



US 20200370047A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2020/0370047 A1**

Kmiec et al. (43) **Pub. Date: Nov. 26, 2020**

(54) **GENE KNOCKOUT OF NRF2 FOR TREATMENT OF CANCER**

(71) Applicant: **Christiana Care Health Services, Inc.**, Newark, DE (US)

(72) Inventors: **Eric Kmiec**, Middletown, DE (US); **Pawel Bialk**, Wilmington, DE (US)

(73) Assignee: **Christiana Care Health Services, Inc.**, Newark, DE (US)

(21) Appl. No.: **16/881,919**

(22) Filed: **May 22, 2020**

Related U.S. Application Data

(60) Provisional application No. 62/852,076, filed on May 23, 2019.

Publication Classification

(51) **Int. Cl.**
C12N 15/113 (2006.01)
C12N 9/22 (2006.01)
A61K 31/7105 (2006.01)

(52) **U.S. Cl.**
CPC *C12N 15/113* (2013.01); *C12N 9/22* (2013.01); *C12N 2800/80* (2013.01); *C12N 2310/20* (2017.05); *A61K 31/7105* (2013.01)

(57) **ABSTRACT**

The disclosure provides a guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complementary to a target domain from an NRF2 gene. The disclosure also provides nucleic acid sequence encoding the gRNA. The disclosure further provides a method of treating cancer in a subject comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease and a guide RNA that is complementary to a target domain from an NRF2 gene in the subject. Methods of treating cancer comprising administering a pharmaceutical composition comprising: a DNA sequence encoding a guide RNA that is complementary to a target domain from an NRF2 gene in the subject; and a nucleic acid sequence encoding a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease, are also provided.

Specification includes a Sequence Listing.

FIGURE 1A

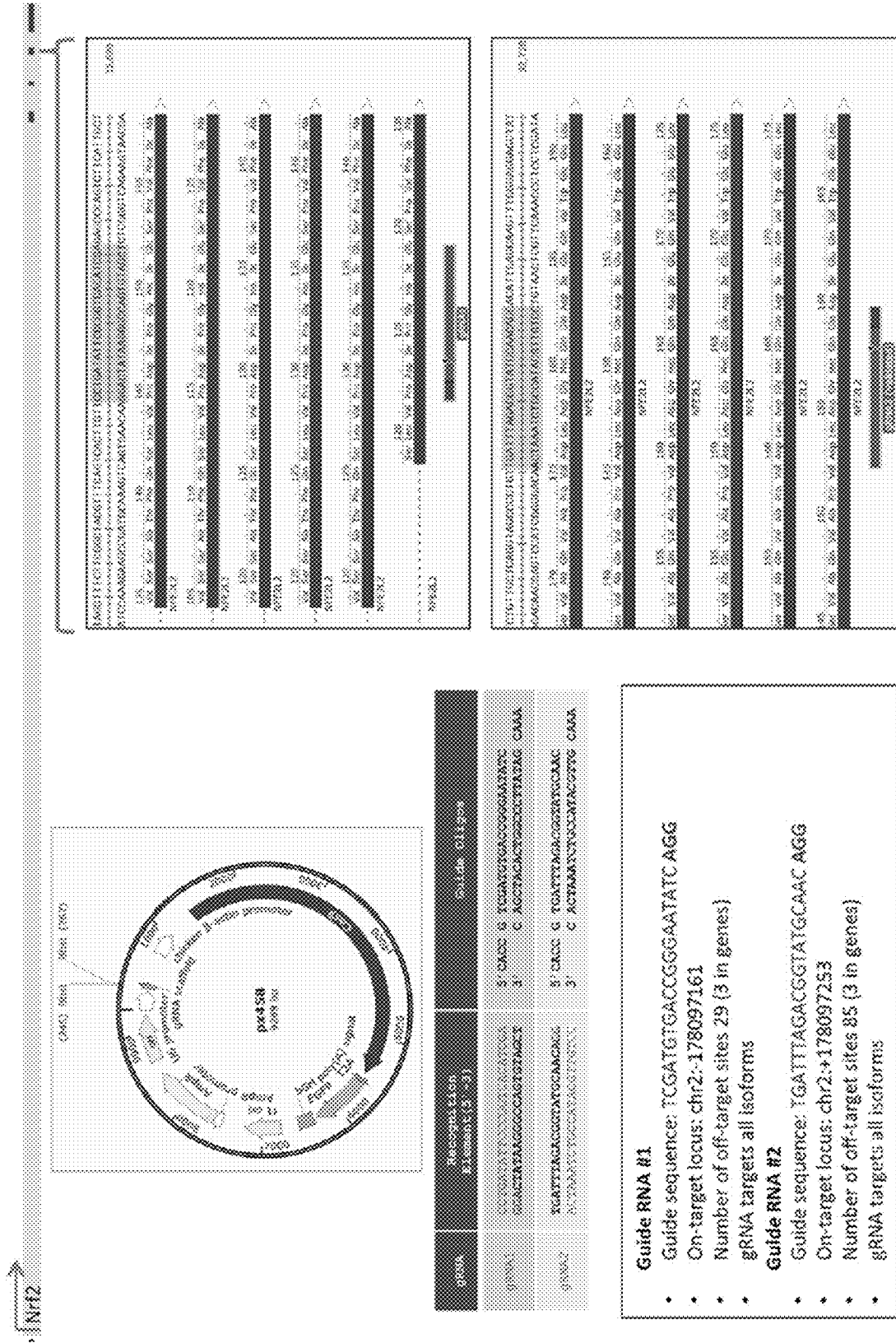


FIGURE 1B

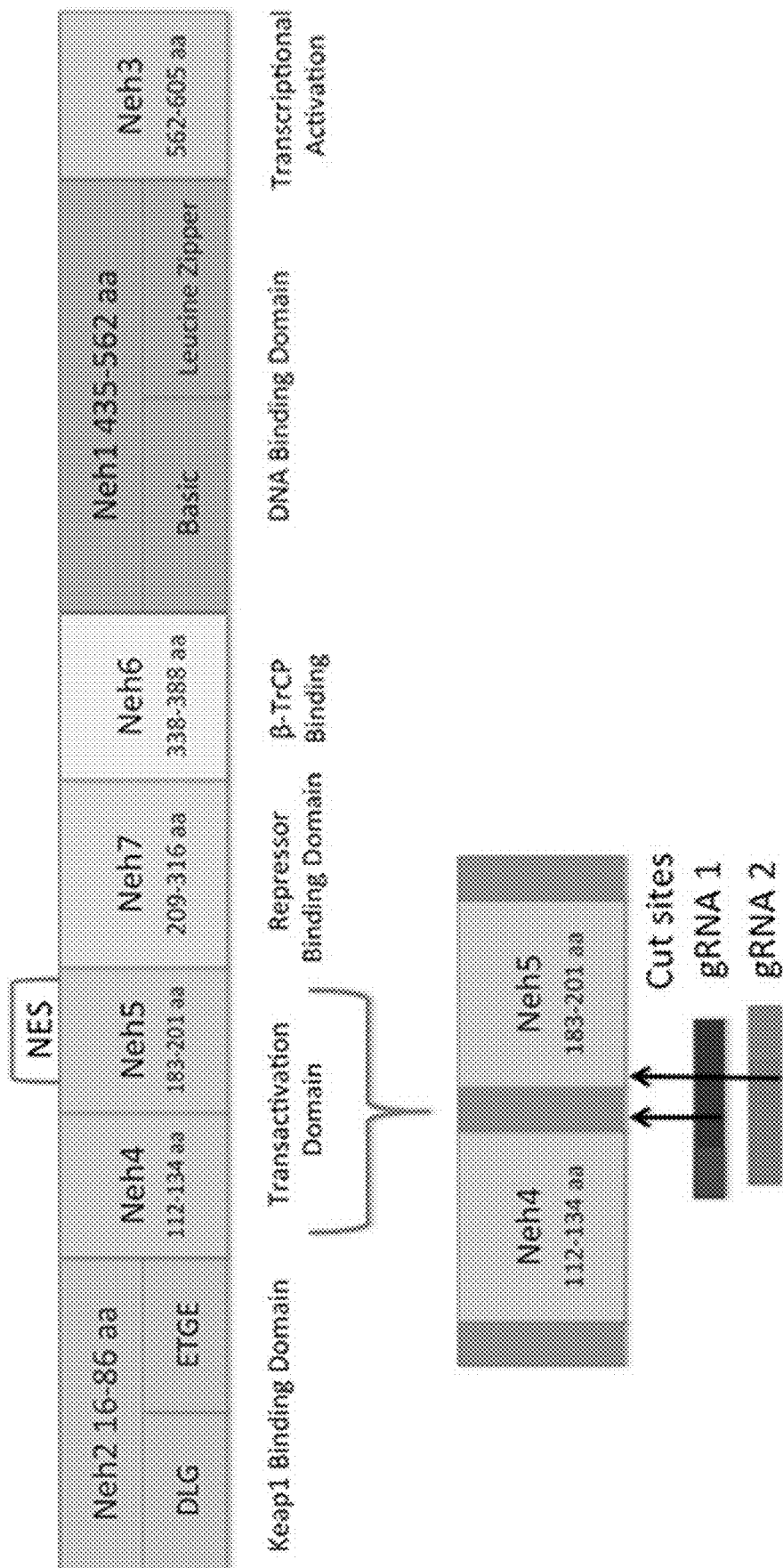


FIGURE 1C

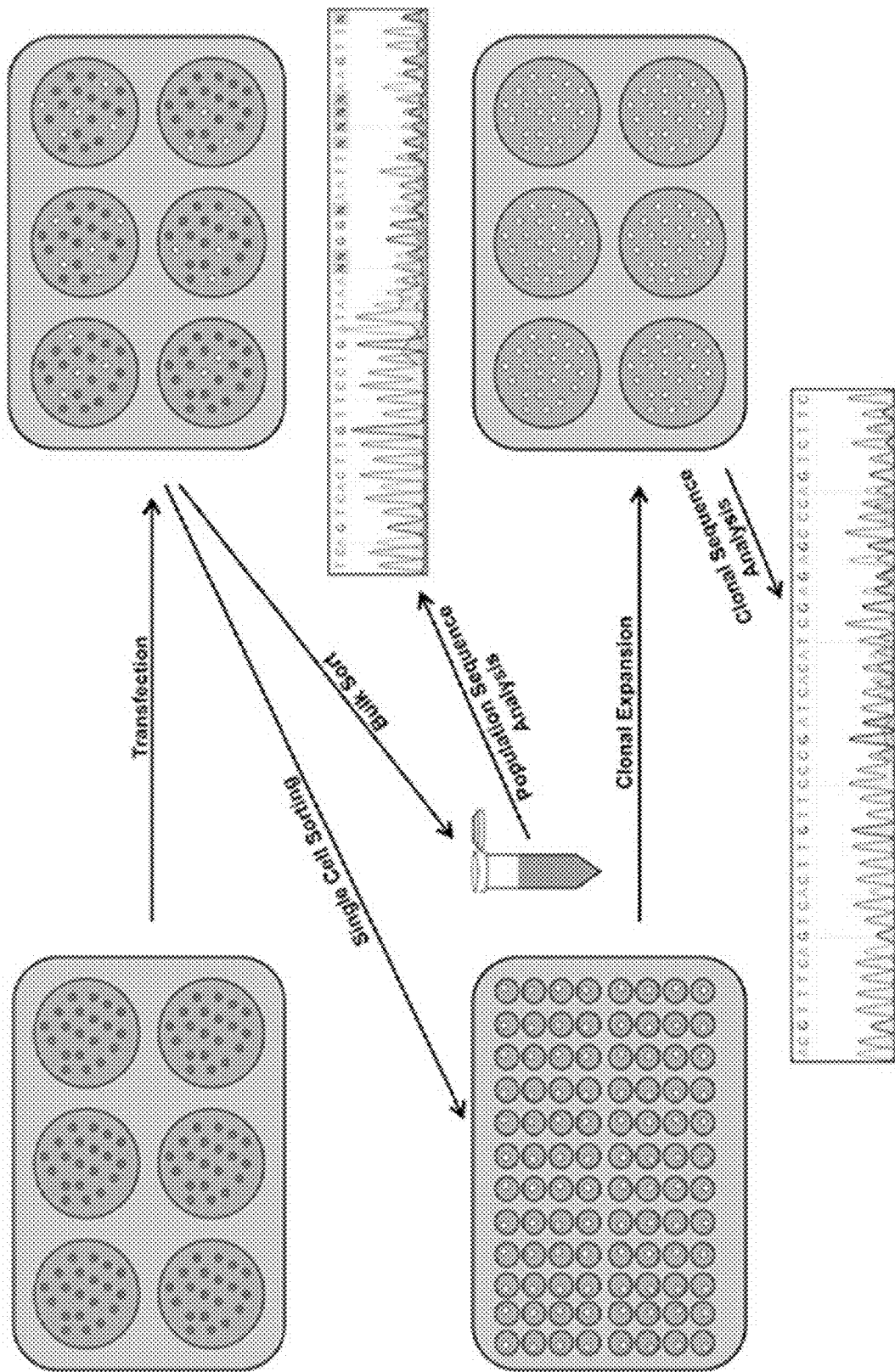


FIGURE 2A

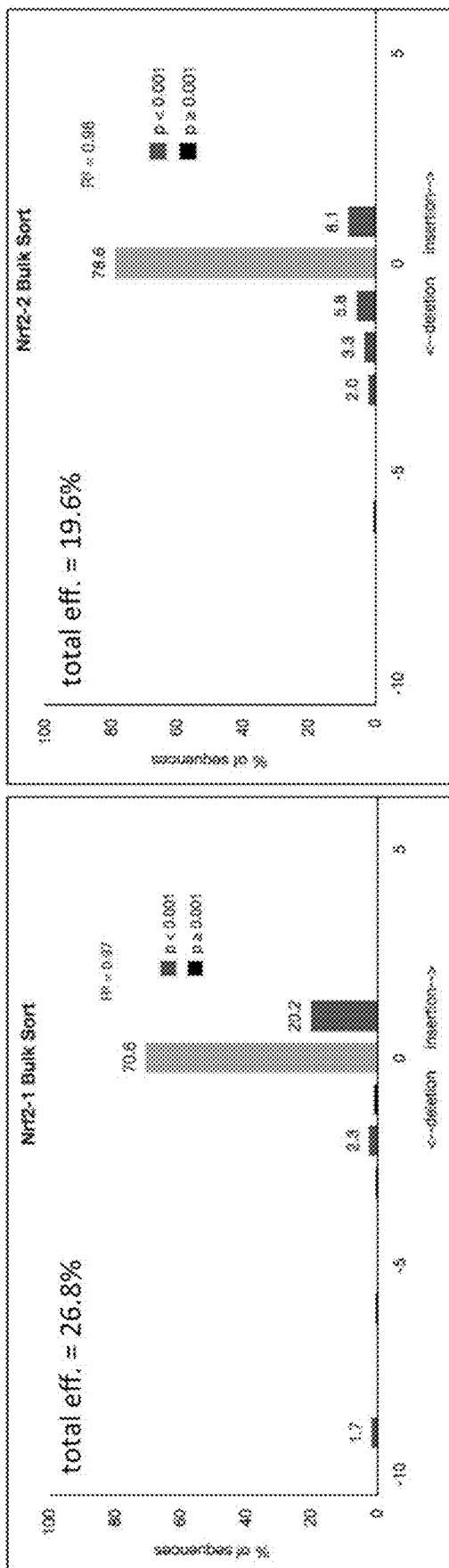


FIGURE 2B

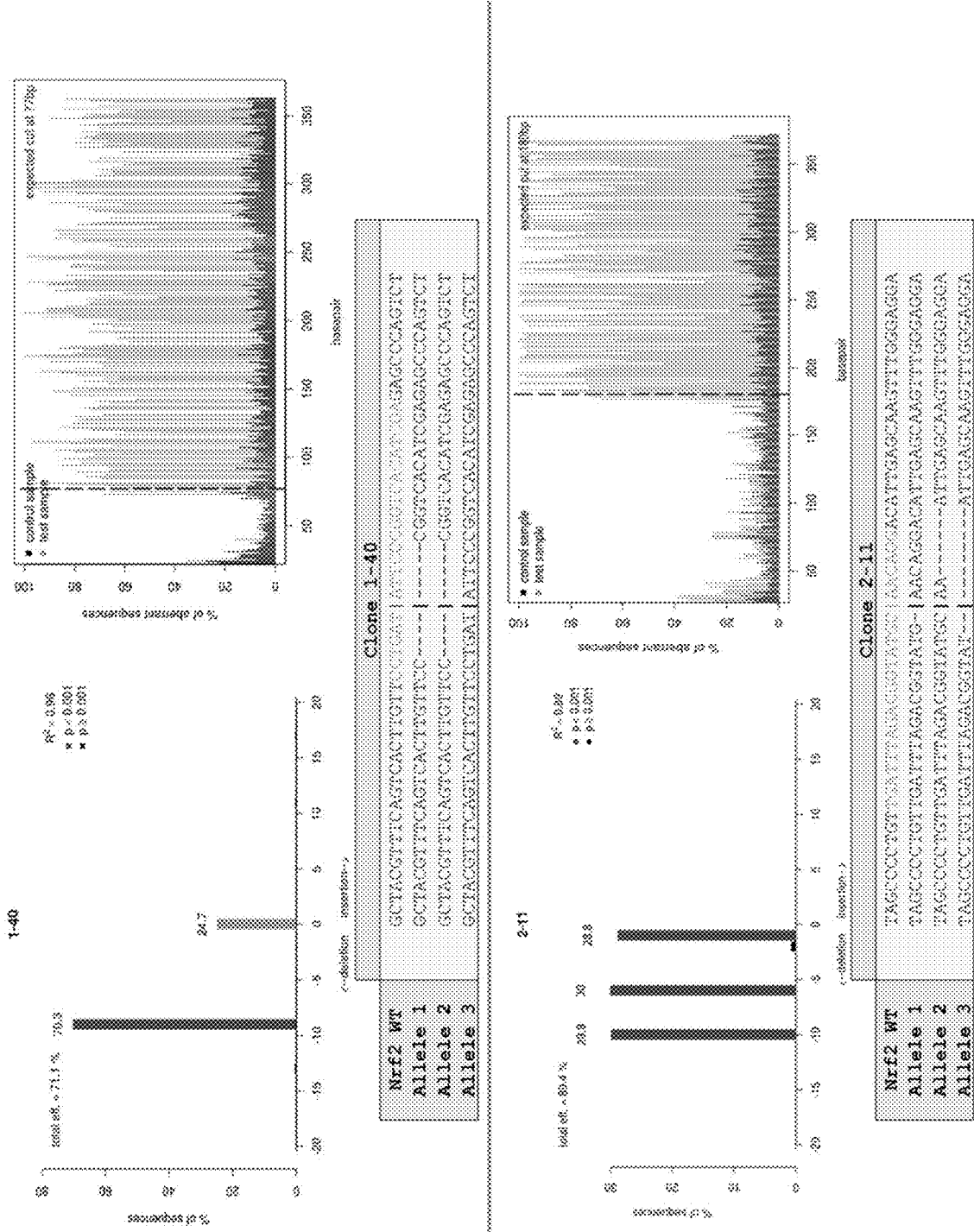


FIGURE 3A

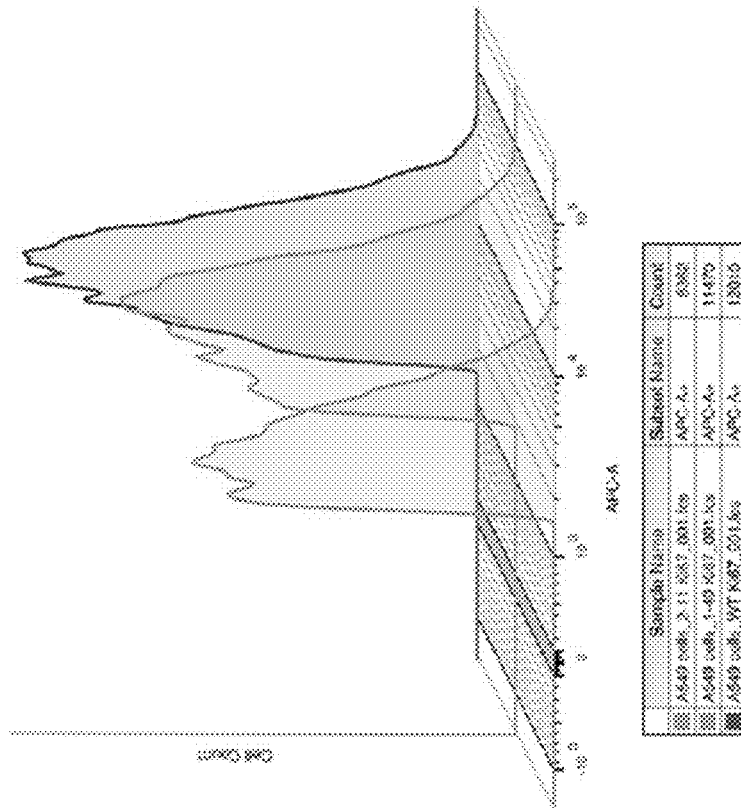
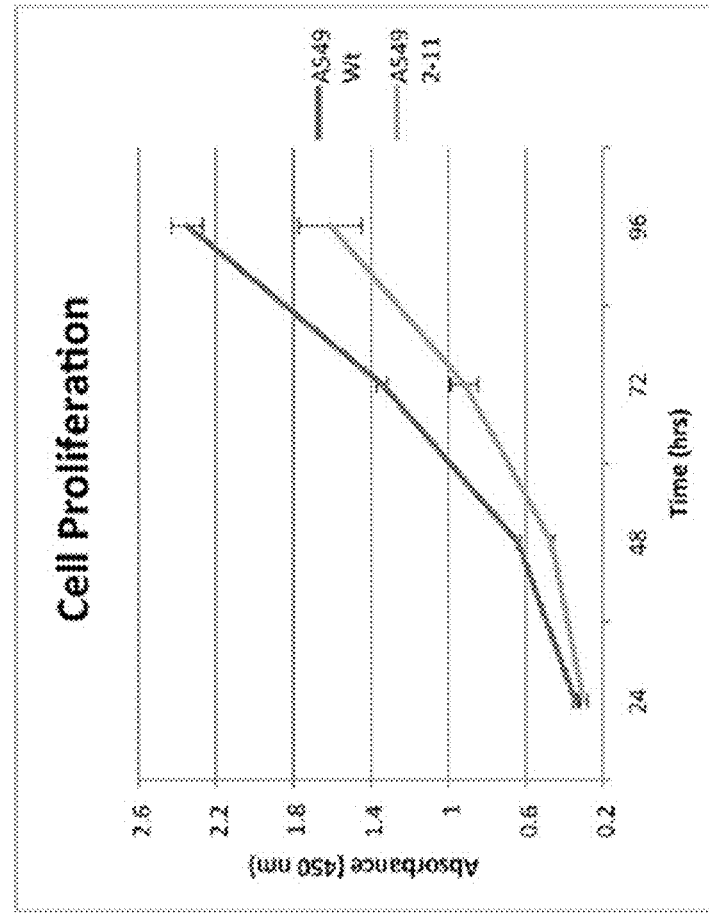
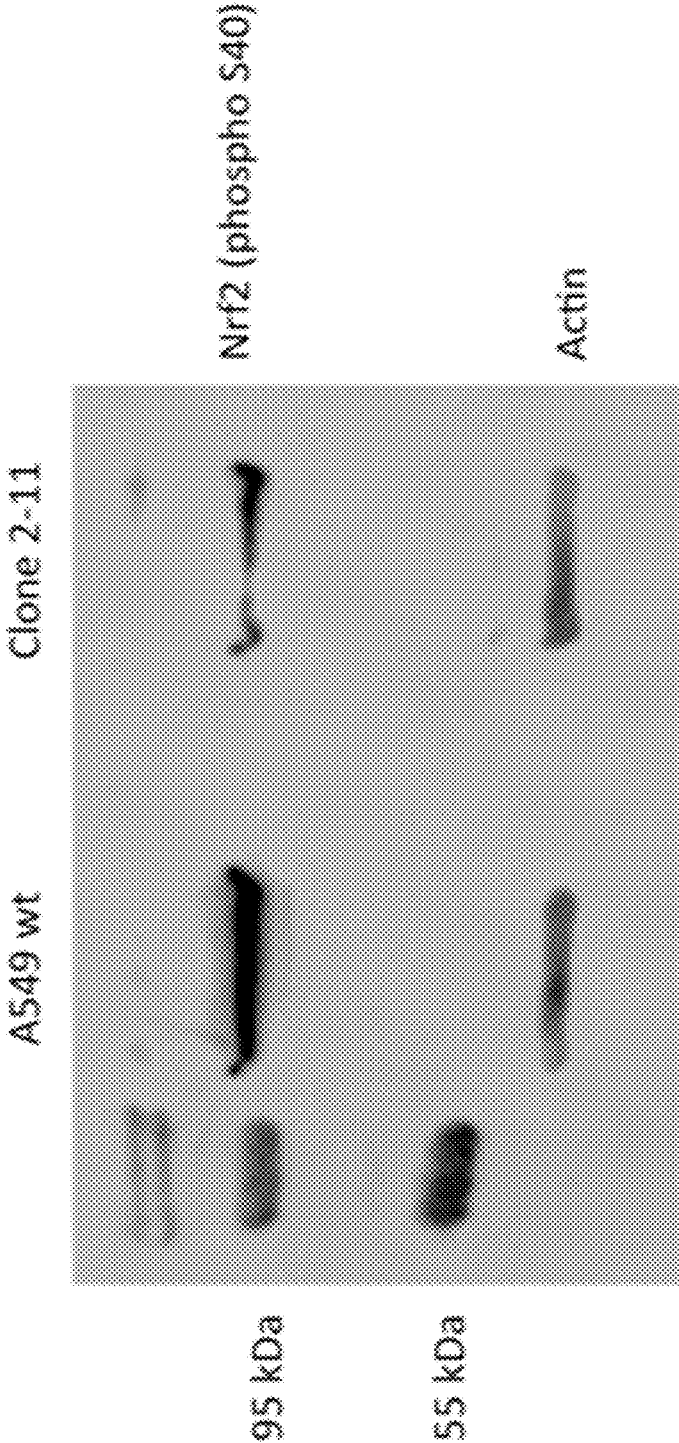


FIGURE 3B



	Nrf2 Signal	Actin Signal	Normalized to actin	%
A549 wt	4.45	1.41	3.1560	100
Clone 2-11	1.28	1.26	1.0158	32

