**Supplementary Information**

# CRISPRi-mediated functional analysis of NKX2-1-binding sites in the lung

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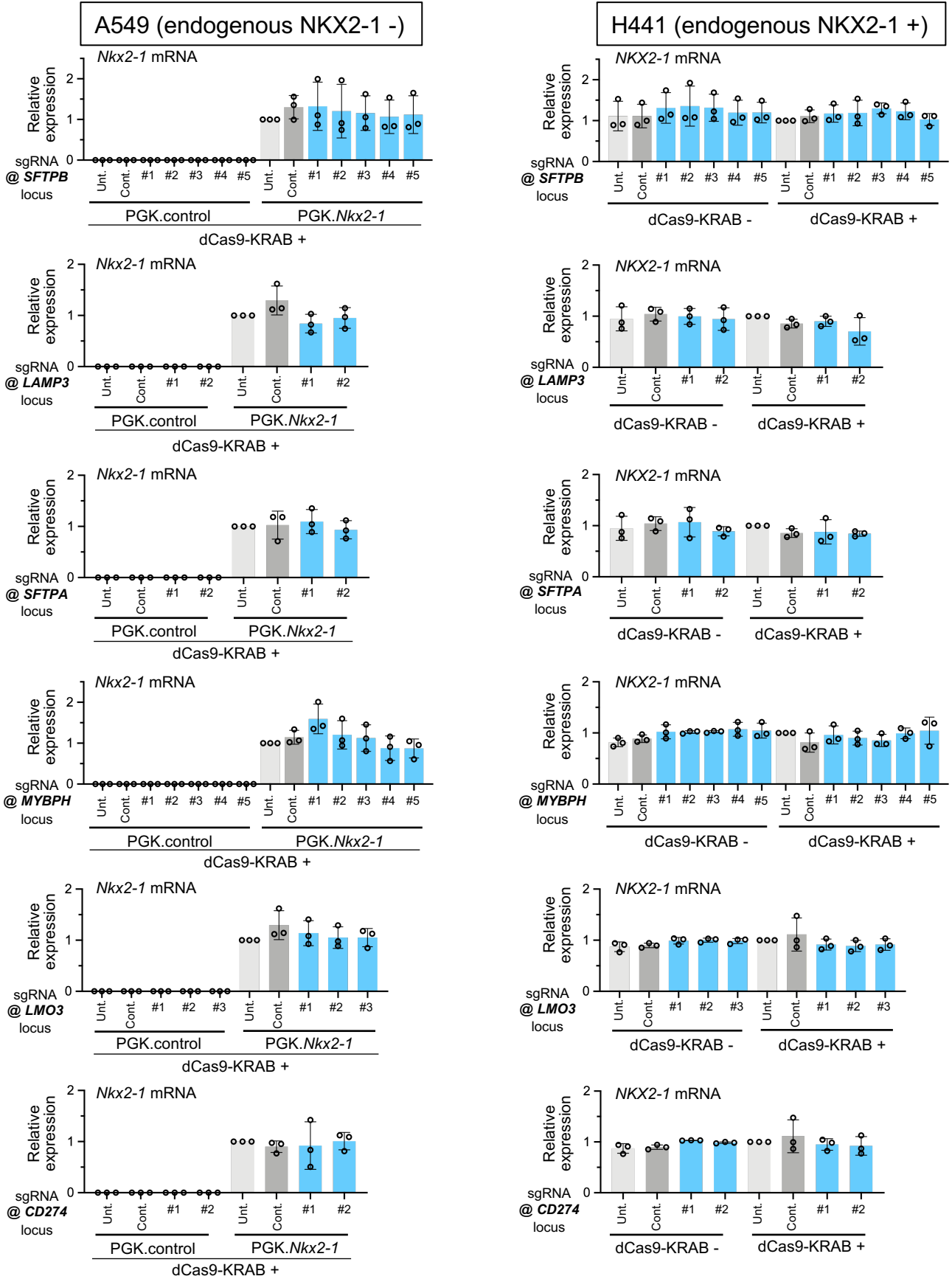
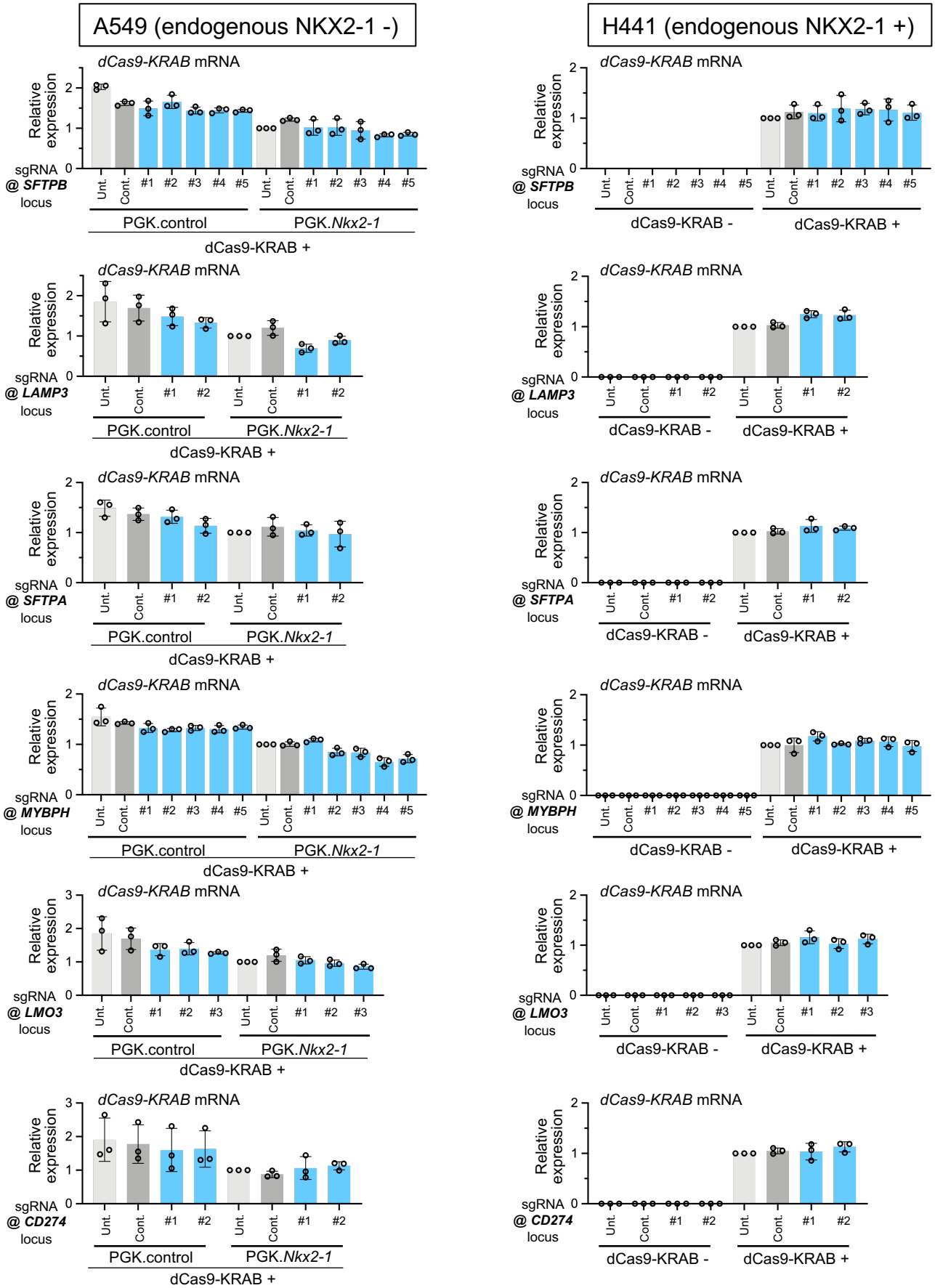



**Supplementary Fig. 1. The expression of *Nkx2-1/NKX2-1* is not altered by synthetic sgRNAs in the presence of dCas9-KRAB in A549 and H441 lung epithelial cells**

Shown is gene expression of rat *Nkx2-1* in A549 cells and human *NKX2-1* in H441 cells, both of which stably express dCas9-KRAB. A549 cells expressing dCas9-KRAB, which we previously created (1), were infected with lentivirus carrying rat *Nkx2-1* (PGK.*Nkx2-1*) or empty (PGK.control). Likewise, H441 cells expressing dCas9-KRAB were created. TaqMan gene expression analysis was performed using mRNA from these cells as described in Methods. The expression of *Nkx2-1*/*NKX2-1* was not influenced by indicated synthetic sgRNAs. Results are expressed as the mean ± SD of the triplicates for each group. Unt., untransfected. Cont., non-targeted control sgRNA.




