1 **Loss of 9p21 regulatory hub promotes kidney cancer progression by upregulating HOXB13**

2 Running title: Loss of 9p21 regulatory hub promotes kidney cancer

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23 Abstract

24 Loss of chromosome 9p21 is observed in one-thirds of clear cell renal cell carcinoma (ccRCC) and associated
25 with poorer patient survival. Unexpectedly, 9p21 loss-of-heterozygosity (LOH) does not lead to decreased
26 expression of the 9p21 tumor suppressor genes, *CDKN2A* and *CDKN2B*, suggesting alternative mechanisms of
27 9p-mediated tumorigenesis. Concordantly, CRISPR-mediated 9p21 deletion promotes growth of immortalized
28 human embryonic kidney epithelial cells independently of the *CDKN2A/B* pathway inactivation. The 9p21
29 locus has a highly accessible chromatin structure, suggesting that 9p21 loss might contribute to kidney cancer
30 progression by dysregulating genes distal to the 9p21 locus. We identified several 9p21 regulatory hubs by
31 assessing which of the 9p21-interacting genes are dysregulated in 9p21-deleted kidney cells and ccRCCs. By
32 focusing on the analysis of the *HOXB13* locus, we found that 9p21 loss relieves the *HOXB13* locus, decreasing
33 *HOXB13* methylation and promoting its expression. Upregulation of *HOXB13* facilitates cell growth and is
34 associated with poorer survival of ccRCC patients.

35 **Implications:** The results of our study propose a novel tumor suppressive mechanism based on coordinated
36 expression of physically associated genes, providing a better understanding of the role of chromosomal
37 deletions in cancer.

38 Introduction

39 Chromosomal rearrangements such as copy number variations (CNVs) arise from deletions, duplications,
40 translocations and inversions are characteristics of cancer cells. Expression of genes located within affected
41 chromosomal regions often positively correlates with gene copy number alteration (1). However, genes involved
42 in CNVs not always show a significant change in expression or even an inverse correlation. This could be
43 partially explained by dosage compensation or a buffering effect. Genes flanking but not physically affected by
44 CNVs can also show altered expression profiles due to the abrogation of long-range interactions occurring
45 between a CNV affected region and neighboring genes (2).

46 Multiple reports demonstrated higher order compartmentalization of chromatin into distinct active and inactive
47 chromosome territories (3). A mapping of long-range interactions revealed that cis-interactions frequently occur
48 within active chromosome territories, decreasing proportionally with the genomic distance from their locus
49 control region (4). Furthermore, chromosome interactions are not limited to cis, but can also extend to
50 interactions with alternate chromosomes in trans (4,5). Large-scale chromosomal deletions may disrupt a

51 number of spatial interactions causing differential expression of genes mapped to alternate chromosomes. In
52 fact, there are numerous examples of CNVs affecting the expression of genes situated throughout the whole
53 genome (6). However, the contribution of cancer-associated chromosomal deletions on global gene expression
54 is still unclear. We hypothesized that the deletions could affect gene expression and contribute to tumorigenic
55 transformation by disrupting chromosomal interactions.

56 The 9p deletion is one of the most common alterations in human cancers (7). The most commonly deleted 9p21
57 region includes genes encoding for p16/CDKN2A, p14/CDKN2A, and p15/CDKN2B as well as non-coding
58 RNAs *CDKN2A-AS1* and *CDKN2B-AS1* (*ANRIL*). The 9p tumor suppressor p16/CDKN2A and p14/CDKN2A
59 are critical regulators of cell cycle progression. CDK4/6 inhibition provided by p16/CDKN2A or p15/CDKN2B
60 prevents Retinoblastoma (Rb) phosphorylation, thus retaining the transcription factor E2F1 in complex with Rb
61 and blocking G1-S phase cell cycle transition. Inactivation of the E3 ubiquitin ligase MDM2 by p14ARF leads
62 to stabilization of the tumor suppressor p53 (8). *ANRIL* was implicated in the regulation of *CDKN2A* and
63 *CDKN2B* expression (9). In our study, we examined how dysregulation of genes interacting with the 9p21 locus
64 may contribute to tumor development and progression of clear cell renal cell carcinoma (ccRCC).

65 **Materials & Methods**

66 **TCGA copy number alteration, gene expression, and survival analyses of ccRCCs**

67 To identify the frequency of 9p21 loss in ccRCCs, we downloaded the normalized level 3 Genome Wide SNP
68 6.0 segmented copy number data from the TCGA portal. An in-house perl script parsed copy number segments
69 for each patient across chromosome 9. Patients were stratified according the Log2Ratio of the segment
70 overlapping *CDKN2A*, *CDKN2A-AS1*, *CDKN2B* and *CDKN2A-AS1*. The Log2Ratio stratification levels cutoffs
71 were defined as follows: $-0.1 < WT < 0.1$, homozygous deletion < -1 , and $-1 < LOH \leq -0.1$. RNA-seq read count
72 data for ccRCCs were downloaded from the TCGA. Differential gene expression analysis was performed using
73 the DESeq2 package (10). Genes were considered as differentially expressed with a q-value < 0.05 and a
74 basemean value > 50 . Survival analysis of the TCGA ccRCCs was performed in R using the survival package.
75 Tabulated survival data were run in R generating Kaplan-Meier curves and log-rank comparison.