FIGURE 4

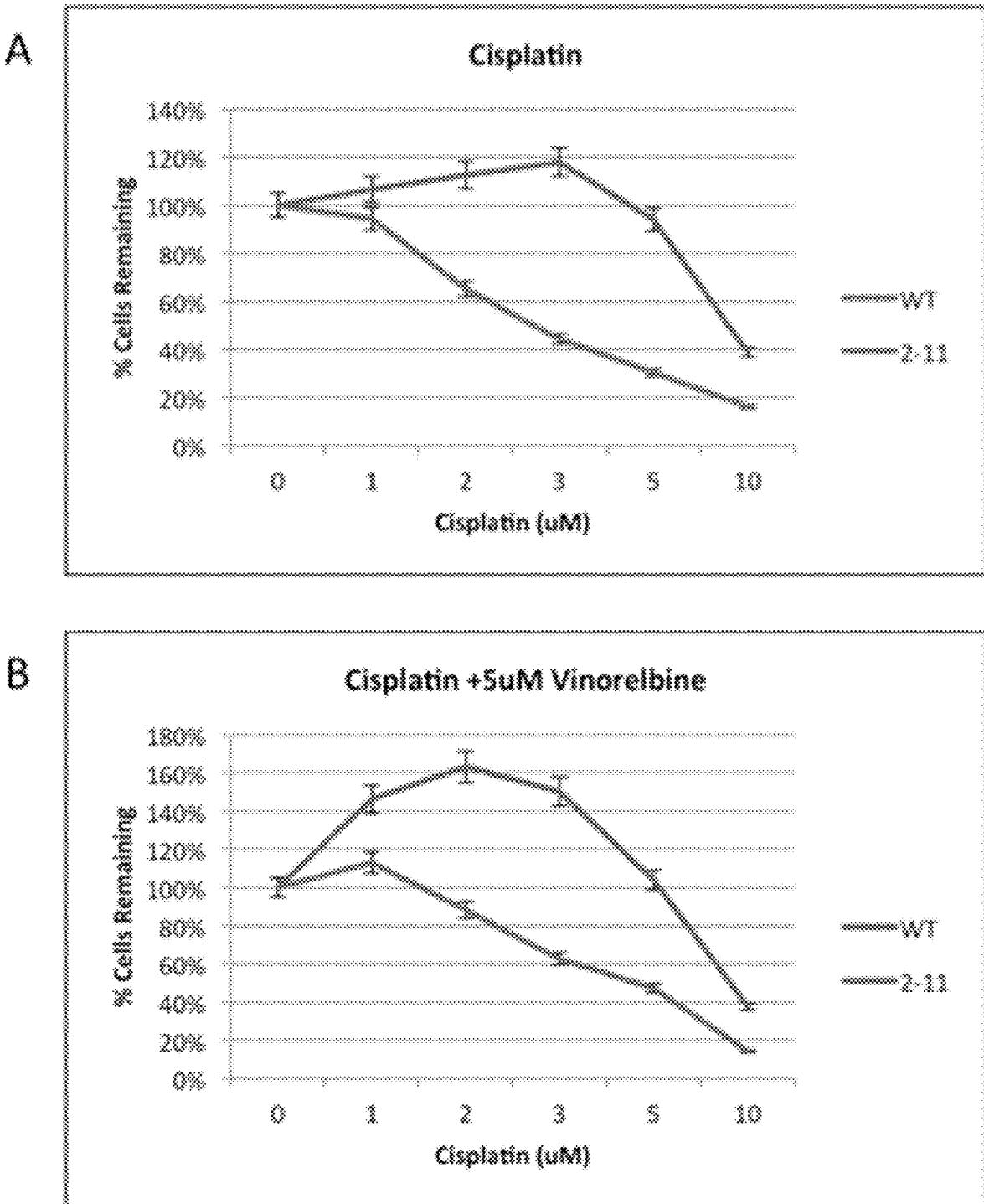


FIGURE 5A

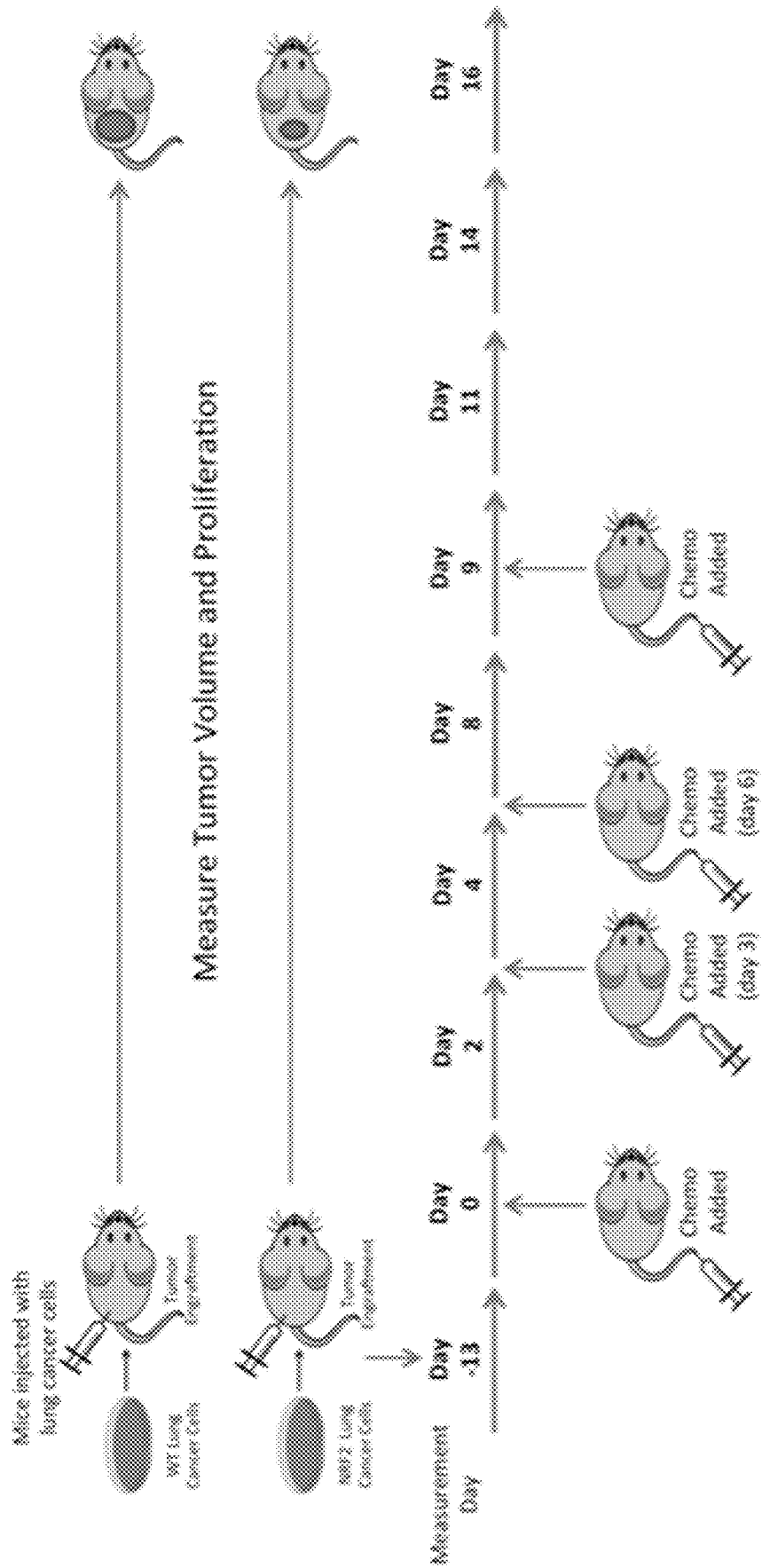


FIGURE 5B

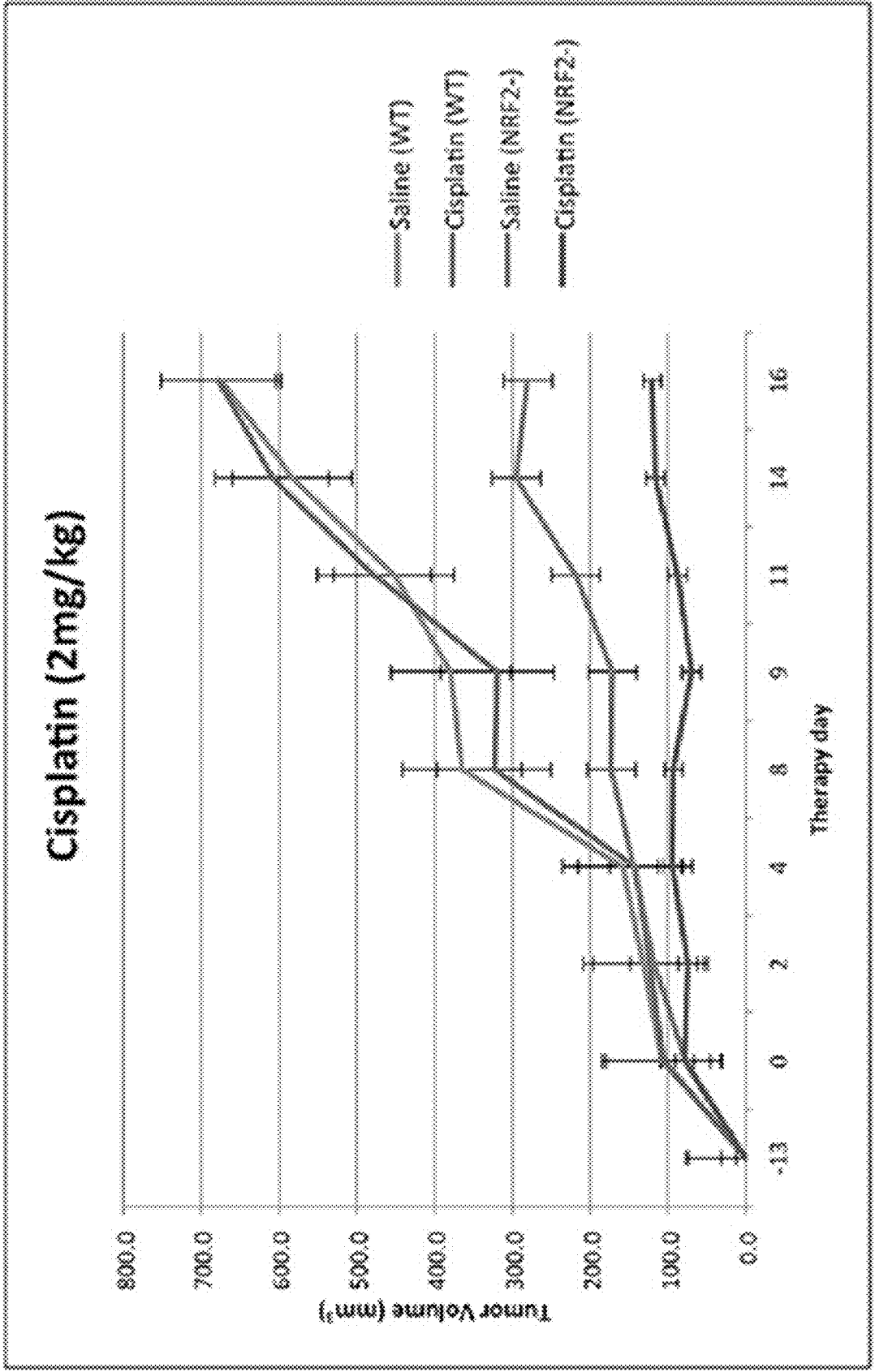


FIGURE 5C

**Cisplatin + Vinorelbine
(5mg/kg + 5mg/kg)**

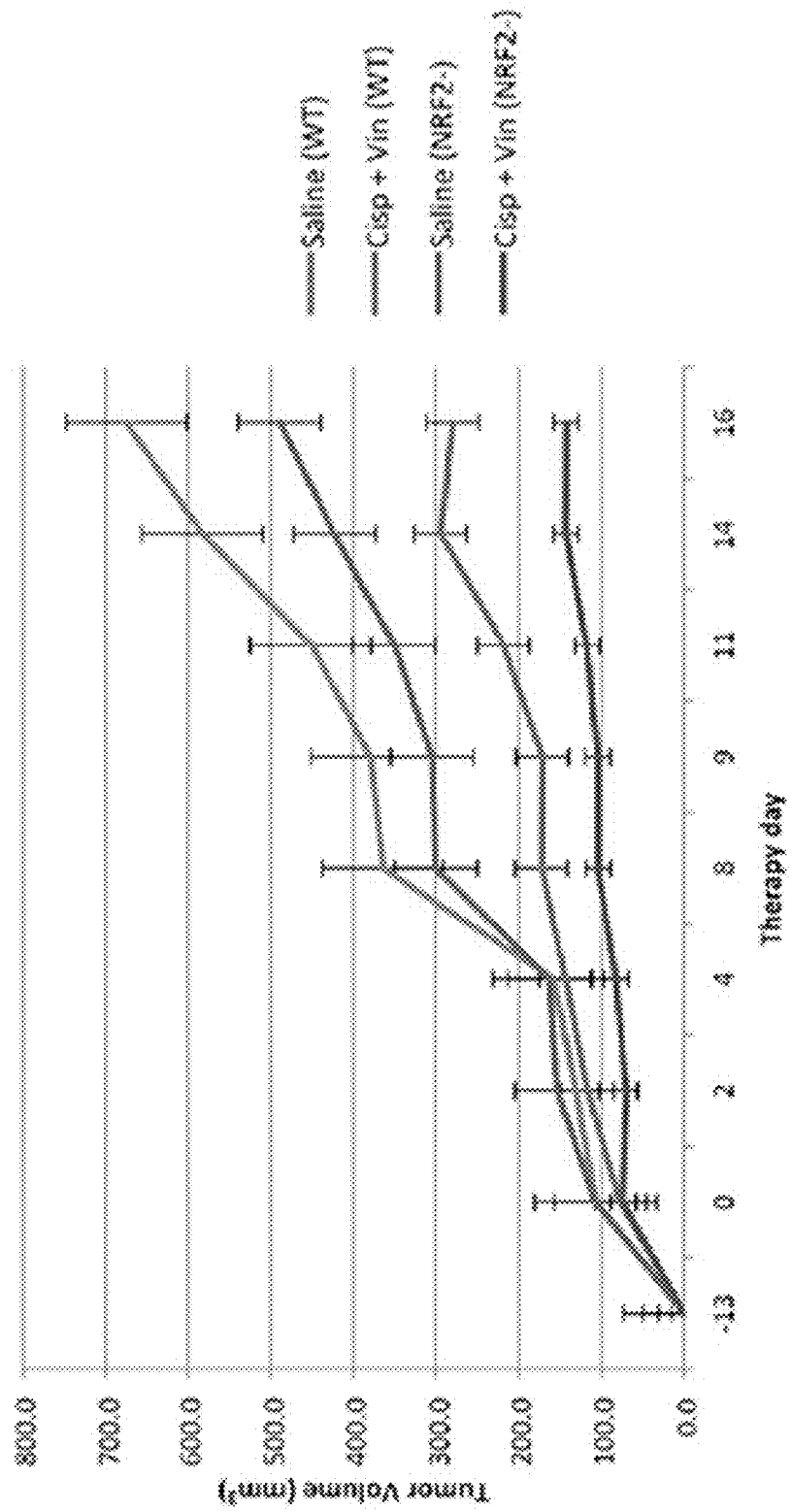


FIGURE 5D

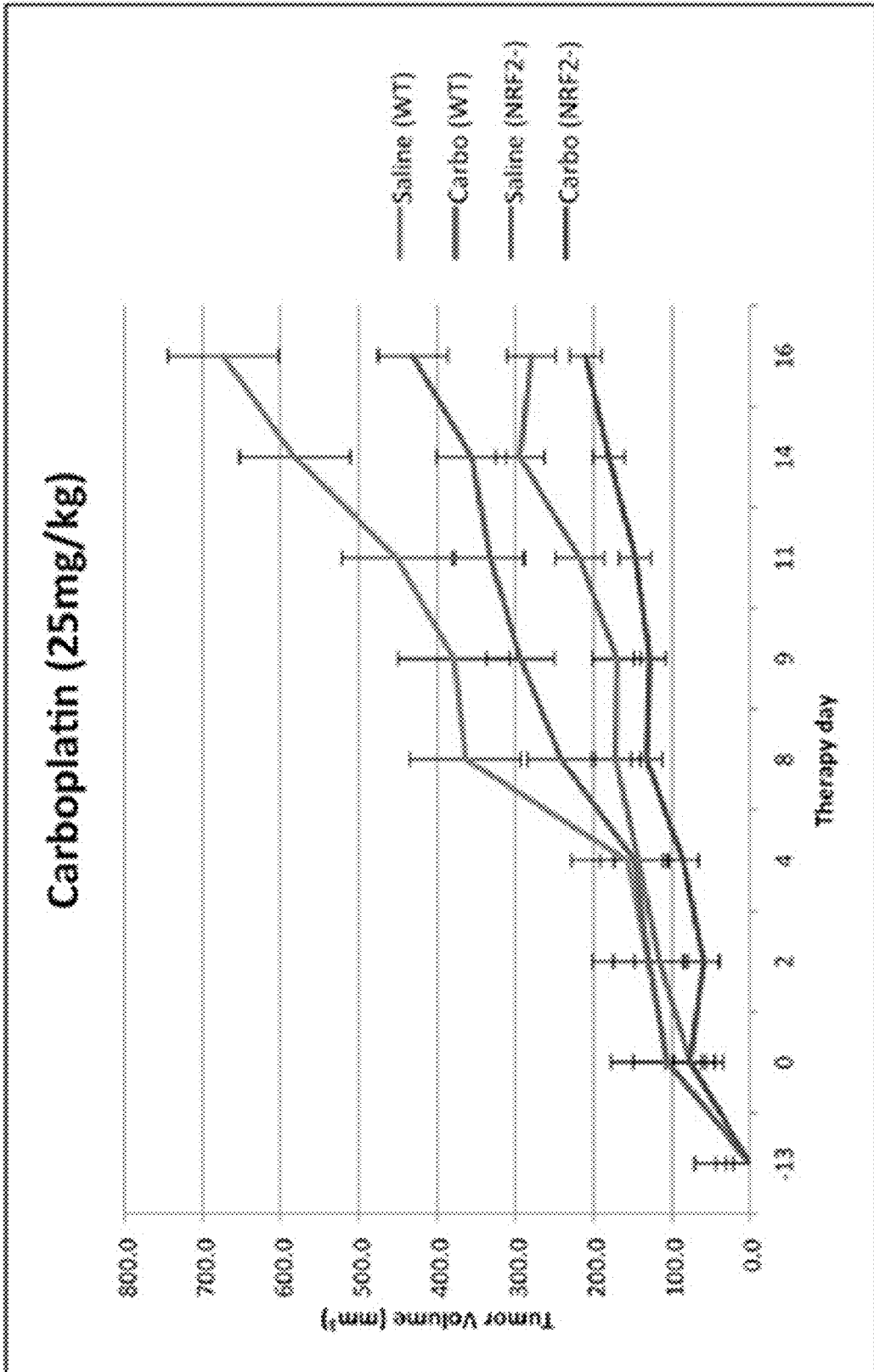
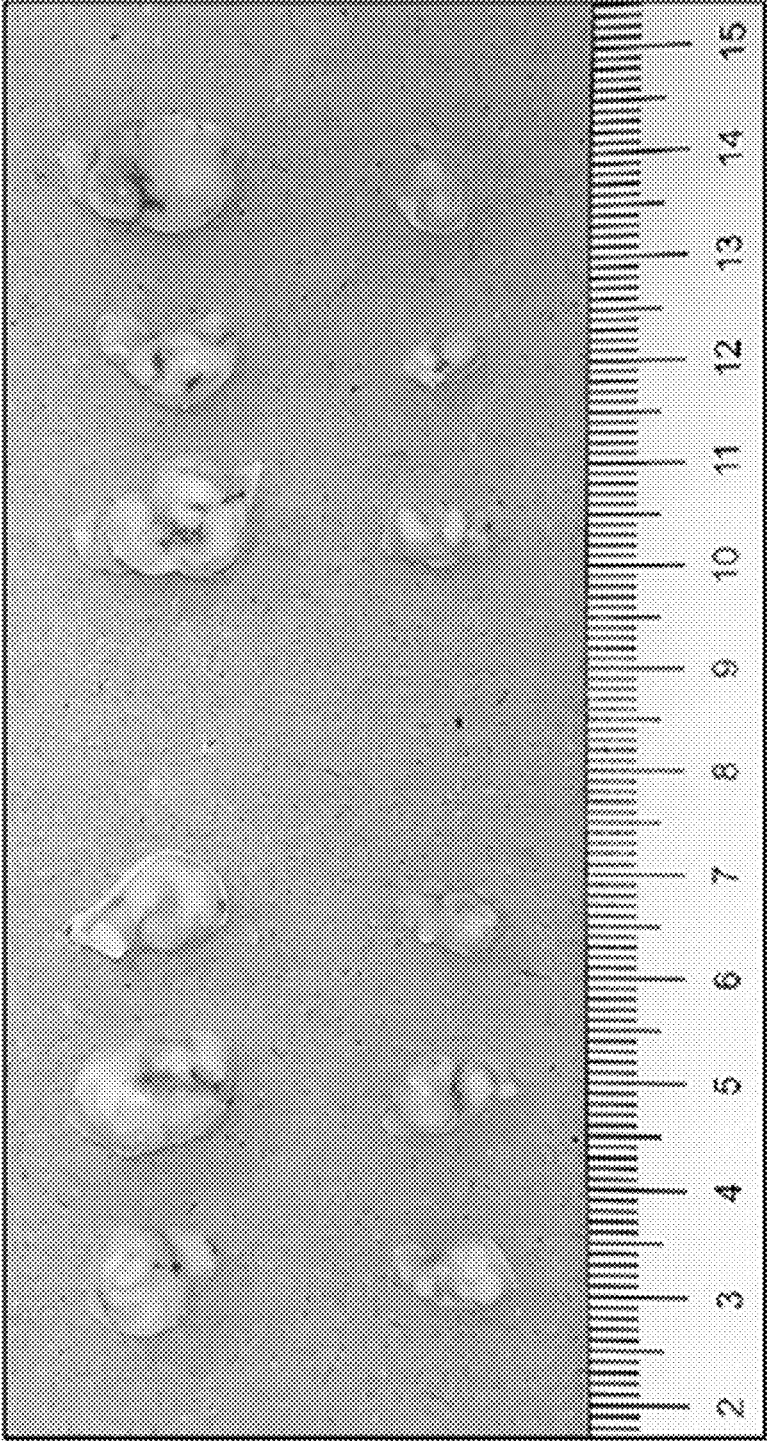


FIGURE 5E

Treatment

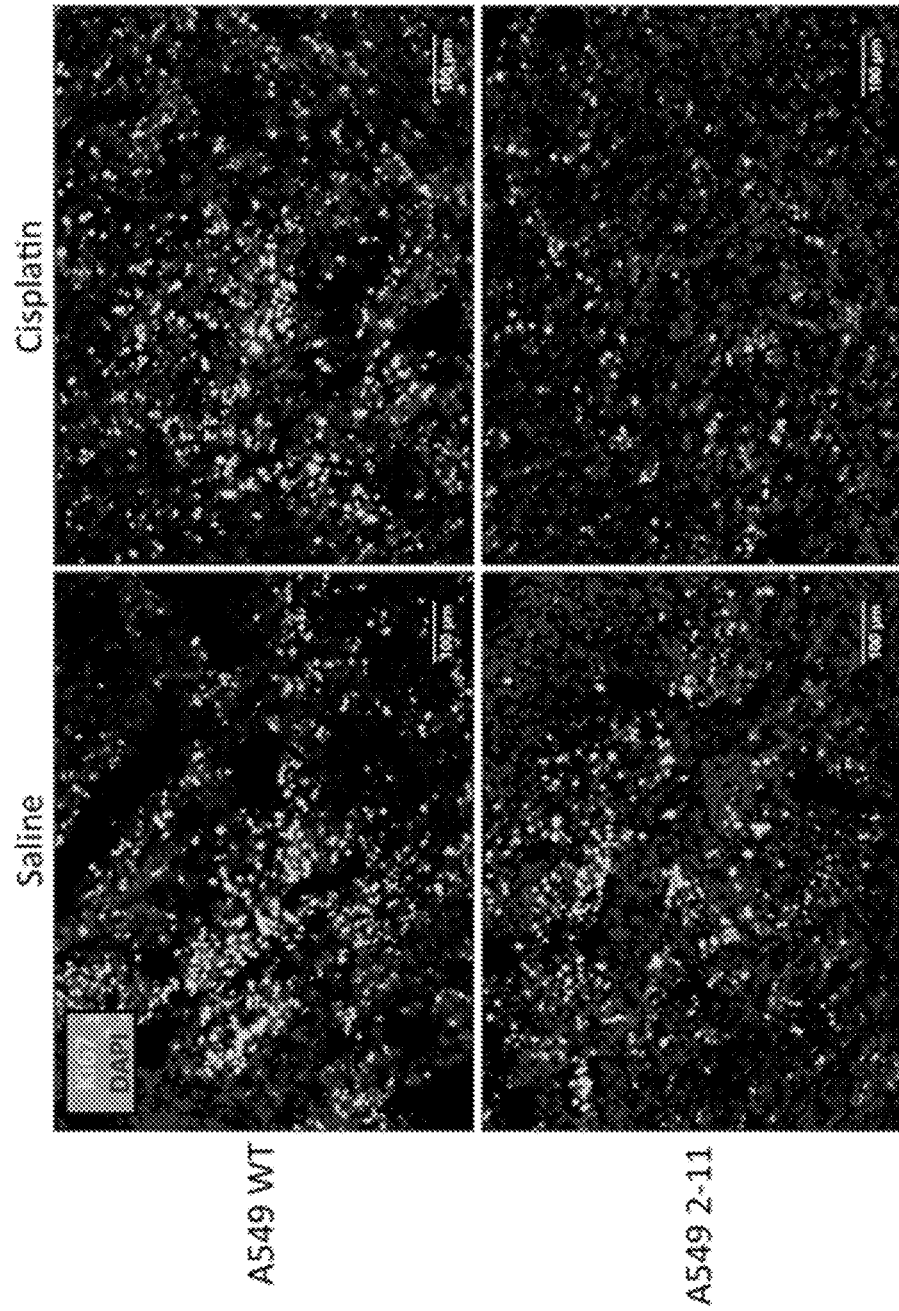
Saline Cisplatin



A549 WT

A549 2-11

FIGURE 6



Intensity Mean Value			
	DAPI	Ki67	Ki67/DAPI
A549 WT Saline	789.304	357.806	0.447627
A549 WT Cisplatin	885.689	376.553	0.425148
A549 2-11 Saline	1068.138	301.688	0.282443
A549 2-11 Cisplatin	835.693	214.252	0.256376

FIGURE 7A

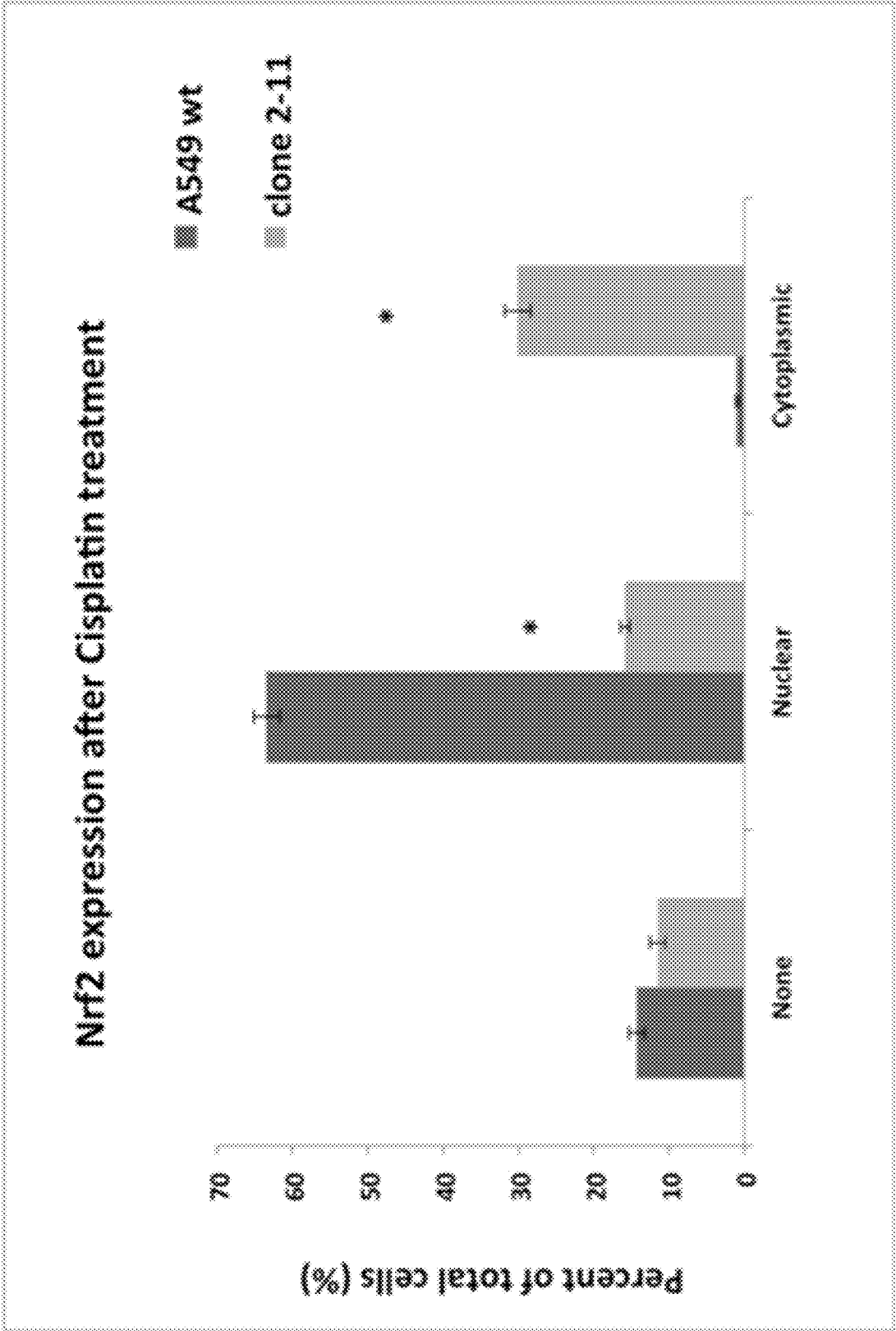
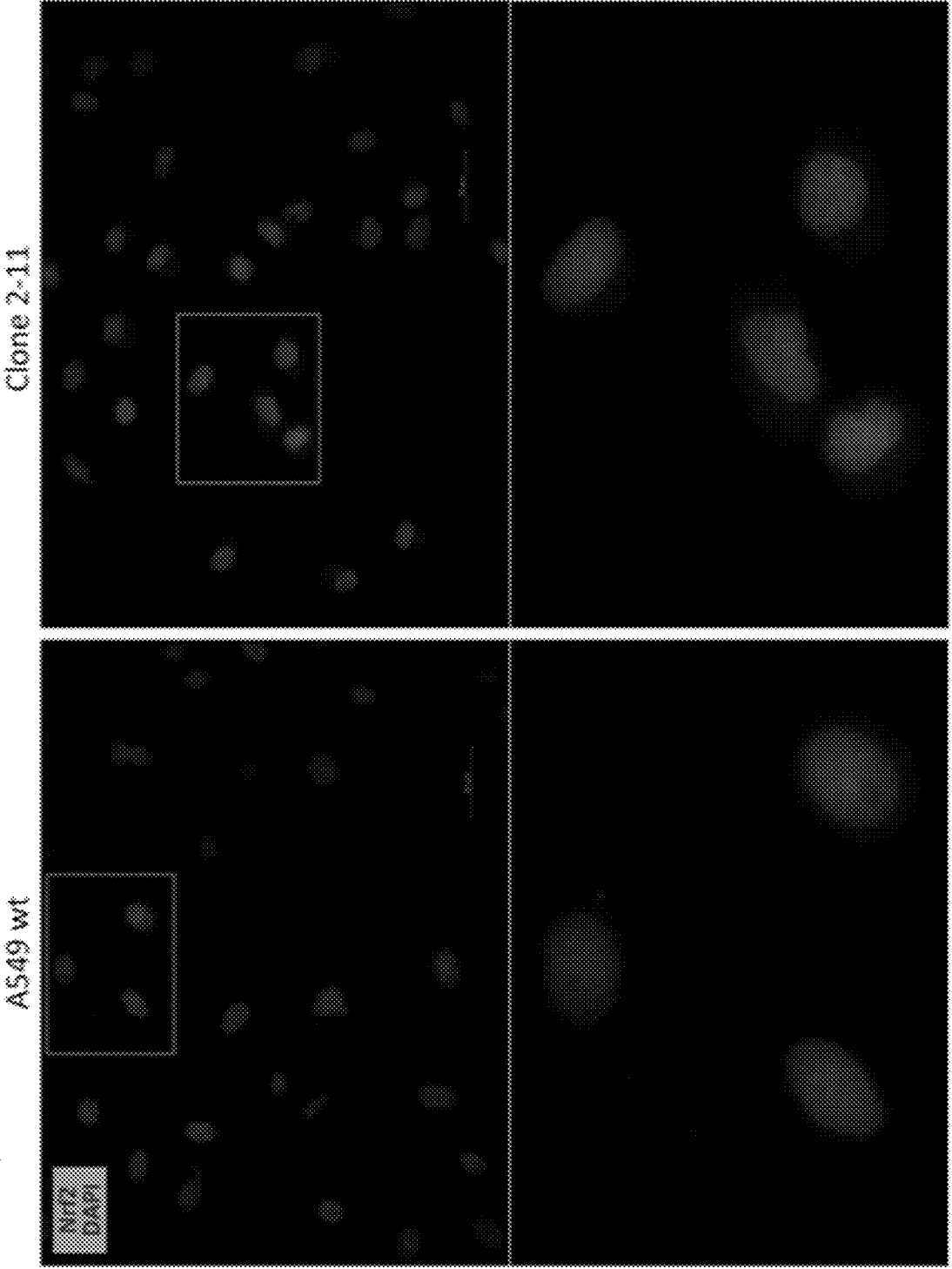
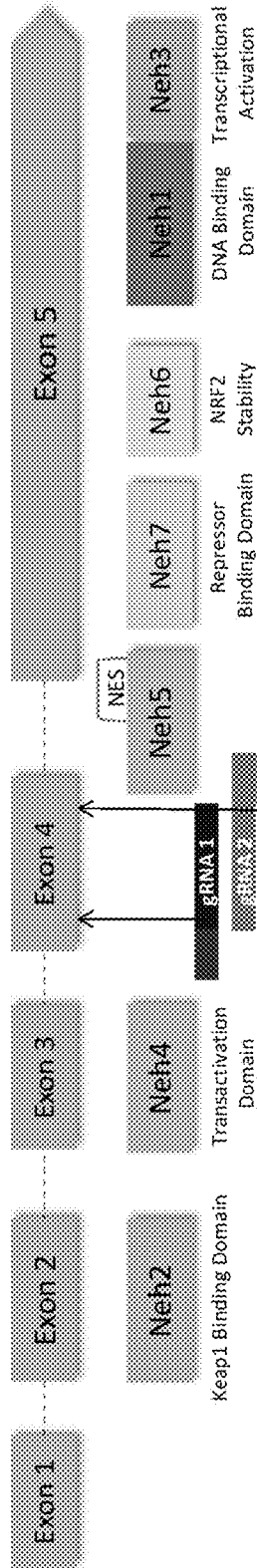