**Supplementary Fig. 2. The expression of *dCas9-KRAB* is not altered by synthetic sgRNAs in A549 and H441 lung epithelial cells**
Shown is gene expression of *dCas9-KRAB* in A549 cells stably expressing *dCas9-KRAB* with or without *Nkx2-1* and H441 cells stably expressing *dCas9-KRAB*. TaqMan gene expression analysis was performed using mRNA from these cells as described in Methods. *dCas9-KRAB* expression was not influenced by indicated synthetic sgRNAs. Results are expressed as the mean ± SD of the triplicates for each group. Unt., untransfected. Cont., non-targeted control sgRNA.

**Supplementary Fig. 3. sgRNA target sequences for regions that harbor NKX2-1-binding sites**

NKX2-1-binding motif: **cttg** or **caag**
sgRNA target sequence: xxxxxxxxxxxxxxxxxxxx
PAM sequence: xxx
SNP: x

*SFTPB* locus

Upstream region #1

cctcatcatgcccctcaggactcca**cttg**aggcctcttacttcagggagagac**caag**aag

First intronic region #2

SNP rs3024791 (C/T or G/A): g

gtaaggaggtggtgagagtgtcctgggtctgcccttccaggg**cttg**ccctgggttaagagccaggcaggaagctct**c**
**aag**agcattgct**caa**gagtagaggggcctgggaggcccagggaggggatgggaggggaacacccaggctgccccca
accagatgccctccaccctcctcaacctccctcccacggcctggagaggtgggaccaggtatggagg**cttg**agagcc
cctggttggaggaagcca**caag**tccaggaacatgggagtctgggcaggggcaaaggaggcaggaacaggccatcag
ccaggacaggtggtaaggcaggcaggagtgttcctgctgggaaaaggtgggat**caag**cacctggagggctcttcaga
gcaaagacaaacactgaggtcgctgccactcctac

Ninth intronic region #3

gctgagtgagcaccagg**caag**ccttcagaggcctggttatctaccaggagatgagtaatgctagggccagtt**caag**c
caggaaagggactagccttctctccagggtcctgatccctttactgcccccacactcct**caag**gtgtgactcactca
ggacaaacccatggcaaaaggagagggctgga**cttg**aaggtcctagggcc**cttg**ccaatactcagtcaatgacagg
aaattccctttttttttttttttttttttttttgagatggagttttgct**cttg**ttgcccaggctggagtgcaatgg
cacaat**cttg**gctcactgcaacctctgcctccgggttcaggcgattctcctgcctcagcct**cttg**agtagctgggat
tacaggcatgtgctaccaggcccggctaatttttg

Proximal downstream region #4

gtaagagcccacggttggggatgaatctgtgacagatttagtggat**cttg**agatgagcagaaatcagatgtgtagca
gtgatggca**caag**aagatgatgatcagg**caag**acgagagacactccagagagacctggatggccagggaatgcttag
tccccagtgcctctcctcaccgcagggcaatctgtaaatcattcccagggaaaggggaaagaggattcagtggttag
tcagc**caag**

Distal downstream region #5

cctgtaatcccagtactttaggaggccgaggcgggcagatca**cttg**agctcagaagtttgaggccagcctgggcaac
atggtgaaacaccaccactacaaaaaaaatcaaaacaattagcccagtgtagtggtg**caag**cctgtgggcccagcta
ctcaggaggctgaggtgggaggatgg**cttg**agcccaggaggcggagattgcagtgagc**caag**atcatgccactacac
tccagcctg
ggcgacagtgccagaccctgtctcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaagtagaaacttcctgataaattag
gctagcagctgaggatttca**cttg**ctatcctaagagccagtgaagtgtctttaagcatgagatcagagtatgggggga
gaaatgactcaggtttgtgttttaaaagattactctgactaaagcatggataaactgggggtaggaggcaaagaaat
gtggagagacctattgagagcactgcaaacagaaactggcaatagcttaaactaaaatgatagtggcaaagggccag
tacagaagtctgatggat**cttg**gagaacgaccgtggactatcgtaaacttaa

*LAMP3* locus

Third intronic region #1

ctccttctgtgcagcccaaaaagggcttcagggcctcttatgggaga**cttg**acctctggggaacgtaaccacccgtg
ccctttgcaaaccagccaaaggaggaagttgtgcagatgaata

Fifth intronic region #2

gacaatctgatatgaggacccgggagttccccaaagagactcgggcttaacagcaagag**cttg**gctttggagagctg
tgatctccttccacctcctccttcaaactggcacctgctccaggctcccctgtgtccagtgttaagggcactcgaaa
gttctgtagggcagagga**caag**gtgtttattcaccagccactcactcttctgcagaatgattaatgtaagatttacc
ttctagcatgtggaaatttctcccctagcagatgaaa**caag**aagcagaatttaaaat**caag**tatagagg

*SFTPA* (*SFTPA1* and *SFTPA2*) locus

Proximal upstream region #1

gtgcctgtgtaagggctctcggctttcactgctaaggag**cttg**ctaggggcagggtgggtgacctcagc**caag**gggg
ggatttctcttcttacagacctggagttcctctttcgcaggttctgtgctcccct**caag**ggtccttgagatcctcc
agcctgagtgct**cttg**gggaaacatg

Distal upstream region #2

aggtggggagtggggtgggtcacttccctgtgactctgc**cttg**tgataggcattttggcttct**caag**ggtcctcacc
**cttg**tgtactcatgtcatcaggccctgtccagcggccctcccggttcccattcaggggcctgccaggcac**caag**ag
gtgcttccgtggtaaagaagatccctct**caag**gctgtgtctcctgatgccattgacacaatgttgaagagcccatag
gcccagagg

*MYBPH* locus

Far distal upstream region #1

cgggactggagtccctgcagagtg**cttg**agagatggccctccagcctcccagtgaggggcagccgattac**cttg**gaa
gcaacccattccgtggctgggctgaaatctgtcaatagtcctagtgctgggcttagccctttgttctgttcacccct
cccctaacactcatccctccacccattcagagatcaaagtacctctgct**caag**ctggcctgagataggaggtcggga
ggatgtgat

Distal upstream region #2

cccaccccaccacacaggcatctttgaaaccccagggctgcaagcc**cttg**tgggcagccaactgccctcccctgggg
ccacccttcccccagccggggacaggctctccagggctgactcagtgtcctgtggggttagtcacattcacttatag
ctgcagagtcagaggc**caag**gatggagatccagtctccaggacccactccctgcctgg**caag**acgtggagtcaccta
gcagagggc

Proximal upstream region #3

agatggatgaggaagtgactcct**caag**gggcc**cttg**tggttcctcccccagagctattcctggcctgggcgcctctc
caccctccagtcctcctg**cttg**acctgaccccacagctgggaa