76 **Circularized Chromosome Conformation Capture (4C)**

77 Viewpoints for the 9p region of interest encompassing *CDKN2A* – *CDKN2B-AS1* were chosen based on
78 several criteria. First, we searched for 9p21 regions enriched in histone marks using the ENCODE ChIP-Seq

79 data of human mammary epithelial cells (HMEC) (Supplementary Fig. S1). To select such regions, we used the
80 ENCODE data available for primary human mammary epithelial cells. We next designed primers based on the
81 presence of cutting sites for the restriction enzymes, CviQI and DpnII, that were used for the 4C analysis. Finally,
82 we ended up with the viewpoints, for which primer design and the coverage along the region of interest were
83 optimal (Supplementary Table S4). 4C-seq analysis was performed to assess the 9p21 interaction profile.
84 Briefly, cells nuclei were cross-linked with formaldehyde and treated with a primary 4-bp restriction
85 endonuclease, DpnII. Ligation of digested DNA was followed by removal of chromatin crosslinks. A final
86 round of digestion was performed using a secondary 4-bp restriction endonuclease, CviQI, trimming the DNA.
87 Finally, ligation of the DNA fragments circular DNA was performed. Captured DNA containing target loci was
88 amplified by inverse PCR, using primers designed for the flanking sequences of each viewpoint, and sent for
89 next-generation sequencing generating approximately 1 million different 50bp single end reads. Primers are
90 listed in Supplementary Table S4.

91 We used PeakC (11) for calling of near-cis interaction and our custom scripts for calling of far-cis (>5 MB from
92 the viewpoint; https://github.com/ZhaoPeiHua/LongRangeInteraction_from_4C-seq) and trans interaction.
93 PeakC was incorporated in the pipeline ‘pipe4C’ (12). The pipeline performed reads demultiplexing, trimming,
94 mapping, and counting in one step directly from FASTQ files. The default settings were used. The pipeline
95 stored the reads mapping statistics as RDS file, which was further processed by the function ‘doPeakC’ from the
96 pipeline for the near-cis peak calling. The far-cis (>5 MB from the viewpoint) and trans interactions were
97 analyzed using our custom scripts. The reads were demultiplexed by the reading primer in the 5’ end. Each PCR
98 primer represented a single 4C experiment. The reading primer was then trimmed from the reads. Primer
99 trimmed-reads were mapped to hg38 assembly by STAR (13), and the multi-mapping reads were excluded from
100 the bam files. A library of restriction fragment ends was constructed based on DpnII restriction sites. The
101 mapped reads that do not start at DpnII restriction sites were discarded. The reads were counted per fragment
102 end, and only fragment ends with at least two mapped reads were considered as an observed one. To correct for
103 PCR artifacts, fragment ends coverages were binarized (observed and non-observed fragment end is set to 1 and
104 0, respectively). To identify significant interactions, a sliding window of 400 fragment ends was used to count
105 observed fragment ends and the whole chromosome was used as background window to count the observed
106 fragment end, a binomial test was applied to test the null-hypothesis that the proportion of observed fragment

ends in a window was below or equal to the background proportion. The regions near (<5 MB) the viewpoint were masked out. P-values were adjusted using the Benjamini-Hochberg procedure. Windows with an adjusted p-value<0.05 were scored as significant interactions. Circos plots for each of the viewpoints significant interactions were plotted using RCircos (version 1.1.2).

Generation of 9p-deleted single cell-derived clones

gRNAs targeting 9p21 were designed using the Zhang lab web resources (<https://zlab.bio/guide-design-resources>). 48 hours after transfection, single GFP-positive (for the Nuclease-deleted cells) or double GFP/RFP-positive cells (for the Nickase-deleted cells) were sorted using FACS (FACSAria III; BD Biosciences). Genomic DNA was extracted from cells using QIAamp DNA Mini kit (Qiaagen). The amplicons encompassing the conjunctive regions of the targeted 9p deletions were amplified by nested PCR using Mytaq polymerase (Gentaur).

Single cells from positive pools were plated into 96-well plates using FACSAria III. Genomic DNA was extracted from single-cell clones using the lysis kit for blood (Sigma-Aldrich). PCR amplification of the purified genomic DNA was performed in 96-well format and analyzed using QIAxcel system (Qiagen). The PCR amplicons were then sequenced by Sanger sequencing. Genomic DNA from each 9p21-deleted single cell clone was sequenced using low coverage sequencing at the Genomics core KU Leuven.

Primers

Primers for genomic PCR or 4C-seq library preparation were designed using Primer3 (<http://primer3.ut.ee>) and verified using the *In Silico* PCR from UCSC (<http://rohsdb.cmb.usc.edu/GBshape/cgi-bin/hgPcr>) or CLC Main Workbench 7 (QIAGEN Aarhus A/S). Primers for qRT-PCR validation were designed with Primer3 or with the IDT DNA PrimeTime tool. Primer sequences are listed in Supplementary Table S4.

ChIP-seq Analysis

The mapped files (.BED) of Adult Kidney Histone H3K4me3 and H3K9me3 ChIP-Seq Data were downloaded from BI Human Reference Epigenome Mapping Project(14). To assess the enrichment, we generated a list of random sites, which matched with chromosome distribution and length with interacting sites. The bedtools were used to count the number of reads overlapping with interacting and random regions.

