

SUPPLEMENTAL MATERIAL

Methods

Primer Sequences

Human real-time PCR primers:

β-ACTIN Fwd: 5'-CCTGGCACCCAGCACAAT
Rev: 5'-GCCGATCCACACGGAGTACT

RASA1 Fwd: 5'-GGCCGGGAAGAAGATCCAC
Rev: 5'-GCAGACTTGACCAACTGTCATT

SPRED1 Fwd: 5'-AAGGATGCCCCGAATCAAAAA
Rev: 5'-GGCTTGGCTTTGCATGTAGAC

SPRED2 Fwd: 5'-ACTGGTGGTATTGGAATGCTATG
Rev: 5'-CAGCTTCATTATGGATGGTGGAA

SPRY1 Fwd: 5'-TTCGGTGGTGAAAAGACCTGC
Rev: 5'-CCCTGGCATTACTTGGGAGT

SPRY3 Fwd: 5'-CCGAAGAGAGGTGGAGAAACC
Rev: 5'-GTTTTAAGTCAGGGTGGCACTT

SPRY4 Fwd: 5'-AGCTGGAGGAAGAGGTTGCG
Rev: 5'-ATTTGTACCTGTGGGGAGGG

Mouse real-time PCR primers:

β-actin Fwd: 5'-CCTGAACCCTAAGGCCAACC
Rev: 5'-ATGGCGTGAGGGAGAGCATA

Rasa1 Fwd: 5'-TGTGGTGATTACTACATTGGTGG
Rev: 5'-CGCCTTCTATCTTCTACTGGCTC

Spred1 Fwd: 5'-GAGATGACTCAAGTGGTGGATG
Rev: 5'-TCTGAAAGGTAAGGCCAAACTTC

Spred2 Fwd: 5'-TGAAGGCAACGGACGAAGC
Rev: 5'-TCCAATGATGAAACGTCGGATT

Spry1 Fwd: 5'-AGTTCCAGCAGTCAGCGG
Rev: 5'-GCTGAATCACCCTAGCGAAGT

Spry3 Fwd: 5'-CTTGTCATCATTTGTTGATTCTACC
Rev: 5'-TCATCTATCACTGTGGCATCC

Spry4 Fwd: 5'-TTCGGGGATTTACACAGACG
Rev: 5'-CTGCTGTCAAGGAGGGGC

Histology/Pathology

The proliferative and neoplastic lesions were evaluated in the lungs by a board certified veterinary pathologist (K.L.B.). Hyperplasia, atypical adenomatous hyperplasia (AAH), adenoma, and adenocarcinoma were scored as previously described (Nikitin *et al.*, 2004).

Specifically, AAH had focal and diffuse lesions involving alveoli and terminal bronchioles and consisting of relatively uniform atypical cuboidal to columnar cells with dense chromatin. Degrees of cellular hypertrophy and hyperchromasia were variable. Cellular and nuclear atypia were the distinctive features as compared with hyperplasia. AAH with anaplasia had similar morphologic characteristics of AAH but with prominent nuclear pleomorphism and variable cell size. Adenomas were well circumscribed areas consisting of cuboidal to columnar cells lining alveoli. The tumor size was less than 5mm in diameter and retained preexisting alveolar structure. There was no invasion of vessels, airways, or pleura and minimal variation in cell size and nuclear size. Adenoma with anaplasia had similar morphologic characteristics of adenoma but with prominent nuclear pleomorphism, variable cell size, and focal areas where the tumor is poorly circumscribed. Adenomas in the mice that overexpressed miR-31 frequently had cells that were pleomorphic and had focal loss of demarcation of the borders and these were classified as adenomas with atypia. Adenocarcinomas were cytological atypia, and had increased frequency of mitoses, regional variation in growth pattern, more papillary structures, with sizes over 5mm in diameter, and showed invasion of vessels, large airways or pleura, as well as lymphatic and hematogenous metastases.

Detailed information of TCGA data analysis

The samples with miR-31 expression and clinicopathological characteristics available were selected resulting in 349 lung adenocarcinoma and 22 normal lung tissue samples for figure 1D-G, tables 1, S2, and S3, and 474 lung adenocarcinoma and 58 normal for figure 9C and S8. Non-parametric Wilcoxon rank-sum tests were used to determine significance for miRNA expression between normal and adenocarcinoma. Survival curves were calculated using the Kaplan-Meier

method and compared using the log-rank test. Univariate and multivariate Cox proportional hazards regression analysis were conducted for overall survival outcomes of lung adenocarcinoma patients based on miR-31 expression. For univariate and multivariate analyses, we considered stratified tumor stages [grouped by lower stages (stage I, IA/B, IIA/B) and higher stages (stage IIIA/B and IV)], stratified tumor sizes [grouped by smaller sizes (T1, T1a, T1b, T2, T2a, and T2b) and larger sizes (T3 and T4)], stratified lymph node metastasis [grouped by no lymph node metastasis (N0) and lymph node metastasis (N1, N2, N3)], and patient's age at the time of initial diagnosis. Patients were divided into lower age group and higher age group by calculating the median age. Survival analyses for figure 1F, 1G, and S1 were performed using the R package (Therneau and Grambsch, 2014). The “days to death” column was used to consider event (vital status=dead) and ‘days to last follow up’ column to consider the data as censor (vital status= alive). Patients were censored from statistical analysis if they were alive and had <5 year of clinical follow up. Among the 349 lung adenocarcinoma patients, 19 had neither the ‘days to death’ nor the ‘days to last follow up’ information in the lung adenocarcinoma clinical data. Furthermore, 14 patients were excluded from the analysis because the reported event/censor information was not within the time range. In total 316 patients were eligible for survival analysis among them mortality occurred in 73 (23.10%). We divided the patient samples based on miR-31 expression.

BrdU and propidium iodide (PI) analysis.

A549 cells were transfected with miR-31 inhibitor or inhibitor control. For BrdU analysis, 48 hours after transfection, BrdU (1mM) was added to cultures and cells harvested at intervals. BrdU incorporation was determined by flow cytometry following manufacturer's protocol (BD

Biosciences BrdU flow kit; San Diego, CA). For cell cycle analysis, 48 hours after transfection, cells were harvested, DNA was stained with PI, and samples evaluated by flow cytometry. Flow cytometry data was analyzed using FlowJo Analysis Software (Ashland, OR).

Supplemental References

Nikitin AY, Alcaraz A, Anver MR, Bronson RT, Cardiff RD, Dixon D, Fraire AE, Gabrielson EW, Gunning WT, Haines DC, *et al.* Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. *Cancer Res* 2004; (64):2307-16.

Therneau T, Grambsch P. *Modeling Survival Data: Extending the Cox Model*. New York: Springer 2014.

Supplemental Tables**Table S1. Clinicopathological features of Vanderbilt lung adenocarcinoma cohort**

Category	# of Samples (out of 94 total)
Age at initial pathologic diagnosis:	
≤ 65	50
> 65	44
Gender:	
Male	47
Female	47
Smoking History:	
Current	15
Reformed	68
Never	11
Pack years smoked:	
≤ 20	24
> 20	70
Tumor stage:	
I	21
II	13
III	13
IV	10
Normal Lung	37

Table S2. Increased miR-31 in all stages of lung adenocarcinoma¹

	Stage I	Stage II	Stage III	Stage IV
Normal	3.981e-07	1.504e-06	1.510e-07	3.66e-08
Stage I		0.506	0.101	0.013
Stage II			0.105	0.012
Stage III				0.054

¹Wilcoxon rank-sum test (one-tailed).

Table S3. Inverse correlation of miR-31 and target mRNA expression

miRNA	mRNA	Spearman	P-value
miR-31	<i>RASA1</i>	0.016	0.613
miR-31	<i>SPRED1</i>	-0.108	0.025
miR-31	<i>SPRED2</i>	-0.157	0.002
miR-31	<i>SPRY1</i>	-0.166	0.001
miR-31	<i>SPRY3</i>	0.044	0.788
miR-31	<i>SPRY4</i>	-0.204	9.502e-05

Supplemental Figure Legends

Figure S1. Increased miR-31 expression significantly correlates to decrease 3-year survival of lung adenocarcinoma patients. Kaplan-Meier 3 year survival analysis of miR-31 expression in 278 lung adenocarcinoma patients in TCGA. Median miR-31 expression determined high (n=139) and low (n=139) patient groups, p=0.00085, log-rank test.