Third intronic region #4

ggggccctgtccaggatc**cttg**gctgctcagctgtctctc**cttg**gccacctggagctgc**caag**catt**cttg**accccc
tgcagctgtccctagagcattccatgcctgagctcccagagc**caag**tgcccactcagctaatcctcagagacaccgt
gttccttctcactgt**cttg**tcctcct

Sixth intronic region #5

acctgactccctaggaggcccttag**caag**actcttcctctctggacctcagatgcctcctgaagactgtgaagaggg
ctggcccgggctgtctgaagcctttt**caag**ttctggagccatgcttctcaaatcttaatgtccacatgagtcacctgg
ggag**cttg**ctaagagg cagagtctga

*LMO3* locus

Second intronic region #1

ttgaaac**caag**ccctaa**cttg**ttgaggctc**cttg**accctactgaacatcagcctgccatc

Second intronic region #2

tttatagttttattctatgccaaaaatag**cttg**ttaggaatataaaagaaattaataaagagtagtaatacaattat
taaaatatatatgctttagagttcagcattcatatgaagaaatctttttaaaggaaaactctgctgaaaaaaaagtc
ctctatttttctgttcttccctgctagataagtatcacagaaaaatctcaatgagactgatggtaagcctgttataa
tagcaatggaattttgagtgtttgttttaacattgtgaaagaaagtcaacagaatgaaactcagatcatggcaaata
ttt**cttg**aggatttactctataatgggtagtgttataggaactgaggctccagcagttaaaaaaatggacagaaata
tttgcctttatgtatcttatattttagtattatttttcaggtattctcaatgagttatatatgtgctaagtgctgta
cagatgtaatctcctctagcc**cttg**ggaactacagtattattaaacccattttagagaagaaggcactgaggctgag
g

Second intronic region #3

a**caag**cactacaaa**caag**aattta**caag**gatctccagcttaaatggaaaagtggatctatgataactttataaacga
gtgtgggagtaaagagtgcaggaggaagaggtta**caag**aattat

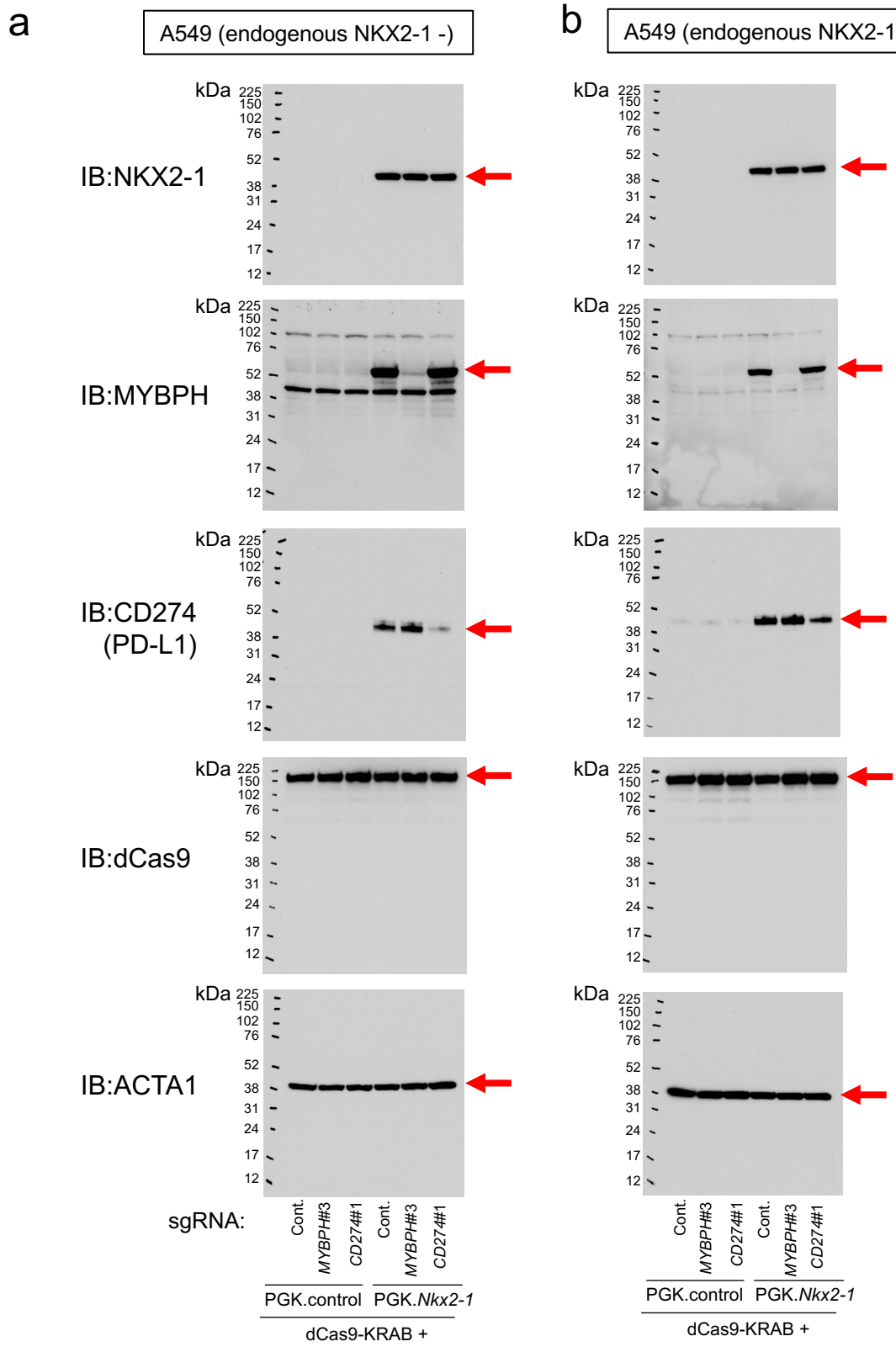
*CD274/PD-L1* locus

Upstream region #1

aagat**caag**aacatttactggaaattgctccttcaccaggaatttgctcacatctcttcaggtccacttataagat**c**
**ttg**aaatcagtcctcctgagatcagtacaaacgaggatcatctctcctgctttagaagcaaaagtggcaagcagggt
agacagaggcagaaggaaggatggta

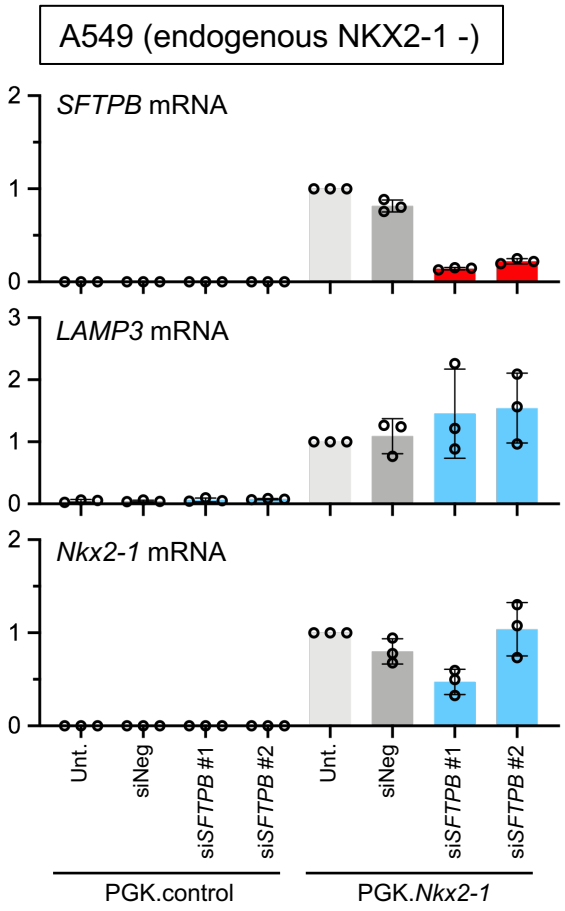
First intronic region #2

ggtgctcaatcagtgtttgctaaacgaaataattagtcacattt**caag**caggatgactaaatgaagaatagaatcta
ggcagatactctggaagagtggctgtgagtcattcatatctta