FIGURE 7B



Structural domains and targeting of NRF2

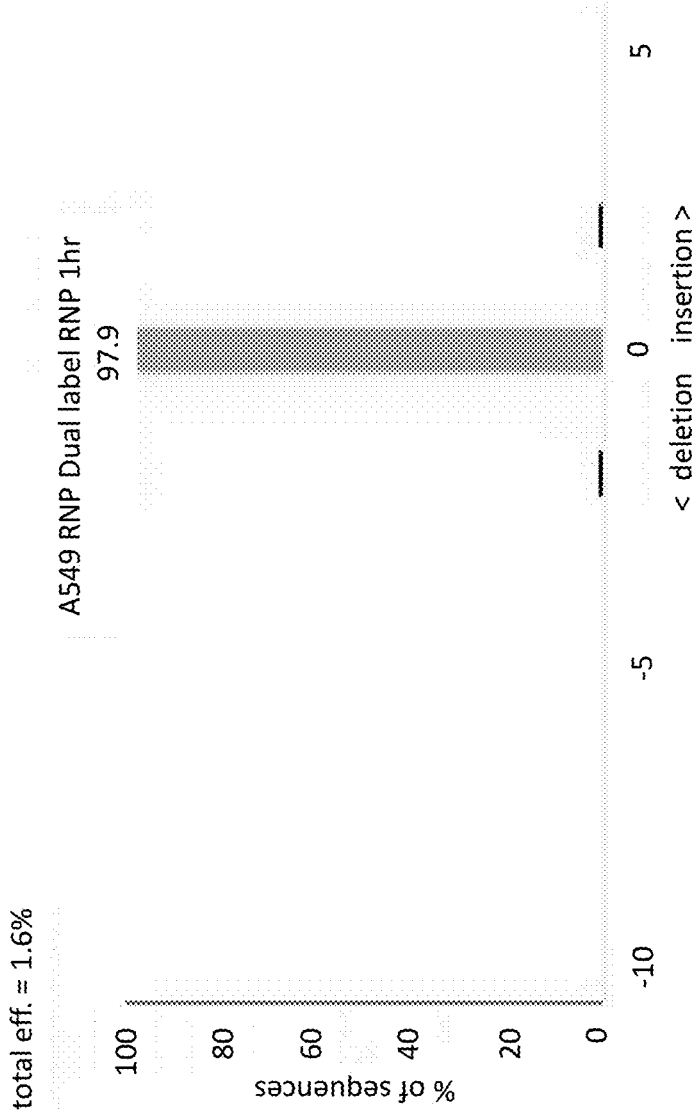
FIGURE 8



Clone	Sequence
Clone 15	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 103 bp KO ----- ACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 102 bp KO ----- AACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 21	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 103 bp KO ----- ACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 102 bp KO ----- AACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 29	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 103 bp KO ----- ACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 102 bp KO ----- AACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 31	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGAT ----- 102 bp KO ----- CAACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 32	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 104 bp KO ----- CAACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 103 bp KO ----- ACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 33	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 101 bp KO ----- CAACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 35	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGAT ----- 113 bp KO ----- TGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 102 bp KO ----- AACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 39	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 104 bp KO ----- CAACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTG ----- 104 bp KO ----- CAACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 43	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 103 bp KO ----- ACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT GTTTCTCGGCTACGTTTCAGTCACTGTTCTGATA ----- 102 bp KO ----- AACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT
Clone 44	GTTTCTCGGCTACGTTTCAGTCACTGTTCTGAT ----- 102 bp KO ----- CAACAGGACATTGAGCAAGTTTGGGAGGAGCTATTATCCATT

FIG. 9A

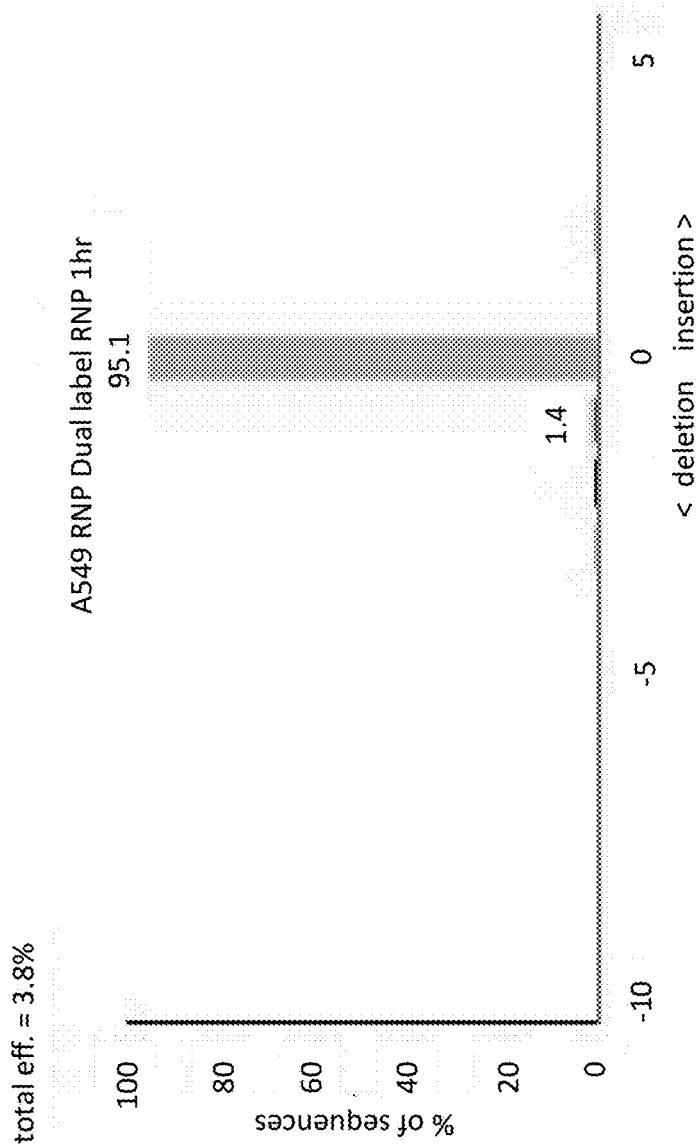
1 hour



FACS: 53.6 % Indel: 1.6%

FIG. 9B

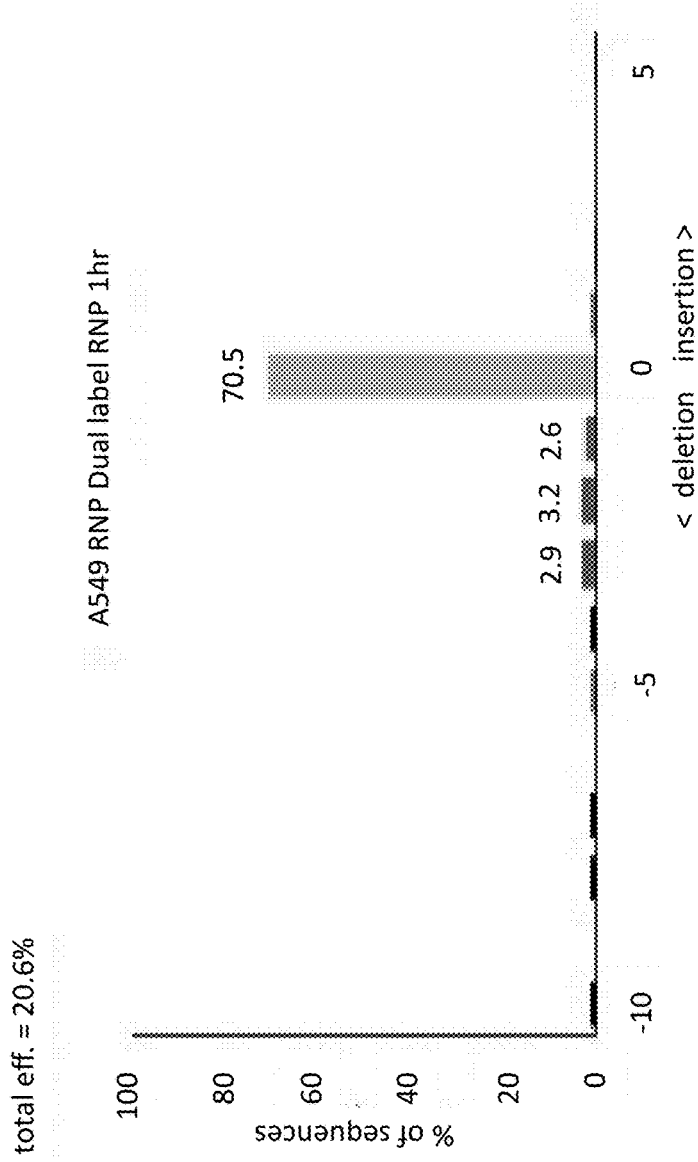
4 hours



FACS: 29.8 % Indel: 3.8%

FIG. 9C

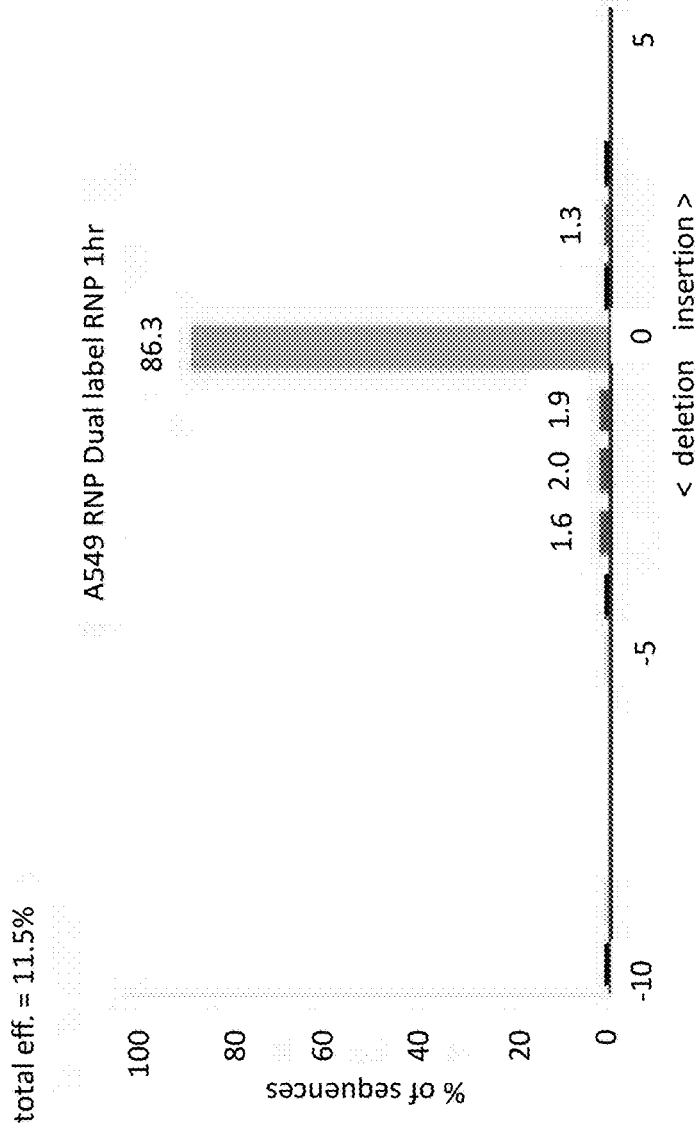
8 hours



FACS: 10.2 % Indel: 20.6%

FIG. 9D

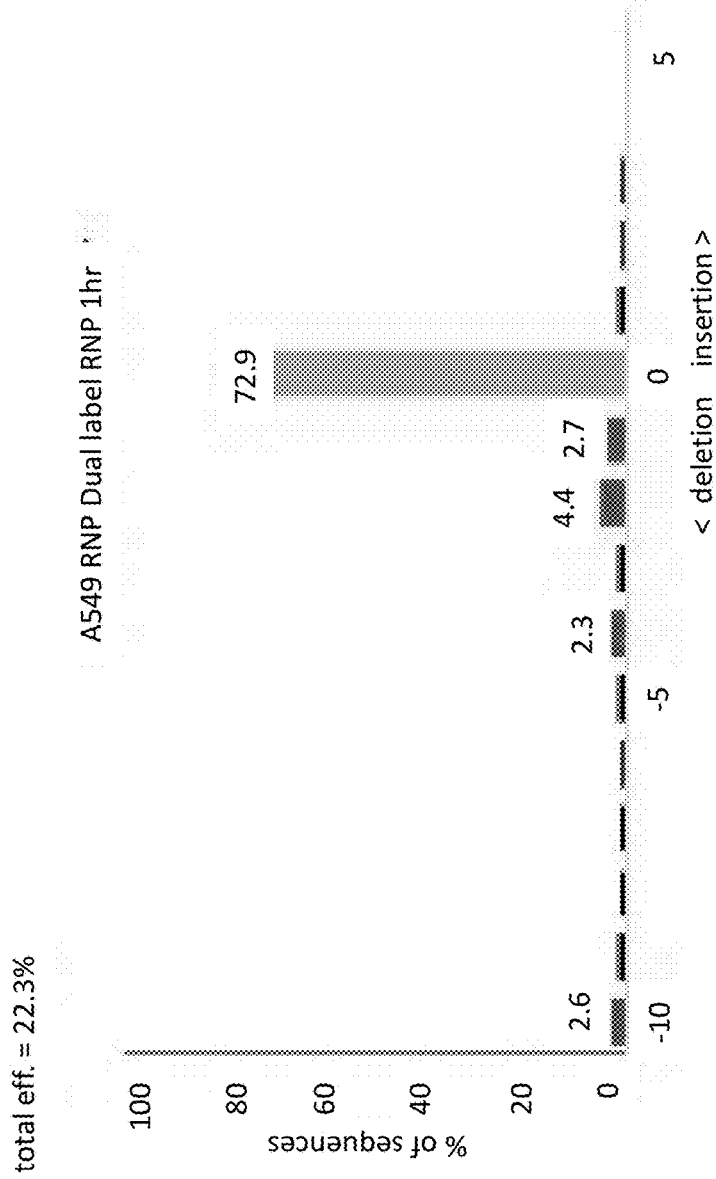
12 hours



FACS: 8.5 % Indel: 11.5%

FIG. 9E

24 hours



FACS: 4.5 % Indel: 22.3%

FIG. 9F

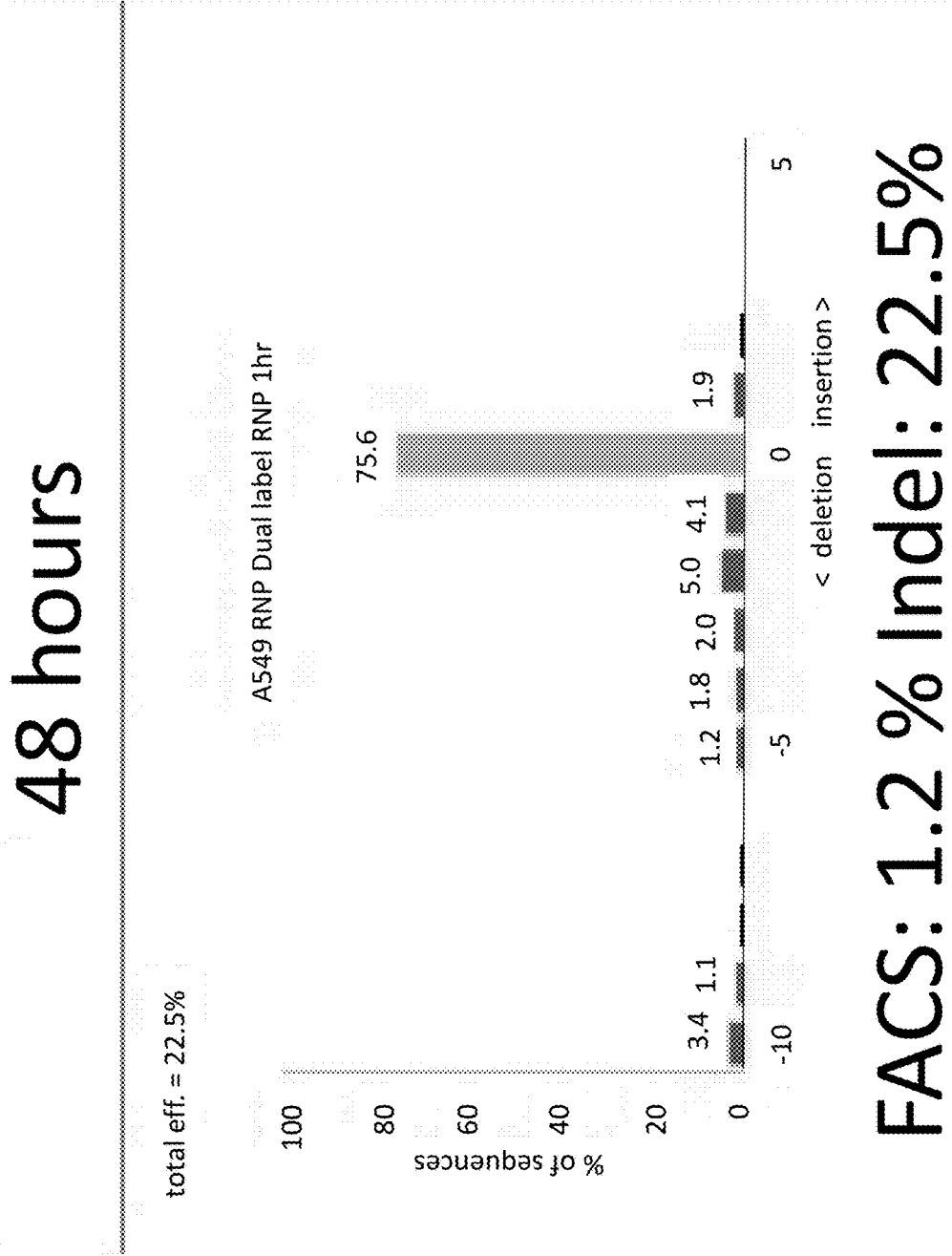
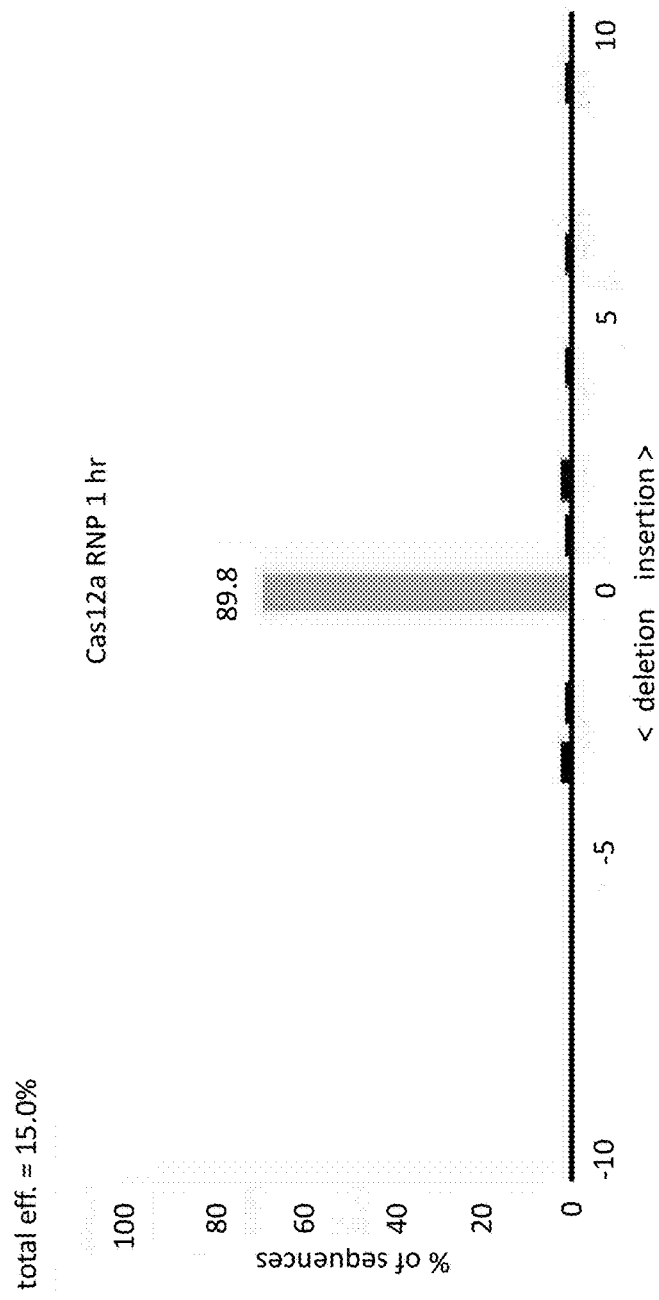


