LOSS OF P130 ACCELERATES TUMOR DEVELOPMENT IN A MOUSE MODEL FOR HUMAN SMALL CELL LUNG CARCINOMA – SCHAFFER, PARK ET AL.

Supplementary Data

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1: Generation of a conditional mutant allele of the mouse p130 gene

A, a modified *lox* site (*lox2722*) was used to flank exon 2 of the mouse *p130* gene by homologous recombination. This *lox2722* site does not recombine with *loxP* sites, preventing chromosomal events between the *Rb* and *p130* genes in double conditional mutant cells (1). The $p130^{lox}$ allele generated can be used to conditionally delete *p130* in cells expressing the Cre recombinase. Deletion of the *lox* sites results in an out-of-frame mutation producing a truncated protein of 79 amino acids of unknown stability, compared to the normal size of 1135 residues for wild-type mouse p130. The pocket domain and other functional domains of p130 are absent in the truncated protein (2, 3). *B,* levels of the p130 protein are similar in wild-type and *p130* conditional mutant mouse embryonic fibroblasts (MEFs) grown in 10% serum (proliferating) and 0.1% serum (quiescent), indicating that the *lox2722* sites do not affect the expression of *p130*. *C,* disappearance of the p130 protein by immunoblot with extracts from $p130^{2lox/2lox}$ MEFs in culture only in cells infected with an adenovirus expressing Cre, and not in cells infected with Ad-GFP or not infected. * non-specific bands. Controls include MEFs from germline knockout *Rb* and *p130* (4, 5). See (6) for methods related to experiments in MEFs. See (7) for an example of efficient deletion of the *Rb* and *p130* conditional alleles *in vivo*.

Figure S2: Immunoblot analysis of p107 and p130 in primary SCLC

Expression of p130, p107, and Karyopherin β1 (loading control) was analyzed by immunoblot analysis of protein extracts from double (*Rb/p53*) and triple (*Rb/p53/p130*) mutant tumors. *Rb* family triple knock-out MEFs (TKO) provided a negative control for

p130 and p107 expression. One normal adult lung sample (CTRL) was used as a positive control for p130; note that p107 levels were low in this sample, probably because most of the cells in an adult lung are quiescent and p107 levels are well known to correlate with cell proliferation. *, non-specific band.

Figure S3: Immunofluorescence analysis for cell cycle markers in double and triple mutant tumors.

Representative immunostaining for Ki67, PCNA, and MCM6 (red) on lung tumor sections from *Rb/p53/p130* conditional mice 6 months after Ad-Cre infection and from lung tumor sections from *Rb/p53* conditional mice 9 months after Ad-Cre infection. DAPI stains the DNA (blue). Bar: 25 μm. Note that the majority of tumor cells in both cases are positive for these proliferation markers.

Figure S4: Histology of the lung tumors in Rb/p53/p130 conditional mice after Ad-Cre infection

A-I, hematoxylin and eosin (H&E) staining of lung tumor sections from *Rb/p53/p130* conditional mice 6 months after Ad-Cre infection. Similar observations were made with tumors from double mutant mice (not shown). *A*, neuroendocrine hyperplasia (arrows) in bronchioles. *B-F*, classic SCLC. *B* and *C*, SCLC growing in sheets (*B*) and nests (*C*) with abundant mitotic and apoptotic figures. *B-F*, tumors have small cells with a high nucleus/cytoplasmic ratio and finely granular chromatic with inconspicuous to absent nucleoli; mitotic figures are abundant. *B, D, E*, asterisks denote areas of necrosis. *E* and *F*, tumor cells form Flexner-Wintersteiner rosettes (*E*) and perivascular pseudorosettes (*F*),

denoted by circles. *G*, large cell neuroendocrine carcinoma, composed of undifferentiated nests of large malignant cells with high mitotic activity, prominent nuclear molding, apoptosis and necrosis. *H*, large cell carcinoma composed of undifferentiated nests of larger malignant cells with high mitotic activity and prominent nucleoli and without nuclear molding. *I,* adenocarcinoma with neuroendocrine features, with malignant gland-like structures composed of cells with neuroendocrine-type chromatin and nuclear molding. *J-O*, analysis of metastatic lesions in *Rb/p53/p130* mutant mice 6 months after Ad-Cre infection. *J-K*, whole-mount images of the livers of double (*Rb/p53*, *J*) and triple (*Rb/p53/p130*, *K*) mutant mice. Arrows indicate the metastatic lesions. *L-M*, H&E staining of liver sections from double (*Rb/p53*, *L*) and triple (*Rb/p53/p130*, *M*) mutant mice. The lesion shown in *M* is composed of small cells with a high nuclear-cytoplasmic ratio typical of SCLC. *N-O* H&E staining of sections from a pulmonary lymph node and a kidney, respectively, from triple mutant mice. In *N*, the dotted lines demarcate the border between the metastases and resident lymphocytes. Asterisks indicate metastases. Bars: 100 μm.