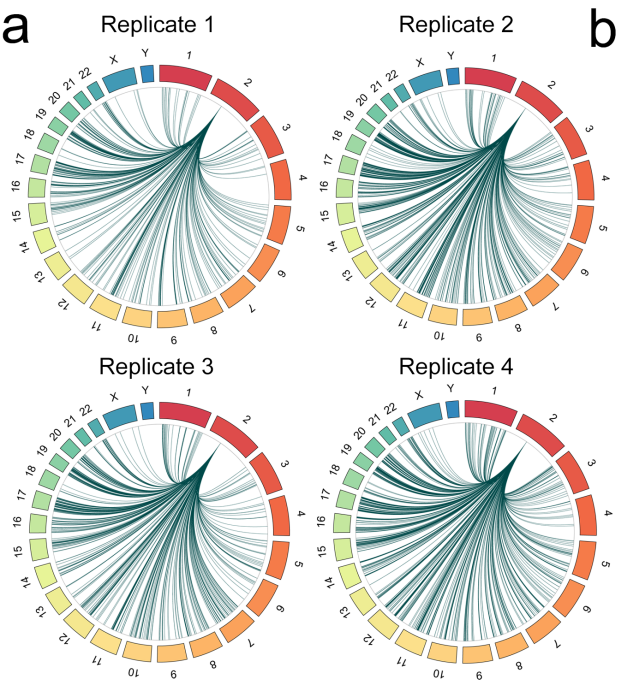

**Supplementary Fig. 4. CRISPRi targeting NKX2-1-binding sites, including ones at loci of *MYBPH* and *CD274*, represses the expression of MYBPH and CD274 at the protein level in A549 lung epithelial cells that express NKX2-1**

Shown are immunoblots of indicated proteins using protein extracts from A549 cells with or without NKX2-1 (PGK.rat *Nkx2-1* or PGK.control) that were performed independently twice (a and b). Red arrows indicate protein bands targeted by antibodies specific to the proteins. ACTA1 was used as a loading control. SgRNAs used in this analysis are indicated at the bottom. Cont., non-targeted control sgRNA.




**Supplementary Fig. 5. The expression of *SFTPB* does not affect the expression of *LAMP3* in A549 lung epithelial cells that express *Nkx2-1***

Shown is the expression of *Nkx2-1* and its downstream target genes, *SFTPB* and *LAMP3*, in A549 cells with or without *Nkx2-1* (lentiviral PGK.*Nkx2-1* or PGK.control, respectively), which were transiently transfected with two independent siRNAs targeting endogenous *SFTPB* (si*SFTPB* #1 or si*SFTPB* #2) or non-targeted negative control siRNA (siNeg). Unt. indicates untransfected. The data points are obtained from three independent experiments as described in Methods. Results are expressed as the mean ± SD of the triplicates for each group. Only $P<0.05$ and more than 2-fold suppression are considered significant siRNA-mediated suppression (highlighted in red).




b

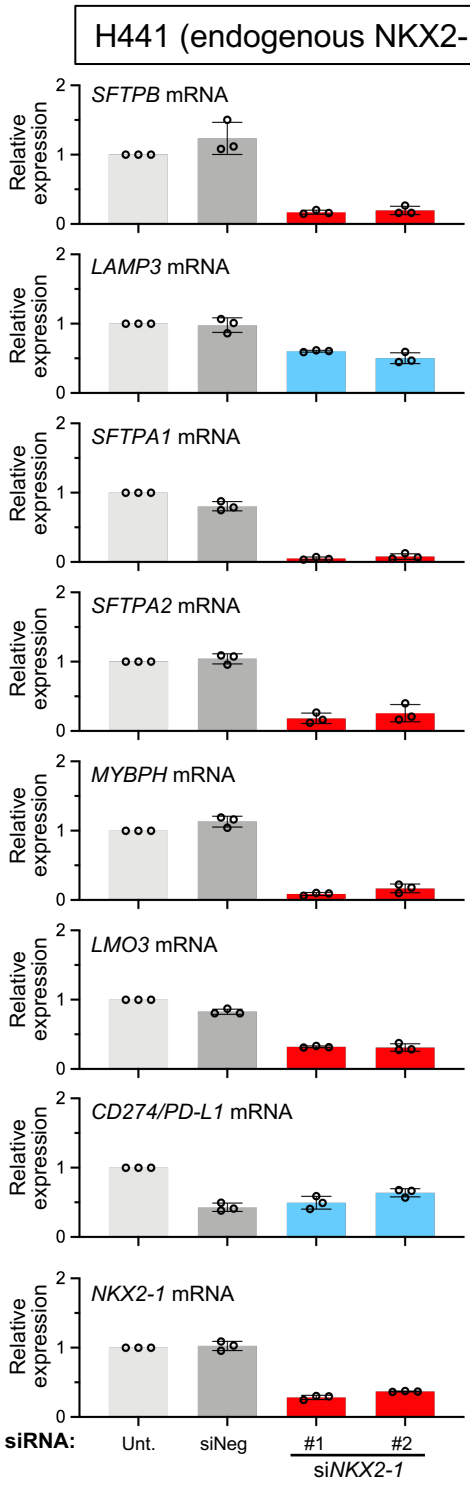
Number of Hi-C interactions with *SFTPB* locus in each chromosome

| Chromosome | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 |
|---|---|---|---|---|
| 1 | 20 | 38 | 31 | 43 |
| 3 | 17 | 22 | 16 | 33 |
| 4 | 3 | 14 | 10 | 12 |
| 5 | 17 | 20 | 23 | 32 |
| 6 | 6 | 17 | 9 | 20 |
| 7 | 24 | 31 | 41 | 37 |
| 8 | 18 | 35 | 31 | 43 |
| 9 | 21 | 42 | 20 | 36 |
| 10 | 15 | 33 | 33 | 37 |
| 11 | 27 | 47 | 27 | 51 |
| 12 | 15 | 62 | 42 | 46 |
| 13 | 4 | 9 | 8 | 16 |
| 14 | 11 | 29 | 20 | 33 |
| 15 | 18 | 40 | 27 | 35 |
| 16 | 33 | 58 | 43 | 44 |
| 17 | 64 | 109 | 84 | 108 |
| 18 | 3 | 5 | 4 | 13 |
| 19 | 48 | 91 | 63 | 76 |
| 20 | 24 | 47 | 39 | 50 |
| 21 | 5 | 2 | 4 | 9 |
| 22 | 10 | 12 | 15 | 16 |
| X | 4 | 6 | 4 | 5 |
| Y | 0 | 0 | 0 | 0 |

**Supplementary Fig. 6. The locus of human *SFTPB* on chromosome 2 interacts with genomic regions located on different chromosomes in A549 lung epithelial cells**