133 **RNAseq Analysis**

134 Total RNA was extracted using the RNeasy Mini kit (Qiagen). RNAseq was performed using Illumina
135 HiSeq2000 sequencing platforms. Approximately 2-30 million de-multiplexed 50bp single end were deposited
136 in Fastq format and inputted into a pre-processing pipeline. Reads containing barcodes post de-multiplexing
137 were processed with CutAdapt (Version 1.4.1). The quantification of RNA-seq was done by RSEM software
138 package (15). The STAR (13) was used to align fastq reads. The index was built using human genome (hg38)
139 with GENCODE v22 for a gene annotation. RSEM outputted the genes reads count and TPM (Transcripts Per
140 Million). DESeq2 (10) was employed to calculate differentially expressed genes using the reads count matrix
141 extracted from RSEM result.. Genes were determined as significantly differentially expressed with a p-value
142 ≤ 0.05 and a basemean value ≥ 50 .

143 **Cell culture and lentiviral transduction**

144 Embryonic kidney epithelial cells (HEK TE) immortalized by hTERT and SV40 Large T antigen (LT) were a
145 gift from Dr. W. Hahn (Dana-Farber Cancer Institute, USA)(16). HEK293T and HEK TE were cultured in
146 DMEM-F12 medium (GIBCO). All media were supplemented with 10% fetal bovine serum and penicillin/
147 streptomycin, diluted as recommended by the manufacturer's (GIBCO). Cells were checked for mycoplasma
148 using MycoAlert™ Mycoplasma Detection Kit (Lonza) once a month.

149 Transient transfections were performed using Turbofect (Life Technologies) or XtremeGene9 (Roche).
150 Lentiviral infections were performed as described by the RNAi Consortium (TRC). Infected cells were
151 selected by treatment with 2 µg/ml puromycin (InvivoGen) for 48 hours.

152 Cell growth was measured with the real-time monitoring system Incucyte (Essen Bioscience). 1000 cells were
153 plated in a 96-well plate in triplicates and the cell index was measured in at least three independent
154 experiments. For 2D clonogenic assay, 1000 cells per well of a 6well-dish were plated in triplicates and
155 allowed to grow for a week. Colonies were stained with 0.1% Crystal Violet solution in 35% methanol and
156 then quantified using ImageJ software (National Institutes of Health USA).

157 For inhibition of the DNMT enzymes cells were incubated with 1µM 5-Aza-2'-deoxycytidine (Sigma, A3656).

158 **Expression vectors and antibodies**

159 Full-length cDNAs coding for *HOXB13*, *APOL1* and *PLCG2* were cloned into the inducible pLenti-HA
160 lentiviral vector. The pLKO.1-puro shGFP and pLKO.1-puro vectors containing shRNAs targeting *HOXB13*,
161 *APOL1*, *LGALS3BP*, *PLCG2*, *CDKN2A* and *CDKN2B* were purchased from Sigma-Aldrich. pLenti-
162 CRISPRv2 with or without gRNA targeting *CDKN2A* were purchased from Genescript. shRNA and gRNA
163 sequences are listed in Supplementary Table S4.

164 Antibodies were purchased from different vendors and diluted in 3% bovine albumin serum/Tris-buffered
165 solution containing 0.1% Tween (TBS-T). Mouse monoclonal anti-HA (3F10), anti-vinculin (Sigma-Aldrich,
166 clone hVIN-1), anti-HOXB13 (D7N8O, Cell Signaling); anti-p16 (G175-405, BD Pharmingen), for IHC anti-
167 HOXB13-HRP (F-9, Santa Cruz) were used.

168 **Tumor sample analysis**

169 Fresh surgical biopsies of primary kidney tumors were collected from 22 patients treated at the University
170 Hospital Leuven, Belgium and from whom written informed consent was obtained. The studies were
171 conducted according to recognized ethical guidelines and the protocol approved by the Medical Ethical
172 Commission UZ/ KU Leuven (ref. S59806). Tumors were diagnosed as conventional ccRCC using recent
173 WHO classification criteria. Cytogenetic analysis was performed using short-term primary tumor's tissue
174 culture. A sample of the tumor tissue was disaggregated overnight by collagenase treatment and cultured for 5
175 to 10 days. Chromosome analysis was performed on G-banded metaphases. Karyotypes were described using
176 ISCN criteria (Supplementary Table S2).

177 For *ex-vivo* assay, primary cells isolated from collagenase disaggregated tumor specimens were used for the
178 short-term cell cultures. Cells were grown for three days in DMEM supplemented with 10% fetal bovine
179 serum, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate.

180 **FISH and Immunohistochemistry**

181 FISH analysis was performed using fluorescent probes specific for *CDKN2A*, *APOL1*, *HOXB13* and *LGALS3BP*
182 purchased from Empire Genomics (USA) according to manufacturer's protocol. Briefly, HEK TE cells were
183 fixed in cold Methanol:Acetic acid solution 3:1 and spread on a glass slide. Cells were first digested with pepsin
184 0.015% in 0.01N HCl at 37°C and then fixed with 1% formaldehyde/0.05% MgCl₂ in PBS. After dehydration
185 in EtOH series, nuclear DNA and probes were denatured at 73°C and then hybridized overnight at 37°C in a

humid chamber. Aspecific binding was removed by washing at 73°C in 0.4x SSC/0.3%NP40 and in 2x SSC/0.1%NP40 at room temperature. Samples were then mounted in Vectashield containing DAPI.