FIG. 10A

1 hour

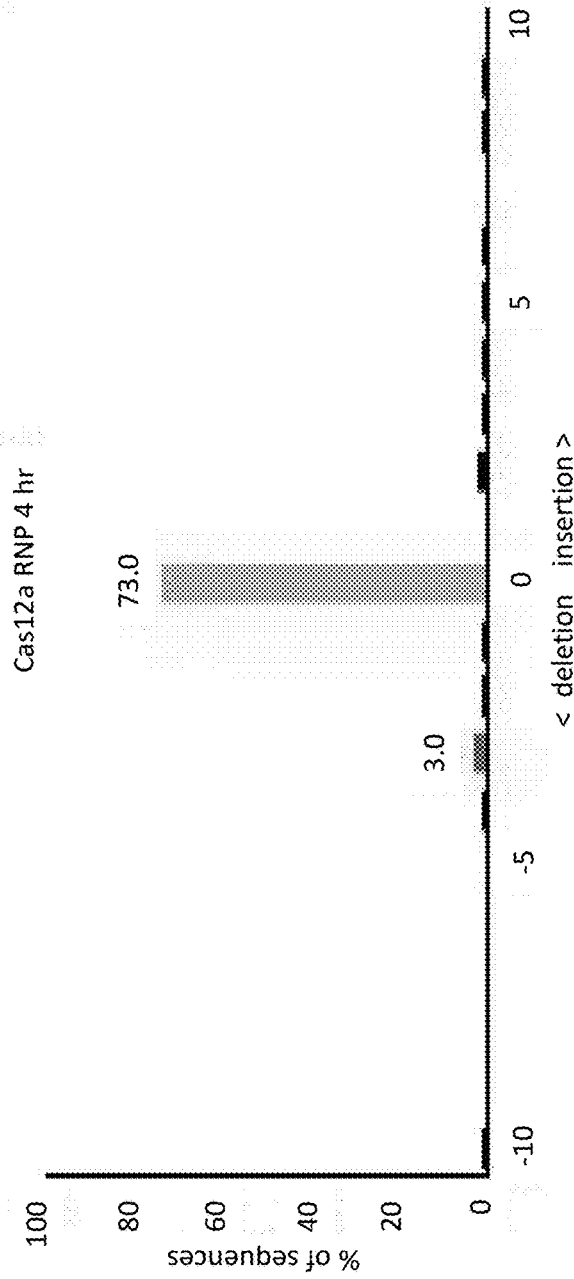


Indel: 15.0%

FIG. 10B

4 hours

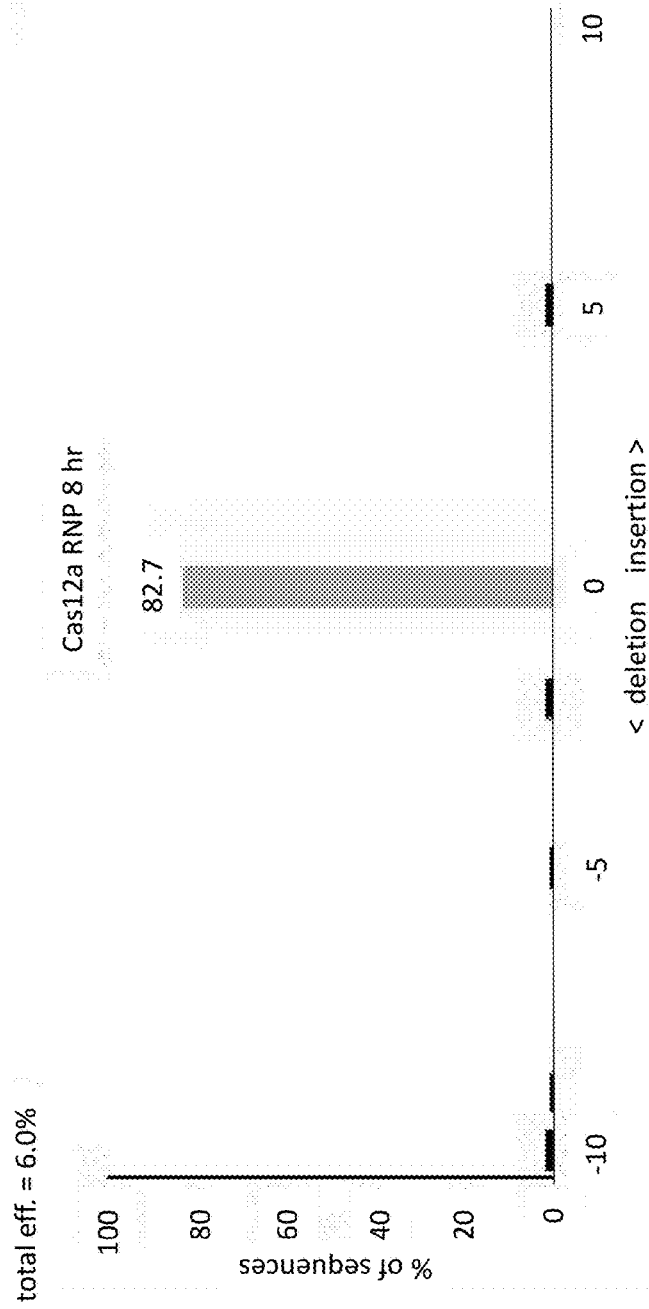
total eff. = 14.9%



Indel: 14.9%

FIG. 10C

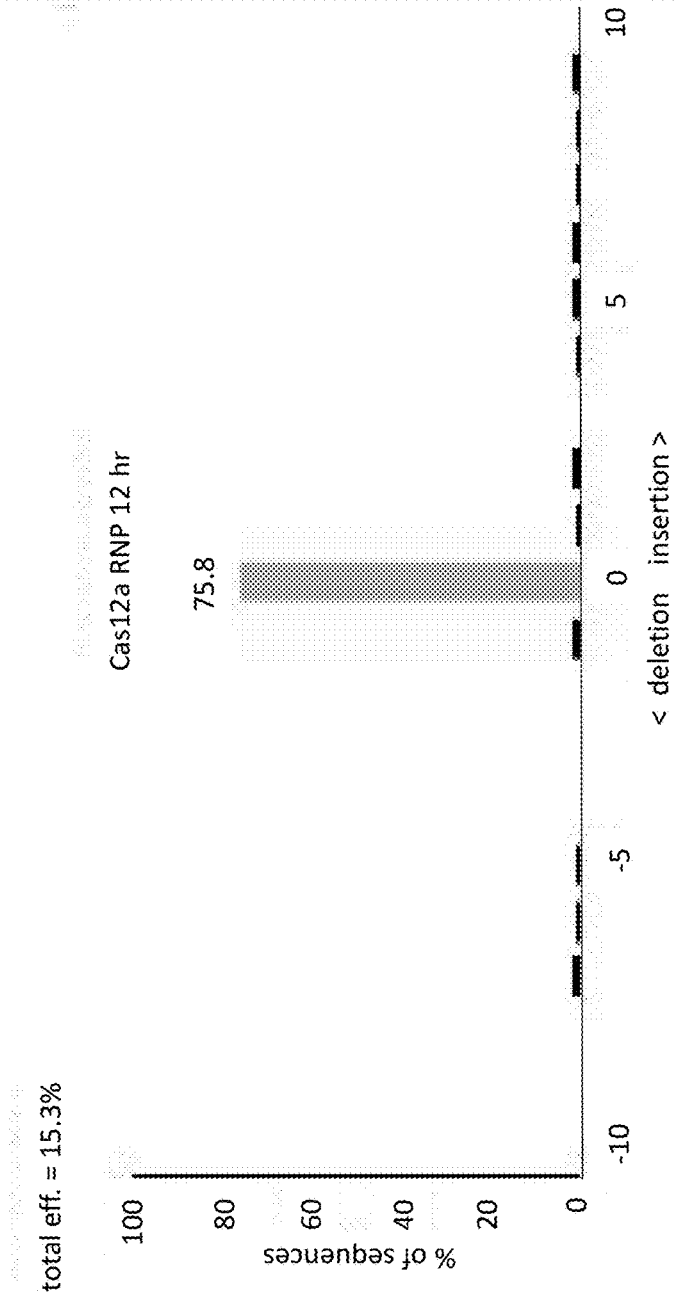
8 hours



Indel: 6.0%

FIG. 10D

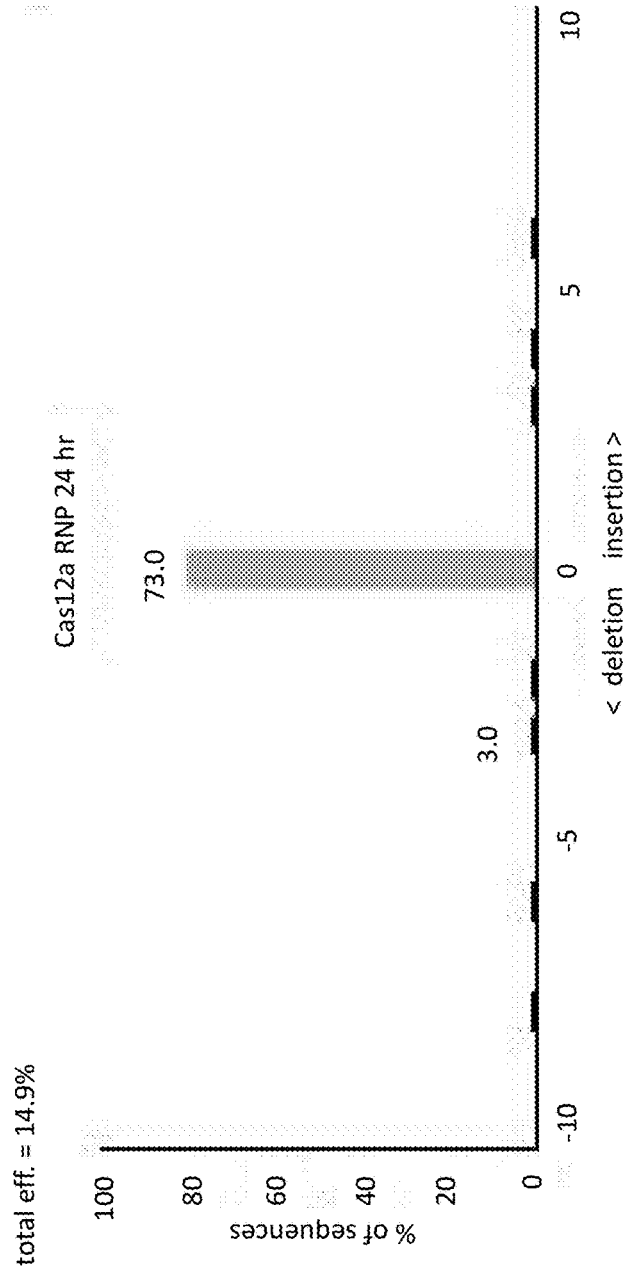
12 hour



Indel: 15.3%

FIG. 10E

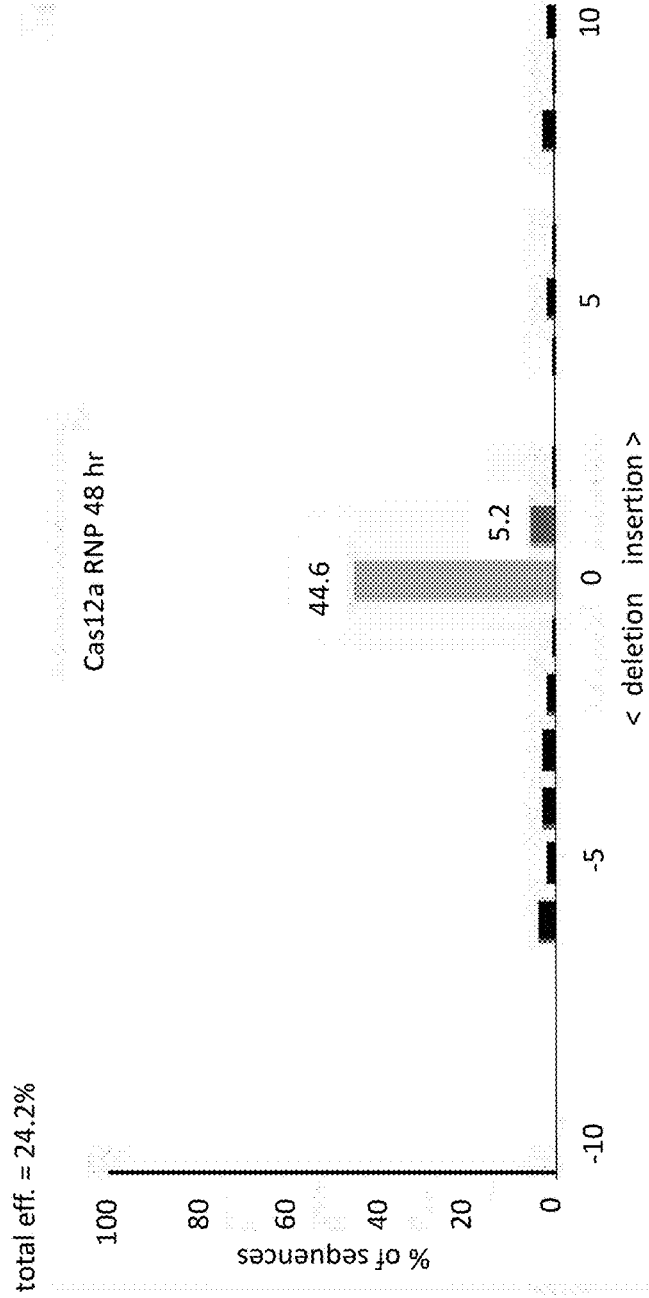
24 hours



Indel: 14.9%

FIG. 10F

48 hours



Indel: 24.2%

FIGURE 11

G9_H1703-26. $R^2 = 0.98$

INDEL	%	P-VALUE
3000	0.00	

G2_H1703_R34G_53. $R^2 = 0.93$

INDEL	%	P-VALUE
3000	0.00	

H1703_R34G_2-31-Neh2FWD.ab1 $R^2 = 0.92$

INDEL	%	P-VALUE
-6	51.2	0.00
-4	48.8	0.00

H1703_R34G_2-91-Neh2FWD.ab1 $R^2 = 0.88$

INDEL	%	P-VALUE
52.2	0.00	
-20	47.8	0.00

GENE KNOCKOUT OF NRF2 FOR TREATMENT OF CANCER

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application 62/852,076, filed May 23, 2019, which is incorporated herein, in its entirety, by reference.

SEQUENCE LISTING

[0002] The Sequence Listing associated with this application is filed in electronic format via EFS-Web and is hereby incorporated by reference into the specification in its entirety. The name of the text file containing the Sequence Listing is 130949_00101_SEQUENCELISTING.TXT. The size of the text file is 13 KB, and the text file was created on May 22, 2020.

FIELD

[0003] The present disclosure relates to compositions and methods for knocking out NRF2 to treat cancer using Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/endonuclease gene editing.

BACKGROUND

[0004] Cancer is presently one of the leading causes of death in developed nations. A diagnosis of cancer traditionally involves serious health complications. Cancer can cause disfigurement, chronic or acute pain, lesions, organ failure, or even death. Commonly diagnosed cancers include lung cancer, pancreatic cancer, breast cancer, melanoma, lymphoma, carcinoma, sarcoma leukemia, endometrial cancer, colon and rectal cancer, prostate cancer, and bladder cancer.

[0005] Traditionally, many cancers are treated with surgery, chemotherapy, radiation, or combinations thereof. Chemotherapeutic agents used in the treatment of cancer are known to produce several serious and unpleasant side effects in patients. For example, some chemotherapeutic agents cause neuropathy, nephrotoxicity, stomatitis, alopecia, decreased immunity, anemia, cardiotoxicity, fatigue, neuropathy, myelosuppression, or combinations thereof. Oftentimes, chemotherapy is not effective, or loses effectiveness after a period of efficacy, either during treatment, or shortly after the treatment regimen concludes. Thus a need exists for improved methods of treating cancer.

SUMMARY

[0006] One aspect is for a method of reducing NRF2 expression or activity in a cell comprising introducing into the cell (a) one or more DNA sequences encoding one or more guide RNAs (gRNAs) that are complementary to one or more target sequences in the NRF2 gene and (b) a nucleic acid sequence encoding a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease, whereby the one or more gRNAs hybridize to the NRF2 gene and the CRISPR-associated endonuclease cleaves the NRF2 gene, and wherein NRF2 expression or activity is reduced in the cell relative to a cell in which the one or more DNA sequences encoding the one or more gRNAs and the nucleic acid sequence encoding the CRISPR-associated endonuclease are not introduced. In some embodiments, the one or more gRNAs are complementary to one or more target sequences in exon 1, 2, 3, 4,

or 5, or spanning across two exons of the NRF2 gene. In some embodiments, the one or more gRNAs comprise a trans-activated small RNA (tracrRNA) and a CRISPR RNA (crRNA). In some embodiments, the one or more gRNAs are one or more single guide RNAs. In some embodiments, the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease, and in some embodiments, the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a. In some embodiments, expression of one or more allele(s) of the NRF2 gene is reduced in the cell. In some embodiments, NRF2 activity is reduced in the cell. In some embodiments, NRF2 expression or activity is not completely eliminated in the cell. In some embodiments, NRF2 expression or activity is completely eliminated in the cell. In some embodiments, the cell is a eukaryotic cell; in some embodiments, the eukaryotic cell is a mammalian cell; in some embodiments, the mammalian cell is a human cell.

[0007] An additional aspect is for a cell comprising a mutated NRF2 gene produced by the aforementioned method.

[0008] Another aspect is for a method of reducing NRF2 expression or activity in a cell comprising introducing into the cell (a) one or more guide RNAs (gRNAs) that are complementary to one or more target sequences in the NRF2 gene and (b) a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease, whereby the one or more gRNAs hybridize to the NRF2 gene and the CRISPR-associated endonuclease cleaves the NRF2 gene, and wherein NRF2 expression or activity is reduced in the cell relative to a cell in which the one or more gRNAs and the CRISPR-associated endonuclease are not introduced. In some embodiments, the one or more gRNAs are complementary to one or more target sequences in exon 1, 2, 3, 4, or 5, or spanning across two exons of the NRF2 gene. In some embodiments, the one or more gRNAs comprise a trans-activated small RNA (tracrRNA) and a CRISPR RNA (crRNA). In some embodiments, the one or more gRNAs are one or more single guide RNAs. In some embodiments, the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease, and in some embodiments, the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a. In some embodiments, expression of one or more allele(s) of the NRF2 gene is reduced in the cell. In some embodiments, NRF2 activity is reduced in the cell. In some embodiments, NRF2 expression or activity is not completely eliminated in the cell. In some embodiments, NRF2 expression or activity is completely eliminated in the cell. In some embodiments, the cell is a eukaryotic cell; in some embodiments, the eukaryotic cell is a mammalian cell; in some embodiments, the mammalian cell is a human cell.

[0009] An additional aspect is for a cell comprising a mutated NRF2 gene produced by the aforementioned method.

[0010] A further aspect is for a guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complementary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4. In some embodiments, the gRNA is complementary to a target sequence in exon 1, 2, 3, 4, or 5, or spanning across two exons of the NRF2 gene. In some embodiments, the DNA-binding domain comprises the nucleic acid sequence of SEQ

ID NO:2 or a biologically active fragment thereof. In some embodiments, the gRNA comprises a trans-activated small RNA (tracrRNA) and a CRISPR RNA (crRNA). In some embodiments, the gRNA is a single guide RNA.

[0011] An additional aspect is for a pharmaceutical composition comprising the aforementioned gRNA. In some embodiments, the pharmaceutical composition further comprises a Clustered Regularly

[0012] Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease; in some embodiments, the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease; and in some embodiments, the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a.

[0013] Another aspect is for a ribonucleoprotein (RNP) complex comprising the aforementioned gRNA and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease. In some embodiments, the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease, and in some embodiments, the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a.

[0014] A further aspect is for a pharmaceutical composition comprising the aforementioned RNP complex.

[0015] An additional aspect is for a DNA sequence encoding the aforementioned gRNA or a biologically active fragment thereof. In some embodiments, the biologically active fragment is a tracrRNA or a crRNA, and in some embodiments, the biologically active fragment is crRNA comprising the nucleic acid sequence of SEQ ID NO: 1. In some embodiments, the DNA sequence comprises the nucleic acid sequence of SEQ ID NO:1.

[0016] Another aspect is for a vector comprising the aforementioned DNA sequence. In some embodiments, the vector is an adeno-associated virus (AAV) vector. In some embodiments, the vector further comprises a nucleic acid sequence that encodes a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein; in some embodiments, the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease; and in some embodiments, the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a.

[0017] A further aspect is for a pharmaceutical composition comprising the aforementioned DNA sequence or the aforementioned vector. In some embodiments, the pharmaceutical composition further comprises a nucleic acid sequence that encodes a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein; in some embodiments, the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease; and in some embodiments, the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a.

[0018] An additional aspect is for a pharmaceutical composition comprising the aforementioned DNA sequence, further comprising a nucleic acid sequence that encodes a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein.

[0019] Another aspect is for a method of treating cancer in a subject comprising administering to the subject a therapeutically effective amount of the aforementioned pharmaceutical composition. In some embodiments, the cancer is resistant to one or more chemotherapeutic agents. In some embodiments, the cancer is selected from the group consisting of lung cancer, melanoma, esophageal squamous cancer (ESC), head and neck squamous cell carcinoma (HNSCC), and breast cancer; in some embodiments, the lung cancer is

non-small-cell lung cancer (NSCLC); and in some embodiments, the NSCLC is adenocarcinoma, squamous cell carcinoma, or large cell carcinoma. In some embodiments, the method further comprises administering one or more chemotherapeutic agents to the subject; in some embodiments, the one or more chemotherapeutic agents are selected from the group consisting of cisplatin, vinorelbine, carboplatin, and a combination thereof; in some embodiments, the pharmaceutical composition is administered in an amount sufficient to reduce proliferation of cells of the cancer relative to cancer cells that are treated with the at least one chemotherapeutic agent but are not treated with the pharmaceutical composition; and in some embodiments, the pharmaceutical composition is administered in an amount sufficient to reduce tumor growth relative to a tumor that is treated with the at least one chemotherapeutic agent but is not treated with the pharmaceutical composition. In some embodiments, the pharmaceutical composition is administered in an amount sufficient to reduce proliferation of cells of the cancer relative to cancer cells that are not treated with the pharmaceutical composition. In some embodiments, the pharmaceutical composition is administered in an amount sufficient to reduce tumor growth relative to a tumor that is not treated with the pharmaceutical composition. In some embodiments, the subject is a human.

[0020] A further aspect is for a method of reducing resistance to a chemotherapeutic agent in a cancer comprising administering to the subject a therapeutically effective amount of the aforementioned pharmaceutical composition.

[0021] Other objects and advantages will become apparent to those skilled in the art upon reference to the detailed description that hereinafter follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIG. 1A, 1B and 1C show CRISPR design and NRF2 knockout experimental workflow. NRF2 coding regions containing the six known genetic isoforms were utilized for targeting by CRISPR/Cas9. The gRNA sequences, along with their chromosomal loci and cloning details are displayed (A). The structural domains and location of CRISPR-directed gene editing of the NRF2 protein (B). The experimental workflow for testing the efficiency of CRISPR/Cas9 knockout of NRF2 in a targeted population and in isolated and expanded clonal cell lines (C).

[0023] FIG. 2A and 2B show genomic analyses of NRF2 knockout clones. Bulk sorted GFP+A549 cells transfected with either gRNA1 or gRNA2 were Sanger sequenced and analyzed for indel activity by TIDE (A). Clonally isolated NRF2 targeted cells were genomically analyzed for CRISPR/Cas9 induced NHEJ activity. Genomic DNA was Sanger sequenced and TIDE was used to develop the indel spectrums, sequence decompositions and allelic patterns of NRF2 as shown for clones 1-40 and 2-11 (B).

[0024] FIG. 3A and 3B show the cellular proliferation profile of NRF2 knockout A549 cells and western blot analysis. Cells were fixed with ethanol for 72 hours and stained with Alexa Fluor 647 Anti-Ki67. Fluorescence-activated cell sorting (FACS) was used to capture the intensity of Ki67-stained cells and plotted as a histogram using FlowJo software (left panel) (A). Cell proliferation was measured via bioreduction of MTS to a formazan product, plotted as mean raw absorbance values (right panel) and error bars represent \pm SEM (A). Western blot analysis of

wild type A549 cells and NRF2 knockout 2-11 cells using an antibody for phosphorylated NRF2 (B).

[0025] FIG. 4A and 4B show the proliferation capacity of wild type and NRF2 modified A549 cells (2-11) in response to chemotherapeutic drugs. Proliferation was measured via bioreduction of MTS to a formazan product. Cells were treated with increasing dosages of cisplatin (A) and increasing dosages of cisplatin with 5 μ M vinorelbine (B) for 72 hours, then evaluated for cell proliferation. Error bars represent \pm SEM.

[0026] FIG. 5A, 5B, 5C, 5D and 5E show restored chemosensitivity in mice with NRF2 knockout in tumors. Experimental workflow of mouse xenograft. Athymic nude mice were subcutaneously injected with either wild type A549 cells or NRF2 knockout A549 cells and once established tumors reach 100 mm³ were treated with the first dose of chemotherapy on Day 0. Mice were subsequently treated with chemo on days 3, 6, 9. Tumor volumes were measured daily for 16 days until tumors reached 2000 mm³ (A). Wild type A549 or NRF2 Knockout A549 tumors were treated with either 2mg/kg of cisplatin (B), 5mg/kg of cisplatin with 5mg/kg of vinorelbine (C), 25mg/kg of carboplatin (D), or saline and tumor size was measured for 16 days. Error bars represent \pm SEM. Tumors (treated with 2mg/kg of cisplatin or saline) were extracted from both the wild type A549 and NRF2 knockout A549 (2-11) implanted mice. Representative tumors from each group are shown (n=3) (E).

[0027] FIG. 6 shows proliferation of xenograft tumors. Representative images of xenograft tumors extracted from mice implanted with either wild type A549 or NRF2 knockout A549 cells (2-11) 16 days after initial treatment of either 2mg/kg cisplatin or saline were sectioned and stained with Ki67 (green) and DAPI (blue). Fluorescence intensity mean values for DAPI and Ki67 were obtained for the images using the Zeiss Zen software and relative values were obtained for fluorescence intensity of Ki67. Scale bar represents 100 μ m.

[0028] FIG. 7A and 7B show cisplatin-induced nuclear and cytoplasmic localization of NRF2 in wildtype A549 cells and clone 2-11. Cells were treated with 2 μ M Cisplatin, fixed and stained for NRF2. Immunocytochemistry was performed using fluorescence microscopy. Random fields were imaged and the total number of cells/field was counted. The percent of NRF2 positive stained cells over the total cells analyzed in each category was plotted in this graph. Error bars represent \pm SEM and * denotes a significant p value that is <0.05 (Student's t-test) (A).

[0029] Representative images of nuclear and cytoplasmic localization of NRF2 in wild type A549 and NRF2 knockout A549 (2-11) (B). Scale bar represents 50 μ m.

[0030] FIG. 8 shows clonal analysis using two CRISPR plasmid constructs to cleave 103 bases in exon 4 of NRF2. The top panel displays the structural domains and target region of gRNA1 and gRNA2 designed and used by Bialk et al., *Mol. Ther.—Oncolytics* 11, 75-89 (2018). The lower panel displays the genetic analysis of various clones recovered by fluorescence activated single cell sorting (FACS). Sequences in green indicate a wildtype sequence and bases highlighted in red indicate a frameshift resulting in a stop codon.

[0031] FIG. 9(A-F) shows population sequence analysis of CRISPR/Cas9-targeted cells. The A549 cell line was transfected with a RNP complex targeting the Neh2 domain in Exon 2 using gRNA 5' TGGATTGATTGACATACTTTGG

3'. Cells were collected at 1 (A), 4 (B), 8 (C), 12 (D), 24 (F), 48 h (G) post transfection. DNA was isolated, sequenced (across exon 2 of NRF2) and analyzed using Tracking of Indels by DEcomposition (TIDE). The indel efficiencies (%) are listed in the figure.

[0032] FIG. 10(A-F) shows Population Sequence Analysis of CRISPR/Cas12a-targeted cells. The

[0033] A549 cell line was transfected with a RNP complex targeting the Neh2 domain in Exon 2 using gRNA 5' TTTGATTGACATACTTTGGAGGCAA 3'. Cells were collected at 1 (A), 4 (B), 8 (C), 12 (D), 24 (F), 48 h (G) post transfection. DNA was isolated, sequenced (across exon 2 of NRF2) and analyzed using TIDE. The indel efficiencies (%) are listed in the figure.

[0034] FIG. 11 shows genetic Analysis of H1703 NRF2 KO clonal-derived cell lines. The H1703 cell line was transfected with Exon 2 gRNA 3 and R34G ssDNA template. Cells were expanded and analyzed. Several NRF2 KO clones were selected for further characterization.

DETAILED DESCRIPTION

[0035] This disclosure is based at least in part on the discovery that successful knockout of the NRF2 gene using CRISPR/Cas9 in chemo-resistant A549 lung cancer cells decreased proliferation of the cancer cells and increased effectiveness of the anticancer drugs cisplatin, carboplatin and vinorelbine in both in vitro culture and a xenograft mouse model. The overall strategy was to design and utilize a CRISPR/Cas gene editing tool to disable the NRF2 gene in cancer cells rendering it incapable of producing a functional protein. The CRISPR/Cas9 complex aligns in homologous register with the target gene, which enables it to execute a double-stranded DNA break. This action is followed by an attempt by the cell to reclose scission, most often through a process known as nonhomologous end joining (NHEJ). The re-closure is often imperfect and unfaithful as a number of nucleotides are lost during the process resulting in a genetic frameshift and the subsequent production of nonfunctional transcripts, a gene knockout of NRF2.

[0036] The compositions described herein include nucleic acids encoding a CRISPR-associated endonuclease (e.g., Cas9) and a guide RNA that is complementary to an NRF2 gene (e.g. exon 1, 2, 3, 4, or 5 of an NRF2 gene). Compositions comprising the guide RNA and the CRISPR-associated endonuclease are also described, as well as methods of administering the compositions to a subject for the treatment of cancer.

Definitions

[0037] Applicants specifically incorporate the entire contents of all cited references in this disclosure. Further, when an amount, concentration, or other value or parameter is given as either a range or a list of upper values and lower values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or value and any lower range limit or value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the present disclosure be limited to the specific values recited when defining a range.

[0038] The indefinite articles “a” and “an”, as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one”.

[0039] The phrase “and/or”, as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified unless clearly indicated to the contrary. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A without B (optionally including elements other than B); in another embodiment, to B without A (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[0040] As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of”, or, when used in the claims, “consisting of”, will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e., “one or the other but not both”) when preceded by terms of exclusivity, “either”, “one of”, “only one of”, “exactly one of”. “Consisting essentially of”, when used in the claims, shall have its ordinary meaning as used in the field of patent law.

[0041] The term “about” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, or $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

[0042] A “Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain” or “Cas binding domain” refers to a nucleic acid element or domain within a nucleic acid sequence or polynucleotide sequence that, in an effective amount, will bind or have an affinity for one or a plurality of CRISPR-associated endonuclease (or functional fragments thereof). In some embodiments, in the presence of the one or a plurality of proteins (or functional fragments thereof) and a target sequence, the one or plurality of proteins and the nucleic acid element forms a biologically active CRISPR complex and/or can be enzymatically active on a target sequence. In some embodiments, the CRISPR-associated endonuclease is a class 1 or class 2 CRISPR-associated endonuclease, and in some embodiments, a Cas9 or Cas12a endonuclease. The Cas9 endonuclease can have a nucleotide sequence identical to the wild type *Streptococcus pyogenes* sequence. In some embodiments, the CRISPR-associated endonuclease can be a sequence from other species, for example other *Streptococcus* species, such as *thermophilus*; *Pseudomona aeruginosa*, *Escherichia coli*, or other

sequenced bacteria genomes and archaea, or other prokaryotic microorganisms. Such species include: *Acidovorax avenae*, *Actinobacillus pleuropneumoniae*, *Actinobacillus succinogenes*, *Actinobacillus suis*, *Actinomyces* sp., *Cytophila denitrificans*, *Aminomonas paucivorans*, *Bacillus cereus*, *Bacillus smithii*, *Bacillus thuringiensis*, *Bacteroides* sp., *Blastopirellula marina*, *Bradyrhizobium* sp., *Brevibacillus laterosporus*, *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, *Candidatus punicispirillum*, *Clostridium cellulolyticum*, *Clostridium perfringens*, *Corynebacterium accolens*, *Corynebacterium diphtheria*, *Corynebacterium matruchotii*, *Dinoroseobacter shibae*, *Eubacterium dolichum*, *Gammaproteobacterium*, *Gluconacetobacter diazotrophicus*, *Haemophilus parainfluenzae*, *Haemophilus sputorum*, *Helicobacter canadensis*, *Helicobacter cinaedi*, *Helicobacter mustelae*, *Ilyobacter polytropus*, *Kingella kingae*, *Lactobacillus crispatus*, *Listeria ivanovii*, *Listeria monocytogenes*, *Listeriaceae bacterium*, *Methylocystis* sp., *Methylosinus trichosporium*, *Mobiluncus mulieris*, *Neisseria bacilliformis*, *Neisseria cinerea*, *Neisseria flavescens*, *Neisseria lactamica*, *Neisseria meningitidis*, *Neisseria* sp., *Neisseria wadsworthii*, *Nitrosomonas* sp., *Parvibaculum lavamentivorans*, *Pasteurelna multocida*, *Phascolarctobacterium succinatutens*, *Ralstonia syzygii*, *Rhodopseudomonas palustris*, *Rhodovulum* sp., *Simonsiella muelleri*, *Sphingomonas* sp., *Sporolactobacillus vineae*, *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Streptococcus* sp., *Subdoligranulum* sp., *Tistrella mobilis*, *Treponema* sp., and *Verminephrobacter eiseniae* (or functional fragments or variants of any of the aforementioned sequences that have at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of the aforementioned Cas9 endonucleases). In some embodiments, the CRISPR-associated endonuclease can be a Cas12a nuclease. The Cas12a nuclease can have a nucleotide sequence identical to a wild type Prevotella or Francisella sequence (or functional fragments or variants of any of the aforementioned sequences that have at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to any of the aforementioned Cas12 endonucleases).

[0043] In some embodiments, the terms “(CRISPR)-associated endonuclease protein-binding domain” or “Cas binding domain” refer to a nucleic acid element or domain (e.g. and RNA element or domain) within a nucleic acid sequence that, in an effective amount, will bind to or have an affinity for one or a plurality of CRISPR-associated endonucleases (or functional fragments or variants thereof that are at least about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% homologous to a CRISPR-associated endonuclease). In some embodiments, the Cas binding domain consists of at least or no more than about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210,

215, 220, 225, 230, 235, 240, 245, or 250 nucleotides and comprises at least one sequence that is capable of forming a hairpin or duplex that partially associates or binds to a biologically active CRISPR-associated endonuclease at a concentration and within a microenvironment suitable for CRISPR system formation.

[0044] The “Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)—CRISPR associated (Cas) (CRISPR-Cas) system guide RNA” or “CRISPR-Cas system guide RNA” may comprise a transcription terminator domain. The term “transcription terminator domain” refers to a nucleic acid element or domain within a nucleic acid sequence (or polynucleotide sequence) that, in an effective amount, prevents bacterial transcription when the CRISPR complex is in a bacterial species and/or creates a secondary structure that stabilizes the association of the nucleic acid sequence to one or a plurality of Cas proteins (or functional fragments thereof) such that, in the presence of the one or a plurality of proteins (or functional fragments thereof), the one or plurality of Cas proteins and the nucleic acid element forms a biologically active CRISPR complex and/or can be enzymatically active on a target sequence in the presence of such a target sequence and a DNA-binding domain. In some embodiments, the transcription terminator domain consists of at least or no more than about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, or 250 nucleotides and comprises at least one sequence that is capable of forming a hairpin or duplex that partially drives association of the nucleic acid sequence (sgRNA, crRNA with tracrRNA, or other nucleic acid sequence) to a biologically active CRISPR complex at a concentration and microenvironment suitable for CRISPR complex formation.

[0045] The term “DNA-binding domain” refers to a nucleic acid element or domain within a nucleic acid sequence (e.g., a guide RNA) that is complementary to a target sequence (e.g., an NRF2 gene). In some embodiments, the DNA-binding domain will bind or have an affinity for an NRF2 gene such that, in the presence of a biologically active CRISPR complex, one or plurality of Cas proteins can be enzymatically active on the target sequence. In some embodiments, the DNA binding domain comprises at least one sequence that is capable of forming Watson Crick basepairs with a target sequence as part of a biologically active CRISPR system at a concentration and microenvironment suitable for CRISPR system formation.

[0046] “CRISPR system” refers collectively to transcripts or synthetically produced transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated (“Cas”) genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a “direct repeat” and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a “spacer” in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type

III CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a

[0047] CRISPR complex, “target sequence” refers to a nucleic acid sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as

[0048] DNA or RNA polynucleotides. In some embodiments, the target sequence is a DNA polynucleotide and is referred to a DNA target sequence. In some embodiments, a target sequence comprises at least three nucleic acid sequences that are recognized by a Cas-protein when the Cas protein is associated with a CRISPR complex or system which comprises at least one sgRNA or one tracrRNA/crRNA duplex at a concentration and within an microenvironment suitable for association of such a system. In some embodiments, the target DNA comprises at least one or more proto-spacer adjacent motifs which sequences are known in the art and are dependent upon the Cas protein system being used in conjunction with the sgRNA or crRNA/tracrRNAs employed by this work. In some embodiments, the target DNA comprises NNG, where G is an guanine and N is any naturally occurring nucleic acid. In some embodiments the target DNA comprises any one or combination of NNG, NNA, GAA, NNAGAAW and NGGNG, where G is an guanine, A is adenine, and N is any naturally occurring nucleic acid

[0049] In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

[0050] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. In some embodiments, the tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of a CRISPR complex. As with the target sequence, it is believed that complete complementarity is not needed, provided there is sufficient to be functional (bind the Cas protein or functional fragment thereof). In some embodiments, the tracr sequence has at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%,

69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that the presence and/or expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. With at least some of the modification contemplated by this disclosure, in some embodiments, the guide sequence or RNA or DNA sequences that form a CRISPR complex are at least partially synthetic. The CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. In some embodiments, the disclosure relates to a composition comprising a chemically synthesized guide sequence. In some embodiments, the chemically synthesized guide sequence is used in conjunction with a vector comprising a coding sequence that encodes a CRISPR enzyme, such as a class 2 Cas9 or Cas12a protein. In some embodiments, the chemically synthesized guide sequence is used in conjunction with one or more vectors, wherein each vector comprises a coding sequence that encodes a CRISPR enzyme, such as a class 2 Cas9 or Cas12a protein. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more additional (second, third, fourth, etc.) guide sequences, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR enzyme, one or more additional guide sequence, tracr mate sequence, and tracr sequence are each a component of different nucleic acid sequences. For instance, in the case of a tracr and tracr mate sequences and in some embodiments, the disclosure relates to a composition comprising at least a first and second nucleic acid sequence, wherein the first nucleic acid sequence comprises a tracr sequence and the second nucleic acid sequence comprises a tracr mate sequence, wherein the first nucleic acid sequence is at least partially complementary to the second nucleic acid sequence such that the first and second nucleic acid for a duplex and wherein the first nucleic acid and the second nucleic acid either individually or collectively comprise a DNA-targeting domain, a Cas protein binding domain, and a transcription terminator domain. In some embodiments, the CRISPR enzyme, one or more additional guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter. In some embodiments, the disclosure relates

to compositions comprising any one or combination of the disclosed domains on one guide sequence or two separate tracrRNA/crRNA sequences with or without any of the disclosed modifications. Any methods disclosed herein also relate to the use of tracrRNA/crRNA sequence interchangeably with the use of a guide sequence, such that a composition may comprise a single synthetic guide sequence and/or a synthetic tracrRNA/crRNA with any one or combination of modified domains disclosed herein.

