Supplemental Information

Supplemental Results

Histological characterization of lung tumors. Lung tumors from NNK-treated control-Dox and lung-*Cpr*-null-Dox mice (three mice per group) were submitted to the Pathology Laboratory of the Wadsworth Center for histological examination. All lung lobes contain at least one focus of type II pneumocyte (presumptive) hyperplasia or adenoma; these foci consist of irregularly oval to round, subpleural or peribronchial, approximately 1 mm nodules of cuboidal cells which line preexisting alveolar walls; while most trabeculae are lined by two layers of cuboidal cells (one layer on each side of an alveolar wall, presumptive), some are more solid, with multiple layers of cuboidal cells, typical of adenoma. The tumors are non-encapsulated. Where they are subpleural, they push up the pleura slightly, but there is no pleural reaction. There are sloughed epithelial cells or pulmonary alveolar macrophages between cords of tumor cells. There are sometimes lymphoid infiltrates in the tumor stroma; also, nodules of lymphocytes are found in moderate numbers (in one lung-*Cpr*-null-Dox and two control-Dox mice), scattered in the alveolar parenchyma; all mice have increased perivascular lymphoid cuffs.

Supplemental Materials and Methods

Chemicals and reagents. NNK was obtained from Chemsyn Science Laboratories (Lenexa, KS). NNAL, 4-(methylnitrosamino)-1-(3-[2,4,5,6-D_4]-pyridyl)-1-butanone (D_4-NNK), and 4-(methylnitrosamino)-1-(3-[2,4,5,6-D_4]-pyridyl)-1-butanol (D_4-NNAL) (isotopic purity >99% for both compounds) were obtained from Midwest Research Institute (Kansas City, MI).

Genotype analysis. Genomic DNA was prepared from mouse tail (or toe) biopsies, which were obtained at the ages of 10 to 14 days. Primers 5'-tacaatggaccaggctctgc-3' (forward) and 5'-aagagggacaaagagcacc-3' (reverse) were used for genotyping Cpr^{lox} and Cpr^{+} alleles (1). *CCSP-rtTA* (2) and *Cre* (3) transgenes were detected as described.

Quantitative PCR analysis of the abundance of the Cpr^{-} and Cpr^{lox} alleles. AECII cells were isolated according to published method (4, 5). The extent of enrichment of AECII cells was estimated by

the fold of enrichment of SP-C in the isolated cell preparations relative to the whole lung (6). Genomic DNA was prepared from lung tissues and isolated AECII cell preparations using DNeasy Tissue Kit (Qiagen; Valencia, CA), with RNase I treatment. Genomic DNA was used as template for the determination of relative levels of the Cpr^{-} and Cpr^{lox}. Real-time DNA-PCR was performed using a LightCycler™ (Roche Applied Science). Primers CPRF1 (5'-ccttctctcataccggtctct-3') and CPRR1 (5'-cattgcccctgtttcactatcc-3') were used to amplify the Cpr^{-} allele, whereas primers CPRF2 (5'-tcccattggatctcccattggc-3') and CPRR2 (5'-atatgcctcgaatcaccagtgcg-3') were use to amplify the Cpr^{lox} and Cpr^{+} allele. The PCR mixtures contained 1 µl of FastStart DNA Master SYBR Green I (Roche Applied Science), 3 (for CPRF1/R1) or 4 (for CPRF2/R2) mM $MgCl_2$, 0.4 µM each primer, and 2 µl of DNA template (up to 20 ng) in a total volume of 10 µl. PCR was monitored for 40 cycles, with annealing temperature at 66 ^{o}C. At the end of the PCR cycles, melting curve analysis was performed, in order to assess the purity of PCR products. PCR specificity was confirmed by electrophoresis of PCR products on agarose gels. Negative control reactions (complete reaction minus DNA template) were routinely included to monitor potential contamination of reagents.