ccRCC tissues microarrays (TMA) (purchased from US Biomax, USA) were immunostained for HOXB13 (sc-28333 HRP, Santa Cruz) after epitope retrieval induced by heat in Tris-EDTA (pH 9). Visualization was done with Diaminobenzidine (Dako, K3468) and the sections were counterstained with hematoxylin according to standard procedures. HOXB13 immunohistochemistry was evaluated using a semi-quantitative approach that combines intensity and distribution of immunoreactivity in the epithelial tumor cells. Arrays were scored in a blinded manner for HOXB13 nuclear intensity on a scale of negative, low, moderate. TMA were prepared for FISH analysis following deparaffinization, dehydration and digestion with 0.2% pepsin in 0.01N HCL at 37°C. After dehydration in EtOH series, samples were denatured at 83°C, hybridized with the probes and processed as described above. The number of FISH signals per cell was counted in two separate fields for each duplicate of the ccRCC tissue microarrays. ccRCC samples were considered as harboring 9p LOH if the percentage of cells with a single FISH signal (9p LOH) vs. cells with double/ multiple FISH signal (wt-9p) was above 52%. The cut-off was set based on the mean percentage of cells with aberrant FISH signal in normal kidney tissue + 2SD.

Methylation analysis

Methylation analysis was performed by methylation specific PCR using primers specific for *HOXB13* promoter (Epitect II, Qiagen) following the manufacturer's protocol.

ChIP

ChIP was performed using the Magna ChIP kit (Millipore) following the manufacturer's protocol. Briefly, cells were fixed in 1% formaldehyde, cells were subsequently pelleted at 800 x g at 4°C for 5 minutes and lysed in cell lysis buffer on ice for 15 minutes. After centrifugation at 800 x g at 4°C for 5 minutes, nuclei were extracted resuspending the pellet in nuclear lysis buffer as provided by the kit. Cross-linked chromatin was sonicated to obtain optimal DNA fragments size. Immunoprecipitation was performed by incubating 2µg of DNA material using 3µg of antibody anti-DNMT3B or IgG and protein-A/G coupled magnetic beads overnight at 4°C. Elution, reversal of cross-linking of protein/ DNA complexes and DNA extraction was done following the kit's

212 protocol. qPCR analysis was performed using primers specific to *HOXB13* promoter region (Supplementary
213 Table S4).

214 **Luciferase-based enhancer assay**

215 For enhancer assays, we cloned the 9p21 viewpoints upstream of a minimal promoter driving Firefly luciferase
216 expression (17). Viewpoints genome references are listed in Supplementary Table S4. As a negative control, we
217 used a vector containing 1.6 kb insert that was also used in the enhancer assay experiments in (17). Cells were
218 then transfected with the different constructs together with 1:10 of a vector expressing Renilla luciferase (pRL-
219 TK vector, Promega) to control for transfection efficiency. 24 hours after transfection, luciferase activity was
220 measured using the Duo-Glo kit (Promega).

221 **Statistical analysis**

222 Quantifications were calculated as percentages from at least three independent experiments. The error bars
223 indicate the standard error of the mean (s.e.m.). All data were analyzed using Graphpad Prism for Apple Mac
224 (Version 6.0f). Correlation analysis was performed in R using Spearman's correlation.

225 **Results**

226 *Heterozygous loss of chromosome 9p21 is associated with poor survival of ccRCC patients*

227 The TCGA data analysis of ccRCC patients demonstrated that heterozygous loss of 9p21 (LOH) accounted for
228 approximately 30% of ccRCCs, whereas homozygous loss was found in less than 1% of ccRCCs (Fig. 1A). To
229 pinpoint potential 9p drivers of cancer development, we employed the Genomic Identification of Significant
230 Targets in Cancer (GISTIC) approach. The GISTIC analysis of 528 ccRCCs revealed a focal 9p21.3 deletion
231 (chr9:21931610-9, q-value=1.79E-20), whereas survival analysis demonstrated that 9p21 LOH was associated
232 with poorer survival of ccRCC patients (Fig. 1B). However, 148 out of 159 of 9p21.3 deleted samples also
233 present a deletion of the whole 9p arm, making it difficult to discriminate between the contribution of 9p21.3
234 loss and the deletion of the whole chromosome arm to the poor patient survival.

235 A focal 9p21.3 deletion contains four genes, *CDKN2A*, *CDKN2B*, *CDKN2B-AS1*, *CDKN2A-AS1*. However, the
236 expression of none of these genes was down-regulated as a result of 9p21 LOH (Fig. 1C). On the contrary, we
237 observed up-regulation of *CDKN2A* in 9p21 LOH samples. Point mutations of the 9p21 genes are also quite rare

238 in ccRCCs (Fig. 1A). Moreover, lower expression of either *CDKN2A*, or *CDKN2B* was not associated with
239 poorer survival of ccRCC patients (Fig. 1D). Given that 9p21 LOH in ccRCC does not lead to down-regulation
240 of the 9p21 tumor suppressors, we hypothesized that 9p21 LOH may contribute to ccRCC development through
241 mechanisms other than the loss of well-known 9p21 tumor suppressor genes, *CDKN2A* and *CDKN2B*.