Figure S2. Inhibition of miR-31 results in decreased proliferation and reduced cell cycle progression in lung cells. (A, B) Beas-2B, H1437, and H460 cells were transfected with either miR-31 mimic or RNA control. Viable cells were counted (A) and MTT assays (B) were performed at intervals (triplicate). (C) H1437 and H460 were infected with empty or miR-31 encoded retrovirus and viable cells counted at intervals (triplicate). (D) H1993 cells were transfected with either miR-31 inhibitor or inhibitor control and viable cells counted at intervals (triplicate). (E) 16HBE were transfected with miR-31 mimic or RNA control and A549 cells were transfected with miR-31 inhibitor or inhibitor control. Percent viability was determined by Trypan blue dye exclusion. (F) A549 cells were transfected with miR-31 inhibitor or inhibitor control. BrdU incorporation was determined by flow cytometry at intervals after incubation with BrdU 48 hours after transfection. (G) A549 cells were transfected with either miR-31 inhibitor or inhibitor control, and 72 hours later DNA was stained with propidium iodide and analyzed by flow cytometry. Watson Pragmatic cell cycle analysis was performed. (H) Beas2B cells were infected with empty or miR-31 encoded retrovirus and 500 cells/well were placed in 6 well plates in triplicate. Colonies were counted after 14 days, p=0.0032. (I) H1993, H1437, and H460 cells infected with either a miR-31 encoded retrovirus or an empty retrovirus (vector) were injected subcutaneously into nude mice and allowed to grow (see Figure 3). Following sacrifice

and tumor removal, miR-31 levels were measured in individual tumors (left) or the mean of the tumors (right) by qRT-PCR. miR-31 expression is relative to RNU6B, SEM.

Figure S3. Generation and initial characterization of miR-31 transgenic mice (A) Schematic representation of the cross between *tet-O-mmu-miR-31* and *CCSP-rtTA* transgenic mice and the induction of miR-31 transgene with doxycycline (dox). (B) Mendelian inheritance of *tet-O-mmu-miR-31* transgene. PCR analysis of *tet-O-mmu-miR-31* and *CCSP-rtTA* transgenes from genomic DNA from two litters of mice. The CCSP PCR product is 500bp and the miR-31 PCR product is 735 bp. (C) miR-31 expression measured by qRT-PCR (triplicate) in the lungs of littermate matched wild-type (WT) and single and double transgenic mice of the indicated genotype given control water or doxycycline containing water for two weeks. miR-31 expression is relative to snoRNA-202 levels, SEM.

Figure S4. miR-31 expression in the lungs of miR-31 transgenic mice. miR-31 levels in the lungs of a subset of miR-31/CCSP and the indicated control mice (littermate matched) from figure 4 were quantified by qRT-PCR. miR-31 expression is relative to snoRNA-202 levels, SEM. Mice were provided dox in their drinking water to induce miR-31 for 2 months (top) and 12 months (bottom).

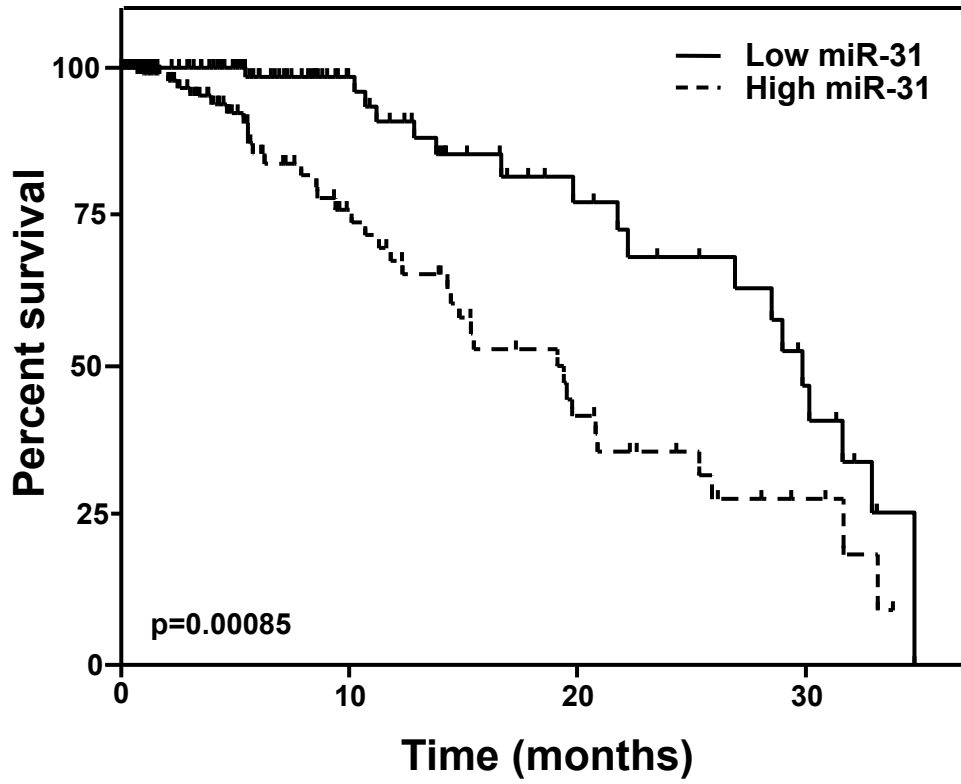
Figure S5. miR-31 transgenic mouse lung histology. (A) Enlarged H&E images for figure 4A. (B) Representative pictures of Ki67 IHC for figure 4B. (C) Enlarged H&E images for figure 4C. (D, page 1) Enlarged H&E images for figure 4G (top three) and figure 4H (bottom two). Higher magnification (10X and 40X) H&E images for figure 4G also included. (D, page 2) An

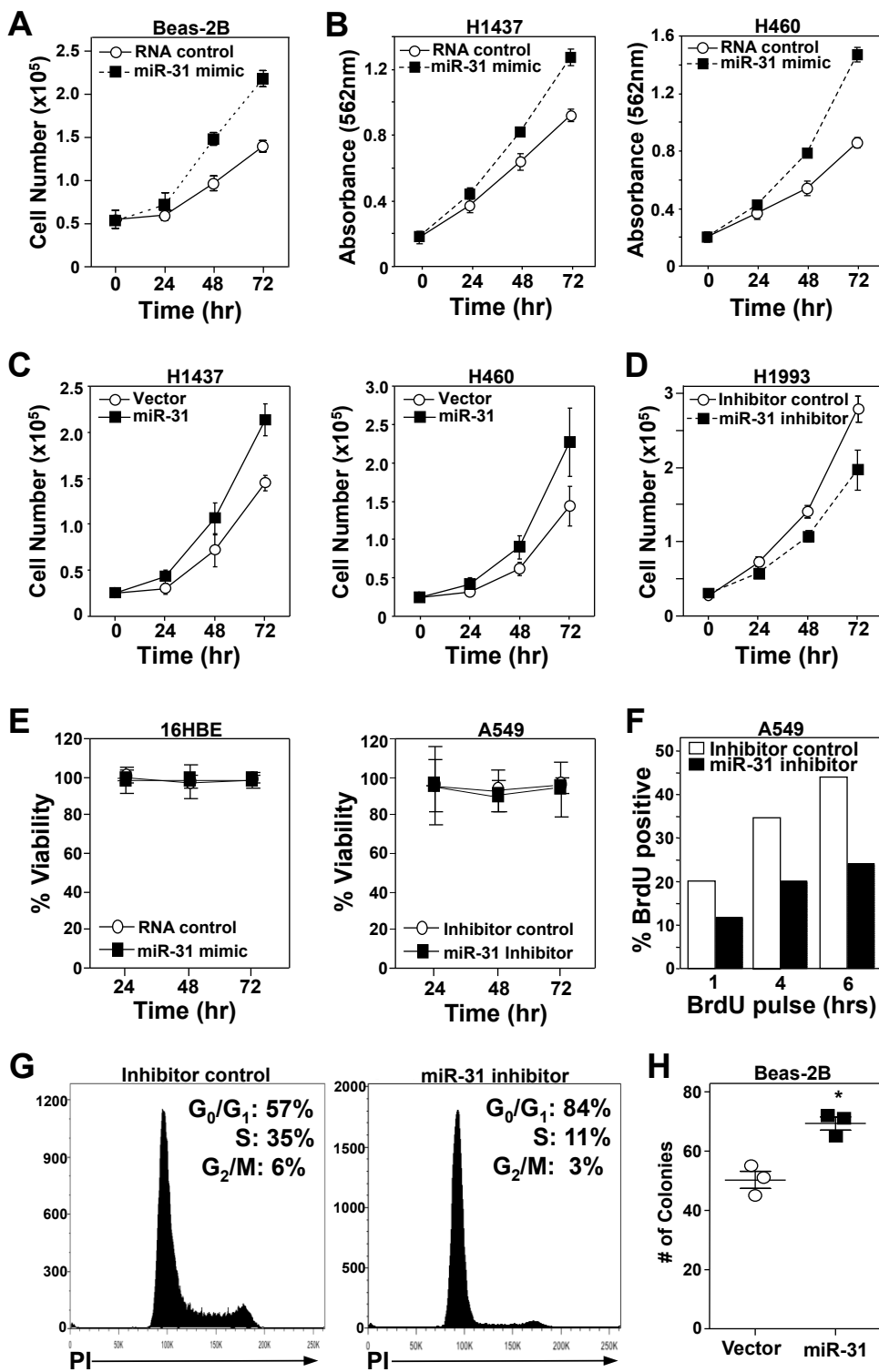
additional representative H&E image of a lung adenocarcinoma from a miR-31/CCSP mouse given dox for 18 months (top). An enlarged H&E image for figure 4I (middle left) and an additional image from the same mouse showing another example of an adenocarcinoma invading into a blood vessel (middle right). An H&E image of a lung adenocarcinoma invading into a blood vessel from another miR-31/CCSP mouse given dox for 18 months (bottom).