The relative abundance of the Cpr^{-} and Cpr^{lox} alleles in the whole lung or in the AECII cell preparations was determined with use of hepatic DNA of adult *Alb-Cre/*$Cpr^{lox/+}$ mice as the standard. In the livers of adult *Alb-Cre/*$Cpr^{lox/+}$ mice, the Cpr^{lox} allele is converted to the Cpr^{-} allele because of the CRE-mediated recombination, whereas the Cpr^{+} allele is intact. Therefore, the DNA samples prepared from these livers have approximately equal proportions of the Cpr^{+} and Cpr^{-} alleles. Hepatic genomic DNA was prepared from five individual, 2-month-old *Alb-Cre/*$Cpr^{lox/+}$ mice. One of the hepatic DNA samples, which was serially diluted to 10, 2.5, 0.63, and 0.16 ng/µl, was used in PCR to generate standard curves, for which a 1:1 ratio was set as the relative abundance of the Cpr^{-} and Cpr^{+} alleles. The other four DNA samples were used as positive controls. The percentage of the Cpr^{-} allele in a given DNA sample was calculated [Cpr^{-}/(Cpr^{-} and Cpr^{lox}) x 100%] from at least triplicate determinations.

RNA preparation and quantitative RNA-PCR. Total RNA was isolated from mouse lung and isolated AECII cell preparations using RNeasy Mini Kit (Qiagen). RNA concentrations were determined

spectrally. Up to 0.5 μg of the total RNA was used for reverse transcription (RT) using the SuperScript™ III First-Strand Synthesis System (Invitrogen). Contaminating genomic DNA was removed using DNase I before the RT reaction. Negative controls for the RT reaction consisted of complete reaction mixture minus reverse transcriptase. The relative mRNA levels were determined for SP-C using a model 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Real-time PCR was preformed using the SYBR@ Green PCR Core Reagent (Applied Biosystems). Primers 5'-catcgttgtgtatgactaccagcg-3' (forward) and 5'-gaatcggactcggaaccagtatc-3' (reverse) were used to amplify SP-C cDNA (7). The levels of β-actin, which was used for normalization of total RNA input in RT reactions, were determined with use of primers 5'-gcccagagcaagagaggtat-3' (forward) and 5'-ggccatctcctgctcgaagt-3' (reverse) (8). The PCR mixtures contained 2 μl of 10x SYBR Green PCR buffer, 3 mM $MgCl_2$ and 0.3 nM each primers for SP-C or 2 mM $MgCl_2$ and 0.4 nM each primers for β-actin, 0.2 mM each dNTP, 0.2 μl of AmpErase (1 U/ml), 0.1 μl of Ampli Tag Gold (5 U/ml), and 4 μl of RT reaction mix in a total volume of 20 μl. Reactions were monitored for 40 cycles with annealing temperature at 60 ^{o}C. At the end of the amplification program, melting curve analysis was performed to assess the purity of PCR products. PCR products were also analyzed by electrophoresis on agarose gels to confirm PCR specificity. Negative control reactions for PCR (complete reaction minus DNA template) were routinely included to monitor potential contamination of reagents. For each primer pair, serial dilutions of a common lung RNA sample from a control mouse were used to construct standard curves for quantification of the relative amounts of SP-C and β-actin.

Immunohistochemical analysis. Paraffin sections (4~5 μm) of lungs were analyzed using conditions described in a recent study (9). The antibody-binding sites were visualized using Alexa-488 or Alexa-594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR); both fluorescently labeled antibodies were used at 1:200 dilution, and the incubations were preformed at room temperature for 1 h. Sections, mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories), were stored in the dark at 4 ^{o}C until they were examined (within one week). Fluorescent signals were detected using a

Nikon 50i microscope (Nikon Inc., Melvile, NY) equipped with a Quad Fluor epi-fluorescence attachment and a 20× objective lens. The Quad-Fluor filter sets used were B-2E/C for Alexa-488, UV-2E/C for Alexa-594, and G-2E/C for DAPI. The color images were captured digitally using a Spot CCD camera (Diagnostic Imaging, Sterling Heights, MI). Contrast enhancement and montaging were done in Adobe PhotoShop software, version 6.0 (Adobe Inc., San Jose, CA). The primary antibodies used included rabbit anti-CPR (1:2000; Stressgen, San Diego, CA), rabbit anti-prosurfactant protein C (Clone:AB3786, 1:2000; Chemicon, Temecula, CA), and rabbit anti-CCSP (1:2000; Chemicon). For negative controls, the primary antibodies were replaced with goat serum. The proximal airway region was identified by where the trachea enters the lung, whereas the terminal airway region was identified by where the bronchioles branch into alveolar ducts.

