93) and Furin mRNA and protein levels are significantly decreased in the brain of Alzheimer’s disease patients (94). Additionally, in hepatocellular carcinoma, Furin overexpression associates with better prognosis (95).

In sum, our understanding on the regulation of Furin and its importance remains still incomplete. However, it will be critical to continue work on the subject as better understanding of this matter may potentially have clinical value in understanding the pathogenesis and treatment strategies for a wide range of detrimental diseases.

DATA AVAILABILITY STATEMENT
The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE158456.

AUTHOR CONTRIBUTIONS
ZO organized experiments and participated in generating EL-4 clones, carried out and analyzed RNA sequencing, carried out ELISA, and drafted the manuscript. AG and ML designed and cloned gRNAs. MK-A transfected EL-4 cells and carried out cell viability assays, determined alive/dead cell counts, carried out qRT PCR. IH generated EL-4 cell clones. MP supervised research and reviewed data and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.630389/full#supplementary-material

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Conflict of Interest: MP was affiliated with Fimlab Ltd without employment.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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