[0051] In some embodiments, a guide RNA can be a short, synthetic, chimeric tracrRNA/crRNA (a "single-guide RNA" or "sgRNA"). A guide RNA may also comprise two short, synthetic tracrRNA/crRNAs (a "dual-guide RNA" or "dgrRNA").

[0052] The terms "cancer" or "tumor" are well known in the art and refer to the presence, e.g., in a subject, of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, decreased cell death/apoptosis, and certain characteristic morphological features.

[0053] As used herein, "cancer" refers to all types of cancer or neoplasm or malignant tumors found in humans, including, but not limited to: leukemias, lymphomas, melanomas, carcinomas and sarcomas. As used herein, the terms or language "cancer," "neoplasm," and "tumor," are used interchangeably and in either the singular or plural form, refer to cells that have undergone a malignant transformation that makes them pathological to the host organism. Primary cancer cells (that is, cells obtained from near the site of malignant transformation) can be readily distinguished from non-cancerous cells by well-established techniques, particularly histological examination. The definition of a cancer cell, as used herein, includes not only a primary cancer cell, but also cancer stem cells, as well as cancer progenitor cells or any cell derived from a cancer cell ancestor. This includes metastasized cancer cells, and in vitro cultures and cell lines derived from cancer cells. In certain embodiments, the cancer is a blood tumor (i.e., a non-solid tumor). In some embodiments, the cancer is lymphoid neoplasm diffuse large B-cell lymphoma, cholangiocarcinoma, uterine carcinosarcoma, kidney chromophobe, uveal melanoma, mesothelioma, adrenocortical carcinoma, thymoma, acute myeloid leukemia, testicular germ cell tumor, rectum adenocarcinoma, pancreatic adenocarcinoma, pheochromocytoma and paraganglioma, esophageal carcinoma, sarcoma, kidney renal papillary cell carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, kidney renal clear cell carcinoma, liver hepatocellular carcinoma, glioblastoma multiforme, bladder urothelial carcinoma, colon adenocarcinoma, stomach adenocarcinoma, ovarian serous cystadenocarcinoma, skin cutaneous melanoma, prostate adenocarcinoma, thyroid carcinoma, lung squamous cell carcinoma, head and neck squamous cell carcinoma, brain lower grade glioma, uterine corpus endometrial carcinoma, lung adenocarcinoma, or breast invasive carcinoma (see, e.g., Kerins et al., *Sci. Rep.* 8:12846 (2018)).

[0054] In certain embodiments, the cancer is a solid tumor. A "solid tumor" is a tumor that is detectable on the basis of tumor mass; e.g., by procedures such as CAT scan, MR imaging, X-ray, ultrasound or palpation, and/or which is detectable because of the expression of one or more cancer-

specific antigens in a sample obtainable from a patient. The tumor does not need to have measurable dimensions.

[0055] Specific criteria for the staging of cancer are dependent on the specific cancer type based on tumor size, histological characteristics, tumor markers, and other criteria known by those of skill in the art. Generally, cancer stages can be described as follows:

[0056] Stage 0—Carcinoma in situ

[0057] Stage I, Stage II, and Stage III—Higher numbers indicate more extensive disease: Larger tumor size and/or spread of the cancer beyond the organ in which it first developed to nearby lymph nodes and/or tissues or organs adjacent to the location of the primary tumor

[0058] Stage IV—The cancer has spread to distant tissues or organs

[0059] As used herein, the terms “treat,” “treating” or “treatment” refer to an action to obtain a beneficial or desired clinical result including, but not limited to, alleviation or amelioration of one or more signs or symptoms of a disease or condition (e.g., regression, partial or complete), diminishing the extent of disease, stability (i.e., not worsening, achieving stable disease) of the state of disease, amelioration or palliation of the disease state, diminishing rate of or time to progression, and remission (whether partial or total). “Treatment” of a cancer can also mean prolonging survival as compared to expected survival in the absence of treatment. Treatment need not be curative. In certain embodiments, treatment includes one or more of a decrease in pain or an increase in the quality of life (QOL) as judged by a qualified individual, e.g., a treating physician, e.g., using accepted assessment tools of pain and QOL. In certain embodiments, a decrease in pain or an increase in the QOL as judged by a qualified individual, e.g., a treating physician, e.g., using accepted assessment tools of pain and QOL is not considered to be a “treatment” of the cancer.

[0060] “Chemotherapeutic agent” refers to a drug used for the treatment of cancer. Chemotherapeutic agents include, but are not limited to, small molecules, hormones and hormone analogs, and biologics (e.g., antibodies, peptide drugs, nucleic acid drugs). In certain embodiments, chemotherapy does not include hormones and hormone analogs.

[0061] A “cancer that is resistant to one or more chemotherapeutic agents” is a cancer that does not respond, or ceases to respond to treatment with a chemotherapeutic regimen, i.e., does not achieve at least stable disease (i.e., stable disease, partial response, or complete response) in the target lesion either during or after completion of the chemotherapeutic regimen. Resistance to one or more chemotherapeutic agents results in, e.g., tumor growth, increased tumor burden, and/or tumor metastasis.

[0062] A “therapeutically effective amount” is that amount sufficient, at dosages and for periods of time necessary, to achieve a desired therapeutic result, such as for treatment of a disease (e.g. cancer), condition, or disorder, and/or pharmacokinetic or pharmacodynamic effect of the treatment in a subject. A therapeutically effective amount can be administered in one or more administrations. The therapeutically effective amount may vary according to factors such as the disease state, age, sex, and weight of the subject.

NRF2

[0063] Nuclear Factor Erythroid 2- Related Factor (NRF2) is considered the master regulator of 100-200 target genes involved in cellular responses to oxidative/electrophilic

stress. Targets include glutathione (GSH) mediators, anti-oxidants and genes controlling efflux pumps. Hayden, et al., *Urol. Oncol. Semin. Orig. Investig.* 32, 806-814 (2014). NRF2 is also known to regulate expression of genes involved in protein degradation and detoxification and is negatively regulated by Kelch-like ECH-associated protein 1 (KEAP1), a substrate adapter for the Cul3-dependent E3 ubiquitin ligase complex. Under normal conditions, Keap 1 constantly targets NRF2 for ubiquitin-dependent degradation maintaining low expression of NRF2 on downstream target genes. However, chemotherapy has been shown to activate transcriptional activity of the NRF2 target genes often triggering a cytoprotective response; enhanced expression of NRF2 occurs in response to environmental stress or detrimental growth conditions. Other mechanisms that lead to NRF2 upregulation include mutations in KEAP1 or epigenetic changes of the promoter region. The upregulation of NRF2 expression leads to an enhanced resistance of cancer cells to chemotherapeutic drugs, which by their very action induce an unfavorable environment for cell proliferation. Indeed, Hayden et al. (*ibid*) have clearly demonstrated that increased NRF2 expression leads to the resistance of cancer cells to chemotherapeutic drugs including cisplatin. Singh et al. (2010, *Antioxidants & Redox Signaling* 13) also showed that constitutive expression of NRF2 leads to radioresistance, and inhibition of NRF2 causes increased endogenous reactive oxygen species (ROS) levels as well as decreased survival. Recently, Torrente et al. (*Oncogene* (2017). doi: 10.1038/onc.2017.221) identified crosstalk between NRF2 and the homeodomain interacting protein kinase two, HIPK2, demonstrating that HIPK2 exhibits a cytoprotective effect through NRF2.

CRISPR/Endonucleases

[0064] CRISPR/endonuclease (e.g., CRISPR/Cas9) systems are known in the art and are described, for example, in U.S. Pat. No. 9,925,248, which is incorporated by reference herein in its entirety. CRISPR-directed gene editing can identify and execute DNA cleavage at specific sites within the chromosome at a surprisingly high efficiency and precision. The natural activity of CRISPR/Cas9 is to disable a viral genome infecting a bacterial cell. Subsequent genetic reengineering of CRISPR/Cas function in human cells presents the possibility of disabling human genes at a significant frequency.

[0065] In bacteria, the CRISPR/Cas loci encode RNA-guided adaptive immune systems against mobile genetic elements (viruses, transposable elements and conjugative plasmids). Three types (I-III) of CRISPR systems have been identified. CRISPR clusters contain spacers, the sequences complementary to antecedent mobile elements. CRISPR clusters are transcribed and processed into mature CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) RNA (crRNA) containing a DNA binding region (spacer) which is complementary to the target gene. The CRISPR-associated endonuclease, Cas9, belongs to the type II CRISPR/Cas system and has strong endonuclease activity to cut target DNA. Cas9 is guided by a mature crRNA that contains about 20 base pairs (bp) of unique target sequence (called a spacer) and a trans-activated small RNA (tracrRNA) that serves as a guide for ribonuclease III-aided processing of pre-crRNA. The crRNA:tracrRNA duplex directs Cas9 to target DNA via complementary base pairing between the spacer on the crRNA and the complementary

sequence (called protospacer) on the target DNA. Cas9 recognizes a trinucleotide (NGG) protospacer adjacent motif (PAM) to specify the cut site (the 3rd nucleotide from PAM).

[0066] The compositions described herein can include a nucleic acid encoding a CRISPR-associated endonuclease. The CRISPR-associated endonuclease can be, e.g., a class 1 CRISPR-associated endonuclease or a class 2 CRISPR-associated endonuclease. Class 1 CRISPR-associated endonucleases include type I, type III, and type IV CRISPR-Cas systems, which have effector molecules that comprise multiple subunits. For class 1 CRISPR-associated endonucleases, effector molecules can include, in some embodiments, Cas2 and Cas5, along with, in some embodiments, SS (Cas11) and Cas8a1; Cas8b1; Cas8c; Cas8u2 and Cas6; Cas3" and Cas10d; Cas SS (Cas11), Cas8e, and Cas6; Cas8f and Cas6f; Cas6f; Cas8-like (Csf1); SS (Cash) and Cas8-like (Csf1); or SS (Cas11) and Cas10. Class 1 CRISPR-associated endonucleases also be associated with, in some embodiments, target cleavage molecules, which can be Cas3 (type I) or Cas10 (type III) and spacer acquisition molecules such as, e.g., Cas 1, Cas2, and/or Cas4. See, e.g., Koonin et al., *Curr. Opin. Microbiol.* 37:67-78 (2017); Strich & Chertow, *J. Clin. Microbiol.* 57:1307-18 (2019).

[0067] Class 2 CRISPR-associated endonucleases include type I, type V, and type VI CRISPR-Cas systems, which have a single effector molecule. For class 2 CRISPR-associated endonucleases, effector molecules can include, in some embodiments, Cas9, Cas12a (cpf1), Cas12b1 (c2c1), Cas12b2, Cas12c (c2c3), Cas12d (CasY), Cas12e (CasX), Cas12f1 (Cas14a), Cas12f2 (Cas14b), Cas12f3 (Cas14c), Cas12g, Cas12h, Cas12i, Cas12k (c2c5), Cas13a (c2c2), Cas13b1 (c2c6), Cas13b2 (c2c6), Cas13c (c2c7), Cas13d, c2c4, c2c8, c2c9, and/or c2c10. See, e.g., Koonin et al., *Curr. Opin. Microbiol.* 37:67-78 (2017); Strich & Chertow, *J. Clin. Microbiol.* 57:1307-18 (2019); Makarova et al., *Nat. Rev. Microbiol.* 18:67-83 (2020).

[0068] In some embodiments, the CRISPR-associated endonuclease can be a Cas9 nuclease. The Cas9 nuclease can have a nucleotide sequence identical to the wild type *Streptococcus pyogenes* sequence. In some embodiments, the CRISPR-associated endonuclease can be a sequence from other species, for example other *Streptococcus* species, such as *thermophilus*; *Pseudomonas aeruginosa*, *Escherichia coli*, or other sequenced bacteria genomes and archaea, or other prokaryotic microorganisms. Such species include: *Acidovorax avenae*, *Actinobacillus pleuropneumoniae*, *Actinobacillus succinogenes*, *Actinobacillus suis*, *Actinomyces* sp., *Cytophila denitrificans*, *Aminomonas paucivorans*, *Bacillus cereus*, *Bacillus smithii*, *Bacillus thuringiensis*, *Bacteroides* sp., *Blastopirellula marina*, *Bradyrhizobium* sp., *Brevibacillus laterosporus*, *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, *Candidatus pumiceispirillum*, *Clostridium cellulolyticum*, *Clostridium perfringens*, *Corynebacterium accolens*, *Corynebacterium diphtheria*, *Corynebacterium matruchotii*, *Dinoroseobacter shibae*, *Eubacterium dolichum*, *Gammaproteobacterium*, *Gluconacetobacter diazotrophicus*, *Haemophilus parainfluenzae*, *Haemophilus sputorum*, *Helicobacter canadensis*, *Helicobacter cinaedi*, *Helicobacter mustelae*, *Ilyobacter polytropus*, *Kingella kingae*, *Lactobacillus crispatus*, *Listeria ivanovii*, *Listeria monocytogenes*, *Listeriaceae bacterium*, *Methylocystis* sp., *Methylosinus trichosporium*, *Mobiluncus mulieris*, *Neisseria bacilliformis*, *Neisseria cinerea*, *Neisseria flavescens*, *Neisseria lactamica*, *Neisseria menin-*

gitidis, *Neisseria* sp., *Neisseria wadsworthii*, *Nitrosomonas* sp., *Parvibaculum lavamentivorans*, *Pasteurella multocida*, *Phascolarctobacterium succinatutens*, *Ralstonia syzygii*, *Rhodopseudomonas palustris*, *Rhodovulum* sp., *Simonsiella muelleri*, *Sphingomonas* sp., *Sporolactobacillus vineae*, *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Streptococcus* sp., *Subdoligranulum* sp., *Tistrella mobilis*, *Treponema* sp., and *Verminephrobacter eiseniae*.

[0069] Alternatively, the wild type *Streptococcus pyogenes* Cas9 sequence can be modified. The nucleic acid sequence can be codon optimized for efficient expression in mammalian cells, e.g., human cells. A Cas9 nuclease sequence codon optimized for expression in human cells can be for example, the Cas9 nuclease sequence encoded by any of the expression vectors listed in Genbank accession numbers KM099231.1 GI:669193757; KM099232.1 GI:669193761; or KM099233.1 GI:669193765. Alternatively, the Cas9 nuclease sequence can be, for example, the sequence contained within a commercially available vector such as pX458, pX330 or pX260 from Addgene (Cambridge, Mass.). In some embodiments, the Cas9 endonuclease can have an amino acid sequence that is a variant or a fragment of any of the Cas9 endonuclease sequences of Genbank accession numbers KM099231.1 GI:669193757; KM099232.1 GI:669193761; or KM099233.1 GI:669193765 or Cas9 amino acid sequence of pX458, pX330 or pX260 (Addgene, Cambridge, Mass.). The Cas9 nucleotide sequence can be modified to encode biologically active variants of Cas9, and these variants can have or can include, for example, an amino acid sequence that differs from a wild type Cas9 by virtue of containing one or more mutations (e.g., an addition, deletion, or substitution mutation or a combination of such mutations). One or more of the substitution mutations can be a substitution (e.g., a conservative amino acid substitution). For example, a biologically active variant of a Cas9 polypeptide can have an amino acid sequence with at least or about 50% sequence identity (e.g., at least or about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity) to a wild type Cas9 polypeptide.

[0070] In some embodiments, the CRISPR-associated endonuclease can be a Cas12a nuclease. The Cas12a nuclease can have a nucleotide sequence identical to a wild type *Prevotella* or *Francisella* sequence. Alternatively, a wild type *Prevotella* or *Francisella* Cas12a sequence can be modified. The nucleic acid sequence can be codon optimized for efficient expression in mammalian cells, e.g., human cells. A Cas12a nuclease sequence codon optimized for expression in human cells sequence can be for example, the Cas9 nuclease sequence encoded by any of the expression vectors listed in Genbank accession numbers MF193599.1 GI: 1214941796, KY985374.1 GI: 1242863785, KY985375.1 GI: 1242863787, or KY985376.1 GI: 1242863789. Alternatively, the Cas12a nuclease sequence can be, for example, the sequence contained within a commercially available vector such as pAs-Cpf 1 or pLb-Cpf 1 from Addgene (Cambridge, Mass.). In some embodiments, the Cas12a endonuclease can have an amino acid sequence that is a variant or a fragment of any of the Cas12a endonuclease sequences of Genbank accession numbers

MF193599.1 GI: 1214941796, KY985374.1 GI: 1242863785, KY985375.1 GI: 1242863787, or KY985376.1 GI: 1242863789 or Cas12a amino acid sequence of pAs-Cpfl or pLb-Cpfl (Addgene, Cambridge, Mass.). The Cas12a nucleotide sequence can be modified to encode biologically active variants of Cas12a, and these variants can have or can include, for example, an amino acid sequence that differs from a wild type Cas12a by virtue of containing one or more mutations (e.g., an addition, deletion, or substitution mutation or a combination of such mutations). One or more of the substitution mutations can be a substitution (e.g., a conservative amino acid substitution). For example, a biologically active variant of a Cas12a polypeptide can have an amino acid sequence with at least or about 50% sequence identity (e.g., at least or about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity) to a wild type Cas12a polypeptide.

[0071] The compositions described herein may also include sequence encoding a guide RNA (gRNA) comprising a DNA-binding domain that is complementary to a target domain from an NRF2 gene (e.g., a target domain from exon 1, 2, 3, 4, or 5 of an NRF2 gene), and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain. The guide RNA sequence can be a sense or anti-sense sequence. The guide RNA sequence may include a proto-spacer adjacent motif (PAM). The sequence of the PAM can vary depending upon the specificity requirements of the CRISPR endonuclease used. In the CRISPR-Cas system derived from *S. pyogenes*, the target DNA typically immediately precedes a 5'-NGG proto-spacer adjacent motif (PAM). Thus, for the *S. pyogenes* Cas9, the PAM sequence can be AGG, TGG, CGG or GGG. Other Cas9 orthologs may have different PAM specificities. The specific sequence of the guide RNA may vary, but, regardless of the sequence, useful guide

[0072] RNA sequences will be those that minimize off-target effects while achieving high efficiency. In some embodiments, the guide RNA sequence achieves complete ablation of the NRF2 gene.

[0073] In some embodiments, the DNA-binding domain varies in length from about 20 to about 55 nucleotides, for example, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, or about 55 nucleotides. In some embodiments, the Cas protein-binding domain is from about 30 to about 55 nucleotides in length, for example, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, or about 55 nucleotides.

[0074] In some embodiments, the compositions comprise one or more nucleic acid (i.e. DNA) sequences encoding the guide RNA and the CRISPR endonuclease. When the compositions are administered as a nucleic acid or are contained within an expression vector, the CRISPR endonuclease can

be encoded by the same nucleic acid or vector as the guide RNA sequence. In some embodiments, the CRISPR endonuclease can be encoded in a physically separate nucleic acid from the guide RNA sequence or in a separate vector. The nucleic acid sequence encoding the guide RNA may comprise a DNA binding domain, a Cas protein binding domain, and a transcription terminator domain.

[0075] The nucleic acid encoding the guide RNA and/or the CRISPR endonuclease may be an isolated nucleic acid. An "isolated" nucleic acid can be, for example, a naturally-occurring DNA molecule or a fragment thereof, provided that at least one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Isolated nucleic acid molecules can be produced by standard techniques. For example, polymerase chain reaction (PCR) techniques can be used to obtain an isolated nucleic acid containing a nucleotide sequence described herein, including nucleotide sequences encoding a polypeptide described herein. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Various PCR methods are described in, for example, PCR Primer: A Laboratory Manual, Dieffenbach and Dveksler, eds., Cold Spring Harbor Laboratory Press, 1995. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the template to be amplified. Various PCR strategies also are available by which site-specific nucleotide sequence modifications can be introduced into a template nucleic acid.

[0076] Isolated nucleic acids also can be chemically synthesized, either as a single nucleic acid molecule (e.g., using automated DNA synthesis in the 3' to 5' direction using phosphoramidite technology) or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >50-100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector. Isolated nucleic acids also can be obtained by mutagenesis of, e.g., a naturally occurring portion of a Cas9-encoding DNA (in accordance with, for example, the formula above).

[0077] Recombinant constructs are also provided herein and can be used to transform cells in order to express the CRISPR endonuclease and/or a guide RNA complementary to an NRF2 gene. A recombinant nucleic acid construct may comprise a nucleic acid encoding a CRISPR endonuclease and/or a guide RNA complementary to an NRF2 gene, operably linked to a promoter suitable for expressing the CRISPR endonuclease and/or a guide RNA complementary to the NRF2 gene in the cell. In some embodiments the nucleic acid encoding a CRISPR endonuclease is operably linked to the same promoter as the nucleic acid encoding the guide RNA. In other embodiments, the nucleic acid encoding a CRISPR endonuclease and the nucleic acid encoding the guide RNA are operably linked to different promoters. In some embodiments, the nucleic acid encoding a CRISPR endonuclease and/or the nucleic acid encoding a guide RNA are operably linked to a lung specific promoter. Suitable lung

specific promoters include, but are not limited to, Clara cell 10-kDa protein (CC₁₀) (aka Scgblal) promoter (Stripp et al., *J. Biol. Chem.* 267:14703-12 (1992)), SFTPC promoter (Wert et al., *Dev. Biol.* 156:426-43 (1993)), FOXJ1 promoter (Ostrowski et al., *Mol. Ther.* 8:637-45 (2003)), aquaporin (Aqp5) promoter (Funaki et al., *Am. J. Physiol.* 275:C1151-57 (1998)), Keratin 5 (Krt5) promoter (Rock et al., *Dis. Model Mech.* 3:545-56 (2010)), Keratin 14 (Krt14) promoter (Rock et al., *Dis. Model Mech.* 3:545-56 (2010)), cytokeratin 18 (K18) promoter (Chow et al., *Proc. Natl. Acad. Sci. USA* 94:14695-14700 (1997)), surfactant protein B (SP-B) promoter (Strayer et al., *Am. J. Physiol. Lung Cell Mol. Physiol.* 282:L394-404 (2002)), TTF1 gene under the control of human telomerase reverse transcriptase promoter and human surfactant protein A1 promoter (Fukazawa et al., *Cancer Res.* 64:363-69 (2004)), surfactant protein C (SP-C) promoter (Zhuo et al., *Transgenic Res.* 15:543-55 (2006)), insulinoma-associated antigen-1 (INSM1) promoter (Li et al., *Biochem. Biophys. Res. Commun.* 236:776-81 (1997)), and surfactant protein A (SP-A) promoter (Bruno et al., *J. Biol. Chem.* 270:6531-36 (1995)).

[0078] In some embodiments, one or more CRISPR endonucleases and one or more guide RNAs may be provided in combination in the form of ribonucleoprotein particles (RNPs). An RNP complex can be introduced into a subject by means of, e.g., injection, electroporation, nanoparticles, vesicles, and/or with the assistance of cell-penetrating peptides.

[0079] DNA vectors containing nucleic acids such as those described herein also are also provided. A “DNA vector” is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a DNA vector is capable of replication when associated with the proper control elements. Suitable vector backbones include, for example, those routinely used in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs, or PACs. The term “DNA vector” includes cloning and expression vectors, as well as viral vectors and integrating vectors. An “expression vector” is a vector that includes a regulatory region. A wide variety of host/expression vector combinations may be used to express the nucleic acid sequences described herein. Suitable expression vectors include, without limitation, plasmids and viral vectors derived from, for example, bacteriophage, baculoviruses, and retroviruses. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, Wis.), Clontech (Palo Alto, Calif.), Stratagene (La Jolla, Calif.), and Invitrogen/Life Technologies (Carlsbad, Calif.).

[0080] The DNA vectors provided herein also can include, for example, origins of replication, scaffold attachment regions (SARs), and/or markers. A marker gene can confer a selectable phenotype on a host cell. For example, a marker can confer biocide resistance, such as resistance to an antibiotic (e.g., kanamycin, G418, bleomycin, or hygromycin). As noted above, an expression vector can include a tag sequence designed to facilitate manipulation or detection (e.g., purification or localization) of the expressed polypeptide. Tag sequences, such as green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or Flag™ tag (Kodak, New Haven, Conn.) sequences typically are expressed as a fusion with the

encoded polypeptide. Such tags can be inserted anywhere within the polypeptide, including at either the carboxyl or amino terminus.

[0081] The DNA vector can also include a regulatory region. The term “regulatory region” refers to nucleotide sequences that influence transcription or translation initiation and rate, and stability and/or mobility of a transcription or translation product. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, protein binding sequences, 5' and 3' untranslated regions (UTRs), transcriptional start sites, termination sequences, polyadenylation sequences, nuclear localization signals, and introns.

[0082] As used herein, the term “operably linked” refers to positioning of a regulatory region (e.g. a promoter) and a sequence to be transcribed in a nucleic acid so as to influence transcription or translation of such a sequence. For example, to bring a coding sequence under the control of a promoter, the translation initiation site of the translational reading frame of the polypeptide is typically positioned between one and about fifty nucleotides downstream of the promoter. A promoter can, however, be positioned as much as about 5,000 nucleotides upstream of the translation initiation site or about 2,000 nucleotides upstream of the transcription start site. A promoter typically comprises at least a core (basal) promoter. A promoter also may include at least one control element, such as an enhancer sequence, an upstream element or an upstream activation region (UAR). The choice of promoters to be included depends upon several factors, including, but not limited to, efficiency, selectability, inducibility, desired expression level, and cell- or tissue-preferential expression. It is a routine matter for one of skill in the art to modulate the expression of a coding sequence by appropriately selecting and positioning promoters and other regulatory regions relative to the coding sequence.

[0083] Vectors include, for example, viral vectors (such as adenoviruses (“Ad”), adeno-associated viruses (AAV), and vesicular stomatitis virus (VSV) and retroviruses), liposomes and other lipid-containing complexes, and other macromolecular complexes capable of mediating delivery of a polynucleotide to a host cell. Direct injection of adenoviral vectors into lung tumors has been a routine procedure in clinical trials evaluating gene therapy of lung cancer. Dong et al., *J. Int. Med. Res.* 36, 1273-1287 (2008); Li et al., *Cancer Gene Ther.* 20, 251-259 (2013); Zhou, et al., *Cancer Gene Ther.* 23, 1-6 (2016). Vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. As described and illustrated in more detail below, such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such as agents mediating nuclear localization); and components that influence expression of the polynucleotide. Such components also might include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have

components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities. Other vectors include those described by Chen et al; *Bio Techniques*, 34: 167-171 (2003). A large variety of such vectors are known in the art and are generally available.