Liquid chromatography-mass spectrometry (LC-MS) determination of NNK and NNAL. The internal standards, D_4-NNK and D_4-NNAL, were dissolved in H_2O at a concentration of 1 μg/ml. For analysis of NNK and NNAL (unconjugated), 2.5, 5.0 or 10 μl of plasma was mixed with 150 μl of saline and 5 μl of D_4-NNK and D_4-NNAL (1 μg each/ml). The resultant mixture was extracted with Oasis Mixed Mode Cation Exchange (MCX) Cartridge (30-mg size) (Waters; Milford, MA) according to a reported method (10), with modifications. Briefly, the MCX cartridge was first conditioned with 1 ml of methanol and then equilibrated with 1 ml of H_2O, prior to the application of the plasma sample; thereafter, the cartridge was washed sequentially with 1 ml each of 0.1 M HCl, methanol, and a mixture of H_2O:methanol:NH_4OH (90:5:5). NNK and NNAL were eluted with 1 ml of methanol containing 5% of concentrated NH_4OH (28-30%). The solid phase extraction procedure, except for the elution step, was performed using a 12-port manifold (Fisher Scientific), with the flow rate controlled at 1-2 ml/min. The elution step was performed by gravity. The eluted samples were dried in a Savant SpeedVac (GMI Inc., Ramsey, MI). The residues were resuspended in 100 μl of 2 mM ammonium formate containing 15% acetonitrile, and centrifuged at ~10, 000 g for 5 min; the resultant supernatant fractions were used for LC-MS analysis.

For preparation of calibration curves, plasma samples from saline-treated animals were spiked with 5 µl of mixtures of NNK and NNAL in the concentration range of 10 ng/ml to 60 µg/ml. All calibration samples contained the same amounts of D_4-NNK and D_4-NNAL (5 µl of a 1 µg each/ml stock solution), and were extracted and processed as described in the preceding paragraph. Blank controls for solvent and matrix were included in each set of calibration samples.

For LC-MS determination of NNK and NNAL, an Agilent-1100 LC-MSD system (Agilent Technologies, Palo Alto, CA, USA) was used. The LC-MS system was controlled using ChemStation Software (Agilent Technologies). The HPLC solvent conditions were set up according to the method of Byrd and Ogden (11), with modifications. NNK and NNAL were resolved using a Zorbax SB-C_{18} column (2.1 mm × 150 mm, 3.5 µm, Agilent Technologies) preceded by a 2.1 × 15 mm guard column. The mobile phase consisted of 2 mM ammonium formate in 15% acetonitrile (pH 6.8) (A) and 100% acetonitrile (B). The solvent was held at 100% A for the first 3 min, followed by a linear increase from 0 to 30% B from 3 to 10 min at a flow rate of 0.3 ml/min. The MS was operated in the positive ion mode, using electrospray ionization. The protonated molecules $[M+H]^+$ were measured in the selected ion monitoring (SIM) scan mode at *m/z* 210 for NNAL, *m/z* 214 for D_4-NNAL, *m/z* 208 for NNK, and *m/z* 212 for D_4-NNK. The parameters for the electrospray chamber were as follows: fragmentor at 100 v, capillary voltage at 3000 v, nitrogen sheath gas temperature at 350 ^oC; drying gas speed at 9.0 *l*/min; and nebulizer pressure at 40 psig. The column was flushed with 6 ml of 100% acetonitrile every 5~10 injections. The autosampler needle was washed with 65% acetonitrile between injections.