242 ***9p21 loss triggers cell growth independently of CDKN2A/CDKN2B function***

243 To assess the contribution of 9p21 LOH in ccRCC development, we introduced a targeted 9p21 LOH into
244 immortal but non-tumorigenic human kidney epithelial cells expressing SV40 large T (LT) and the telomerase
245 catalytic subunit hTERT (HEK TE cells), which are genetically stable across passages (16). To model 9p21 loss,
246 we generated two sets of HEK TE clones harboring 9p21 deletion by using either nuclease, or nickase CRISPR-
247 CAS9 approaches. Guide RNAs (gRNAs) were designed to induce the deletion of the chr9:21957626-22130154
248 region (Fig. 2A). After co-transfection of either wild-type, or D10A mutant CAS9 with 9p21 targeting gRNAs,
249 we isolated single-cell clones and screened for the loss of the 9p21 locus using high-throughput PCR screening
250 of genomic DNA (Fig. 2B). Sequence analysis of 9p21 deleted clones also detected the gRNA target sites as
251 well as non-homologous end-joining-mediated indels at the breakpoint junction (Fig. 2C). CNV-seq revealed
252 heterozygous loss of the 9p21 loss in the generated clones and showed that the integrity of the remaining
253 genome was mostly preserved (Fig. 2D, Supplementary Fig. S2). The additional alterations could be due to
254 intrinsic genomic differences between the single-cell clones, co-occurring copy number alterations accumulating
255 during the screening process or due to intrinsic genetic instability of the single-clones harboring 9p deletion.

256 The HEK TE clones stably expressed SV40 LT, which inactivates the pRB suppressor pathway downstream of
257 the CDK4/6 inhibitors *CDKN2A* and *CDKN2B* (8). Thus, this model allowed us to assess *CDKN2A/CDKN2B*-
258 independent effects of 9p21 loss on tumorigenic phenotypes. We confirmed that suppression of either *CDKN2A*,
259 or *CDKN2B* in wt-9p21 HEK TE cells did not increase cell growth (Supplementary Fig. S3). Moreover, 9p
260 deletion only slightly decreased p16 protein expression in HEK TE clones (Fig 2E). Nonetheless, both isogenic
261 clones lacking 9p21 region showed increased cell growth and 2D colony formation (Fig. 2F,G). Altogether,
262 these data indicate that 9p21 loss facilitates the growth of kidney epithelial cells independently of *CDKN2A* or
263 *CDKN2B* loss.

264 ***9p21 loss alters expression of the 9p21-interacting genes***

265 The 9p21 locus is amenable to functional chromatin interactions(18). To identify genes, whose expression could
266 be dysregulated by 9p21 LOH due to disruption of chromosomal interactions, we investigated the 9p21
267 interaction network in HEK TE cells. Using the circularized chromosome conformation capture (4C) technique,
268 we analyzed the interaction frequency of distally located genomic loci to the five designated 9p21 viewpoints
269 (VP). Viewpoints were chosen based on the concentration of the predicted ENCODE histone marks and the
270 feasibility of primer design for reverse-PCR for 4C-seq analysis (Fig. 3A). As expected, the concentration of
271 4C-seq coverage adjacent to the viewpoints was higher and decreased proportionally with the distance from the
272 viewpoints (Supplementary Fig. S4B). We confirmed the reproducibility of the 4C-Seq experiments by
273 performing a correlation analysis. We found a significant correlation across four biological replicates of VP10,
274 with an average Spearman's correlation coefficient of 0.815 (Supplementary Fig. S4A).

275 We identified significant 9p21 interactions by using PeakC (11) and our custom script. The circos plots for each
276 viewpoint showed that the number of detected interactions was highly viewpoint specific (Fig. 3B). To identify
277 putative genes interacting with the 9p21 region, we examined the flanking regions from the midpoint of each
278 significant interaction (Fig. 3B, Supplementary Table S1). Of the identified cis-interacting genes *IFNA21*,
279 *MTAP*, *DMRTA1*, *CDKN2B* and *CDKN2A* were previously reported to interact with the 9p21 locus (18).

280 To elucidate whether 9p loss might affect expression of 9p21interacting genes, we assessed which of the
281 putative interacting genes were differentially expressed in both 9p21-deleted HEK TE cells and TCGA ccRCCs
282 harboring 9p21 LOH. To exclude the possibility that these genes were dysregulated due to *CDKN2A* loss of
283 function, we also tested whether expression of these genes was affected by *CDKN2A* suppression. We found
284 that out of 890 of putative 9p21 interacting genes, expression of 6 genes (1 cis- and 5 trans-interacting) was
285 altered in the same direction in both 9p-deleted cells and ccRCCs harboring 9p21 LOH, but was not affected by
286 *CDKN2A* suppression (Fig 4A,B). qRT-PCR analysis confirmed that expression of the identified 9p21 trans-
287 interacting genes, except *KCNS3*, was dysregulated in 9p21-deleted HEK TE cells, but not affected by shRNA
288 or gRNA targeting *CDKN2A* (Fig. 4C; Supplementary Fig. S5A,B). Furthermore, concordantly to the 4C data,
289 FISH analysis demonstrated a close proximity of the 9p21 locus with *HOXB13*, *LGALS3BP*, and *APOL1*
290 containing regions in HEK TE cells (Fig. 4D; Supplementary Fig. S5C). Expression of the up-regulated genes
291 was highly correlated in ccRCCs, whereas *PLCG2* expression showed a negative correlation with the up-

regulated genes (Supplementary Fig. S5D), suggesting that the identified genes might be co-regulated due to the physical interaction with the 9p21 loci.