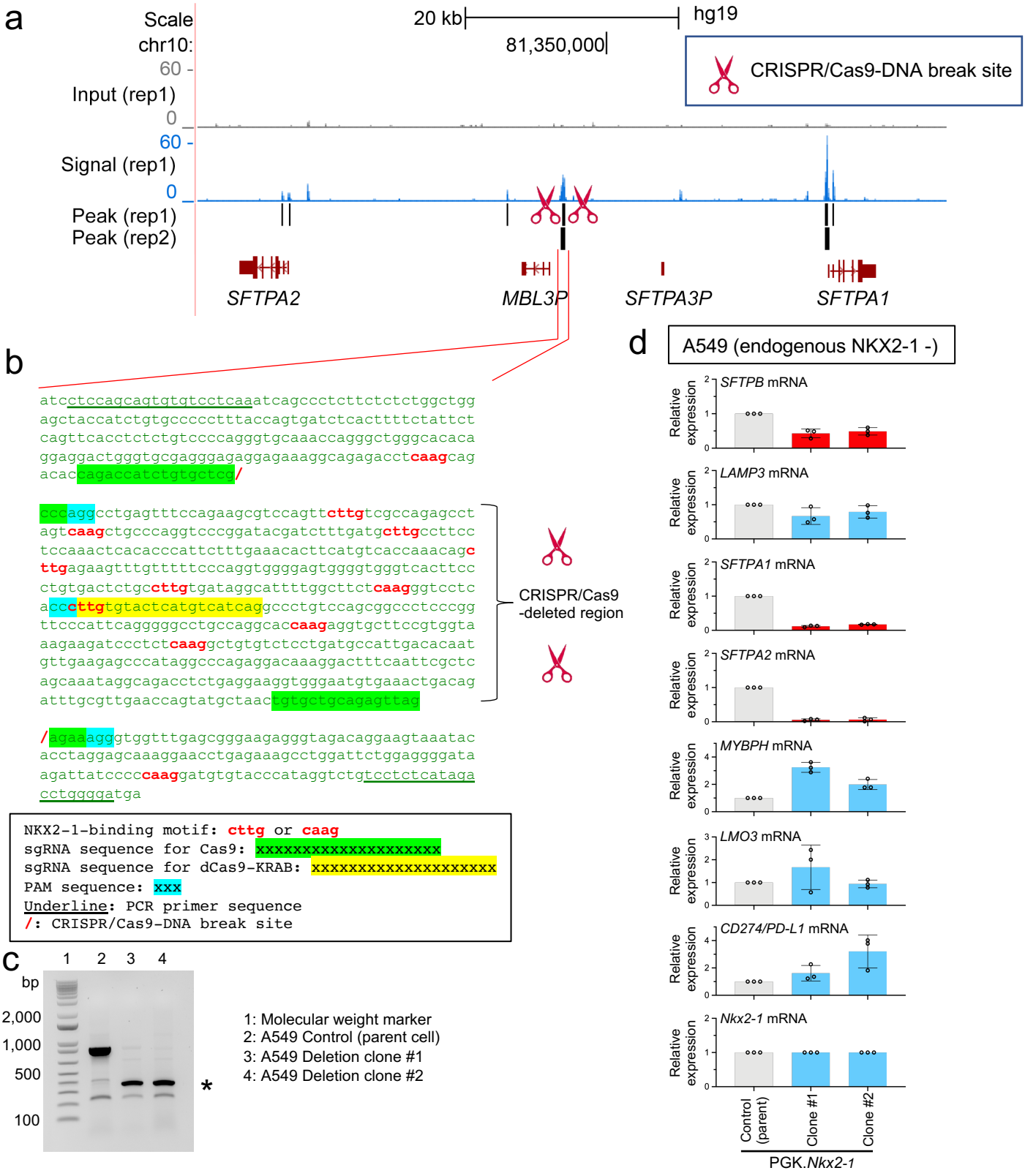
**a.** Shown are Hi-C data of four isogenic replicates that indicate the interaction of the *SFTPB* locus with genomic regions located on different chromosomes. Hi-C data were obtained from ENCODE.

**b.** Shown are distributions of inter-chromosomal interactions in individual Hi-C data replicates. Inter-chromosomal interactions with the *SFTPB* locus (including 10 kb upstream of transcription start site and 10 kb downstream of transcription end site) were extracted at the resolution of 50 kb. Interactions with at least 2 observed counts were included in the analysis.




**Supplementary Fig. 7. SiRNA-mediated suppression of *NKX2-1* indicates that NKX2-1 is required for the expression of *SFTPB*, *SFTPA1*, *SFTPA2*, *MYBPH* and *LMO3* in H441 lung epithelial cells**

Shown is the expression of endogenous *NKX2-1* and genes in H441 cells, which are induced by rat *Nkx2-1* in A549 lung epithelial cells, transfected with two independent siRNAs (#1 and #2) targeting endogenous *NKX2-1*. siNeg is a negative control siRNA and Unt. indicates untransfected. The data points are obtained from three independent experiments as described in Methods. Of note, the expression of *LAMP3* and *CD274/PD-L1* is regulated by NKX2-1 in A549 cells but not in H441 cells. Results are expressed as the mean ± SD of the triplicates for each group. Only $P<0.05$ and more than 2-fold suppression are considered significant siRNA-mediated suppression (highlighted in red).



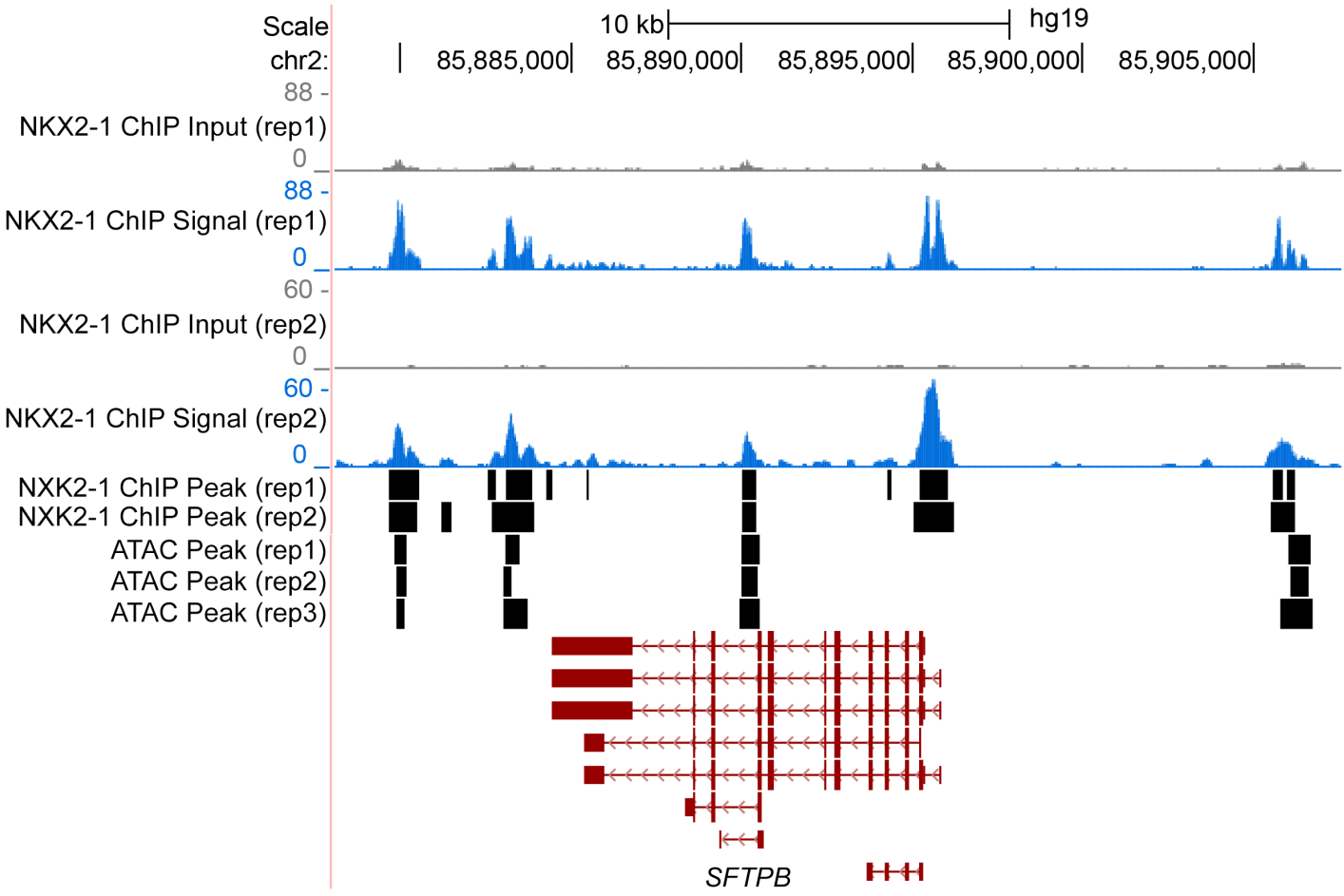

**Supplementary Fig. 8. CRISPR/Cas9-mediated deletion of an NKX2-1-binding region located at an intergenic region between *SFTPA1* and *SFTPA2* represses the expression of *SFTPA1* and *SFTPA2* in A549 lung epithelial cells**