The specificity of the LC-MS analysis of NNK and NNAL was confirmed by analyzing the same samples under two different solvent systems, which produced differing retention times for the analytes. All samples were analyzed with the neutral pH solvent system as described above. Selected samples were also analyzed with an acidic pH solvent system (pH 2.7), which consisted of 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B), with a linear gradient of B from 0 to 50% over 20 min. The MS operating conditions were set up the same as those for the neutral solvent system as described above.

LS-MS determination of O^6-mG. The internal standard O^6-CD_3-dG was synthesized according to a reported method (12, 13). Genomic DNA samples (0.2-0.4 mg each), fortified with 10 μl of 400 nM O^6-CD_3-dG, were hydrolyzed in 0.1 N HCl at 80 ^oC for 90 min in a total volume of 300 μl, according to Chung et al. (14). Following hydrolysis, 270 μl of the reaction mixtures were extracted with Oasis MCX cartridge (Waters) for detection of O^6-mG, as described above for the extraction of plasma NNK, except that the column was not washed with the mixture of H_2O:methanol:NH_4OH (95:5:5). O^6-mG was eluted by gravity in 1 ml of methanol containing 5% of concentrated NH_4OH (28-30%). The eluted fractions were dried with the Savant SpeedVac. The residues were resuspended in 40 μl of 15 mM ammonium acetate, and centrifuged at ~10, 000 g for 10 min; the resultant supernatant was used for LC-MS analysis.

Genomic DNAs from tissues of saline-treated animals or salmon sperm DNA (Stratagene, La Jollas, CA) were used for preparation of calibration curves for the quantification of O^6-mG. Briefly, 0.2 mg of control genomic DNAs, fortified with 10 μl of 400 nM O^6-CD_3-dG, were spiked with 10 μl of O^6-CH_3-dG in the concentration range of 40 to 3200 nM. The calibration samples were then hydrolyzed, extracted, and processed as described above. Blank controls for solvent and matrix were included in each set of calibration samples.

The LC-MS method for detection of O^6-mG was modified from Guza et al (15). The LC-MS system and column were the same as those for the analysis of NNK and NNAL. The mobile phase consisted of 15 mM ammonium acetate (pH 6.8) (A) and 100% acetonitrile (B) with a linear gradient of 0 to 15% of B over 20 min at a flow rate of 0.3 ml/min. The MS was operated in the positive ion mode, using electrospray ionization, and the analytes were measured by the SIM scan mode as $[M+H]^+$ at *m/z* 166 for O^6-mG and at *m/z* 169 for O^6-CD_3-G. The column used was the same as that used for NNK and NNAL. The parameters for the electrospray chamber were as follows: fragmentor at 120 v, capillary voltage at 2000 v, nitrogen sheath gas temperature at 350 ^oC; drying gas speed at 12.0 *l*/min; and nebulizer pressure at 50 psig. The column was flushed with 3 ml of 100% acetonitrile, and the autosampler needle was washed with 65% acetonitrile, between injections.

Determination of guanine. Following a mild acidic hydrolysis of genomic DNA (14), 10 μl of the reaction mixture was neutralized with 7 μl of 0.1M NH_4OH, and diluted 1:500 with H_2O. Five microliters of the diluted sample was injected for the detection of guanine using an HPLC system with a diode array detector (Agilent 1100). The mobile phase consisted of 98% of 15 mM ammonium acetate and 2% of acetonitrile at a flow rate of 0.3 ml/min. The column used was the same as used for NNK and NNAL measurement. The chromatogram was recorded at 254 nm. The Quantification of guanine was achieved with a standard curve in the range of 0.78 to 25 μM.

Other Methods. Statistical analyses were performed with use of SigmaStat software (version 2.0). The tests used include Student's *t* test for comparison between two groups with equal variance; Mann-Whitney Sum Rank Test for comparison between two groups with unequal variance; One-Way ANOVA for comparison among three or more groups, and chi-squared test for genotype distribution and tumor frequency (percentage of mice with tumors).