9p21-mediated up-regulation of HOXB13 induces tumorigenesis

We next assessed whether dysregulation of the 9p21 interacting genes might contribute to ccRCC development. Homeobox gene 13 (HOXB13) was previously linked to oncogenic transformation in different cancer types, although its function is still controversial (19-23). The apolipoprotein family member APOL1 is associated with prostate cancer and breast cancer (24,25) and plays a role in the kidney pathogenesis (26). LGALS3BP, a secreted tumor antigen, was reported to promote the tumor progression of different cancer types (27,28), whereas downregulation of PLCG2 sensitizes human cervical adenocarcinoma cells to doxorubicin and paclitaxel (29).

We found that higher expression of *HOXB13*, *APOL1* and *LGALS3BP*, and lower expression of *PLCG2* in ccRCC patients correlated with poorer overall survival (Fig. 5A). Furthermore, suppression of *HOXB13* or *APOL1* in 9p21-deleted HEK TE cells resulted in decreased cell growth and 2D colony formation; *LGALS3BP* depletion only inhibited the ability to form 2D colonies; whereas suppression of *PLCG2* in 9p21-wt HEK TE cells had no effect on cell growth (Fig. 5B-D; Supplementary Fig. S6A-H). Only overexpression of HOXB13 to a level comparable to the one observed in 9p21-deleted cells increased cell growth (Fig. 5I,J). In contrast, overexpression of APOL1 or PLCG2 did not affect cell growth (Supplementary Fig. S6I-K). Altogether, these data indicate that whereas both HOXB13 and APOL1 were essential for cell growth, only up-regulation of HOXB13 was sufficient to promote cell growth of kidney cells.

We decided to further focus on *HOXB13*, which encodes for a transcription factor of the Homeobox family. Gene expression analysis of ccRCC short-term cultures confirmed that 9p21 loss was associated with increased expression of *HOXB13*, but did not affect expression of 9p21-located genes (Fig. 5E, Supplementary Table S2). Furthermore, HEK TE cells lacking 9p21 showed higher HOXB13 protein expression (Fig. 5F). Immunohistochemistry analysis of a ccRCC panel revealed that ccRCCs harboring 9p21 LOH showed low or moderate HOXB13 levels, whereas the majority of the 9p-wt samples were stained negatively for HOXB13, (Fig. 5G, Supplementary Fig. S7A,B).

Genes up-regulated in 9p-deleted HEK TE cells and the TCGA ccRCCs significantly overlapped with genes identified by *HOXB13* ChIP-seq analysis of kidney tumor G401 cells (ref GSM1594132, $p=0.024$, Fisher's

exact test; Fig. 5H), confirming 9p-mediated HOXB13 upregulation in kidney cancer. Several HOXB13-target genes have been implicated in promoting tumorigenic phenotypes, supporting association of 9p loss to more aggressive ccRCC phenotypes (Supplementary Table S3). Specifically, *FOSL1*, *HMGA1*, *CDC20* induce cell growth; *HMGA1*, *CDC20*, *CDCA7* and *PLAU* promote cell motility (30-36); whereas *ADAMTS6* codes an extracellular metalloproteinase that inhibits cell-cell and focal adhesions (37). *NFE2L3* and *SEMA4B* are associated with poor survival in ccRCCs (38,39). Overall, our data indicate that 9p21 loss in kidney cells provides a growth advantage by relieving inhibition of *HOXB13* expression.

9p21 LOH decreases methylation of the HOXB13 promoter

We next assessed a regulatory effect of the 9p21 locus on transcription of the interacting genes. We cloned the 9p21 viewpoints upstream of a minimal promoter driving luciferase expression. Luciferase assay revealed that viewpoints 6, 8 and 9 showed a significant inhibitory effect on luciferase expression in HEK TE cells (Fig. 6A). As VP6 interacted with the loci containing *HOXB13* and *APOL1* (Supplementary Table S1), we hypothesized that 9p21 loss might reduce the inhibitory effect on the 9p21 interacting gene expression, resulting in upregulation of the interacting gene.

Multiple studies demonstrated that the 9p21 locus is highly methylated (7). Consistently, we found that the *CDKN2A* promoter (cg14069088) was methylated in 440 of 485 of ccRCC samples (beta value ≥ 0.3). Moreover, ChIP-seq analysis of normal kidney tissue showed higher levels of H3K9me3 histone mark in the identified 9p21-interacting regions compared to random regions (Fig. 6B), suggesting that the 9p21 interaction might lead to increased methylation levels. Consistent to this idea, we found decreased methylation of the *HOXB13* promoter in 9p21-deleted cells compared to wt-9p HEK TE cells (Fig. 6C).

A previous study demonstrated binding of the DNA methyltransferase DNMT3B to *HOXB13* and *CDKN2A* promoters (19), suggesting that the interaction of *HOXB13* and *CDKN2A*-containing regions promotes DNMT3B-mediated methylation and inhibition of both genes. Concordantly, ChIP analysis revealed binding of DNMT3B to the *HOXB13* promoter in HEK TE cells, whereas 9p21 loss reduced recruitment of DNMT3B to the *HOXB13* promoter (Fig. 6D). To confirm the role of CpG island methylation in inducing *HOXB13* expression, we used the methyl-transferase inhibitor 5-Aza-2'-deoxycytidine. Inhibition of DNMT enzymes led to an increased *HOXB13* expression in 9p-wt HEK TE cells, but did not affect further *HOXB13* expression in 9p-deleted cells (Fig. 6E). Altogether these data suggest that 9p21 LOH relieves 9p21-dependent inhibition of

348 *HOXB13* expression, leading to decreased methylation of the *HOXB13* promoter and increased expression of
349 *HOXB13*.