**a.** Shown is ChIP-seq data indicating that NKX2-1 binds to an intergenic region of human *SFTPA1* and *SFTPA2*. Significant ChIP-seq peaks are described as in Fig. 5a. *MBL3P* and *SFTPA3P* are pseudogenes. The region of CRISPR/Cas9-mediated deletion is indicated by two scissors.

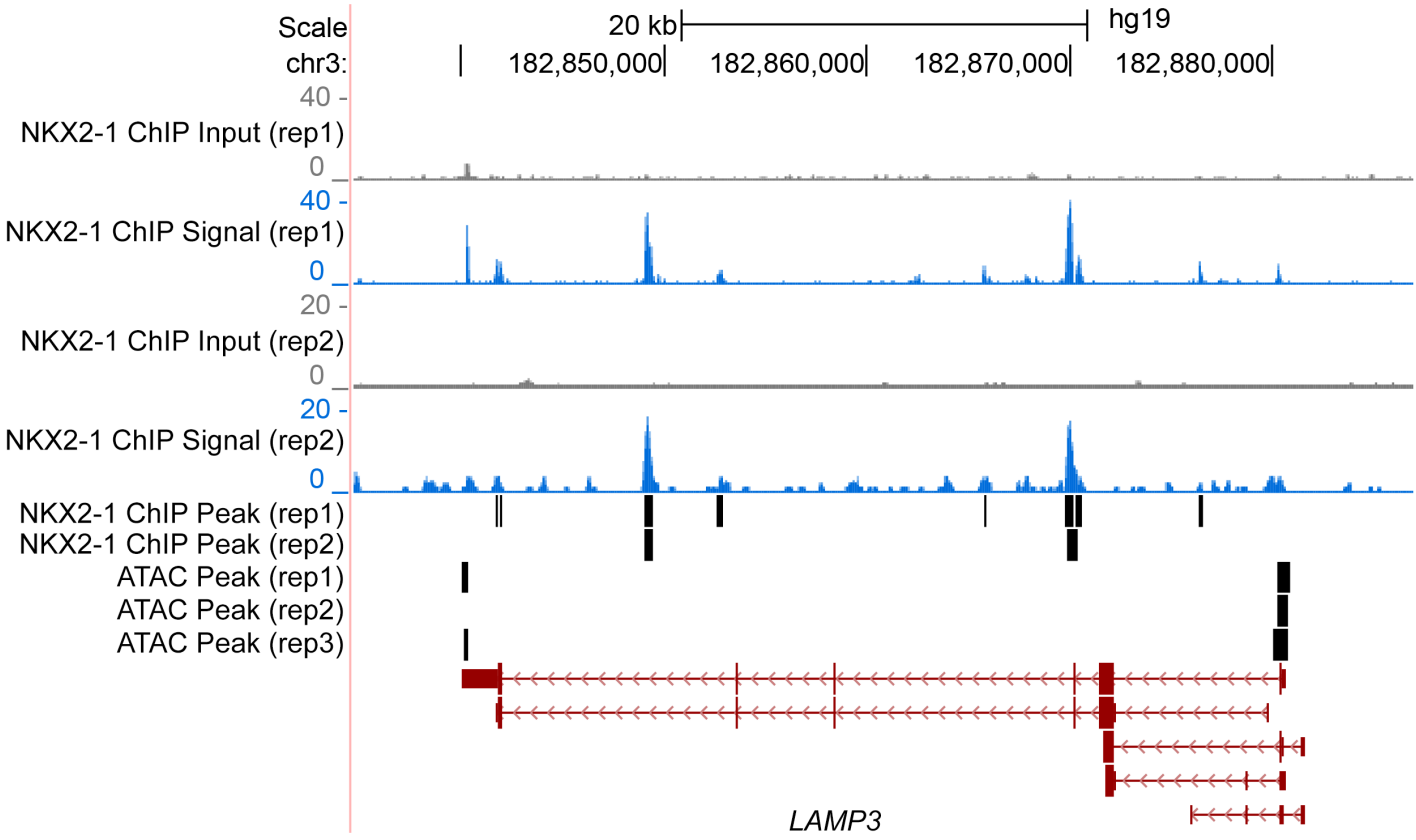
**b.** Shown is the sequence of genomic DNA that is deleted by CRISPR/Cas9. SgRNA sequence information is indicated.

**c.** Deletion was confirmed by PCR amplification using primers described in b. * indicates the deletion. Two independent clones were obtained as described in Methods.

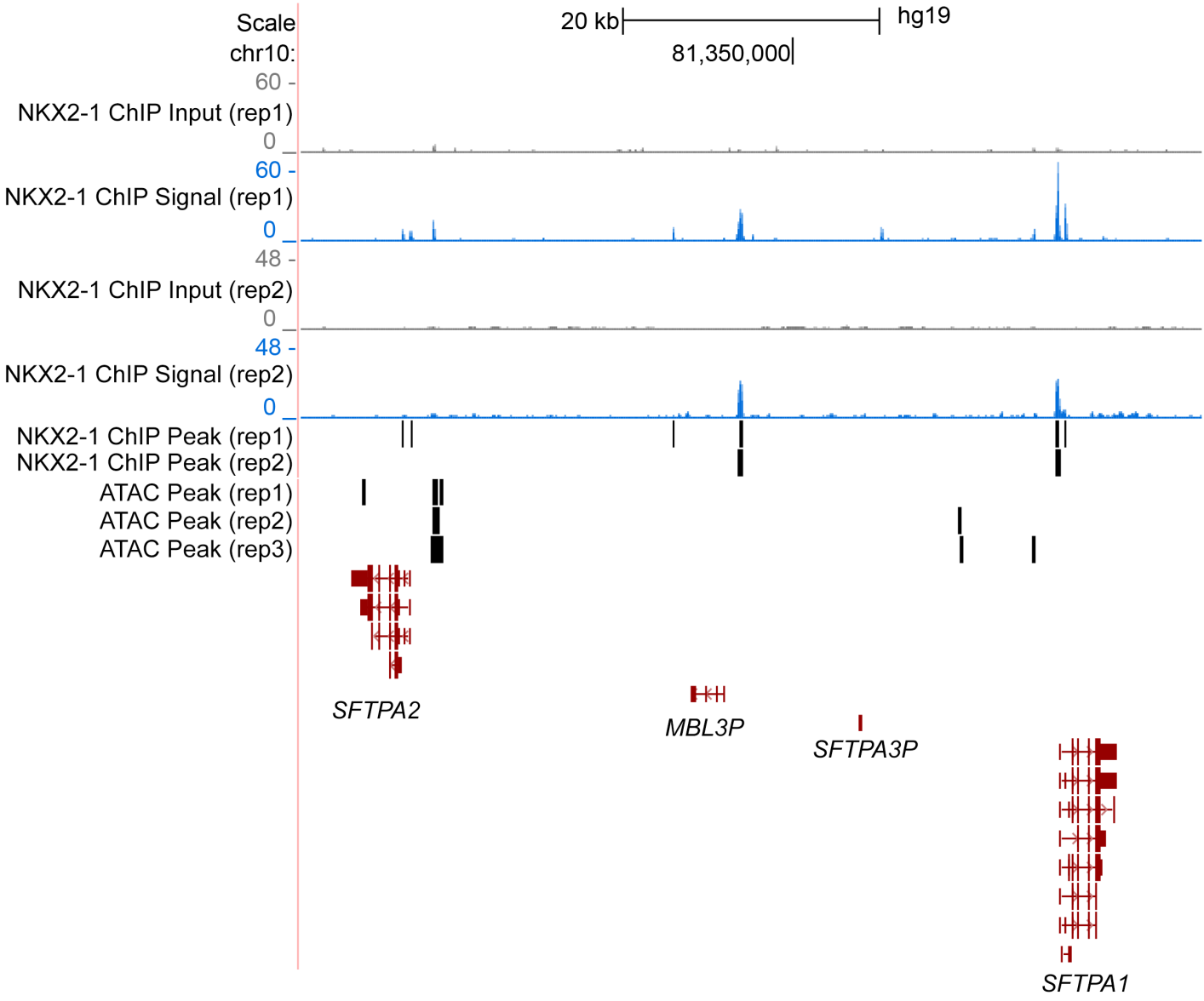
**d.** Shown is gene expression analyzed as described in Fig. 2b using mRNA obtained from A549 cells (described in c) that were infected with lentivirus carrying rat *Nkx2-1*. Expression was normalized to the expression of *GAPDH* and *Nkx2-1*. Statistical significance was determined using Student's *t*-test with Welch's correction for unequal variances. Results are expressed as the mean ± SD of the triplicates for each group. Only $P<0.05$ and more than 2-fold suppression are considered significant (highlighted in red).