Figure S6. miR-31 expression induces infiltrative lung tumor growth in KRas^{G12D} mice. (A) H&E sections of lungs from the indicated mice administered doxycycline (CCSP/KRas n=15, miR-31/CCSP/KRas n=16) for 2 months were evaluated for hyperplasia and adenoma. (B) Enlarged H&E images for figures 5E. (C) Representative photographs of H&E stained lung sections from the genotype indicated after two months of miR-31 induction. Infiltrative growth and dysplasia observed in the miR-31/CCSP/KRas lungs. (D, E) *KRas^{G12D}* (D) and miR-31 (E) levels in the lungs of the indicated genotype of mice (littermate matched) administered doxycycline or control water for 2 months were measured in triplicate by qRT-PCR. *KRas^{G12D}* expression is relative to *β-actin* levels and miR-31 expression is relative to snoRNA-202 levels. (F) miR-31 expression was measured in triplicate by qRT-PCR from the indicated genotype of transgenic mice (littermate matched) that were injected once intraperitoneally with 1 g/kg urethane and provided with doxycycline containing water for 6 weeks. miR-31 levels are relative to snoRNA-202 levels. Number of mice indicated by n; error bars are SEM, *p=0.000007, t-test. (G) Lung adenocarcinoma and normal lung with accompanying miR-31 expression and genomic data were acquired from the TCGA. miR-31 expression in adenocarcinoma with mutant KRAS (G12A, G12C, G12D, G12R, G12S, G12V, Q61L) and adenocarcinoma with wild-type KRAS and no other known driving mutation.

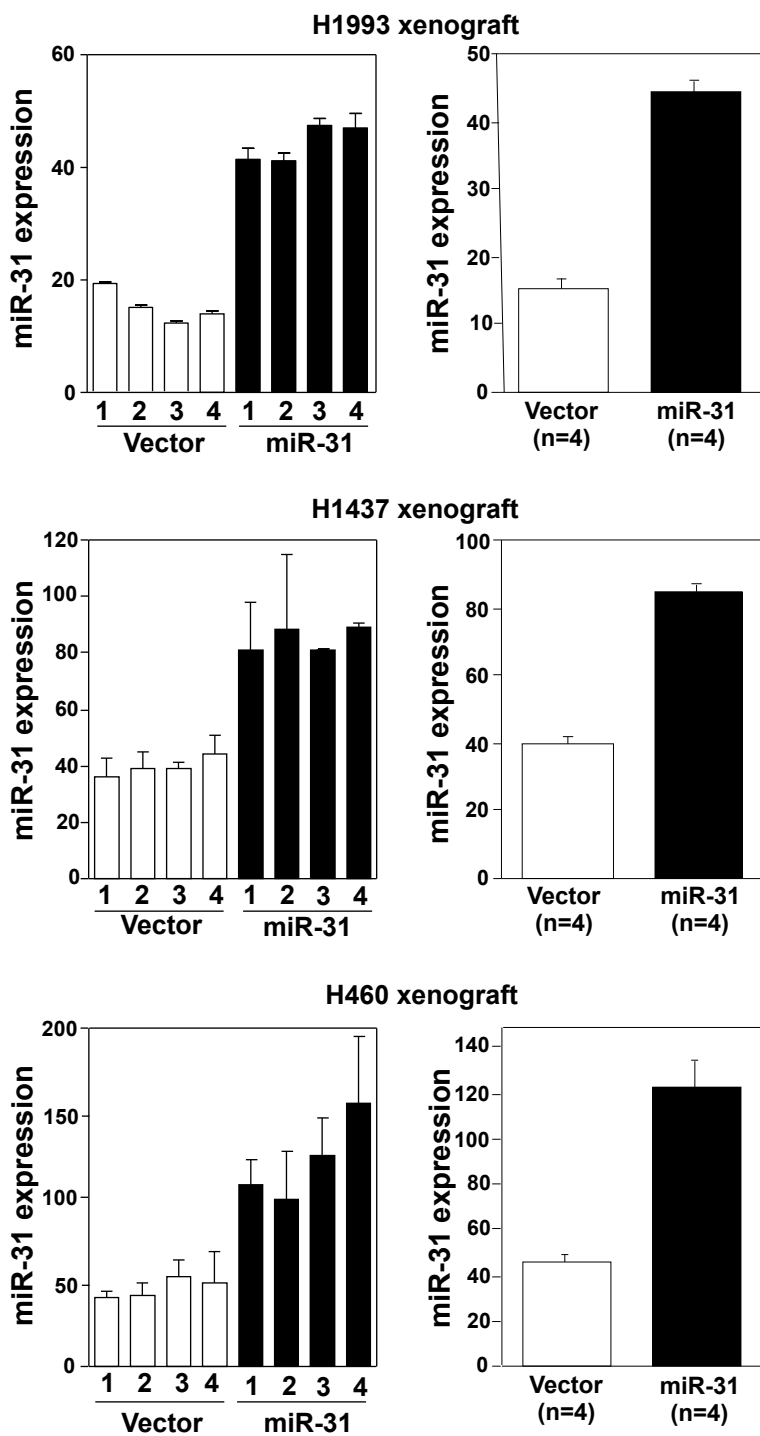
Figure S7. Putative miR-31 targets are modulated by miR-31 levels. (A, B) Beas-2B cells were transfected with miR-31 mimic or RNA control (A) and A549 adenocarcinoma cells were transfected with miR-31 inhibitor or inhibitor control (B). qRT-PCR (triplicates) for putative miR-31 target mRNA; values normalized to levels of β -*ACTIN*, SEM. A, *p=0.0001, **p=0.0006, ***p=0.007; B, *p=0.0002, **p=0.0003, ***p=0.001, t-tests. (C) Densitometry for western blot analysis performed in figure 7D. Values normalized to Tubulin levels and then the samples transfected with RNA control were set at 1 for each. (D) MTT analysis (triplicates) for Beas-2B cells transfected with either miR-31 mimic or RNA control and treated with either vehicle (DMSO) control or 100 nM of the MEK inhibitor Trametinib (Trem) or Selumetinib (Sel), SEM.