350 **Discussion**

351 Efficient transcription requires local concentrations of the relevant trans-acting factors. Spatial clustering of
352 chromatin-associated binding sites for trans-acting factors provides an effective mean to locally increase the
353 concentration of such factors. Genes located on separate chromosomes physically associating in the nucleus
354 may coordinate their expression (40). For example, the role of three-dimensional genome organization has been
355 documented for the regulation of expression of the β -globin locus (41). The large-scale chromosomal
356 rearrangements that commonly occur in cancer genomes (1) disorganize the 3D genome conformation and
357 might contribute to cancer development by altering gene transcription (42).

358 9p21, one of the most frequently altered loci in human disease (43), is known to harbor variants that correlate
359 with high risk of cancer, myocardial infarction, coronary artery disease (CAD) (44,45) and type -2 diabetes (46).
360 The majority of these variants are single-nucleotide polymorphisms located outside protein-coding sequences.
361 The 9p21 locus is highly enriched in enhancer elements (18), suggesting that 9p21 alterations may affect
362 expression of non-proximal genes.

363 By applying 4C-seq together with a CRISPR-engineered cell model, we identified chromosomal interactions of
364 the 9p21-locus and genes dysregulated by 9p21 locus loss. We identified multiple cis-interactions, including the
365 cis-interaction between *IFNA21* and the 9p21 locus previously described (18). However, 9p21 loss in kidney
366 cells did not affect expression of the interferon locus. All of the differentially regulated genes that interact with
367 9p21, except *DMRTA1*, are distal genes. The wild-type copy of 9p21 is highly methylated and leads to the
368 silencing of the 9p-located tumor suppressor genes as well as the 9p21 interacting genes (47). Mechanistically,
369 9p21 deletion correlates with reduced recruitment of the methyltransferase DNMT3B to the 9p21 interacting
370 genes, including *HOXB13*. Moreover, DNMT inhibition increases *HOXB13* expression in 9p-wt HEK TE cells,
371 but does not affect *HOXB13* expression in 9p-deleted cells, confirming the role of 9p-dependent DNA
372 methylation in the regulation of *HOXB13* expression in kidney cells. Interestingly, a recent study demonstrated
373 that expression of the *Hoxb13* locus in mouse embryonic stem cells is regulated through homotypic chromatin
374 interactions (48).

375 In ccRCCs, increased *HOXB13* expression correlated with poorer prognosis and higher proliferative rate in
 376 samples harboring 9p21 LOH. Several studies found an association between *HOXB13* alterations and increased
 377 risk of onset of prostate cancer (22), ovarian cancer (21), cervical cancer, and oral cancer (49). *HOXB13*
 378 upregulation was shown to promote anchorage independent growth and decrease apoptosis levels in ovarian
 379 cancer cells (22). Recently BRD4-mediated *HOXB13* upregulation has been also reported to promote castration-
 380 resistant prostate cancer, whereas its inhibition suppresses cancer cell proliferation and induces apoptosis (22).
 381 In line with these studies, *HOXB13* upregulation in HEK TE cells increased their growth rate. However,
 382 overexpression of SV40 LT in our cell model significantly perturbs apoptotic pathways (50), thus limiting our
 383 ability to study the anti-apoptotic properties of *HOXB13* in ccRCCs.

384 Additional studies are needed to address the tumorigenic mechanisms linked to the dysregulation of *HOXB13*
 385 expression and its contribution in ccRCC development and progression. In addition, we could not also exclude
 386 that there are additional 9p21-interacting genes still to be identified might contribute to 9p21-associated
 387 phenotypes. Taken together, our results propose a novel tumor suppressive mechanism based on coordinated
 388 expression of physically associated genes.

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Figures and Tables

Fig. 1. 9p21 loss is associated with poorer survival of ccRCC patients. (A) CNVs of 9p locus and mutations of the 9p21 located genes in the TCGA ccRCC cohort. LOH, Loss of heterozygosity; HOM, homozygous deletion. (B) Kaplan-Meier curve of the TCGA ccRCCs stratified according to the 9p21 CNV. (C) Box-Whisker plots of the normalized expression profile of the 9p21 located genes in the TCGA ccRCCs. Adjusted p-values were determined by DESeq2. ns, non-significant. (D) Kaplan-Meier curve of the TCGA ccRCCs stratified according to their CDKN2A and CDKN2B expression levels. The mean of mRNA expression was used as a cutoff to define patients with high and low CDKN2A/B expression. **B, D**, p-values were determined by the log-rank test. n, number of patients.

Fig. 2. 9p21 loss increases the cell growth independently of CDKN2A/B activity. (A) Scheme of the CRISPR-mediated 9p21 chromosomal deletion. (B) PCR of the 9p21 CRISPR-targeted region in single-cell-derived HEK TE clones. PCR product indicates CRISPR-Cas9 cut and end joining. (C) Sanger sequencing of 230 kb breakpoint junction in single-cell-derived HEK TE clones. Sequencing primers were designed using sequences that are separated by the targeted chromosomal segment. (D) Copy number status of the 9p21 locus in single-cell derived HEK TE clones. Read depth coverage per window is compared to blood normal, plotted as red boxes. (E) Immunoblot analysis of p16 expression in wt-9p (3ED4 and A6A6) and 9p-deleted (3AD8 and A6A7) HEK TE clones. (F) Relative cell growth of HEK TE clones with different 9p21 status. (G) Relative 2D clonogenic growth of HEK TE clones with different 9p21 status. **E,F,G**, Data are shown as mean±SEM, n≥3. p-values were calculated by two-tailed t-test.