Figure S5: Characterization of cell lines derived from double and triple mutant SCLC

A, microphotograph of representative cell lines from double (*Rb/p53*) and triple (*Rb/p53/p130*) mutant tumors. Magnification: 40x. *B,* immunoblot analysis of protein extracts from *Rb/p53* and *Rb/p53/p130* mutant cell lines. *Rb* family triple knock-out MEFs (TKO) provided negative controls for p130 and p107 expression. Protein extracts from lung adenocarcinoma LKR cells (murine Non Small Cell Lung Cancer cells) (8) were used as an additional negative control for the expression of the neuroendocrine marker SYP (Synaptophysin). *, non-specific band. Karyopherin β1, loading control.

Figure S6: List of the genes that are differentially expressed Rb/p53/p130 and Rb/p53 mouse tumors

Gene list extracted from a file generated by SAM analysis with 3 triple mutant tumors and 10 double mutant tumors. Cutoff fold change: 1.5 - False discovery rate: 10%. The genes in black have functional implication in cell cycle regulation (from DAVID analysis).

Figure S7: Expression levels of cell cycle and cell death genes in double and triple knock-out tumors.

A, the levels of expression of classical E2F target genes (17 cell cycle genes and 4 cell death genes) were obtained from the raw microarray data and graphed. CTRL, 3 wild-type lungs; DKO: 10 *Rb/p53* double mutant tumors; TKO: 3 *Rb/p53/p130* triple mutant tumors. No significant difference was observed. *B*, quantitative RT-PCR analysis of genes involved in cell cycle progression in wild-type lungs (n=3), double knock-out tumors (n=5), and triple knock-out tumors (n=3). c-*Yes* and *Rad54l* were found to be significant by SAM analysis (Fig. S5). Genes coding for PCNA, B-Myb, and Cyclin E (*Ccne*) are classical cell cycle genes and E2F targets. ns, not significant; **, $p<0.01$; ***, $p<0.001$.

Figure S8: Gene Set Enrichment Analysis: Enrichment of human SCLC genes in Rb/p53/p130 tumors

A, the top 10% of genes overexpressed in human SCLC is enriched in *Rb/p53/p130* mutant tumors. NOM p-val: nominal p-value; FDR q-value: false discovery rate q-value. *B,* list of the genes whose expression is enriched both in *Rb/p53/p130* mutant tumors and human SCLC tumors.

SUPPLEMENTARY TABLES

Supplementary Table S1: primers for genotyping mice and tumors

	Forward	*Reverse*
Rb^{lox}	CTC TAG ATC CTC TCA TTC TTC CC	CCT TGA CCA TAG CCC AGC AC
Rb^{Δ}	CTC TAG ATC CTC TCA TTC TTC CC	GCAGGAGGCAAAAATCCACATAAC
$p53^{lox}$	CAC AAA AAC AGG TTA AAC CCA G	AGC ACA TAG GAG GCA GAG AC
$p53^{\Delta}$	CAC AAA AAC AGG TTA AAC CCA G	GAA GAC AGA AAA GGG GAG GG
	AGC ACA TAG GAG GCA GAG AC	
$p130^{lox}$	GTG TTG TAA CAT TCT CGT GGG	GAC TGC TGG TAT TAG AAC CC
$p130^{\Delta}$	GTG TTG TAA CAT TCT CGT GGG	CGG AGG CTA AGA GCA TTT GTT G

All PCR were run at an annealing temperature of 55ºC, except the $p53^{\Delta}$ (58ºC); denaturation and elongation for all were 94ºC and 72ºC, respectively.