Figure S8. miR-31 down-regulates the six negative regulators of RAS/MAPK signaling, which may reduce lung adenocarcinoma patient survival. (A) Kaplan-Meier analysis from TCGA lung adenocarcinoma data on each of the six negative regulators of RAS/MAPK signaling. Median (left, n=247 high and 248 low) and the bottom (Q1) and top (Q4) quartiles (right) of expression of the indicated gene are graphed; n=495 total; p-values from log-rank tests. (B) H1993 cells were infected with an empty retrovirus (vector) or a retrovirus encoding miR-31. qRT-PCR (triplicate) was performed for the mRNA indicated; values are relative to the levels of β -*ACTIN*.

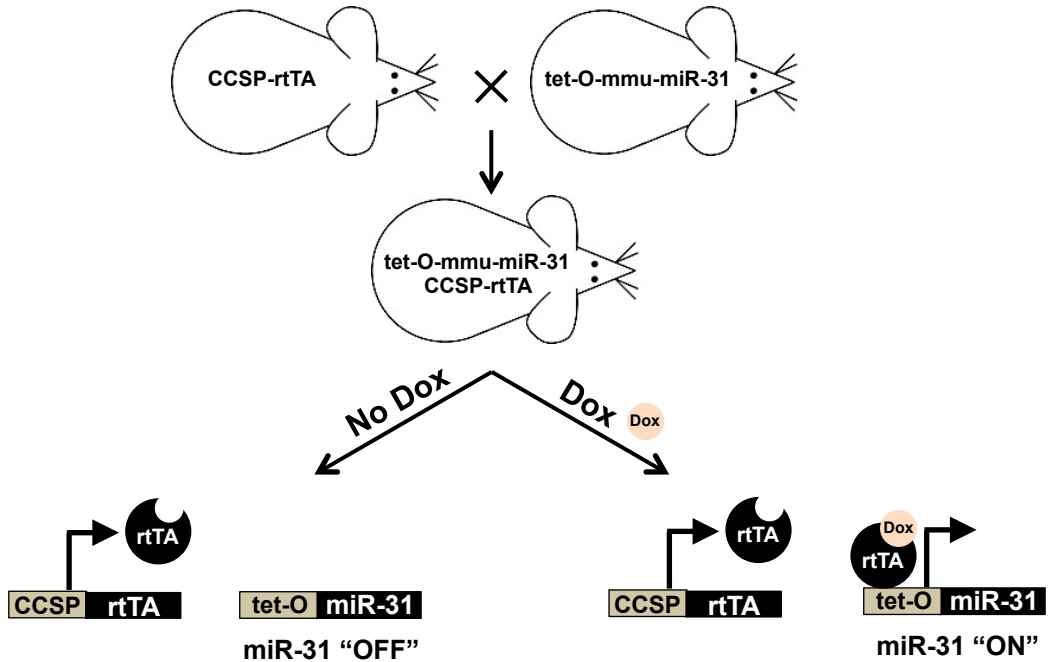




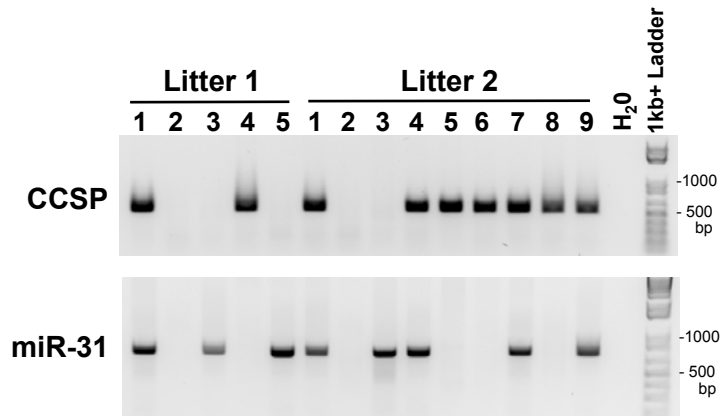
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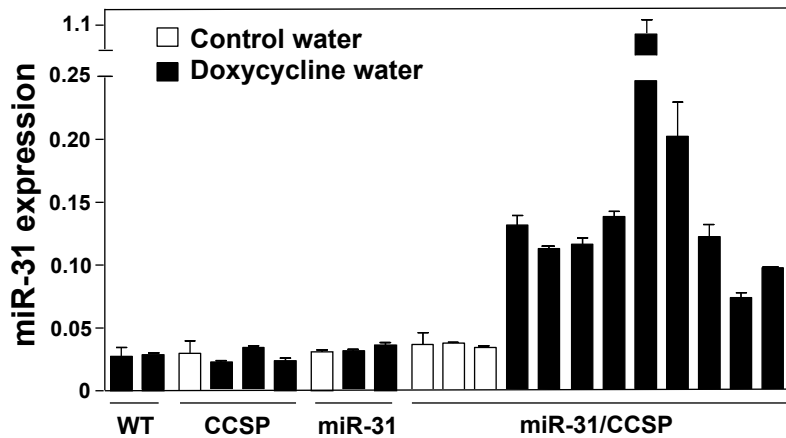
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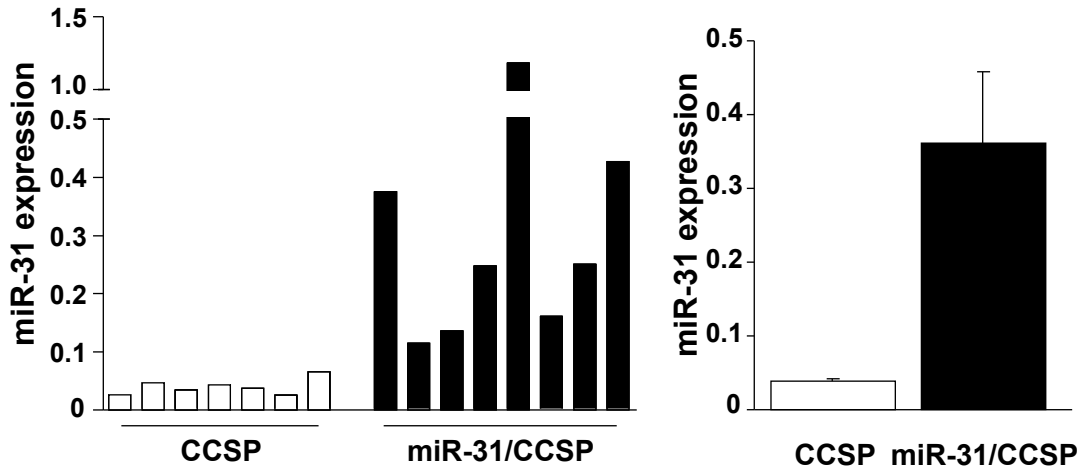
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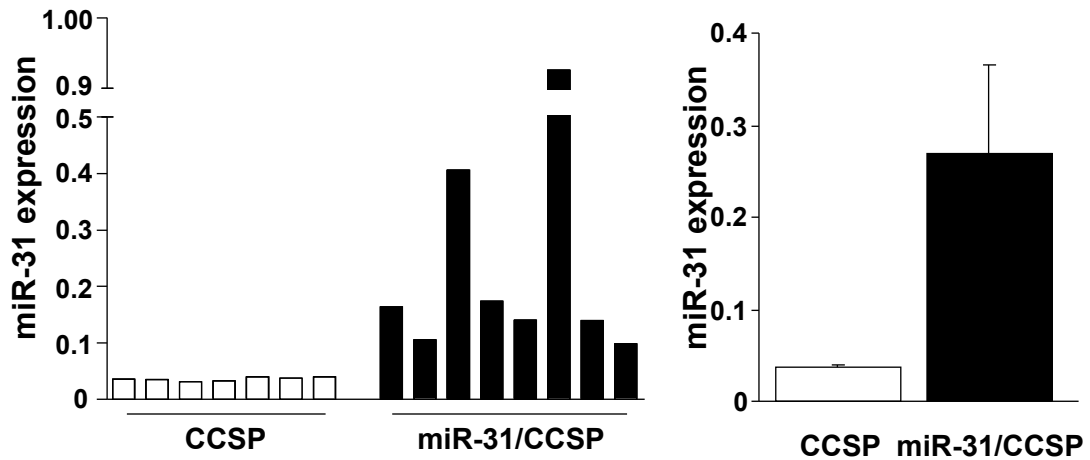
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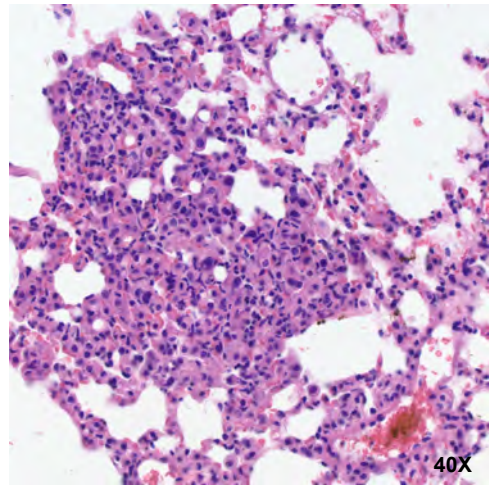
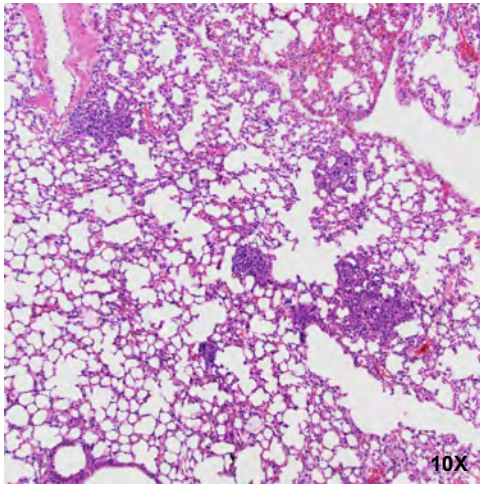
2 months