Fig. 3. The 9p21 interaction network identified by the 4C approach. (A) Diagram shows the 5 investigated viewpoints (red bars) located within the 9p21.3 locus. 9p21.3 located genes are indicated by grey transcripts. (B) Circos plots indicating the number of significant interactions identified for each viewpoint. Red and blue lines denote cis and trans interactions, respectively. Significant interactions were determined by PeakC and our

549 custom scripts. Putative cis and trans interaction genes located ± 400 Kb from the center of predicted interaction
550 sites across each viewpoint.

551 **Fig. 4. Loss of 9p21 alters expression of the 9p21-interacting genes.** (A) Heatmap of RNA-seq analysis of
552 HEK TE clones with different 9p21 status. (B) Expression analysis of the 9p21-interacting genes in the TCGA
553 ccRCCs with different 9p21 status. Boxplots represent the 25th to 75th percentile group of values with whiskers
554 expanding to 1.5 times the interquartile range of the data. The line indicates the median expression value.
555 Outline samples are indicated by dots. Adjusted p-values were determined by DESeq2. (C) RT-qPCR analysis
556 of the 9p21-interacting gene expression in wt-9p (3ED4) and 9p-deleted (3AD8) HEK TE clones. Data are
557 shown as mean $\pm$ SEM, n>3. p-values were calculated by two-tailed t-test. (D) FISH analysis of wt-9p HEK TE
558 clone (3ED4) using probes for *CDKN2A* (red) and the 9p21-interacting gene (green). Scale bar, 10 μ m.

559 **Fig. 5. HOXB13 up-regulation upon 9p21 loss promotes kidney cell growth.** (A) Survival analysis of the
560 TCGA ccRCCs with different expression of the 9p21-interacting genes. The mean of *APOL1*, *HOXB13*, or
561 *LGALS3BP* expression was used as a cutoff to define patients with high and low expression; the median of
562 mRNA *PLCG2* expression was used as a cutoff to define patients with high and low expression. p-values were
563 determined using log-rank test. n, number of patients. (B) RT-qPCR analysis of *HOXB13* gene expression in 9p-
564 deleted 3AD8 cells expressing shRNA targeting GFP or HOXB13. (C) Growth curve of 9p-deleted 3AD8 cells
565 expressing shRNA targeting GFP or HOXB13. (D) 2D clonogenic assay of 9p-deleted 3AD8 cells expressing
566 shRNA targeting GFP or HOXB13. (E) RT-qPCR analysis of expression of the indicated genes in short-term
567 ccRCC tissues. (F) Immunoblot analysis of HOXB13 protein expression in single-cell wt-9p (3ED4) and 9p-
568 deleted (3AD8) HEK TE clones. (G) IHC analysis of ccRCCs with different 9p status immunostained for
569 HOXB13. 9p CNV was determined by FISH (Supplementary Fig. S7). n, number of patients, p-value was
570 determined by Chi-square test. (H) Overlap between HOXB13 target genes in G401 cells and the genes up-
571 regulated in 9p-deleted HEK TE cells and TCGA ccRCCs. Overlapped genes are listed in Supplementary Table
572 S3. (I) Immunoblot analysis of HOXB13 expression in single-cell 3ED4 and 3AD8 HEK TE clones expressing
573 an empty vector (EV) and 3ED4 clone expressing HA-HOXB13. (J) Growth curve of wt-9p 3ED4 cells
574 expressing an empty vector (EV) or HA-HOXB13. **B-E, J**, Data are shown as mean $\pm$ SEM, n $\geq$ 3. p-values were
575 calculated by two-tailed t-test.

576 **Fig. 6. 9p21 loss induces HOXB13 expression due to decreased methylation of the *HOXB13* promoter.** (A)
577 Luciferase reporter enhancer assay of the 9p21 viewpoints. (B) H3K9me3 status of the 9p interacting regions
578 and random genomic region in normal kidney tissue(14). Box plots show the number of ChIP-seq reads within
579 interacting and random regions. p value was determined by two-tailed t-test. (C) Methylation of the *HOXB13*
580 promoter detected by methylation specific PCR. (D) ChIP analysis of DNMT3B recruitment to the *HOXB13*
581 promoter region in wt-9p (3ED4) and 9p-deleted (3AD8) HEK TE clones. (E) RT-qPCR analysis of *HOXB13*
582 expression in wt-9p (3ED4) and 9p-deleted (3AD8) HEK TE clones incubated with or without the DNA methyl-
583 transferase inhibitor 5-Aza-2'-deoxycytidine (1 μ M, 72 hours). A, C-E, Data are shown as mean $\pm$ SEM, n>3. p-
584 values were calculated by two-tailed t-test.

585 **Supplementary Materials**

586 Supplementary Fig. S1 to S7

587 Supplementary Table S1 to S4

figure 1

figure 2

figure 3

figure 4

figure 5

figure 6