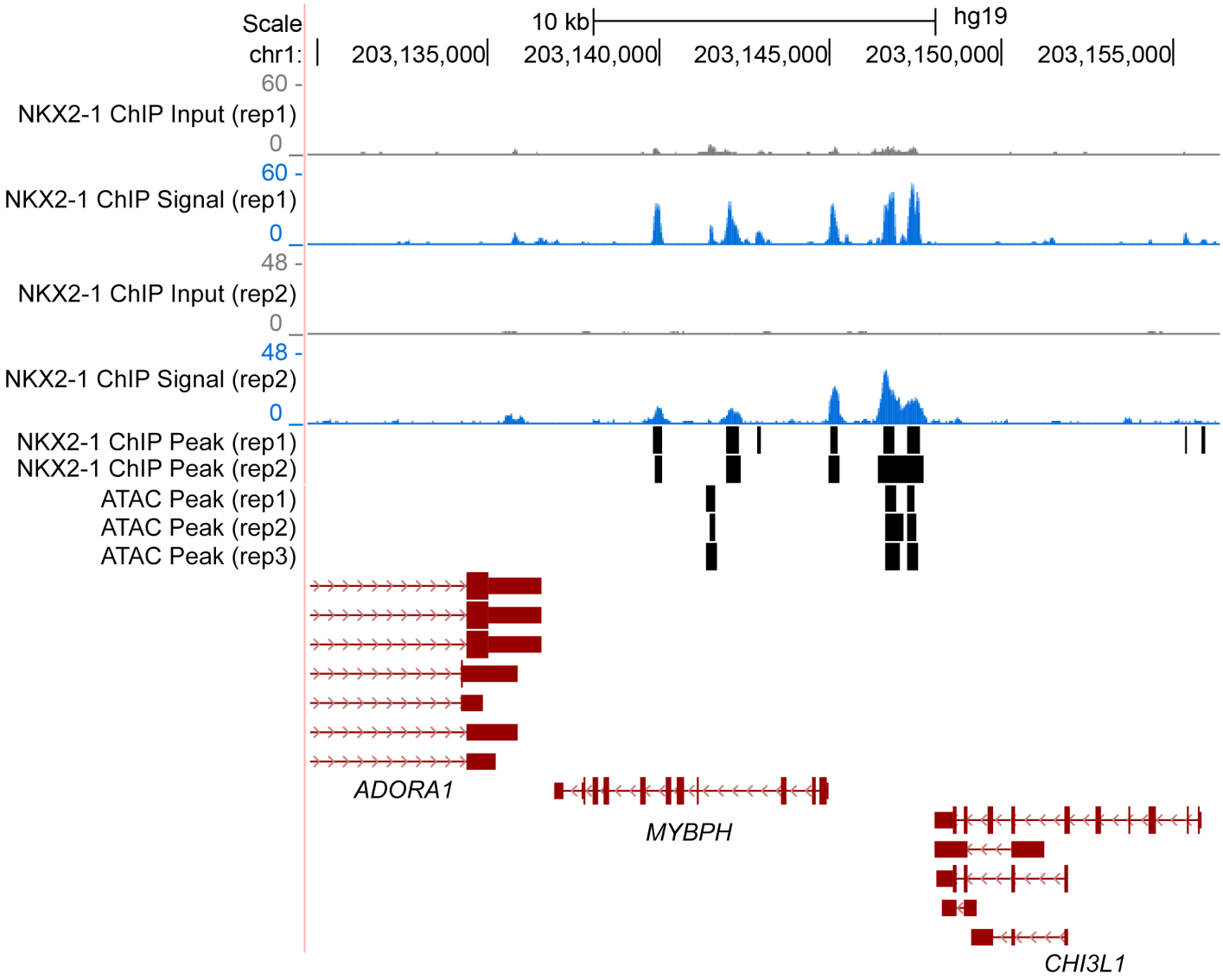

**Supplementary Fig. 9. NKX2-1-binding sites at the human *SFTPB* locus were identified by two independent ChIP-seq analyses**
Shown are ChIP-seq and ATAC-seq data from two or three independent experiments along with different isoforms of *SFTPB* as described in Fig. 2a.