12 months



A

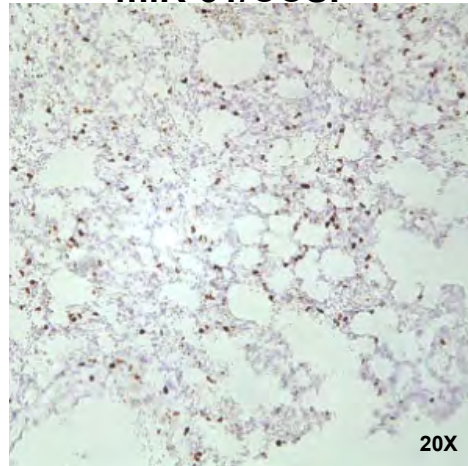
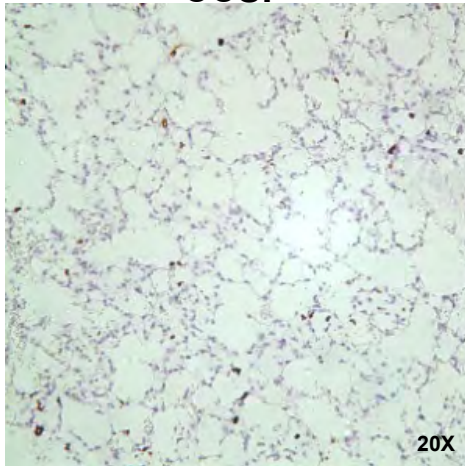


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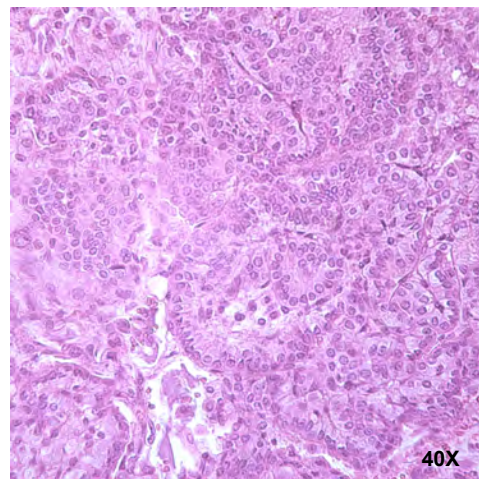
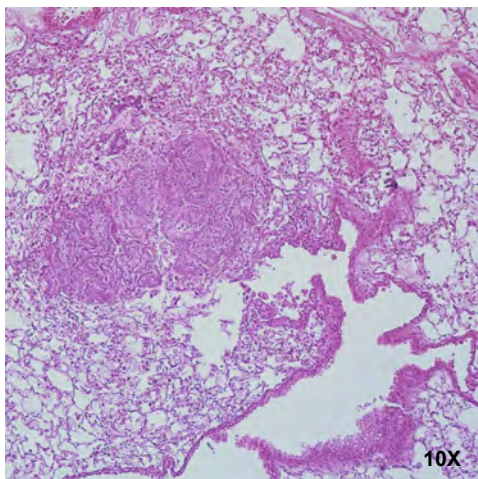
CCSP

miR-31/CCSP

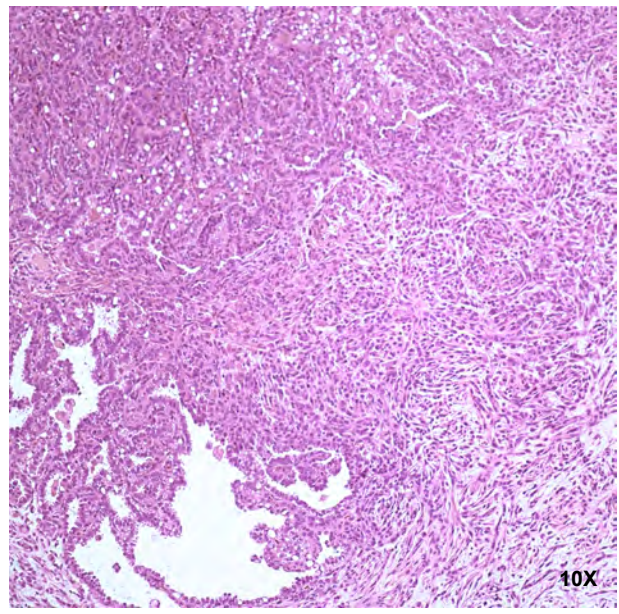
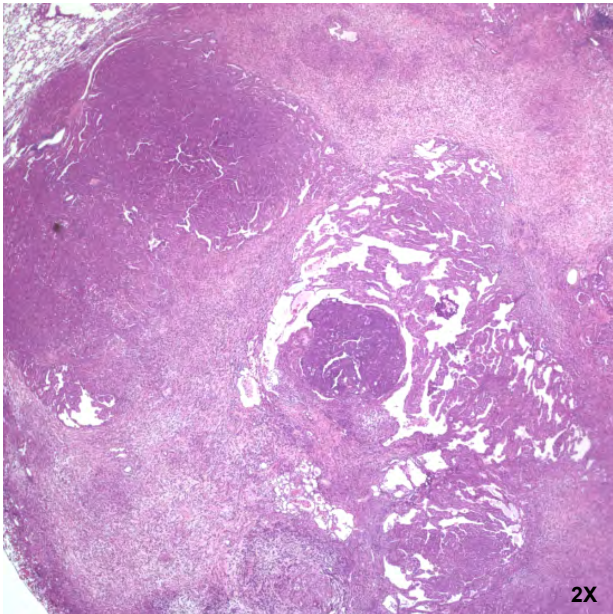
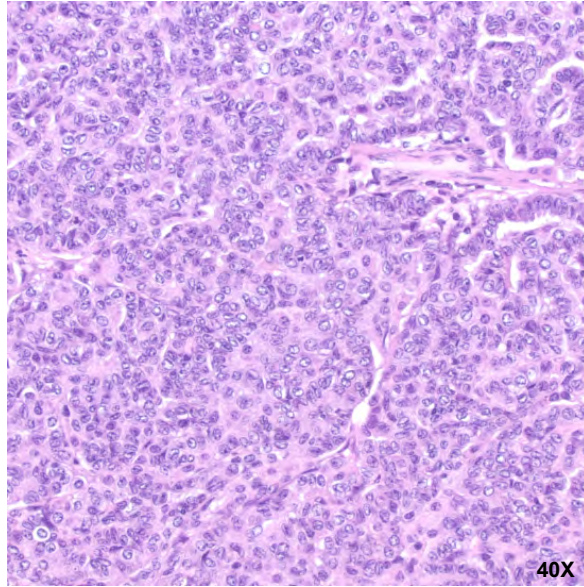
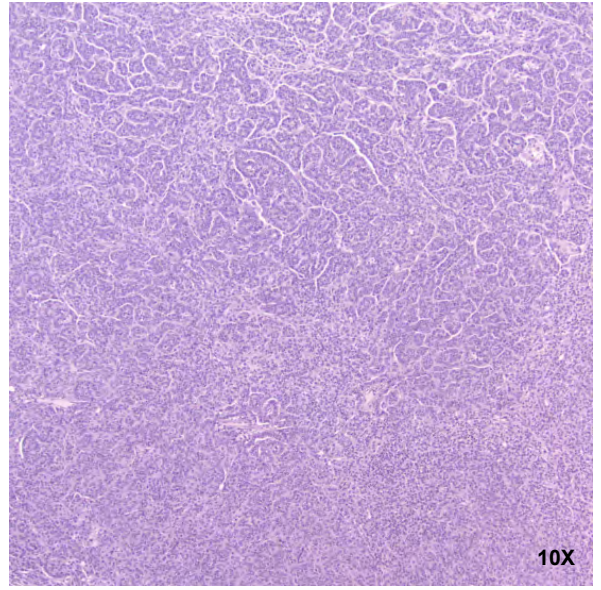
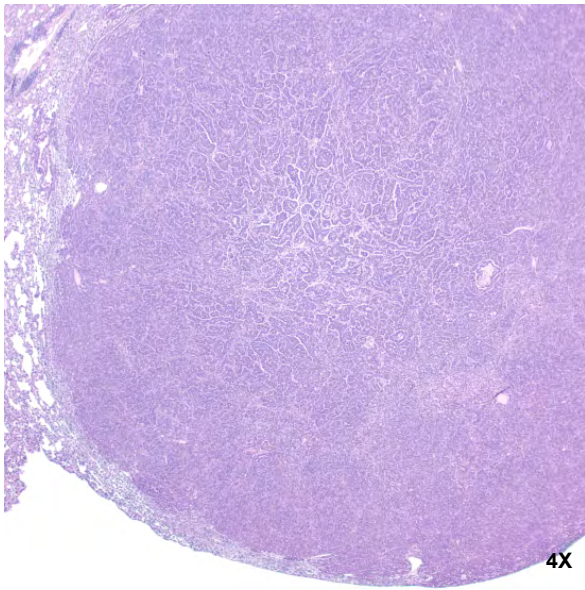
Ki67