Supplemental Reference

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Supplemental Table S1. Genotype distribution among pups resulting from intercrosses either between lung-Cpr-null and *Cpr*$^{lox/lox}$, or between *CCSP-rtTA*^{hemi}/*Cpr*$^{lox/lox}$ and *tetO-Cre*^{hemi}/*Cpr*$^{lox/lox}$ mice

	CCSP-rtTA^{hemi}/*tetO-Cre*^{hemi}/*Cpr*$^{lox/lox}$ (lung-*Cpr*-null)	*CCSP-rtTA*^{hemi}/*Cpr*$^{lox/lox}$	*tetO-Cre*^{hemi}/*Cpr*$^{lox/lox}$	*Cpr*$^{lox/lox}$
Number of pups with each genotype	55	52	62	50
Genotype frequency (% of total)	25	24	23	28

The 219 pups came from 15 breeding pairs on an A/J-N_3 genetic background. The size of the litters was 5.6 ± 2.1 mice/litter (means ± S. D., n=39), which is similar to that of wild-type A/J strain (6.3 ± 0.7 mice/litter; www.jax.org). There was no significant difference between the observed genotype frequency and that predicted from Mendelian distribution (25% each) ($P>0.05$, chi-squared test), indicating that the lung-*Cpr*-null genotype was not associated with any embryonic lethality.

Supplemental Table S2. Body and organ weights of the lung-*Cpr*-null-Dox and control-Dox mice

Animals	Body weight (g)	Lung weight (g)	Liver weight (g)
Lung-*Cpr*-null-Dox	21.4 ± 0.4 (n = 22)	0.17 ± 0.02 (n = 6)	0.98 ± 0.07 (n = 6)
Control-Dox	20.6 ± 0.4 (n = 23)	0.17 ± 0.01 (n = 6)	0.91 ± 0.06 (n = 6)

The body and organ weights were determined for Dox-treated female mice, at the age of 2 months. The values shown are means ± SEM. There was no significant difference ($p>0.05$) between the two groups in either body weights or organ weights.

Supplemental Table S3. Relative abundance of the *Cpr*$^{-}$ allele in the lung and AECII of the lung-*Cpr*-null mice

Dox treatment [*]	Cpr^{-}%[†]	
	Whole lung	AECII
None (n=4)	6.2 ± 1.8	17.4 ± 3.9 [§]
1-2m (n=3)	13.9 ± 0.8 [‡]	25.0 ± 2.9 [§]
E0-2m (n=4)	18.2 ± 2.7 [‡]	32.2 ± 3.7 [‡, §]

[*]None means without Dox treatment; 1-2m and E0-2m means the animals were kept on Dox-containing food from the age of 1 month or from E0, respectively. Animals were sacrificed at the age of 2 months for DNA isolation. [†]The relative abundance of the *Cpr*$^{-}$ allele was determined by quantitative DNA-PCR analysis of PCR products representing the *Cpr*$^{-}$ and *Cpr*^{lox} alleles, as described in Supplemental Methods. The extent of *Cpr* deletion was expressed as a percentage of the amount of *Cpr*$^{-}$ allele relative to the total amount of *Cpr*$^{-}$ and *Cpr*^{lox} amplicon in a given genomic DNA preparation. The values shown are means ± SEM. [‡]Significantly higher than those of untreated lung-*Cpr*-null mice within the same column ($P<0.05$); [§]Significantly higher than those of whole lung with the same treatment.

Supplemental Table S4. Pharmacokinetic parameters for NNK and NNAL in the lung-*Cpr*-null mice and control littermates

Animal groups	Tmax (min)	Cmax (μg/ml)	$t_{1/2}$ (min)	AUC (min·μg/ml)	Cl (ml/min/kg)
NNK					
Control (without Dox)	12.5 ± 4.3	72.7 ± 9.0	41.0 ± 0.4	4520 ± 250	44.6 ± 2.4
Control-Dox	20.0 ± 0.0	73.7 ± 7.1	45.4 ± 2.6	4620 ± 300	43.8 ± 2.8
Lung-*Cpr*-null-Dox	20.0 ± 0.0	83.3 ± 7.3	41.6 ± 0.9	5140 ± 250	39.2 ± 1.8
NNAL					
Control (without Dox)	60.0 ± 0.0	80.6 ± 2.2	56.5 ± 4.8	10800 ± 300	18.5 ± 0.6
Control-Dox	60.0 ± 0.0	86.2 ± 6.4	67.9 ± 5.2	11900 ± 700	16.9 ± 1.0
Lung-*Cpr*-null-Dox	60.0 ± 0.0	97.4 ± 2.2*	58.4 ± 4.4	13500 ± 300	14.8 ± 0.3