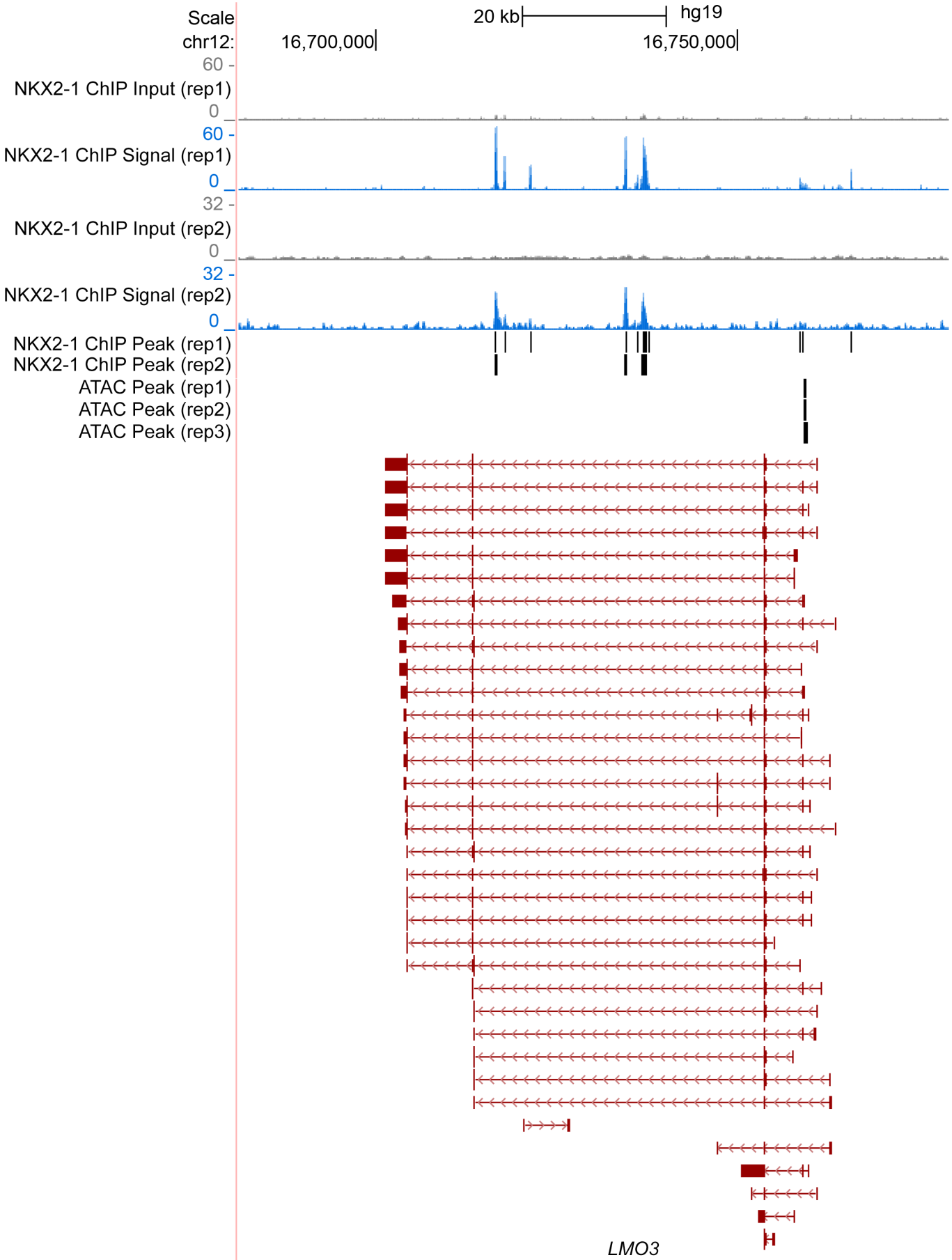

**Supplementary Fig. 10. NKX2-1-binding sites at the human *LAMP3* locus were identified by two independent ChIP-seq analyses**
Shown are ChIP-seq and ATAC-seq data from two or three independent experiments along with different isoforms of *LAMP3* as described in Fig. 4a.



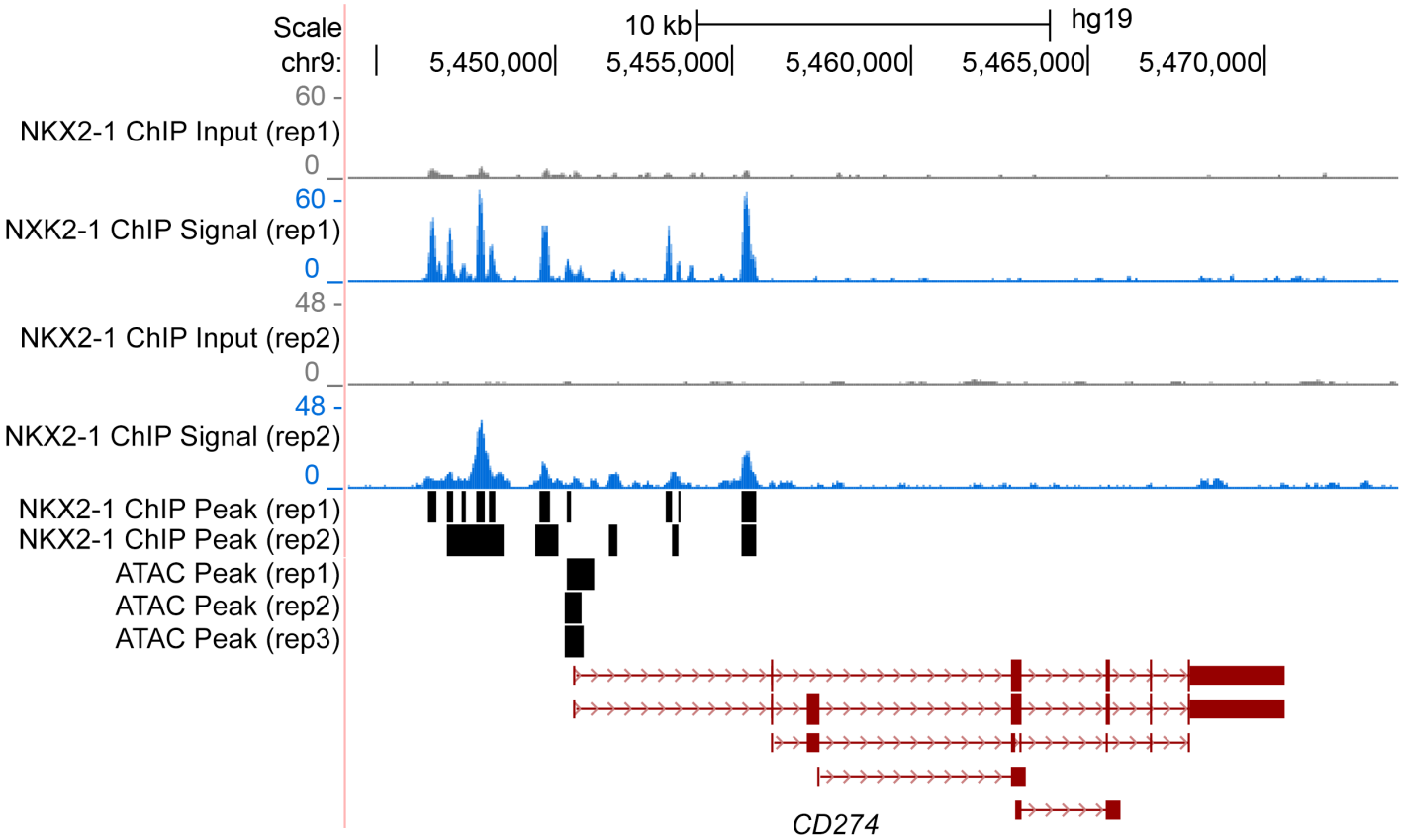

**Supplementary Fig. 11. NKX2-1-binding sites at the human *SFTPA* locus were identified by two independent ChIP-seq analyses**
Shown are ChIP-seq and ATAC-seq data from two or three independent experiments along with different isoforms of *SFTPA1* and *SFTPA2* as described in Fig. 5a. *MBL3P* and *SFTPA3P* are pseudogenes.




**Supplementary Fig. 12. NKX2-1-binding sites at the human *MYBPH* locus were identified by two independent ChIP-seq analyses**
Shown are ChIP-seq and ATAC-seq data from two or three independent experiments along with different isoforms of *MYBPH* as described in Fig. 6a.




**Supplementary Fig. 13. NKX2-1-binding sites at the human *LMO3* locus were identified by two independent ChIP-seq analyses**
Shown are ChIP-seq and ATAC-seq data from two or three independent experiments along with different isoforms of *LMO3* as described in Fig. 7a.




**Supplementary Fig. 14. NKX2-1-binding sites at the human *CD274/PD-L1* locus were identified by two independent ChIP-seq analyses**
Shown are ChIP-seq and ATAC-seq data from two or three independent experiments along with different isoforms of *CD274* as described in Fig. 8a.

**Supplementary Reference**

1. Stuart WD, Guo M, Fink-Baldauf IM, Coleman AM, Clancy JP, Mall MA, Lim FY, Brewington JJ, Maeda Y. CRISPRi-mediated functional analysis of lung disease-associated loci at non-coding regions, NAR Genom Bioinform. 2020 June;2(2):lqaa036. doi: 10.1093/nargab/lqaa036.