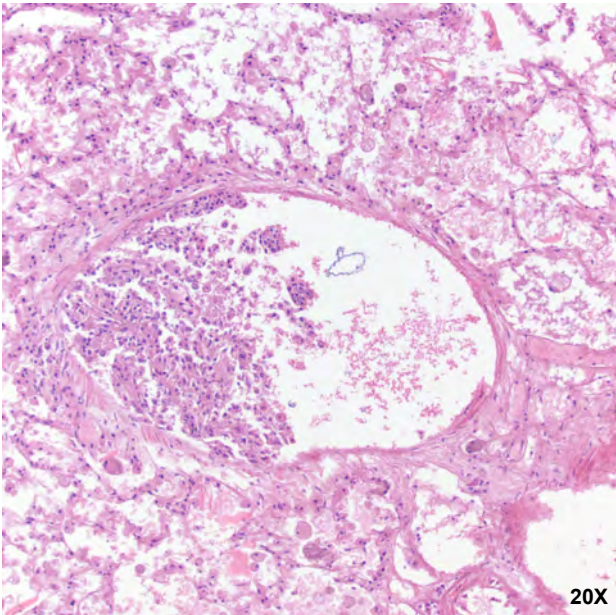
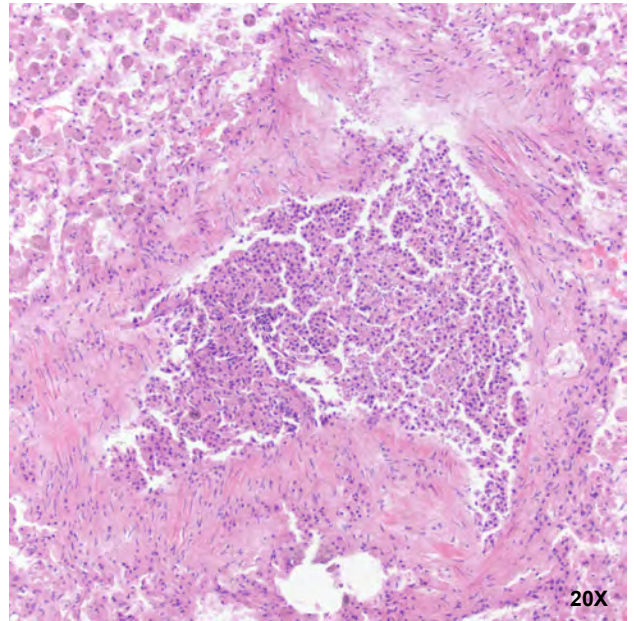
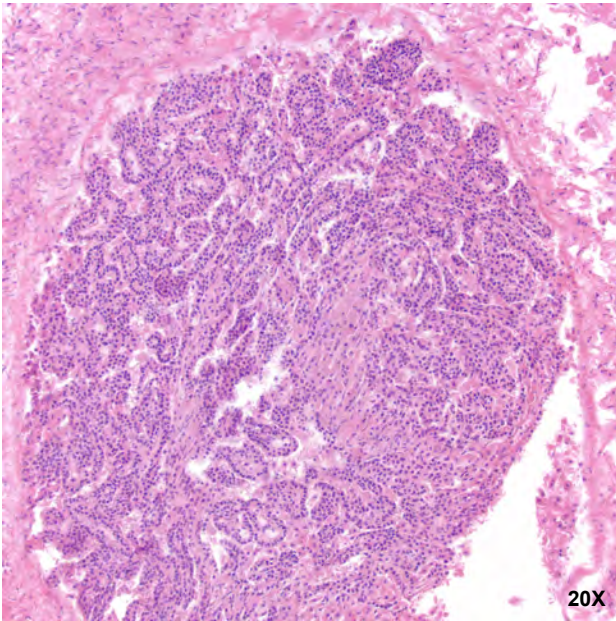
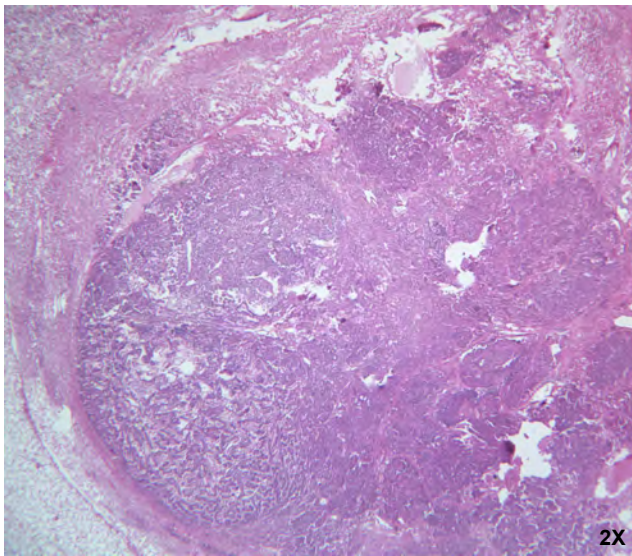