[0084] Suitable nucleic acid delivery systems include recombinant viral vector, typically sequence from at least one of an adenovirus, adenovirus-associated virus (AAV), helper-dependent adenovirus, retrovirus, or hemagglutinating virus of Japan-liposome (HVJ) complex. In such cases, the viral vector comprises a strong eukaryotic promoter operably linked to the polynucleotide e.g., a cytomegalovirus (CMV) promoter. The recombinant viral vector can include one or more of the polynucleotides therein, in some embodiments about one polynucleotide. In embodiments in which the polynucleotide is to be administered with a non-viral vector, use of between from about 0.1 nanograms to about 4000 micrograms will often be useful e.g., about 0.1 ng to about 3900 µg, about 0.1 ng to about 3800 µg, about 0.1 ng to about 3700 µg, about 0.1 ng to about 3600 µg, about 0.1 ng to about 3500 µg, about 0.1 ng to about 3400 µg, about 0.1 ng to about 3300 µg, about 0.1 ng to about 3200 µg, about 0.1 ng to about 3100 µg, about 0.1 ng to about 3000 µg, about 0.1 ng to about 2900 µg, about 0.1 ng to about 2800 µg, about 0.1 ng to about 2700 µg, about 0.1 ng to about 2600 µg, about 0.1 ng to about 2500 µg, about 0.1 ng to about 2400 µg, about 0.1 ng to about 2300 µg, about 0.1 ng to about 2200 µg, about 0.1 ng to about 2100 µg, about 0.1 ng to about 2000 µg, about 0.1 ng to about 1900 µg, about 0.1 ng to about 1800 µg, about 0.1 ng to about 1700 µg, about 0.1 ng to about 1600 µg, about 0.1 ng to about 1500 µg, about 0.1 ng to about 1400 µg, about 0.1 ng to about 1300 µg, about 0.1 ng to about 1200 µg, about 0.1 ng to about 1100 µg, about 0.1 ng to about 1000 µg, about 0.1 ng to about 900 µg, about 0.1 ng to about 800 µg, about 0.1 ng to about 700 µg, about 0.1 ng to about 600 µg, about 0.1 ng to about 500 µg, about 0.1 ng to about 400 µg, about 0.1 ng to about 300 µg, about 0.1 ng to about 200 µg, about 0.1 ng to about 100 µg, about 0.1 ng to about 90 µg, about 0.1 ng to about 80 µg, about 0.1 ng to about 70 µg, about 0.1 ng to about 60 µg, about 0.1 ng to about 50 µg, about 0.1 ng to about 40 µg, about 0.1 ng to about 30 µg, about 0.1 ng to about 20 µg, about 0.1 ng to about 10 µg, about 0.1 ng to about 1 µg, about 0.1 ng to about 900 ng, about 0.1 ng to about 800 ng, about 0.1 ng to about 700 ng, about 0.1 ng to about 600 ng, about 0.1 ng to about 500 ng, about 0.1 ng to about 400 ng, about 0.1 ng to about 300 ng, about 0.1 ng to about 200 ng, about 0.1 ng to about 100 ng, about 0.1 ng to about 90 ng, about 0.1 ng to about 80 ng, about 0.1 ng to about 70 ng, about 0.1 ng to about 60 ng, about 0.1 ng to about 50 ng, about 0.1 ng to about 40 ng, about 0.1 ng to about 30 ng, about 0.1 ng to about 20 ng, about 0.1 ng to about 10 ng, about 0.1 ng to about 1 ng, about 1 ng to about 4000 µg, about 1 ng to about 3900 µg, about 1 ng to about 3800 µg, about 1 ng to about 3700 µg, about 1 ng to about 3600 µg, about 1 ng to about 3500 µg, about 1 ng to about 3400 µg, about 1 ng to about 3300 µg, about 1 ng to about 3200 µg, about 1 ng to about 3100 µg, about 1 ng to about 3000 µg, about 1 ng to about 2900 µg, about 1 ng to about 2800 µg, about 1 ng to about 2700 µg, about 1 ng to about 2600 µg, about 1 ng to about 2500 µg, about 1 ng to about 2400 µg, about 1 ng to about 2300 µg, about 1 ng to about 2200 µg, about 1 ng to about 2100 µg, about 1 ng to about 2000 µg, about 1 ng to about 1900 µg, about 1 ng to about

1800 µg, about 1 ng to about 1700 µg, about 1 ng to about 1600 µg, about 1 ng to about 1500 µg, about 1 ng to about 1400 µg, about 1 ng to about 1300 µg, about 1 ng to about 1200 µg, about 1 ng to about 1100 µg, about 1 ng to about 1000 µg, about 1 ng to about 900 µg, about 1 ng to about 800 µg, about 1 ng to about 700 µg, about 1 ng to about 600 µg, about 1 ng to about 500 µg, about 1 ng to about 400 µg, about 1 ng to about 300 µg, about 1 ng to about 200 µg, about 1 ng to about 100 µg, about 1 ng to about 90 µg, about 1 ng to about 80 µg, about 1 ng to about 70 µg, about 1 ng to about 60 µg, about 1 ng to about 50 µg, about 1 ng to about 40 µg, about 1 ng to about 30 µg, about 1 ng to about 20 µg, about 1 ng to about 10 µg, about 1 ng to about 1 µg, about 1 ng to about 900 ng, about 1 ng to about 800 ng, about 1 ng to about 700 ng, about 1 ng to about 600 ng, about 1 ng to about 500 ng, about 1 ng to about 400 ng, about 1 ng to about 300 ng, about 1 ng to about 200 ng, about 1 ng to about 100 ng, about 1 ng to about 90 ng, about 1 ng to about 80 ng, about 1 ng to about 70 ng, about 1 ng to about 60 ng, about 1 ng to about 50 ng, about 1 ng to about 40 ng, about 1 ng to about 30 ng, about 1 ng to about 20 ng, about 1 ng to about 10 ng, about 10 ng to about 4000 µg, about 20 ng to about 4000 µg, about 30 ng to about 4000 µg, about 40 ng to about 4000 µg, about 50 ng to about 4000 µg, about 60 ng to about 4000 µg, about 70 ng to about 4000 µg, about 80 ng to about 4000 µg, about 90 ng to about 4000 µg, about 100 ng to about 4000 µg, about 200 ng to about 4000 µg, about 300 ng to about 4000 µg, about 400 ng to about 4000 µg, about 500 ng to about 4000 µg, about 600 ng to about 4000 µg, about 700 ng to about 4000 µg, about 800 ng to about 4000 µg, about 900 ng to about 4000 µg, about 1 µg to about 4000 µg, 10 µg to about 4000 µg, 20 µg to about 4000 µg, 30 µg to about 4000 µg, 40 µg to about 4000 µg, 50 µg to about 4000 µg, 60 µg to about 4000 µg, 70 µg to about 4000 µg, 80 µg to about 4000 µg, 90 µg to about 4000 µg, 100 µg to about 4000 µg, 200 µg to about 4000 µg, 300 µg to about 4000 µg, 400 µg to about 4000 µg, 500 µg to about 4000 µg, 600 µg to about 4000 µg, 700 µg to about 4000 µg, 800 µg to about 4000 µg, 900 µg to about 4000 µg, 1000 µg to about 4000 µg, 1100 µg to about 4000 µg, 1200 µg to about 4000 µg, 1300 µg to about 4000 µg, 1400 µg to about 4000 µg, 1500 µg to about 4000 µg, 1600 µg to about 4000 µg, 1700 µg to about 4000 µg, 1800 µg to about 4000 µg, 1900 µg to about 4000 µg, 2000 µg to about 4000 µg, 2100 µg to about 4000 µg, 2200 µg to about 4000 µg, 2300 µg to about 4000 µg, 2400 µg to about 4000 µg, 2500 µg to about 4000 µg, 2600 µg to about 4000 µg, 2700 µg to about 4000 µg, 2800 µg to about 4000 µg, 2900 µg to about 4000 µg, 3000 µg to about 4000 µg, 3100 µg to about 4000 µg, 3200 µg to about 4000 µg, 3300 µg to about 4000 µg, 3400 µg to about 4000 µg, 3500 µg to about 4000 µg, 3600 µg to about 4000 µg, 3700 µg to about 4000 µg, 3800 µg to about 4000 µg, or 3900 µg to about 4000 µg.

[0085] Additional vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include Moloney murine leukemia viruses and HIV-based viruses. One HIV-based viral vector comprises at least two vectors wherein the gag and pol genes are from an HIV genome and the env gene is from another virus. DNA viral vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as a herpes simplex I virus (HSV) vector [Geller, A. I. et al., *J. Neurochem*, 64: 487 (1995); Lim, F., et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995);

Geller, A. I. et al., Proc Natl. Acad. Sci.: U.S.A.:90 7603 (1993); Geller, A. I., et al., Proc Natl. Acad. Sci USA: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., Science, 259:988 (1993); Davidson, et al., Nat. Genet. 3: 219 (1993); Yang, et al., J. Virol. 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M. G., et al., Nat. Genet. 8:148 (1994)].

[0086] If desired, the polynucleotides described here may also be used with a microdelivery vehicle such as cationic liposomes, adenoviral vectors, and exosomes. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, BioTechniques, 6:682 (1988). See also, Feigner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11(2):25 (1989). In some embodiments, exosomes may be used for delivery of a nucleic acid encoding a CRISPR endonuclease and/or guide RNA to a target cell, e.g. a cancer cell. Exosomes are nanosized vesicles secreted by a variety of cells and are comprised of cellular membranes. Exosomes can attach to target cells by a range of surface adhesion proteins and vector ligands (tetraspanins, integrins, CD11b and CD18 receptors), and deliver their payload to target cells. Several studies indicate that exosomes have a specific cell tropism, according to their characteristics and origin, which can be used to target them to disease tissues and/or organs. See Batrakova et al., 2015, J Control Release 219: 396-405. For example, cancer-derived exosomes function as natural carriers that can efficiently deliver CRISPR/Cas9 plasmids to cancer cells. See Kim et al., 2017, J Control Release 266: 8-16.

[0087] Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992);

[0088] Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

[0089] Another delivery method is to use single stranded DNA producing vectors which can produce the expressed products intracellularly. See for example, Chen et al., Bio Techniques, 34: 167-171 (2003), which is incorporated herein, by reference, in its entirety.

Pharmaceutical Compositions

[0090] Any of the pharmaceutical compositions disclosed herein can be formulated for use in the preparation of a medicament, and particular uses are indicated below in the context of treatment, e.g., the treatment of a subject having cancer. When employed as pharmaceuticals, any of the nucleic acids and vectors can be administered in the form of pharmaceutical compositions. Administration may be pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), topical (including ophthalmic and to mucous membranes including intranasal, vaginal and rectal delivery), ocular, oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular administration. Parenteral administration can be in the form of a single bolus dose, or may be, for example, by a continuous perfusion pump. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, supposito-

ries, sprays, liquids, powders, and the like. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

[0091] In some embodiments, pharmaceutical compositions can contain, as the active ingredient, nucleic acids and vectors described herein in combination with one or more pharmaceutically acceptable carriers. The term “pharmaceutically acceptable” refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal or a human, as appropriate. The term “pharmaceutically acceptable carrier,” as used herein, includes any and all solvents, dispersion media, coatings, antibacterial, isotonic and absorption delaying agents, buffers, excipients, binders, lubricants, gels, surfactants and the like, that may be used as media for a pharmaceutically acceptable substance. In making the pharmaceutical compositions disclosed herein, the active ingredient is typically mixed with an excipient, diluted by an excipient or enclosed within such a carrier in the form of, for example, a capsule, tablet, sachet, paper, or other container. When the excipient serves as a diluent, it can be a solid, semisolid, or liquid material (e.g., normal saline), which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), lotions, creams, ointments, gels, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders. As is known in the art, the type of diluent can vary depending upon the intended route of administration. The resulting compositions can include additional agents, such as preservatives. In some embodiments, the carrier can be, or can include, a lipid-based or polymer-based colloid. In some embodiments, the carrier material can be a colloid formulated as a liposome, a hydrogel, a microparticle, a nanoparticle, or a block copolymer micelle. As noted, the carrier material can form a capsule, and that material may be a polymer-based colloid.

[0092] The nucleic acid sequences disclosed herein can be delivered to an appropriate cell of a subject, e.g. a cancer cell. This can be achieved by, for example, the use of a polymeric, biodegradable microparticle or microcapsule delivery vehicle, sized to optimize phagocytosis by phagocytic cells such as macrophages. Delivery of “naked DNA” (i.e., without a delivery vehicle) to an intramuscular, intradermal, or subcutaneous site, is another means to achieve in vivo expression. In the relevant polynucleotides (e.g., expression vectors) the nucleic acid sequence encoding the isolated nucleic acid sequence comprising a sequence encoding a CRISPR-associated endonuclease and a guide RNA can be operatively linked to a promoter or enhancer-promoter combination. Promoters and enhancers are described above.

[0093] In some embodiments, the pharmaceutical compositions can be formulated as a nanoparticle, for example, nanoparticles comprised of a core of high molecular weight linear polyethylenimine (LPEI) complexed with DNA and surrounded by a shell of polyethyleneglycol-modified (PE-Glylated) low molecular weight LPEI.

[0094] The nucleic acids and vectors may also be applied to a surface of a device (e.g., a catheter) or contained within a pump, patch, or other drug delivery device. The nucleic acids and vectors disclosed herein can be administered

alone, or in a mixture, in the presence of a pharmaceutically acceptable excipient or carrier (e.g., physiological saline). The excipient or carrier is selected on the basis of the mode and route of administration. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in Remington's Pharmaceutical Sciences (E. W. Martin), a well-known reference text in this field, and in the USP/NF (United States Pharmacopeia and the National Formulary).

[0095] In some embodiments, the compositions can be formulated as a nanoparticle encapsulating a nucleic acid encoding a CRISPR-associated endonuclease and a guide RNA sequence complementary to an NRF2 gene, or vector comprising a nucleic acid encoding a CRISPR-associated endonuclease and a guide RNA sequence complementary to an NRF2 gene.

Methods of Reducing NRF2 Expression or Activity in a Cell

[0096] In certain aspects, the disclosure relates to a method of reducing NRF2 expression or activity in a cell comprising introducing into the cell (a) one or more DNA sequence(s) encoding a guide RNA (gRNA) that is complementary to a target sequence in the NRF2 gene and (b) a nucleic acid sequence encoding a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease, whereby the gRNA hybridizes to the NRF2 gene and the CRISPR-associated endonuclease cleaves the NRF2 gene, and wherein NRF2 expression or activity is reduced in the cell relative to a cell in which the one or more DNA sequences encoding the gRNA and the nucleic acid sequence encoding the CRISPR-associated nuclease are not introduced.

[0097] Reducing NRF2 expression in the cell may comprise reducing expression of NRF2 mRNA in the cell, reducing expression of the NRF2 protein in the cell, or both. In some embodiments, expression of one or more allele(s) of the NRF2 gene is reduced. In some embodiments, introducing the one or more DNA sequence(s) encoding the gRNA and the nucleic acid sequence encoding a CRISPR-associated endonuclease into the cell reduces NRF2 expression and/or activity in the cell, but does not completely eliminate it. In other embodiments, NRF2 expression and/or activity in the cell are completely eliminated.

[0098] The gRNA may be complementary to a target sequence in an exon of the NRF2 gene. In a particular embodiment, the gRNA is complementary to a target sequence in exon 1, 2, 3, 4, or 5 of the NRF2 gene. In some embodiments, the gRNA is encoded by a single DNA sequence. In other embodiments, the gRNA is encoded by two or more DNA sequences. For example, in some embodiments, the gRNA is encoded by a first DNA sequence encoding a trans-activated small RNA (tracrRNA) and a second DNA sequence encoding a CRISPR RNA (crRNA). The tracrRNA and crRNA may hybridize within the cell to form the guide RNA. Accordingly, in some embodiments, the gRNA comprises a trans-activated small RNA (tracrRNA) and a CRISPR RNA (crRNA).

[0099] In some embodiments, CRISPR-associated endonucleases suitable for use in reducing expression of the NRF2 gene include, but are not limited to, a class 1 CRISPR-associated endonucleases such as, e.g., Cas7 and Cas5, along with, in some embodiments, SS (Cas11) and Cas8a1; Cas8b1; Cas8c; Cas8u2 and Cas6; Cas3" and Cas10d; Cas SS (Cas11), Cas8e, and Cas6; Cas8f and Cas6f;

Cas6f; Cas8-like (Csf1); SS (Cas11) and Cas8-like (Csf1); or SS (Cas11) and Cas10. Class 2 CRISPR-associated endonucleases include type I, type V, and type VI CRISPR-Cas systems, which have a single effector molecule. In some embodiments, CRISPR-associated endonucleases suitable for use in reducing expression of the NRF2 gene include, but are not limited to, class 2 CRISPR-associated endonucleases such as, e.g., Cas9, Cas12a, Cas12b, Cas12c, Cas12d, Cas13a, Cas13b, Cas13c, c2c4, c2c5, c2c8, c2c9, and/or c2c10. In some embodiments, CRISPR-associated endonucleases suitable for use in reducing expression of the NRF2 gene include, but are not limited to, CasX, CasY, and/or MAD6 (see, e.g., Liu et al., Nature 566:218-23 (2019)).

[0100] Any cell containing an NRF2 gene may be suitable for use in the methods of reducing NRF2 expression or activity described herein. In some embodiments, the cell is a eukaryotic cell, e.g. a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the NRF2 gene is a human NRF2 gene.

[0101] In certain aspects, the disclosure also relates to a cell comprising a mutated NRF2 gene produced by the methods of reducing NRF2 expression or activity described herein. In some embodiments, the mutated NRF2 gene comprises an insertion or a deletion relative to the wildtype NRF2 gene. In some embodiments, the insertion or deletion occurs within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotide(s) of a protospacer adjacent motif sequence (PAM) in the NRF2 gene.

Methods for Treatment of Cancer

[0102] In certain aspects, the disclosure relates to a method of treating cancer in a subject comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease and a guide RNA that is complementary to a target domain from an NRF2 gene in the subject.

[0103] In certain aspects, the disclosure relates to a method of treating cancer in a subject comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising: (a) a DNA sequence encoding a guide RNA that is complementary to a target domain from an NRF2 gene in the subject; and (b) a nucleic acid sequence encoding a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease.

[0104] In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer is a non-small cell lung cancer. In certain embodiments, the cancer is treated only with the pharmaceutical composition comprising a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease and a guide RNA that is complementary to a target domain from an NRF2 gene in the subject, or only with the pharmaceutical composition comprising: (a) a DNA sequence encoding a guide RNA that is complementary to a target domain from an NRF2 gene in the subject; and (b) a nucleic acid sequence encoding a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease. In certain embodiments, the cancer is treated with the pharmaceutical compositions as described herein and an additional agent, e.g. a chemotherapeutic agent. In certain embodiments, treatment with the chemotherapeutic agent is initiated at the same time as treatment with the pharmaceutical composition. In certain

embodiments, the treatment with the chemotherapeutic agent is initiated after the treatment with the pharmaceutical composition is initiated. In certain embodiments, treatment with the chemotherapeutic agent is initiated at before the treatment with the pharmaceutical composition.

[0105] In certain embodiments, the pharmaceutical compositions of the present disclosure may be utilized for the treatment of cancer wherein the subject has failed at least one prior chemotherapeutic regimen. For example, in some embodiments, the cancer is resistant to one or more chemotherapeutic agents. Accordingly, the present disclosure provides methods of treating cancer in a subject, wherein the subject has failed at least one prior chemotherapeutic regimen for the cancer, comprising administering the pharmaceutical compositions as described herein to the subject in an amount sufficient to treat the cancer, thereby treating the cancer. The pharmaceutical compositions described herein may also be utilized for inhibiting tumor cell growth in a subject wherein the subject has failed at least one prior chemotherapeutic regimen. Accordingly, the present disclosure further provides methods of inhibiting tumor cell growth in a subject, e.g. wherein the subject has failed at least one prior chemotherapeutic regimen, comprising administering the pharmaceutical compositions described herein to the subject, such that tumor cell growth is inhibited. In certain embodiments, the subject is a mammal, e.g. a human.

[0106] For example, the pharmaceutical compositions described herein may be administered to a subject in an amount sufficient to reduce proliferation of cancer cells relative to cancer cells that are not treated with the pharmaceutical composition. The pharmaceutical composition may reduce cancer cell proliferation by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% relative to cancer cells that are not treated with the pharmaceutical composition.

[0107] In some embodiments, the pharmaceutical composition is administered in an amount sufficient to reduce tumor growth relative to a tumor that is not treated with the pharmaceutical composition. The pharmaceutical composition may reduce tumor growth by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% relative to cancer cells that are not treated with the pharmaceutical composition. In a particular embodiment, administration of the pharmaceutical composition to the subject completely inhibits tumor growth.

[0108] In one embodiment, administration of a pharmaceutical composition as described herein, achieves at least

stable disease, reduces tumor size, inhibits tumor growth and/or prolongs the survival time of a tumor-bearing subject as compared to an appropriate control. Accordingly, this disclosure also relates to a method of treating tumors in a human or other animal, including a subject, who has failed at least one prior chemotherapeutic regimen, by administering to such human or animal an effective amount of a pharmaceutical composition described herein. One skilled in the art would be able, by routine experimentation with the guidance provided herein, to determine what an effective amount of the pharmaceutical composition would be for the purpose of treating malignancies including in a subject who has failed at least one prior chemotherapeutic regimen. For example, a therapeutically active amount of the pharmaceutical composition may vary according to factors such as the disease stage (e.g., stage I versus stage IV), age, sex, medical complications, and weight of the subject, and the ability of the pharmaceutical composition to elicit a desired response in the subject. The dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, the dose may be administered by continuous infusion, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

[0109] In certain embodiments, the methods further include a treatment regimen which includes any one of or a combination of surgery, radiation, chemotherapy, e.g., hormone therapy, antibody therapy, therapy with growth factors, cytokines, and anti-angiogenic therapy.

[0110] Cancers for treatment using the methods of the disclosed herein include, for example, all types of cancer or neoplasm or malignant tumors found in mammals, including, but not limited to: leukemias, lymphomas, melanomas, carcinomas and sarcomas. In one embodiment, cancers for treatment using the methods of disclosed herein include melanomas, carcinomas and sarcomas. In some embodiments, the coenzyme Q10 compositions are used for treatment, of various types of solid tumors, for example breast cancer, bladder cancer, colon and rectal cancer, endometrial cancer, kidney (renal cell) cancer, lung cancer, melanoma, pancreatic cancer, prostate cancer, thyroid cancer, skin cancer, bone cancer, brain cancer, cervical cancer, liver cancer, stomach cancer, mouth and oral cancers, neuroblastoma, testicular cancer, uterine cancer, thyroid cancer, head and neck, kidney, lung, non-small cell lung, melanoma, mesothelioma, ovary, sarcoma, stomach, uterus and medulloblastoma, and vulvar cancer. In certain embodiments, solid tumors include breast cancer, including triple negative breast cancer. In certain embodiments, skin cancer includes melanoma, squamous cell carcinoma, cutaneous T-cell lymphoma (CTCL). In certain embodiments, the cancer includes leukemia. In certain embodiments, the cancer is selected from the group consisting of lung cancer, melanoma, esophageal squamous cancer (ESC), head and neck squamous cell carcinoma (HNSCC), and breast cancer.

[0111] In a particular embodiment, the cancer is lung cancer, e.g. non-small-cell lung cancer (NSCLC). In some embodiments, the NSCLC is adenocarcinoma, squamous cell carcinoma, or large cell carcinoma. Beyond the current well-established combinatorial drug strategies used to treat NSCLC, several different combinatorial approaches are also being investigated for the treatment of cancers. For example, the use of an oncolytic virus that infects tumor cells has been found to enhance the activity of chemotherapy. Infection

with myxoma virus combined with cisplatin or gemcitabine efficiently destroyed ovarian cancer cells at much lower dosages than needed without viral addition. Nounamo et al., *Mol. Ther. Oncolytics* 6, 90-99 (2017). The use of oncolytic virus therapy and cytotoxic chemotherapy for improved effectiveness of cancer treatment is an active area of development. Wennier, et al., *Curr. Pharm. Biotechnol.* 13, 1817-33 (2012); Pandha, et al., *Oncolytic Virotherapy* 5, 1 (2016). Infection with a replication competent virus before treatment with cisplatin markedly enhances the therapeutic benefit of chemotherapy.

[0112] With the usage of targeted therapy (targeting EGFR mutation, ALK rearrangement, etc.) and immunotherapy (checkpoint inhibitors, anti-PD1, anti-CTLA4, etc.), the clinical management of

[0113] NSCLC has greatly improved. Patients can have a longer and better quality of life. However, these therapies cannot solve all the problems. For example, agents that target specific molecules typically have a response rate of ~70%. However, after a median period of 8-16 months, due to the inevitable resistance, relapse happens in almost all patients. Anichini, et al., *Cancer Immunol. Immunother.* 67, 1011-1022 (2018). In regards to immunotherapy, though pembrolizumab (Keytruda) can be used as first treatment in certain lung cancer patients, only a fraction of them will respond. Bianco, et al., *Curr. Opin. Pharmacol.* 40, 46-50 (2018).

[0114] On the other hand, chemotherapy is still indispensable in the lung cancer treatment paradigm. In patients with locoregional NSCLC, chemotherapy is the only systemic therapy proven to improve curability when combined with surgery or radiation. Wang, et al., *Investig.*

[0115] *Ophthalmology Vis. Sci.* 58, 3896 (2017). In patients with metastasis, chemotherapy is still the mainstay of care for those who have developed resistance to targeted therapy agents. Meanwhile, it also has the potential to stimulate the immune system to boost the effectiveness of immunotherapy.

Combination Therapies

[0116] In certain embodiments, the pharmaceutical compositions described herein can be used in combination therapy with at least one additional anticancer agent, e.g., a chemotherapeutic agent.

[0117] Small molecule chemotherapeutic agents generally belong to various classes including, for example: 1. Topoisomerase II inhibitors (cytotoxic antibiotics), such as the anthracyclines/anthracenediones, e.g., doxorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones, e.g., mitoxantrone and losoxantrone, and the podophyllotoxines, e.g., etoposide and teniposide; 2. Agents that affect microtubule formation (mitotic inhibitors), such as plant alkaloids (e.g., a compound belonging to a family of alkaline, nitrogen-containing molecules derived from plants that are biologically active and cytotoxic), e.g., taxanes, e.g., paclitaxel and docetaxel, and the vinka alkaloids, e.g., vinblastine, vincristine, and vinorelbine, and derivatives of podophyllo-toxin; 3. Alkylating agents, such as nitrogen mustards, ethyleneimine compounds, alkyl sulphonates and other compounds with an alkylating action such as nitrosoureas, dacarbazine, cyclophosphamide, ifosfamide and melphalan; 4. Antimetabolites (nucleoside inhibitors), for example, folates, e.g., folic acid, fluopyrimidines, purine or pyrimidine analogues such as 5-fluorouracil, capecitabine, gemcit-

abine, methotrexate, and edatrexate; 5. Topoisomerase I inhibitors, such as topotecan, irinotecan, and 9-nitrocamp-tothecin, camptothecin derivatives, and retinoic acid; and 6. Platinum compounds/complexes, such as cisplatin, oxaliplatin, and carboplatin. Exemplary chemotherapeutic agents for use in the methods of disclosed herein include, but are not limited to, amifostine (ethyol), cisplatin, dacarbazine (DTIC), dactinomycin, mechlorethamine (nitrogen mustard), streptozocin, cyclophosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (adriamycin), doxorubicin lipo (doxil), gemcitabine (gemzar), daunorubicin, daunorubicin lipo (daunoxome), procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil (5-FU), vinblastine, vincristine, bleomycin, paclitaxel (taxol), docetaxel (taxotere), aldesleukin, asparaginase, busulfan, carboplatin, cladribine, camptothecin, CPT-I 1, 10-hydroxy-7-ethyl-camptothecin (SN38), capecitabine, fltorafur, 5'-deoxyflurouridine, UFT, eniluracil, deoxycytidine, 5-azacytosine, 5-azadeoxycytosine, allopurinol, 2-chloro adenosine, trimetrexate, aminopterin, methylene-10-deazaaminopterin (MDAM), oxaplatin, picoplatin, tetraplatin, satraplatin, platinum-DACH, ormaplatin, CI-973 (and analogs thereof), JM-216 (and analogs thereof), epirubicin, 9-aminocamp-tothecin, 10,11-methylenedioxcamptothecin, karenitecin, 9-nitrocamptothecin, TAS 103, vindesine, L-phenylalanine mustard, ifosphamidemefosphamide, perfosfamide, trophosphamide carmustine, semustine, epothilones A-E, tomudex, 6-mercaptopurine, 6-thioguanine, amsacrine, etoposide phosphate, acyclovir, valacyclovir, ganciclovir, amantadine, rimantadine, lamivudine, zidovudine, bevacizumab, trastuzumab, rituximab, Pentostatin, floxuridine, fludarabine, hydroxyurea, ifosfamide, idarubicin, mesna, irinotecan, mitoxantrone, topotecan, leuprolide, megestrol, melphalan, plicamycin, mitotane, pegaspargase, pipobroman, tamoxifen, teniposide, testolactone, thiotepa, uracil mustard, vinorelbine, chlorambucil, mTor, epidermal growth factor receptor (EGFR), and fibroblast growth factors (FGF) and combinations thereof which are readily apparent to one of skill in the art based on the appropriate standard of care for a particular tumor or cancer. In a particular embodiment, the chemotherapeutic agent is selected from the group consisting of cisplatin, vinorelbine, carboplatin, and combinations thereof (e.g., cisplatin and vinorelbine; cisplatin and carboplatin; vinorelbine and carboplatin; cisplatin, vinorelbine, and carboplatin).

[0118] In some embodiments, the pharmaceutical composition is administered in an amount sufficient to reduce tumor growth relative to a tumor that is treated with the at least one chemotherapeutic agent but is not treated with the pharmaceutical composition. The pharmaceutical composition may reduce tumor growth by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% relative to cancer cells that are treated with the at least one chemotherapeutic agent but are not treated with the pharmaceutical composition.

TABLE 1

Description of Sequences		
SEQ ID NO:	Description	Sequence
1	Guide RNA1 (gRNA 1) recognition element	3'-AGTACTCTGG CCCTTATAGTCC-5'
2	Guide RNA1 (gRNA 1) Guide RNA2 (gRNA2) recognition element	5'-UCGAUGUGACC GGGAAUAUCAGG-3'
3	(from Sanjana et al., Nat. Methods 11(8):783-84 (2014))	3'-ACTAAATCTGC CATACTGTGTC-5'
4	Guide RNA2 (gRNA2)	5'-UGAUUUAGACG GUAUGCAACAGG-3'

TABLE 1 -continued

Description of Sequences		
SEQ ID NO:	Description	Sequence
5	PCR primer, forward	GTAGTGGTGCCTTA GAGCTTACTCATCC
6	PCR primer, reverse	CTAGCATGGGCAGT ACTCATGACTAAG
7	human NRF2 DNA sequence	NCBI Reference Sequence NM_006164.5
8	human NRF2 amino acid sequence	NCBI Reference Sequence NP_006155.2

PAM sequence underlined.