Plasma levels of NNK and NNAL (from Fig. 3) were used to calculate the pharmacokinetic parameters, including $t_{1/2}$ (half-life), Tmax (time of peak concentration), AUC (area under the concentration-time curve), Cmax, (maximal concentration), and Cl (clearance). Values shown are means ± SEM (n=4).

*$P<0.05$, t-test, compared to the control-Dox mice.

Supplemental Figure Legend

Supplemental Figure S1. Strategy for generation of the lung-*Cpr*-null mouse on an A/J-N_3 genetic background. The original *CCSP-rtTA/tetO-Cre* mice (on a mixed FVB/N and 129/SvJ background) and $Cpr^{lox/lox}$ mice (on a mixed C57B/6J (B6) and 129/SvJ background) were backcrossed to the A/J mice for three generations, respectively, before they were intercrossed to generate the A/J-N_3 lung-*Cpr*-null mice and control littermates. **CCSP-rtTA* or *tetO-Cre* represents $CCSP\text{-}rtTA^{hemi}$ or $tetO\text{-}Cre^{hemi}$. †Heterozygous for the Cpr^{lox} allele.

Supplemental Figure S2. Histological examination of lung tissue sections. Lungs from 2-month old, female control-Dox (A) and lung-*Cpr*-null-Dox mice (B) were analyzed. Tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Typical results are shown. Scale bar, 40 μm. a.d., alveolar ducts; bo., bronchiole; bc., bronchus.

Supplemental Figure S3. Detection of O^6-mG by LC-MS. Lung DNA was prepared, hydrolyzed, and processed as described in Methods. Lung tissue was obtained from control-Dox (A) and lung-*Cpr*-null-Dox mice (B) at 24 h after a single i.p. dose of NNK at 200 mg/kg, or from the control-Dox at 4 h after saline injection (C). O^6-mG (blue trace) was detected by SIM as $[M+H]^+$, at *m/z* 166. The internal standard O^6-CD_3-G (red trace) was detected by SIM as $[M+H]^+$, at *m/z* 169.

Supplemental Figure S4. Plasma levels of NNK and NNAL in lung-*Cpr*-null mice and control littermates. Two-month-old, female lung-*Cpr*-null-Dox, control-Dox, and control (not treated with Dox) mice were given a single i.p. injection of NNK at 200 mg /kg. Blood samples were collected from individual animals at six time points after the injection. Values shown are means ± SEM (n=4). * or †, values are significantly different between lung-*Cpr*-null-Dox and control-Dox mice ($P<0.05$ or 0.01, respectively, t-test).

Supplemental Figure S5. Detection of NNK and NNAL by LC-MS. HPLC analysis was performed using a neutral (pH 6.8, A and B) or an acidic (pH 2.7, C and D) mobile phase, as described in Supplemental Methods. The plasma sample was collected from a liver-*Cpr*-null mouse at 5 min after a single i.p. dose of NNK at 100 mg/kg. NNK (A,C; blue trace) was detected by SIM as $[M+H]^+$ at *m/z* 208. D_4-NNK (A,C; red trace) was detected by SIM as $[M+H]^+$ at *m/z* 212. NNAL (B,D; blue trace) was detected by SIM as $[M+H]^+$ at *m/z* 210. D_4-NNAL (B,D; red trace) was detected by SIM as $[M+H]^+$ at *m/z* 214. The ratios of the peak areas of NNK/D_4-NNK were 22.3 (A) and 20.9 (C). The ratios of the peak areas of NNAL/D_4-NNAL were 7.8 (B) and 7.5 (D).