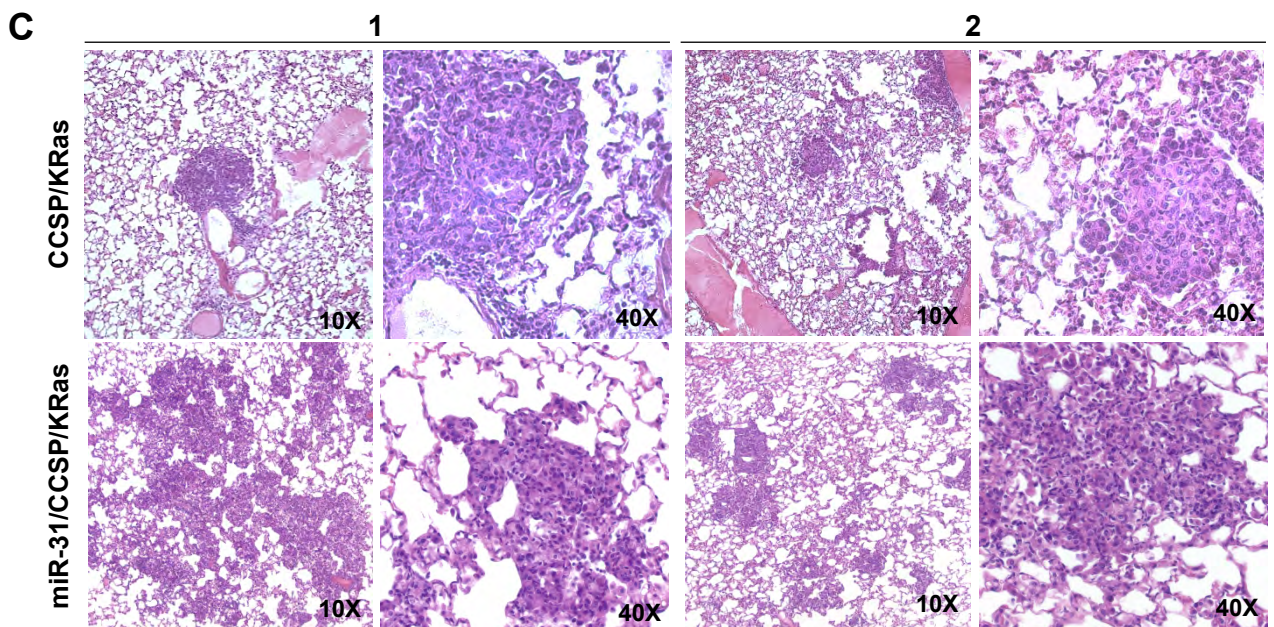
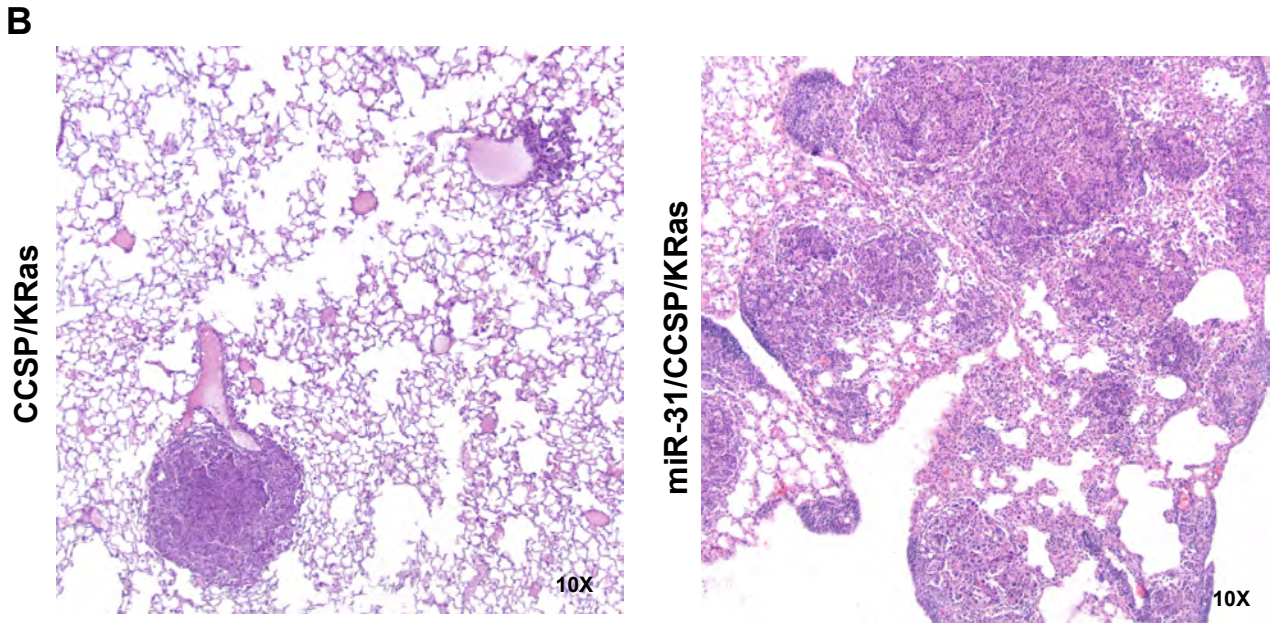
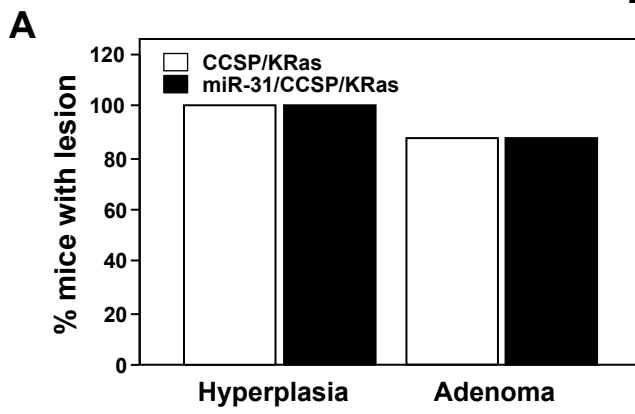
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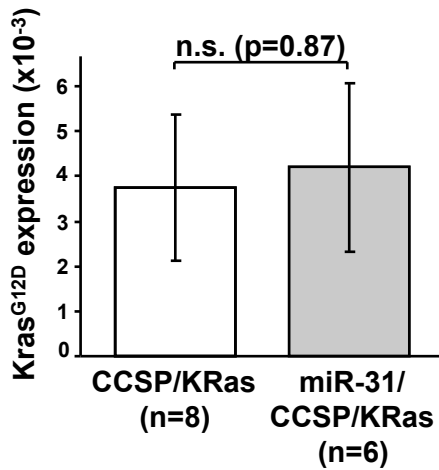
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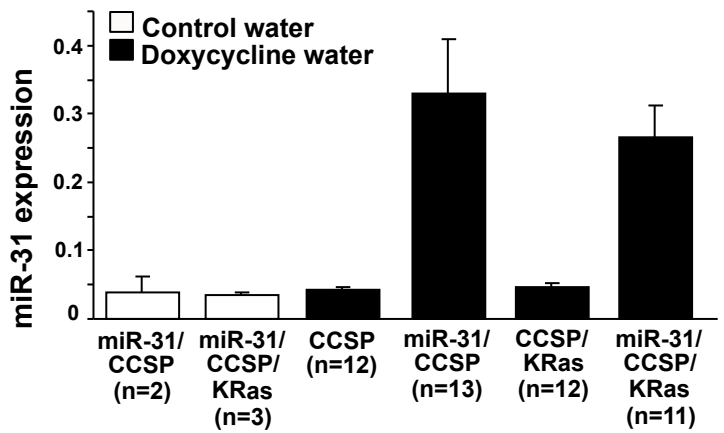