NM_006164.5

(SEQ ID NO: 7)

1 gattaccgag tgccggggag cccggaggag ccgccgacgc agccgccacc gccgcgcgcg
 61 ccgccaccag agccgcctcg tcgcgcgcgc gctcggcag ccggaacagg gccgccgtcg
 121 gggagcccca acacacggtc cacagctcat catgatggac ttggagctgc cgccgcggg
 181 actcccgtcc cagcaggaca tggatttgat tgacatactt tggaggcaag atatagatct
 241 tggagtaagt cgagaagtat ttgacttcag tcagcgacgg aaagagtatg agctggaaaa
 301 acagaaaaaa cttgaaaagg aaagacaaga acaactccaa aaggagcaag agaaaacctt
 361 tttcgtcag ttacaactag atgaagagac aggtgaattt ctcccaattc agccagccca
 421 gcacatccag tcagaaacca gtggatctgc caactactcc caggttgcc acattcccaa
 481 atcagatgct ttgtactttg atgactgcat gcagctttg gcgcagacat tcccgtttgt
 541 agatgacaat gagggttctt cggctacggt tcagtcactt gttcctgata ttcccgggta
 601 catcgagagc ccagtcttca ttgctactaa tcaggctcag tcacctgaaa cttctgttgc
 661 tcaggtagcc cctgttgatt tagacggat gcaacaggac attgagcaag tttgggagga
 721 gctattatcc attcctgagt tacagtgctt taatattgaa aatgacaagc tgggtgagac
 781 taccatgggt ccaagtccag aagccaaact gacagaagtt gacaattatc atttttactc
 841 atctataccc tcaatggaaa aagaagtagg taactgtagt ccacattttc ttaatgcttt
 901 tgaggattcc ttcagcagca tcctctccac agaagacccc aaccagttga cagtgaactc
 961 attaaattca gatgccacag tcaacacaga ttttggtgat gaattttatt ctgctttcat
 1021 agctgagccc agtatcagca acagcatgcc ctcacctgct actttaagcc attcactctc
 1081 tgaacttcta aatgggcccc ttgatgtttc tgatctatca ctttgcaaag ctttcaacca
 1141 aaaccaccct gaaagcacag cagaattcaa tgattctgac tccggcattt cactaacac
 1201 aagtcccagt gtggcatcac cagaacactc agtggaatct tccagctatg gagacacact
 1261 acttggcctc agtgattctg aagtgggaaga gctagatagt gccctggaa gtgtcaaca
 1321 gaatggctcc aaaacaccag tacattcttc tggggatag gtacaaccct tgtcaccatc
 1381 tcaggggcag agcactcagc tgcattgatg ccaatgtgag aacacaccag agaaaagaatt
 1441 gcctgtaagt cctggctcgc ggaaaacccc attcacaaaa gacaaacatt caagccgctt
 1501 ggaggctcat ctcaacaag atgaacttag ggcaaaagct ctccatatcc cattccctgt

-continued

1561 agaaaaaatc attaacctcc ctggtgttga ctccaacgaa atgatgtcca aagagcagtt
 1621 caatgaagct caacttgcat taattcggga tatacgtagg aggggtaaga ataaagtggc
 1681 tgctcagaat tgcagaaaaa gaaaactgga aaatatagta gaactagagc aagatttaga
 1741 tcatttgaaa gatgaaaaag aaaaattgct caaagaaaaa ggagaaaaatg acaaaagcct
 1801 tcacctactg aaaaaacaac tcagcacctt atatctcgaa gttttcagca tgctacgtga
 1861 tgaagatgga aaaccttatt ctctagtga atactccctg cagcaacaa gagatggcaa
 1921 tgttttcctt gttcccaaaa gtaagaagcc agatgttaag aaaaactaga tttaggagga
 1981 tttgaccttt tctgagctag tttttttgta ctattatact aaaagctcct actgtgatgt
 2041 gaaatgctca tactttataa gtaattctat gcaaaatcat agccaaaact agtatagaaa
 2101 ataatacгаа actttaaaaa gcattggagt gtcagtatgt tgaatcagta gtttcacttt
 2161 aactgtaaac aatttcttag gacaccattt gggctagttt ctgtgtaagt gtaaatacta
 2221 caaaaactta ttatactgt tcttatgtca tttgttatat tcatagattt atatgatgat
 2281 atgacatctg gctaaaaaga aattattgca aaactaacca ctatgtactt tttataaat
 2341 actgatgga caaaaaatgg cattttttat attaaattgt ttagctctgg caaaaaaaaa
 2401 aaattttaag agctggctact aataaaggat tattatgact gttaaa

NP_006155.2

(SEQ ID NO: 8)

1 mmdlelpppg lpsqqdmdli dilwrqdidl gvsrevfdfs qrrkeyelek qkklekerqe
 61 qlqkeqekaf faqlqideet geflpiqpaq hiqsetsgsa nysqvahipk sdalyfddcm
 121 qllaqtfpfv dnevssatf qslvpdipgh iespvfiatn qagspetsva qvapvldgm
 181 qqdieqvwee llsipelqcl niendklvet tmvpspeakl tevdnyhfys sipsmekevg
 241 ncsphflnaf edsfsilst edpnqltvns lnsdatvntd fgdefysafi aepsisnmp
 301 spatlshsls ellngpidvs dslckafnq nhpestaefn dsdsgislnt spsvaspehs
 361 vesssygdtl lglsdsevee ldsapgsvkq ngpktpvhss gdmvqplspq qgqsthvha
 421 qcentpekel pvspghrktf ftkdkhssrl eahltrdelr akalhipfpv ekiinlpvvd
 481 fnemmskeqf neaqlalird irrrgknkva aqncrkrkle niveleqld hlkdekekll
 541 kekgendksl hllkkqlstl ylefvsmird edgkypspse yslqqttrdgn vflvpkskkp
 601 dvkkn

EXAMPLES

Example 1

Creation of NRF2 knockout clonal A549 cell lines using a CRISPR-directed Gene Editing Approach

Methods

[0119] Cell culture conditions

[0120] Human lung carcinoma A549 cells were purchased from ATCC (Manassas, VA, USA). A549 is a well-established non-small cell lung adenocarcinoma cell line, which harbors a mutation in the Kelch domain of KEAP1 causing overexpression of NRF2. It has been used often as a standard for the discovery of novel therapeutic agents directed against cancer. Cells were thawed according to manufacturer's protocol and grown in F-12K medium (ATCC, Manassas, Va., USA) supplemented with 10% FBS (ATCC, Manassas,

Va., USA) and 1% Penicillin-Streptomycin Solution (ATCC, Manassas, Va., USA). Cells were cultured and maintained at a concentration between 2×10^3 and 1×10^4 viable cells/cm² and incubated at 37° C. and 5% CO₂. Cell number was determined using a hemacytometer.

Guide RNA Design and Construction

[0121] The NRF2 gene coding sequence was entered into the Zhang lab's online generator (crispr.mit.edu/), and the gRNA with the highest score was chosen for gRNA1 (5'-UCGAUGUGACCGGGAAUAUCAGG) (SEQ ID NO:2) and a previously validated gRNA targeting NRF2 (Sanjana et al., 2014, Nature Methods 11(8); 783-784) was also chosen for gRNA2 (5'-UGAUUUAGACGGUAUGCAACAGG) (SEQ ID NO:4). The CRISPR-directed gene editing system was designed to disable the NES domain of NRF2, which reduces the capacity of the protein to reenter the nucleus and activate the transcription factor. The

CRISPR plasmid was cloned using standard cloning methods with single-step digestion-ligation. The CRISPR guide sequences with appropriate 5' overhangs were cloned into the pX458 backbone vector digested with BbsI (plasmid 48138; Addgene), a human codon optimized pSpCas9 and chimeric guide RNA expression plasmid with a 2AeGFP, purchased through Addgene (addgene.org). The guide RNA sequences were under transcriptional control of the constitutive U6 promoter. See FIG. 1A. Following construction, plasmids were validated by Sanger sequencing (Genewiz Inc., South Plainfield, N.J., USA).

Transfection and Clonal Isolation

[0122] A549 cells were transfected at a concentration of 5×10^5 cells/100 μ l in 4mm gap cuvette (BioExpress, Kaysville, UT, USA). NRF2 targeting pX458 constructs were separately electroporated (250 V, LV, 13 ms pulse length, 2 pulses, 1 s interval) into A549 cells using a Bio-Rad Gene Pulser XCell Electroporation System (Bio-Rad Laboratories, Hercules, Calif., USA). Cells were then recovered in 6-well plates with complete growth media at 37° C. for 72 hours prior to sorting. A549s were sorted into a 96-well plate with a FACS AriaII flow cytometer (BD Biosciences, Franklin Lakes, N.J., USA), with an individual eGFP+cell sorted to each well. Clones were expanded and transferred to larger plates as the individual clones reached confluence, with DNA isolation occurring when cells reached confluence in a six-well plate (1×10^6 cells/mL).

Sequencing and Sequence Analyses

[0123] CRISPR/Cas9 targeted A549 clones were PCR amplified (forward 5'-gtatggtgccttagactactcatcc gtagtggccttagactactcatcc (SEQ ID NO: 5), reverse 5'-ctagcatggcagctactcatgactaag (SEQ ID NO: 6)) using AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems, Foster City, Calif.). Briefly, template DNA, primers, water and master mix were combined and cycled: 95° C. for 10 minutes, 96° C. for 3s, 60° C. for 3s, 68° C. for 5s) x35 cycles, and 72° C. for 10s. The 402 bp products were purified (Qiagen, Hilden, Germany) and Sanger sequenced using the forward PCR primer. Clonal allelic analyses of individual A549 cell clones were analyzed by the software program, Tracking of Indels by DEcomposition (TIDE), to determine the individual sub-sequences within the multi-peaked breakdown product after CRISPR/Cas9 activity. Brinkman, et al., *Nucleic Acids Res.* 42, e168-(2014). The TIDE analyses provide a visual of the sequence decomposition, the indel patterns of the clone, as well as relative ratios of each clonal indel pattern, serving as an intermediate step in determining each allelic profile. By utilizing the indel patterns and their relative ratios provided by TIDE, the control trace sequence and a clonal trace sequence were manually aligned, allowing for the visualization of the indel patterns of each allele of a clone.

Western Blot Analysis

[0124] Total cellular protein was collected from A549 cell lines using a standard RIPA lysis buffer containing a protease inhibitor cocktail. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, Ill., USA). The samples were heated at 95° C. for 10 minutes and then were subjected to SDS-PAGE on a 10% polyacrylamide gel for 90 minutes at 100V.

[0125] The gel was transferred to a nitrocellulose membrane for 1 hour at 100V. The blot was placed in 3% BSA and blocked overnight on a shaker at 4° C. Primary antibody incubation was performed overnight on a shaker at 4° C. for NRF2 (phospho S40) (1:10,000, Abcam ab76026) and 1 hour at room temperature for beta actin (1:8,000, Abcam ab8226), and secondary antibody (Jackson ImmunoResearch, West Grove, Pa., USA) incubations were all done 1 hour at room temperatures, at a 1:10,000 dilution. The protein bands were visualized via chemiluminescence using a Super signal west dura extended duration ECL (Pierce) and detected on the LiCor Odyssey FC. All bands were quantified for densitometry on the Image Studio software system.

Cell Proliferation by FACS Analysis

[0126] A549 cell lines were trypsinized and harvested at 50-70% confluency. Cells were fixed with ice cold 70% ethanol dropwise while vortexing and incubated at 4° C. for a minimum of 72 hours. Fixed cells were pelleted and washed twice with PBS followed by a 30-minute incubation on ice. As indicated in the manufacturer's protocol (BD Biosciences), 20 μ l per 10^6 cells of Alexa Fluor 647 Mouse anti-Ki67 (561126, BD Biosciences) was added to the cells and incubated for 30 minutes. Controls included Alexa Fluor 647 Mouse IgG1 k isotype control (557714, BD Biosciences), at the same dilution. After incubation, cells were washed twice and resuspended in stain buffer (5% BSA in 1x PBS). Cells were analyzed with a FACS AriaII flow cytometer and processed using FlowJo software.

Results

[0127] The strategy was to use CRISPR-directed gene editing to functionally disable NRF2 alleles in A549 lung carcinoma cells. It is critical to establish the fact that a gene editing technology can knockout a target gene. Below are provided the strategy details which were utilized to generate the genetic tools used to disable NRF2 in A549 cells. Figure 1A illustrates the CRISPR/Cas9 machinery designed to target and knockout NRF2. The grey bar running along the top of the panels represents the genomic sequence of NRF2, with the red blocks indicating coding regions. The blue brackets indicate the relative region where each CRISPR/Cas9 is designed to cleave the DNA. Each gRNA was designed to target the fourth exon of NRF2 in a region that contains all known isoforms to ensure complete ablation of the gene (ncbi.nlm.nih.gov/gene/4780). The gRNA with the highest score, according to the Broad Institute's CRISPR Design software (crispr.mit.edu/), was chosen for gRNA1 and a previously validated gRNA (Sanjana et al., *bioRxiv* 006726 (2014). doi:10.1101/006726) was chosen for gRNA2. The gRNAs were assembled by annealing the crRNA oligos and ligating them to complementary restriction site overhangs in plasmid px458 (Addgene #48138) digested with BbsI, as depicted in each panel. FIG. 1B illustrates the functional domains of the NRF2 protein including the KEAP1 Binding Domain, Transactivation Domain, Repressor Binding Domain, β -TrCP Binding Domain, DNA Binding Domain and the Transcriptional Activation Domain. Pandey et al., *Crit. Rev. Oncol. Hematol.* 116, 89-98 (2017); Jung et al., *Molecular Mechanisms to Therapeutic Opportunities.* 26, 57-68 (2018); Namani et al., *Biochim. Biophys. Acta-Mol. Cell Res.* 1843, 1875-1885 (2014). The Neh5 domain spans Exon 4 and 5 and contains

a redox-sensitive nuclear-export signal (NES), which regulates the intracellular localization of NRF2. Jung et al., *Molecular Mechanisms to Therapeutic Opportunities*. 26, 57-68 (2018). In theory, by disrupting the gene/protein within the Neh4 and Neh5 domain, the NES is shifted, rendering it nonfunctional. FIG. 1C exhibits the experimental workflow beginning with the transfection of pX458 containing either gRNA1 or gRNA2 into A549 lung adenocarcinoma cells progressing through to the final step of allelic analyses of an individual clonal population. Importantly, plasmid pX458 contains an eGFP reporter, which allows for isolation of individual transfected cells by FACS. To evaluate the efficiency of CRISPR-directed NRF2 knock-out in the total targeted population, eGFP+ cells were isolated as a population, and the degree of genetic disruption at the NRF2 locus in cells, transfected with either gRNA1 or gRNA2 pX458, was determined. The sorted populations were Sanger-sequenced and the resulting trace files were analyzed for the presence of indels, a marker for gene disruption. These data were obtained using a program known as Tracking of Indels by Decomposition (TIDE). Brinkman, et al., *Nucleic Acids Res.* 42, e168-(2014). As represented in FIG. 2A, both CRISPR/Cas9 designs generated a significant amount of indels, evidenced by TIDE results, indicating a high degree of NRF2 disruption. These results validate the approach and indicate that disruption of the NRF2 is possible in A549 cells via CRISPR/Cas9.

[0128] Next, the same experiment was carried out except in this case, individual cells were isolated by FACS sorting to obtain single cell clonal expansions. When the single cell isolates had expanded to sufficient quantity, half of each clonal population was cryo-preserved and allelic sequence analysis was performed on the other half using the same strategy and method described above. FIG. 2B displays the allelic analyses of two clones, 1-40 and 2-11, derived from gRNA1 and gRNA2 transfected cells respectively, which were chosen from a total of nine (Figure Supplement 1) for subsequent experimentation and analyses. It soon became apparent that all isolated clones generated from this parental lot of A549 cells obtained from ATCC harbored three alleles at the NRF2 locus. The red columns indicate the indel sizes present and their respective representative ratios within that clone. Clone 1-40 contains a 9 bp deletion with a 2:1 ratio to 0 bp indels, revealing a heterozygous KO of NRF2. Clone 2-11 contains a 10 bp deletion, a 6 bp deletion and a 1 bp deletion at a 1:1:1 ratio, a homozygous KO of NRF2. The specific indel patterns on each allele of both clones were characterized by manually aligning the sequence trace files to the wild-type sequence, with the TIDE indel data as a guide. For convenience, we refer to clone 1-40 as a heterozygous knockout and clone 2-11 as a homozygous knockout.

[0129] A fundamental cellular phenotype that could be affected by the lack of an NES is the rate at which cells proliferate in culture. Murakami et al., *Free Radic. Biol. Med.* 88, 168-178 (2015); Mitsuishi et al., *Cancer Cell* 22, 66-79 (2012). Wildtype A549 cells typically have a doubling time of 24 hours, however, it was noted that clones 1-40, but more clearly, 2-11, grew slower in the clonal expansion process (data not shown). This observation prompted us to further investigate the proliferation profile of clones 1-40 and 2-11 by staining the cells with antibodies against Ki67, followed by FACS analysis. Ki67 is a nuclear antigen expressed in actively proliferating cells. Therefore, one

might predict a decrease in Ki67 expression based on the growth characteristics seen in cell culture. Ethanol-fixed cells were stained with Alexa Fluor 647 Anti-Ki67, analyzed by FACS and plotted as a histogram (Left panel, FIG. 3A). Nonspecific binding was controlled for using the mouse IgG1 κ isotype control provided and gated on FlowJo. The x-axis represents fluorescence intensity of the allophycocyanin (APC)-conjugated Anti-Ki67, of which a shift to the left can be seen in clone 2-11, indicating a decrease in fluorescence intensity, correlating to a decrease in cellular proliferation.

[0130] The decrease in proliferation in 2-11 cells was striking while the reduction in proliferation in 1-40 cells was modest at best. Thus, we decided to continue our studies on the effect of NRF2 knockout using 2-11 cells only because CRISPR-directed gene editing succeeded in disrupting function more completely in those cells. Based on growth characteristics in cell culture and the FACS analysis, the MTS assay was utilized to assess the proliferation of 2-11 cells compared to wildtype cells (Right Panel, FIG. 3A). Allelic analysis of clone 2-11 indicated that NRF2 is genetically disabled and when normalized to beta-actin and compared to wildtype A549 cells, clone 2-11 showed a knock-down of -68% (FIG. 3B). Since one of the three alleles in clone 2-11 maintains a functional reading frame, this result is not unexpected. Genetic analysis indicated that the Neh5 domain, which contains the NES, was disrupted. Thus, we moved forward to characterize this clone identifying it as a functional knockout.

Example 2

Chemosensitivity is Increased in NRF2 Knockout A549 Cell Lines

Methods

MTS Cell Proliferation Assay

[0131] Cell viability was evaluated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, Wis.). A549 cell lines were plated at 2×10^3 cells per well and allowed to culture for 24 hours. The cell media was then aspirated, the cells washed with PBS, then exposed to the MTS reagent for 3 hours. After 3 hours of MTS bio-reduction by proliferating cells, the formazan product's absorbance was measured using a 450nm filter on an Infinite 2000 PRO microplate reader (Tecan, Mannadorf, Switzerland). Cell viability after drug exposure was evaluated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. A549 cell lines were plated at 2×10^3 cells per well and allowed to culture for 24 hours. The cells were then treated with cisplatin, carboplatin, or a combination of cisplatin and vinorelbine for three days. The cell media was then aspirated, the cells washed with PBS, then exposed to the MTS reagent for 3 hours. After 3 hours of MTS bio-reduction by proliferating cells, the formazan product's absorbance was measured using a 450nm filter on an Infinite 2000 PRO microplate reader.

Results

[0132] To examine the chemosensitivity of the genetically engineered NRF2-deficient A549 cell lines, the MTS assay, depicted in FIG. 4, was utilized. In 4A, wild type and 2-11 A549 cells were exposed to increasing dosages of cisplatin.

After 72 hours, cisplatin was removed, and the MTS reagent was added for three hours after which time the population was measured for the absorbance of formazan. The data show that, as predicted, wild type A549 cells are resistant to high dosages of Cisplatin. In fact, wild type A549s display a slight increase in cell proliferation up to 3 μM of cisplatin before proliferation is adversely affected at final concentrations of 5 μM and 10 μM , respectively. In the genetically engineered knockout cell lines, we clearly observe an increase in chemosensitivity in a dose-dependent fashion. The 2-11 homozygous knockout cells display a heightened sensitivity, evident even at the lowest dose with a loss of proliferation at concentrations at and above 1 μM . Thus, it is possible that we are observing a gene dosage effect of sorts in that the heterozygous cell line exhibits more resistance to cisplatin than homozygous knockout cells because it contains at least one viable gene copy. In FIG. 4, we display a result from cells being exposed to the same increasing amount of cisplatin as described for Panel A, except Vinorelbine was added to a final concentration of 5 μM . Vinorelbine is an established companion to cisplatin and combinatorial chemotherapeutic regimens NSCLC. Hellmann, et al., *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* 27, 1829-35 (2016). The wild type A549 cells again displayed a dramatic increase in proliferation even at lower doses and did not show elevated sensitivity until the dosage surpassed 5 μM , but the knockout cell line (2-11) displayed increased sensitivity to the combinatorial drug therapy. Carboplatin, a related anticancer drug and commonly used chemotherapy for NSCLC (Hellmann et al., *ibid*) was also evaluated for enhanced chemosensitivity in these genetically engineered A549 cells and the cell killing response reflected what was observed in the experiments using cisplatin (data not shown).

Example 3

Genetically Reengineered A549 Cells Showed Slower Growth Rate and Increased Chemosensitivity in a Xenograft Mouse Model of Lung Cancer

Methods

Animal Experiments and Statistical Analysis

[0133] The animal trials presented herein were carried out at Washington Biotech Inc., Simpsonville Maryland, under animal use and care protocol (SOP 505, SOP 520, SOP 522,

cells (wild type A549 or homozygous knockout (clonal expansion 2-11)) suspended in PBS with 20% Matrigel were injected subcutaneously into right flank of each mouse. Tumor volume was measured three times a week with a digital caliper once palpable, and calculated using the formula, tumor size= $ab^2/2$, where 'a' is the larger and 'b' is the smaller of the two dimensions. When tumors grew up to a mean volume of around 100 mm^3 , A549 tumor bearing mice or A549-2-11 tumor bearing mice were randomly divided into 7 groups (N=5 for each group) respectively and subject to dose/regimen-finding study. They were treated with tail vein injection of (1) Cisplatin (2mg/kg), (2) Carboplatin (25mg/kg), (3) Cisplatin (5mg/kg) and Vinorelbine (5 mg/kg) or (4) saline on day 0, 3, 6 and 9 (day 0 is designated as the day of dose started) (Sanjana et al., 2014, *Nature Methods* 11(8); 783-784). Tumor volume and body weight was closely monitored over time. After 16 days, the animals were sacrificed, with tumor removed, weighed and processed for molecular analysis. Mice were euthanized. The data were expressed as mean \pm SD. Student's t-test and one-way or two-way ANOVA was used to assess the significance of difference. A P value <0.05 was considered significant.

Immunofluorescence Staining

[0134] A549 xenografts were resected on day 16, snap frozen in liquid nitrogen and stored in -80°C . until usage. All immunofluorescence staining was performed as previously described. Wang et al., *Investig. Ophthalmology Vis. Sci.* 58, 3896 (2017). Briefly, tumors were embedded in Optimum Cutting Temperature (Tissue Tek, Torrance, Calif., USA) and 16 μm -thick sections were obtained with a Leica CM3050 cryostat (Leica Microsystems, Buffalo Grove, Ill., USA), and mounted on slides. Slides were fixed and incubated with blocking buffer for 1 hour at room temperature. Sections were then incubated with primary antibody (refer to Table 2 for more details), then washed in PBS and incubated with Alexa Fluor 488 labeled secondary antibody (1:200 dilution; Invitrogen, Grand Island, NY, USA) for 1 hour at RT. Sections were washed in PBS, then mounted with SlowFade Gold antifade mountant with DAPI (Invitrogen, Carlsbad, Calif., USA). Images were obtained with a Zeiss Observer.Z1 microscope (Carl Zeiss, Inc.,

[0135] Gottingen, Germany). The TUNEL assay was conducted with In Situ Cell Death Detection Kit, Fluorescein (Roche, Base, Switzerland), following the manufacturer's instructions.

TABLE 2

Primary antibody	Fixation	Blocking buffer	Dilution buffer	Incubation condition and dilution rate
Ki67 (9129, Cell Signaling)	4% PFA for 30 min at room temperature	5% goat serum, 0.3% Triton X-100 in PBS	2% BSA, 0.3% Triton X-100 in PBS	Overnight at 4°C ., 1:100

SOP 1610, SOP1650) approved by the animal care and use committee of Washington Biotechnology Inc. (AAALAC accredited Animal Welfare Assurance number A4192-01). The human xenograft model was established using methodology reported previously. Kellar et al., *Biomed Res. Int.* 2015, 1-17 (2015). Female athymic nude mice (Envigo, 5-6 weeks old) were used in this study. Approximately 5×10^6

Immunocytochemistry and Image Quantification

[0136] A549 cell lines were seeded in 8-well chamber slides (LabTek II) and allowed to grow for 24 hours. After exposure to 2 μM of cisplatin for 48 hours, cells were washed with PBS, fixed and permeabilized with 4% paraformaldehyde+0.1% Triton X-100 for 45 minutes, while

shaking at room temperature. Cells were washed three times with PBS and blocked with a blocking buffer solution (5% normal goat serum+0.3% Triton X-100 made in 1X PBS) for 2 hours at room temperature. Following blocking, cells were incubated with primary antibody (NRF2 1:500, Abcam ab62352) made in an antibody dilution buffer (1% BSA+0.3% Triton X-100 made in 1X PBS), overnight in a humidified chamber at 4° C. Cells were washed three times with PBS and incubated with a conjugated secondary antibody (goat anti-rabbit Alexafluor 594, Thermo Fisher A-11037) made in an antibody dilution buffer at a concentration of 1:200. Controls included secondary only antibody stains, at the same dilutions. Cells were incubated for one hour at room temperature, in the dark. Cells were washed three times with PBS and the chamber was separated from the glass slide. Immediately following this step, 50 of Slow Fade Gold antifade reagent with DAPI (S36938, Invitrogen) was added to each section of the slide and a coverslip was added and sealed. Slides were imaged on the Zeiss Axio fluorescent observer. Z1 microscope and images were processed on the AxioVision software. Random fields were imaged and the total number of cells/field was counted. Each field was quantified for no staining (none), nuclear staining, or cytoplasmic staining. Two individuals independently counted and quantified the images and values were averaged. The percent of NRF2 positive stained cells over the total cells analyzed in each category was plotted in the graph. Errors bars represent \pm SEM and *denotes a significant p value that is <0.05 (Student's T-test).

Results

[0137] Since CRISPR/Cas9-mediated NRF2 knockdown increased chemosensitivity in A549 cells in vitro, we examine enhanced chemosensitivity driven by gene editing in a xenograft mouse model. The homozygous knockout A549 cells (clone 2-11) and wild type A549 cells (control group) were implanted into the back of a nude mouse and the cells (5×10^6 per cell line) were allowed to proliferate into a tumor with a diameter of approximately 100 mm³. The workflow is depicted in FIG. 5A. As part of the strategy, the chemotherapeutic agent was added at day 0, day 3, day 6 and day 9, respectively, through tail vein injection as indicated in the diagram. Tumor growth through volume and proliferation were measured over the course of 16 days starting at the time of the first injection of the chemotherapeutic agent, day 0, and the results are presented in FIGS. 5B, 5C and 5D.

[0138] FIG. 5B depicts the results of tumor growth over the course of 16 days. As expected, proliferation of wild type A549 cells, treated with either saline or 2 mg/kg of Cisplatin, was not inhibited by the drug, confirming the well-established resistance of A549 cells to cisplatin. The NRF2 knockout xenograft proliferated in the mouse but at a reduced rate even without the addition of Cisplatin. The most dramatic effect is seen when a combinatorial approach is taken wherein NRF2 knockout cells are treated with cisplatin over a period of 16 days. In this case, proliferation of the implanted cells is arrested, and the tumor size is maintained at the same level throughout the course of the experiment, which confirms our previous results generated from experiments conducted in cell culture (FIG. 4).

[0139] FIG. 5C depicts similar results when fixed concentrations of 5 mg/kg cisplatin and 5 mg/kg vinorelbine are used in combination following the same xenograft mouse experimental protocol.

[0140] Interestingly, the wild type A549 cells appear to be more sensitive to this combination of drugs. This observation may reflect the synergistic effect that Vinorelbine has on Cisplatin killing of A549 cells, providing an important internal control that our experimental system recapitulates previously known outcomes. Once again, the homozygous knockout 2-11 cell line proliferates at a slower rate than the wild type cells in the absence of drug treatment, but the combination of

[0141] NRF2 knockout and drug treatment leads to a cessation of tumor growth and maintenance of tumor size over the course of 16 days. The same response is seen once again in the data presented in FIG. 5D wherein 25mg/kg carboplatin is injected in the tail vein; the same reduced proliferation and growth trend described above is reproduced. These results suggest that the combination of gene editing in chemotherapy produces an enhanced chemosensitivity in A549 cells both in cell culture and in a Xenograft mouse model.

Analyses of A549 tumor proliferation

[0142] Representative tumor samples were harvested from four groups (wild type A549 with saline, wild type A549 with cisplatin 2mg/kg, knockout 2-11 with saline, knockout 2-11 with cisplatin 2mg/kg) (N=3 for each group). As shown in FIG. 5E, a distinct difference among the four extracted tumor groups is apparent. As described above, tumors generated from wild type cells proliferate aggressively within the xenograft mouse model in the absence or presence of cisplatin. The NRF2 knockout cell line proliferates more slowly than the wild type even in the absence of the drug. But, the smallest tumors are observed in all the samples from mice bearing NRF2 knockout cells treated with cisplatin.

[0143] Since A549 2-11 knockout xenograft tumors exhibited smaller tumor volume compared to their wild type counterparts, we wanted to examine the proliferative activity within the tumors using Ki67, a well-known marker for proliferation, which presents during all active phases of cell cycle (G1, S, G2 and mitosis). Scholzen et al., J. Cell. Physiol. 182, 311-322 (2000). As shown in FIG. 6, within A549 cells treated with only saline, abundant Ki67-positive cells were observed;

[0144] tumors extracted from mice treated with cisplatin produced similar levels of Ki67 positive cells. In the case of tumors generated from 2-11 cells, Ki67 staining is noticeably decreased and treatment with cisplatin resulted in even lower levels of Ki67, suggesting cisplatin enhances the response of the KO 2-11 cells by slowing down proliferation even further.

[0145] Taken together, the accumulated data build a strong case for clone 2-11 as a functional knockout since these cells enable a higher sensitivity to chemotherapy as compared to the wild-type counterpart. We sought to provide some explanation for this phenotype observed in both cell culture and in the mouse. The effect of the disruption of the NES region located in the Neh5 domain of NRF2 was further characterized using immunocytochemistry. Wild type A549 and clone 2-11 cells were pre-treated with 2 μ M Cisplatin to stimulate NRF2 expression. Random fields of each cell sample were identified, imaged and total cell counts determined. Cells were quantified based on the following observed outcomes: no staining of NRF2, nuclear staining only or cytoplasmic staining only. FIG. 7A represents the average quantification of multiple replicates of several experiments with at least 10 fields of view incorporated into

the data set. We observed a statistically significant difference in the degree of nuclear localization of NRF2 between wildtype A549 and clone 2-11 cells respectively. In wild-type cells, the majority of NRF2 is located in the nucleus while in the functional knockout cell line (2-11), NRF2 is predominantly found in the cytoplasm as seen in FIG. 7B. The images of cisplatin-induced wildtype and knockout cells (FIG. 7B) reflect the data presented in FIG. 7A.

[0146] Cell line 2-11 exhibited a heightened sensitivity to increasing dosages of cisplatin and to a lesser extent in response to increasing concentrations of carboplatin. When cisplatin is combined with vinorelbine, a heightened sensitivity is also observed. Cell killing was determined by the standard MTS assay. Chemosensitivity of the homozygous knockout cell line 2-11 was then evaluated in a xenograft mouse model wherein the cells were implanted in the back of a nude mouse and allowed to proliferate for 16 days. Subsequent tail vein injection of cisplatin, carboplatin or cisplatin and vinorelbine at various days after the tumor had grown to approximately 100 mm³, led to a reduction in tumor proliferation over the course of the next 16 days. Interestingly, cell line 2-11 alone exhibited a slower growth phenotype in a xenograft mouse model even without the addition of chemotherapeutic drugs. This result indicates that the disruption of the NRF2 gene itself reduces proliferative activity to a small degree, although the addition of cisplatin, carboplatin or cisplatin/vinorelbine leads to a significant reduction in tumor cell proliferation.