Supplementary Table S2: primers for quantitative RT-PCR analysis of gene expression

	Forward	*Reverse*
Rb	GCTTGGCTAACTTGGGAG	CAACTGCTGCGATAAAGATG
p53	GCCCATGCTACAGAGGAGTC	AGACTGGCCCTTCTTGGTCT
p130	AAGGCACATGCTAACCAATGAA	GAGCAGTTACCGCAGCATGA
p107	CCGAAGCCCTGGATGACTT	GCATGCCAGCCAGTGTATAACTT
Syp	CTGGCAGACATGGACGTG	CTTGACCACCCGGAACTG
Ascl1	GCTCTCCTGGGAATGGACT	CGTTGGCGAGAAACACTAAAG
TBP	CGGTCGCGTCATTTTCTC	GGGTTATCTTCACACACCATGA
Ncam1	AGGGCAAGGCTGCTTTCT	CCCCATCATGGTTTGGAGT
Cgrp	TGCAGGACTATATGCAGATGAAA	GGATCTCTTCTGAGCAGTGACA
Rad54l	GGAAACCAGAAGACAGATCATCA	TGGACTTGCGTTTCTTAGGAG
c-Yes	TTAAGAAGGGTGAACGATTTCAA	ATTGATCTTGCTTCCCACCA
Pcna	CTAGCCATGGGCGTGAAC	GAATACTAGTGCTAAGGTGTCTGCAT
B-Myb	TTAAATGGACCCACGAGGAG	TTCCAGTCTTGCTGTCCAAA
CcnE	CTGAGAGATGAGCACTTTCTGC	GAGCTTATAGACTTCGCACACCT

SUPPLEMENTARY MATERIAL AND METHODS

Generation of a conditional mutant allele of the mouse p130 gene

J1 ES cells were grown as described (9). The *p130* conditional targeting vector was constructed from the original *p130* knockout vector (5). The strategy was similar to that used for a conditional allele of *Rb* (9). After electroporation, ES cells were selected with 150 μg/ml hygromycin. To delete the hygromycin cassette flanked by *lox2722* sites, ES cells were co-electroporated with a Cre-expressing plasmid and a plasmid expressing the puromycin resistance gene. *lox2722* sites do not recombine with the *loxP* sites used in the *Rb* conditional allele, thus preventing the generation of chromosomal translocations (1). ES cells were selected for 48 h with 2 μg/ml puromycin 24h after electroporation. This brief selection did not kill non-electroporated cells but slowed their growth, leading to enrichment in clones transiently expressing Cre. Details on the cloning strategy, the plasmids, the PCR reactions and the Southern blot analysis to identify candidate clones are available upon request. Mice derived from two of these *p130* conditional knockout ES cell clones and mouse embryonic fibroblasts (MEFs) gave identical results in preliminary experiments. Only one *p130* conditional mutant line was kept and used for further experiments.

Microarray analysis

Data from 3 independent *Rb/p53/p130* triple knock-out tumors and 10 double mutant tumors were analyzed using R (cran.r-project.org) and Bioconductor (www.bioconductor.org) software. Raw data will be available for download from Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo) with the accession number GSE 18534.

Microarray data were normalized with Expression Console software (Affymetrix, Santa Clara), using the RMA algorithm (10). Low signals (below 50) were filtered out using the PreprocessDataset module in GenePattern (http://www.broad.mit.edu/cancer/software/genepattern/). Differentially expressed genes in *Rb/p53/p130* mutant tumors compared to *Rb/p53* mutant tumors were identified using Significance Analysis of Microarrays software (11), with the cutoff false-discovery rate of 10%. To compare the arrays from *Rb/p53/p130* and *Rb/p53* mutant tumors to human lung cancer arrays in GEO database (GSE6044), the pre-processed mouse and GSE6044 data were collapsed to gene symbols, filtered for a minimum fold change of 1.5, followed by standardization (standard deviations from the mean) of each gene symbol in each dataset. We then selected orthologous genes that were present in both arrays based on the mammalian orthology from Mouse Genome Informatics (http://www.informatics.jax.org/). Hierarchical clustering (12) was used to compare standardized array data, using Pearson correlation in sample distance measure. DAVID tools were used as described (13).

Cell culture

SCLC cell lines were established from freshly dissected mouse tumors minced with a razor blade and digested for 30 minutes at 37ºC in dispase (BD Bioscience). Cells were cultured in RPMI1640 supplemented with 10% bovine growth serum (Mediatech) and 1% Penicillin-Streptomycin-Glutamine (Invitrogen). All cell lines tested negative for mycoplasma infection by PCR with the VenorGeM Mycoplasma kit (Sigma).

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