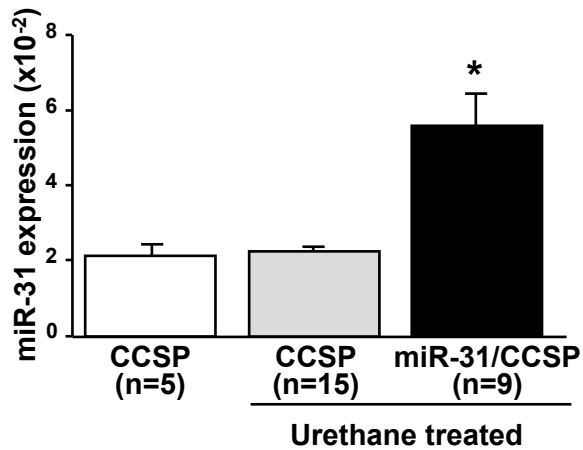
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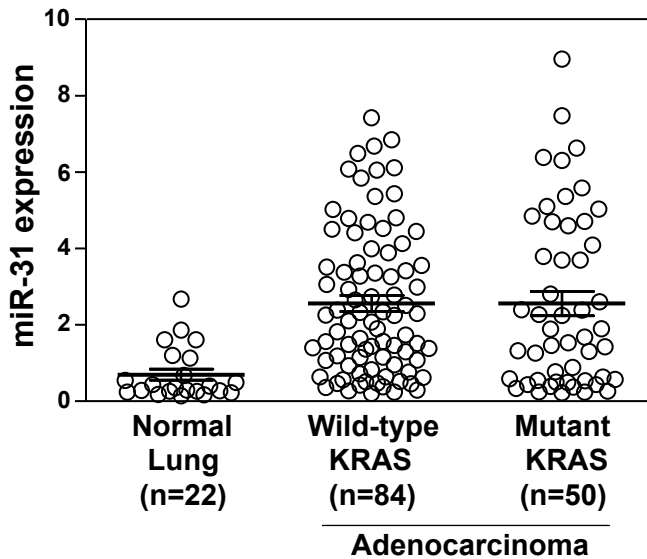
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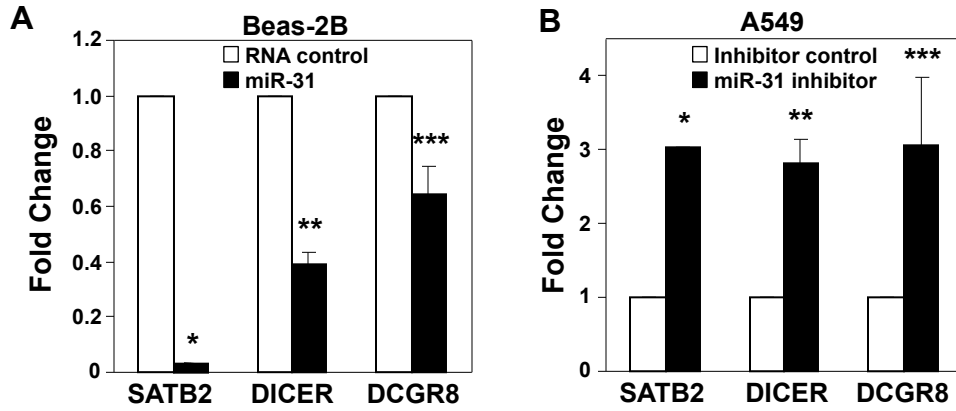


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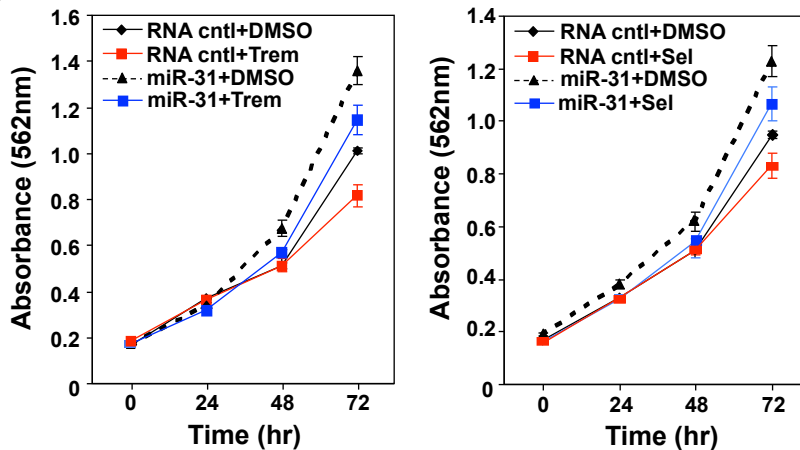




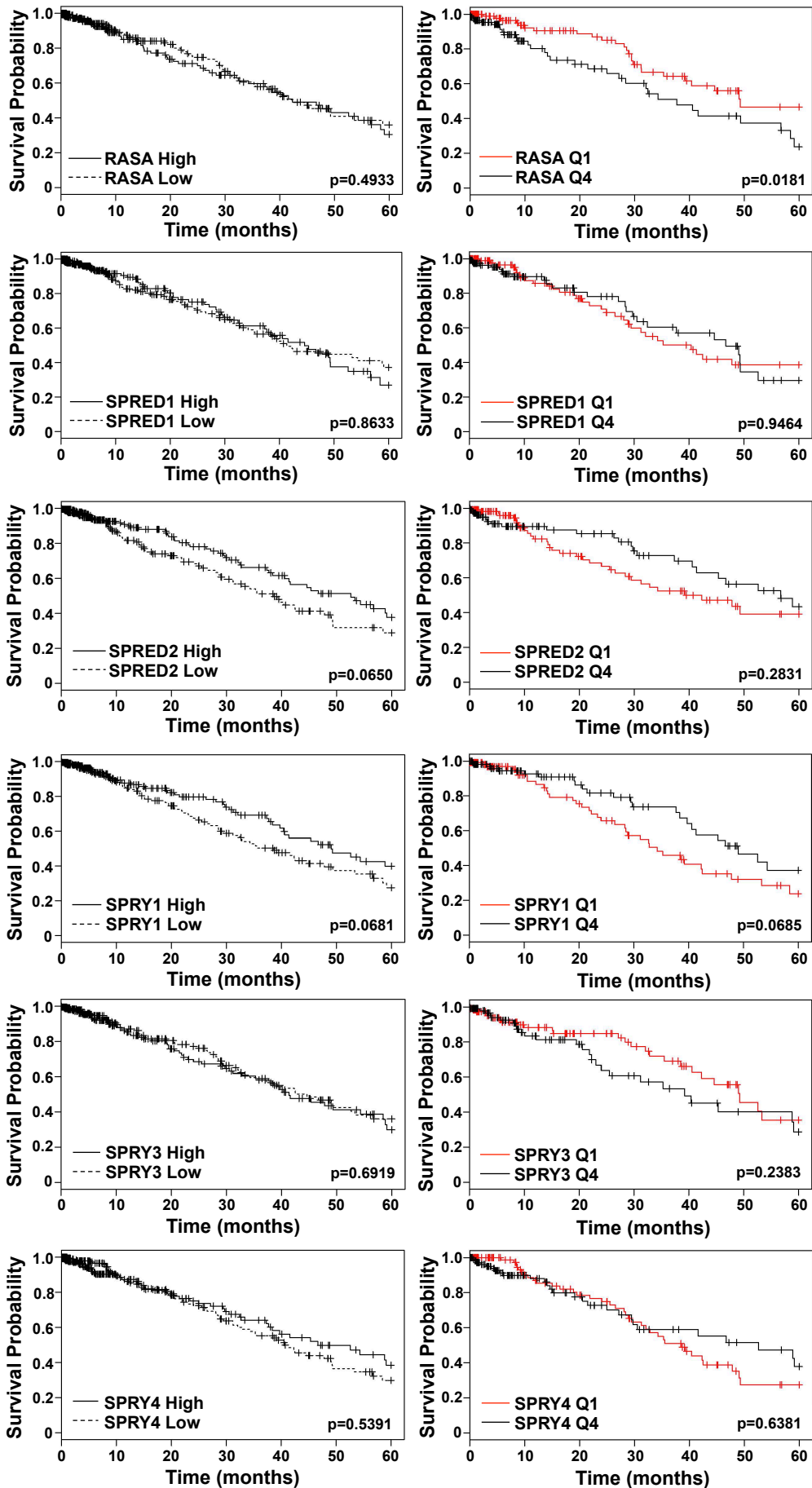
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	Beas2B		A549	
	RNA control	miR-31 mimic	Inhibitor control	miR-31 inhibitor
RASA1	1	0.47	1	3.80
SPRED1	1	0.55	1	2.56
SPRED2	1	0.18	1	1.89
SPRY1	1	0.63	1	2.40
SPRY3	1	0.12	1	3.18
SPRY4	1	0.36	1	2.78

D



A



B