[0147] Tumors isolated from mice implanted with wild type A549 cells or the clonal knockout cells treated with either cisplatin or saline were sectioned and stained for Ki67, a commonly used marker for cell proliferation. Ki67 is strictly associated with cell proliferation and is present during all active phases of the cell cycle, but absent in resting cells. Our results suggest that there is no difference in Ki67 levels in treated or untreated wild type A549 cells grown in the xenograft model, again reflecting the well-known resistance of A549 cells to cisplatin. In contrast, Ki67 levels in NRF2 knockout cells treated with cisplatin were found to be substantially lower when compared to the wild type counterparts. These results provide a plausible explanation for the reduced size of the tumor found in mice implanted with NRF2 knockout cells, a reduction in tumor cell proliferation as a function of CRISPR-directed gene editing. These results reflect those of Velma et al. (Biomark Insights 11, BMI.S39445, 2016) who reported that cisplatin-treated cells are arrested at the G0/G1 border as a function of increasing concentrations.

[0148] Cisplatin reduces proliferation or retardation of cell cycle progression with an impact at the interface between G0 and G1. These data certainly indicate that disruption of NRF2 in A549 cells leads to a reduced proliferative phenotype, which may preclude the appearance of apoptosis in tumors analyzed at 16 days. It is possible that apoptosis may be evident shortly after the introduction of any of the four treatments of cisplatin taking place in the early part of the experiment.

[0149] When stimulated with stressors, functional NRF2 translocates to the nucleus where it binds to the ARE (antioxidant response element) sequence and activates transcription of the various downstream cytoprotective genes. The translocation of NRF2 to the nucleus (appears as purple) can be seen in the images of wildtype A549 cells (FIG. 7B). However, the genetic knockout of NRF2 in clone 2-11

causes loss of NRF2 function and appears to halt translocation of the protein, instead remaining in the cytoplasm, also seen in FIG. 7B. Functional knockouts may have value as CRISPR moves toward clinical application, particularly for cancer therapy.

[0150] Our results provide support for the notion that the combination of gene editing activity and chemotherapy act synergistically to reduce tumor cell growth. In our case, the treatment of A549 cells with CRISPR/Cas9 to disable NRF2 at the level of the gene also led to effective killing at lower dosages of multiple chemotherapeutic agents.

Example 4

Chemosensitivity In vitro and In Xenograft Mouse Models of Melanoma, ESC, HNSCC, and Breast Cancer (Prophetic)

[0151] Following the methods in the examples above, the effects of CRISPR/Cas9-mediated NRF2 knockdown will be evaluated for additional cancers, both in vitro and in xenograft mouse models. For example, following the methods in Example 1 above, the gRNA1 sequence (5'-UCGAUGUGACCGGGAAUAUCAGG) (SEQ ID NO:2) or the gRNA2 sequence (5'-UGAUUUAGACGGUAUGCAACAGG) (SEQ ID NO:4) will be used to generate NRF2 knockdown cell lines of the human malignant melanoma A375 cell line (Wang, et al., 2018, Oxidative Medicine and Cellular Longevity Volume 2018, Article ID 9742154), the esophageal squamous cancer (ESC) cell lines KYSE-30, -50, -70, -110, -140, -150, -170, -180, -220, and -270 (Shibata et al., 2015, Neoplasia 13:864), the head and neck squamous cell carcinoma (HNSCC) HSC-4 cell line (Kitamura & Motohashi, 2018, Cancer Science 109:900), and the breast cancer (adenocarcinoma) cell line MCF7 (Kang et al., 2014, Scientific Reports 4:7201). Chemosensitivity of the genetically engineered NRF2-deficient cancer cell lines will be compared to that of the corresponding wild type cancer cell line in vitro following the methods provided in Example 2 above. For each cell line, chemosensitivity to the following chemotherapeutic agents will be evaluated as shown in Table 3 below.

TABLE 3

Chemotherapeutic Agents for Evaluation of Chemosensitivity	
Cancer Cell lines	Chemotherapeutic Agent
human malignant melanoma (A375)	cisplatin
esophageal squamous cancer (ESC) (KYSE-30, -50, -70, -110, -140, -150, -170, -180, -220, and -270)	5-fluorouracil
head and neck squamous cell carcinoma (HNSCC) (HSC-4)	cetuximab, cisplatin, fluorouracil, carboplatin
breast cancer (MCF7)	doxorubicin

[0152] Wild type and NRF2-deficient cancer cell lines as shown in Table 3 above will be implanted into nude mice and evaluated for chemosensitivity as described in Example 3 above.

Example 5

[0153] NRF2 gRNA designs

px458 plasmid vector
 Exon 4 gRNA1- (SEQ ID NO: 9)
 5' TCGATGTGACCGGGAATATCAGG 3'
 Exon 4 gRNA2- (SEQ ID NO: 10)
 5' TGATTTAGACGGTATGCAACAGG 3'

[0154] The NRF2 gene-coding sequence was entered into the Zhang lab's online generator (<http://crispr.mit.edu/>) and the gRNA with the highest score was chosen for gRNA1 (5' TCGATGTGACCGGGAATATCAGG 3' (SEQ ID NO:9)) and a previously validated gRNA targeting NRF2 was also chosen for gRNA2 (5' TGATTTAGACGGTATGCAACAGG 3' (SEQ ID NO:10)) (1). The CRISPR plasmid was cloned using standard cloning methods with single-step digestion-ligation. The CRISPR guide sequences with appropriate 5' overhangs were cloned into the px458 backbone vector digested with BbsI (plasmid 48138, Addgene). These two plasmids were transfected separately to knockout NRF2 in the A549 cell line, creating cell line 1-40 and 2-11, with small indels at the cleavage site (2).

[0155] Both plasmid constructs, gRNA1 and gRNA2, were transfected (Lipofection) in the A549 cell line targeting NRF2 in order to cleave and remove a 103 base pair fragment. The transfected cells were single-cell sorted and expanded. Clonal populations were initially screened using PCR and gel electrophoresis to visually analyze a shift in amplicon size. Once screened, DNA was sent out for sequenced across exon 4 of NRF2. FIG. 8 shows the ten clones analyzed for INDEL formation.

Exon 3 gRNA3- (SEQ ID NO: 11)
 5' AAGTACAAAGCATCTGATTTGGG 3'
 Exon 3 gRNA4- (SEQ ID NO: 12)
 5' AGCATCTGATTTGGGAATGTGGG 3'

[0156] The previously described experiments were designed to cleave within exon 4. In order to ensure complete knockout of NRF2, gRNA3 (5' AAGTACAAAGCATCTGATTTGGG 3' (SEQ ID NO:11)) and gRNA4 (5' AGCATCTGATTTGGGAATGTGGG 3' (SEQ ID NO:12)) (NRF2 sequence entered into Benchling and gRNA were strategically chosen) were designed to be cloned into the px458 backbone vector to cleave within exon 3. The newly designed gRNAs would be used in conjunction with the previously designed gRNA1 and gRNA2 to cleave from exon 3 to exon 4. gRNA4 was designed to be used with gRNA1 which would remove 782 bases. gRNA3 was designed to be used with gRNA2 which would remove 877 bases. Both of these combinations would result in loss of the majority of exon 3 and exon 4, leaving only the beginning of exon 3 and end of exon 4. (Sanjana et al., Nat. Methods 11:783 (2014); Bialk et al., Mol. Ther.—Oncolytics 11:75-89 (2018)).

Cas9 RNP
 Exon 2 gRNA- (SEQ ID NO: 13)
 5' TGGATTGATTGACATACTTTGG 3'
 (Neh2 for NRF2 KO)
 Exon 5 gRNA- (SEQ ID NO: 14)
 5' GCTTCTACTTTTGGGAACAAGG 3'
 (Neh3 for NRF2 KO)

[0157] The following gRNAs were designed to be complexed with tracrRNA and Cas9 protein to form a ribonucleoprotein (RNP) complex. The gRNAs were designed using the NRF2 sequence in Benchling.

[0158] Exon 2 gRNA (5' TGGATTGATTGACATACTTTGG 3' (SEQ ID NO:13)) was designed to cleave at the beginning of Neh2 in exon 2 in order to knockout NRF2. The RNP was transfected in A549 cells and collected at various times points to assess indel formation (TIDE analysis), shown in FIG. 9(A-F). Cells could be single-cell sorted by FACS to obtain clonal populations to determine the extent of NRF2 KO.

[0159] Exon 5 gRNA (5' GCTTCTACTTTTGGGAACAAGG 3' (SEQ ID NO:14)) was designed to be to cleave at the end of Neh3 in exon 5 and to be used in conjunction with Exon 2 gRNA. By using both gRNAs, the entire NRF2 gene would be removed (3429 bp). Cells would be transfected with both RNP complexes and single-cell sorted by FACS. Clonal populations would be analyzed for NRF2 KO.

Cas12a RNP
 Exon 2 gRNA- (SEQ ID NO: 15)
 5' TTTGATTGACATACTTTGGAGGCAA 3'
 (Neh2 for NRF2 KO)
 Exon 5 gRNA- (SEQ ID NO: 16)
 5' TTTTCCTTGTTCCCAAAGTAAGAA 3'
 (Neh3 for NRF2 KO)

[0160] The following gRNAs were designed to be complexed with tracrRNA and Cas12a protein to form a ribonucleoprotein (RNP) complex. The gRNAs were designed using the NRF2 sequence in Benchling.

[0161] Exon 2 gRNA (5' TTTGATTGACATACTTTGGAGGCAA 3' (SEQ ID NO:15)) was designed to cleave at the beginning of Neh2 in exon 2 in order to knockout NRF2. The RNP was transfected in A549 cells and collected at various times points to assess indel formation, shown in FIG. 10(A-F). Cells could be single-cell sorted by FACS to obtain clonal populations to determine the extent of NRF2 KO.

[0162] Exon 5 gRNA (5' TTTTCCTTGTTCCCAAAGTAAGAA 3' (SEQ ID NO:16)) was designed to cleave at the end of Neh3 in exon 5 and to be used in conjunction with Exon 2 gRNA. By using both gRNAs, the entire NRF2 gene would be removed (3432 bp). Cells would be transfected with both RNP complexes and single-cell sorted by FACS. Clonal populations would be analyzed for NRF2 KO.

Example 6

[0163] H1703 (NCI-H1703) is a lung squamous cell carcinoma cell line with a missense mutation at codon 285 (GAG→AAG) of its p53 gene. You et al., Cancer Res.

60:1009-13 (2000). NCI-H1703 has been shown to primarily express FGFR1c and to induce Erk1/2 phosphorylation upon stimulation with FGF2. Marek et al., Mol. Pharmacol. 75:196-207 (2009).

```

Cas9 RNP
Exon 2 gRNA 3-
                                     (SEQ ID NO: 22)
5' TGGAGGCAAGATATAGATCTTGG 3'
(Neh2 for NRF2 KO)

R34G ssDNA template-
                                     (SEQ ID NO: 23)
5' TTAATAAACATGAGCTCTCTCCTTCCT
TTTTTTGTCTTAAACATAGGACATGGATT
TGATTGACATACTTTGGAGGCAAGATATA
GATCTTGGAGTAAGTGGAGAAGTATTTGA
CTTCAGTCAGCGACGGAAAGAGTATGAGC
TGGAAAAACAGAAAAAAGTGGAAAAAGGAA
AGACAAGAACAACCTCCAAAAGGAGCAAG 3'
(underlined, intended R34G mutation)

```

[0164] The Exon 2 gRNA 3 was designed to cleave at the D29 codon in exon 2 of the NRF2 gene in order to recreate the R34G mutation. The gRNA was complexed with Cas9 protein to form a

[0165] RNP and was used with the R34G template DNA to facilitate DNA repair and introduce an intended R34G mutation in the NRF2 gene. The experiment yielded several clonal-derived cell lines which are being characterized further. FIG. 11 displays the clones that were genetically analyzed by DECODR for indel formation. Clone 26 (G9_H1703-26) contains a homozygous R34G mutation, preceded by a 4 bp deletion at the cleavage site of the gRNA used in the transfection. Clone 53 (G2_H1703_R34G_53) contains a homozygous R34G mutations, preceded by a 2 bp deletion at the cleavage site of the gRNA used in the transfection. Clone 2-31 (H1703_R34G_2-31) contains two wildtype alleles with two distinct indel patterns, a 6 bp deletion on one allele and a 4 bp deletion on the other allele, at the cleavage site of the gRNA used in the transfection. Clone 2-91 (H1703_R34G_2-91) contains a heterozygous R34G mutation along with a wildtype allele containing a 20 bp deletion at the cleavage site of the gRNA used in the transfection. These clones will be used to further characterize the effect of the R34G mutation and NRF2 KO in exon 2 in the H1703 cell line.

[0166] The following gRNAs were designed to be used in the px458 plasmid vector as well as with Cas9 RNP for further experiments in the H1703 cell line. Individual and both RNP complexes is used to create NRF2 KO clonal-derived cell lines for characterization of the NRF2 KO in the H1703 cell line. The clonal-derived cell lines is used for MTS proliferation assay to determine chemosensitivity

```

Cas9 RNP
Exon 4 gRNA1-
                                     (SEQ ID NO: 24)
5' TCGATGTGACCGGGAATATCAGG 3'
(NRF2 KO)

Exon 4 gRNA2-
                                     (SEQ ID NO: 25)
5' TGATTAGACGGTATGCAACAGG 3'
(NRF2 KO)

```

Example 7

(Prophetic) Chemosensitivity is Increased in NRF2 Knockout H1703 Cell Lines

Methods

[0167] MTS cell proliferation assay

[0168] Cell viability will be evaluated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, Wis.). H1703 cell lines will be plated at 2×10^3 cells per well and will be allowed to culture for 24 hours. The cell media will then be aspirated, the cells will be washed with PBS, then will be exposed to the MTS reagent for 3 hours. After 3 hours of MTS bio-reduction by proliferating cells, the formazan product's absorbance will be measured using a 450 nm filter on an Infinite 2000 PRO microplate reader (Tecan, Mannedorf, Switzerland). Cell viability after drug exposure will be evaluated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. H1703 cell lines will be plated at 2×10^3 cells per well and allowed to culture for 24 hours. The cells will then be treated with cisplatin, carboplatin, or a combination of cisplatin and vinorelbine for three days. The cell media will then be aspirated, the cells will be washed with PBS, then will be exposed to the MTS reagent for 3 hours. After 3 hours of MTS bio-reduction by proliferating cells, the formazan product's absorbance will be measured using a 450nm filter on an Infinite 2000 PRO microplate reader.

Results

[0169] To examine the chemosensitivity of the genetically engineered NRF2-deficient H1703 cell lines, the MTS assay will be utilized. Wild type and NRF-2 deficient H1703 cells will be exposed to increasing dosages of cisplatin. After 72 hours, cisplatin will be removed, and the MTS reagent will be added for three hours after which time the population will be measured for the absorbance of formazan. The data will show that wild type H1703 cells are resistant to high dosages of Cisplatin. In the genetically engineered knockout cell lines, we will observe an increase in chemosensitivity in a dose-dependent fashion. The NRF2 knockout cells will display a heightened sensitivity. Thus, it is possible that we will observe a gene dosage effect of sorts in that the heterozygous cell line exhibits more resistance to cisplatin than homozygous knockout cells because it contains at least one viable gene copy.

SEQUENCE LISTING

```

<160> NUMBER OF SEQ ID NOS: 25
<210> SEQ ID NO 1
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

```

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA recognition element

<400> SEQUENCE: 1

agctactctg gcccttatag tcc 23

<210> SEQ ID NO 2
<211> LENGTH: 23
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 2

ucgaugugac cgggaauauc agg 23

<210> SEQ ID NO 3
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA recognition element

<400> SEQUENCE: 3

actaaatctg ccatacggtg tcc 23

<210> SEQ ID NO 4
<211> LENGTH: 23
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 4

ugauuuagac gguaugcaac agg 23

<210> SEQ ID NO 5
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 5

gtagtgtgc cttagagctt actcatcc 28

<210> SEQ ID NO 6
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 6

ctagcatggg cagtactcat gactaag 27

<210> SEQ ID NO 7
<211> LENGTH: 2446
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

gattaccgag tgccggggag cccggaggag ccgccgacgc agccgccacc gccgccgccc 60

-continued

cgcgccaccag agccgcacctg tccgcgccgc gcctcggcag ccggaacagg gccgcctcgc	120
gggagcccca acacacggtc cacagctcat catgatggac ttggagctgc cgccgcggg	180
actcccgctcc cagcaggaca tggatttgat tgacatactt tggaggcaag atatagatct	240
tggagtaagt cgagaagtat ttgacttcag tcagcgcagg aaagagtatg agctggaaaa	300
acagaaaaaa cttgaaaagg aaagacaaga acaactccaa aaggagcaag agaaagcctt	360
tttcgctcag ttacaactag atgaagagac aggtgaattt ctccaattc agccagccca	420
gcacatccag tcagaaaacca gtggatctgc caactactcc caggttgccc acattcccaa	480
atcagatgct ttgtactttg atgactgcat gcagcttttg gcgcagacat tcccgtttgt	540
agatgacaat gaggtttctt cggctacgtt tcagtcactt gttcctgata ttcccggtca	600
catcgagagc ccagttctca ttgctactaa tcaggctcag tcacctgaaa cttctgttgc	660
tcaggtagcc cctgttgatt tagacggat gcaacaggac attgagcaag tttgggagga	720
gctattatcc attcctgagt tacagtgtct taatattgaa aatgacaagc tggttgagac	780
taccatggtt ccaagtccag aagccaaact gacagaagtt gacaattatc atttttactc	840
atctataccc tcaatggaag aagaagtagg taactgtagt ccacattttc ttaatgcttt	900
tgaggattcc ttcagcagca tcctctccac agaagacccc aaccagttga cagtgaactc	960
attaaattca gatgccacag tcaacacaga ttttggtgat gaattttatt ctgctttcat	1020
agctgagccc agtatcagca acagcatgcc ctcacctgct actttaagcc attcactctc	1080
tgaacttcta aatgggcccc ttgatgtttc tgatctatca ctttgcaaag ctttcaacca	1140
aaaccacctt gaaagcacag cagaattcaa tgattctgac tccggcattt cactaaacac	1200
aagtcccagt gtggcatcac cagaacactc agtggaatct tccagctatg gagacacact	1260
acttggcttc agtgattctg aagtgaaga gctagatagt gccctggaa gtgtcaaaaca	1320
gaatggctct aaaacaccag tacattcttc tggggatagc gtacaacctt tgtcaccatc	1380
tcaggggcag agcactcacg tgcattgatc ccaatgtgag aacacaccag agaaagaatt	1440
gcctgtaagt cctggtcacg gaaaaacccc attcacaaaa gacaacatt caagccgctt	1500
ggaggctcat ctcacaagag atgaacttag ggcaaaagct ctccatatcc cattccctgt	1560
agaaaaatc attaacctcc ctgtttgtga cttcaacgaa atgatgtcca aagagcagtt	1620
caatgaagct caacttgcatt taattcggga tatacgtagg aggggtaaga ataaagtggc	1680
tgctcagaat tgcagaaaaa gaaaactgga aatatagta gaactagagc aagatttaga	1740
tcatttgaag gatgaaaaag aaaaattgct caaagaaaaa ggagaaaatg acaaaagcct	1800
tcacctactg aaaaaacaac tcagcacctt atatctcgaa gttttcagca tgctactgta	1860
tgaagatgga aaaccttatt ctccctagtga atactccctg cagcaaaaca gagatggcaa	1920
tgttttcctt gttcccaaaa gtaagaagcc agatgttaag aaaaactaga tttaggagga	1980
tttgacctt tctgagctag tttttttgta ctattatact aaaagctcct actgtgatgt	2040
gaaatgctca tactttataa gtaattctat gcaaaatcat agccaaaact agtatagaaa	2100
ataatacgaac actttaaaaa gcattggagt gtcagtatgt tgaatcagta gtttcacttt	2160
aactgtaaac aatttcttag gacaccattt gggctagttt ctgtgtaagt gtaaaacta	2220
caaaaactta tttatactgt tcttatgtca tttgttatat tcatagattt atatgatgat	2280
atgacatctg gctaaaaaga aattattgca aaactaacca ctatgtactt tttataaat	2340

-continued

 actgtatgga caaaaaatgg cattttttat attaaattgt ttagctctgg caaaaaaaaa 2400

aaattttaag agctggtact aataaaggat tattatgact gttaaa 2446

<210> SEQ ID NO 8

<211> LENGTH: 605

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

 Met Met Asp Leu Glu Leu Pro Pro Pro Gly Leu Pro Ser Gln Gln Asp
 1 5 10 15

 Met Asp Leu Ile Asp Ile Leu Trp Arg Gln Asp Ile Asp Leu Gly Val
 20 25 30

 Ser Arg Glu Val Phe Asp Phe Ser Gln Arg Arg Lys Glu Tyr Glu Leu
 35 40 45

 Glu Lys Gln Lys Lys Leu Glu Lys Glu Arg Gln Glu Gln Leu Gln Lys
 50 55 60

 Glu Gln Glu Lys Ala Phe Phe Ala Gln Leu Gln Leu Asp Glu Glu Thr
 65 70 75 80

 Gly Glu Phe Leu Pro Ile Gln Pro Ala Gln His Ile Gln Ser Glu Thr
 85 90 95

 Ser Gly Ser Ala Asn Tyr Ser Gln Val Ala His Ile Pro Lys Ser Asp
 100 105 110

 Ala Leu Tyr Phe Asp Asp Cys Met Gln Leu Leu Ala Gln Thr Phe Pro
 115 120 125

 Phe Val Asp Asp Asn Glu Val Ser Ser Ala Thr Phe Gln Ser Leu Val
 130 135 140

 Pro Asp Ile Pro Gly His Ile Glu Ser Pro Val Phe Ile Ala Thr Asn
 145 150 155 160

 Gln Ala Gln Ser Pro Glu Thr Ser Val Ala Gln Val Ala Pro Val Asp
 165 170 175

 Leu Asp Gly Met Gln Gln Asp Ile Glu Gln Val Trp Glu Glu Leu Leu
 180 185 190

 Ser Ile Pro Glu Leu Gln Cys Leu Asn Ile Glu Asn Asp Lys Leu Val
 195 200 205

 Glu Thr Thr Met Val Pro Ser Pro Glu Ala Lys Leu Thr Glu Val Asp
 210 215 220

 Asn Tyr His Phe Tyr Ser Ser Ile Pro Ser Met Glu Lys Glu Val Gly
 225 230 235 240

 Asn Cys Ser Pro His Phe Leu Asn Ala Phe Glu Asp Ser Phe Ser Ser
 245 250 255

 Ile Leu Ser Thr Glu Asp Pro Asn Gln Leu Thr Val Asn Ser Leu Asn
 260 265 270

 Ser Asp Ala Thr Val Asn Thr Asp Phe Gly Asp Glu Phe Tyr Ser Ala
 275 280 285

 Phe Ile Ala Glu Pro Ser Ile Ser Asn Ser Met Pro Ser Pro Ala Thr
 290 295 300

 Leu Ser His Ser Leu Ser Glu Leu Leu Asn Gly Pro Ile Asp Val Ser
 305 310 315 320

 Asp Leu Ser Leu Cys Lys Ala Phe Asn Gln Asn His Pro Glu Ser Thr
 325 330 335

-continued

Ala Glu Phe Asn Asp Ser Asp Ser Gly Ile Ser Leu Asn Thr Ser Pro
 340 345 350

Ser Val Ala Ser Pro Glu His Ser Val Glu Ser Ser Ser Tyr Gly Asp
 355 360 365

Thr Leu Leu Gly Leu Ser Asp Ser Glu Val Glu Glu Leu Asp Ser Ala
 370 375 380

Pro Gly Ser Val Lys Gln Asn Gly Pro Lys Thr Pro Val His Ser Ser
 385 390 395 400

Gly Asp Met Val Gln Pro Leu Ser Pro Ser Gln Gly Gln Ser Thr His
 405 410 415

Val His Asp Ala Gln Cys Glu Asn Thr Pro Glu Lys Glu Leu Pro Val
 420 425 430

Ser Pro Gly His Arg Lys Thr Pro Phe Thr Lys Asp Lys His Ser Ser
 435 440 445

Arg Leu Glu Ala His Leu Thr Arg Asp Glu Leu Arg Ala Lys Ala Leu
 450 455 460

His Ile Pro Phe Pro Val Glu Lys Ile Ile Asn Leu Pro Val Val Asp
 465 470 475 480

Phe Asn Glu Met Met Ser Lys Glu Gln Phe Asn Glu Ala Gln Leu Ala
 485 490 495

Leu Ile Arg Asp Ile Arg Arg Arg Gly Lys Asn Lys Val Ala Ala Gln
 500 505 510

Asn Cys Arg Lys Arg Lys Leu Glu Asn Ile Val Glu Leu Glu Gln Asp
 515 520 525

Leu Asp His Leu Lys Asp Glu Lys Glu Lys Leu Leu Lys Glu Lys Gly
 530 535 540

Glu Asn Asp Lys Ser Leu His Leu Leu Lys Lys Gln Leu Ser Thr Leu
 545 550 555 560

Tyr Leu Glu Val Phe Ser Met Leu Arg Asp Glu Asp Gly Lys Pro Tyr
 565 570 575

Ser Pro Ser Glu Tyr Ser Leu Gln Gln Thr Arg Asp Gly Asn Val Phe
 580 585 590

Leu Val Pro Lys Ser Lys Lys Pro Asp Val Lys Lys Asn
 595 600 605

<210> SEQ ID NO 9
 <211> LENGTH: 23
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 9

tcgatgtgac cgggaatc agg

23

<210> SEQ ID NO 10
 <211> LENGTH: 23
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 10

tgatttagac ggtatgcaac agg

23

-continued

<210> SEQ ID NO 11
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 11

aagtacaaag catctgattt ggg 23

<210> SEQ ID NO 12
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 12

agcatctgat ttgggaatgt ggg 23

<210> SEQ ID NO 13
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 13

tggatttgat tgacatactt tgg 23

<210> SEQ ID NO 14
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 14

gcttcttact tttgggaaca agg 23

<210> SEQ ID NO 15
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 15

tttgattgac atactttgga ggcaa 25

<210> SEQ ID NO 16
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 16

tttcccttgt tccc aaaagt aagaa 25

<210> SEQ ID NO 17
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 17

caagatatag atcttggagt aag 23

<210> SEQ ID NO 18
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 18

aagatataga tcttggagta ag 22

<210> SEQ ID NO 19
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 19

gatatagatc ttggagtaag 20

<210> SEQ ID NO 20
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 20

tatagatctt ggagtaag 18

<210> SEQ ID NO 21
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 21

atagatcttg gagtaag 17

<210> SEQ ID NO 22
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

<400> SEQUENCE: 22

tggaggcaag atatagatct tgg 23

<210> SEQ ID NO 23
<211> LENGTH: 200
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ssDNA template

<400> SEQUENCE: 23

-continued

```

ttaaaaaaca tgagctctct ccttcctttt tttgtcttaa acataggaca tggatttgat      60
tgacatactt tggaggcaag atatagatct tggagtaagt ggagaagtat ttgacttcag      120
tcagcgacgg aaagagtatg agctggaaaa acagaaaaaa cttgaaaagg aaagacaaga      180
acaactccaa aaggagcaag                                     200

```

```

<210> SEQ ID NO 24
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

```

```

<400> SEQUENCE: 24

```

```

tcgatgtgac cgggaatadc agg                                             23

```

```

<210> SEQ ID NO 25
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Guide RNA

```

```

<400> SEQUENCE: 25

```

```

tgatttagac ggtatgcaac agg                                             23

```

1. A method of reducing NRF2 expression or activity in a cell comprising introducing into the cell (a) one or more DNA sequences encoding one or more guide RNAs (gRNAs) that are complementary to one or more target sequences in the NRF2 gene and (b) a nucleic acid sequence encoding a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease, whereby the one or more gRNAs hybridize to the NRF2 gene and the CRISPR-associated endonuclease cleaves the NRF2 gene, and wherein NRF2 expression or activity is reduced in the cell relative to a cell in which the one or more DNA sequences encoding the one or more gRNAs and the nucleic acid sequence encoding the CRISPR-associated endonuclease are not introduced.

2. The method of claim 1, wherein the one or more gRNAs are complementary to one or more target sequences in exon 2, exon 4, and/or exon 5 of the NRF2 gene.

3. The method of claim 1, wherein the one or more gRNAs comprise a trans-activated small RNA (tracrRNA) and a CRISPR RNA (crRNA).

4. The method of claim 1, wherein the one or more gRNAs are one or more single guide RNAs.

5. The method of claim 1, wherein the CRISPR-associated endonuclease is a class 2 CRISPR-associated endonuclease.

6. The method of claim 5, wherein the class 2 CRISPR-associated endonuclease is Cas9 or Cas12a.

7. The method of claim 1, wherein expression of one or more allele(s) of the NRF2 gene is reduced in the cell.

8. A method of reducing NRF2 expression or activity in a cell comprising introducing into the cell (a) one or more guide RNAs (gRNAs) that are complementary to one or more target sequences in the NRF2 gene and (b) a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-

associated endonuclease, whereby the one or more gRNAs hybridize to the NRF2 gene and the CRISPR-associated endonuclease cleaves the NRF2 gene, and wherein NRF2 expression or activity is reduced in the cell relative to a cell in which the one or more gRNAs and the CRISPR-associated endonuclease are not introduced.

9. A guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complementary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4.

10. The gRNA of claim 9, wherein the DNA-binding domain comprises the nucleic acid sequence of SEQ ID NO:2 or a biologically active fragment thereof.

11. A ribonucleoprotein (RNP) complex comprising the gRNA of claim 9 and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease.

12. A pharmaceutical composition comprising an RNP complex comprising (a) a guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complementary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4 and (b) a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease.

13. A DNA sequence encoding the gRNA of claim 9, or a biologically active fragment thereof.

14. The DNA sequence of claim **13**, wherein the DNA sequence comprises the nucleic acid sequence of SEQ ID NO:1.

15. A vector comprising a DNA sequence encoding a guide RNA (gRNA) comprising a DNA-binding domain, wherein the DNA-binding domain is complementary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4, or a biologically active fragment thereof.

16. The vector of claim **15**, wherein the gRNA further comprises a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain.

17. The vector of claim **15**, wherein the vector is an adeno-associated virus (AAV) vector.

18. A pharmaceutical composition comprising the vector of claim **15**.

19. A pharmaceutical composition comprising a guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complementary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4.

20. A method of treating cancer in a subject comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complemen-

tary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4.

21. The method of claim **20**, wherein the cancer is resistant to one or more chemotherapeutic agents.

22. The method of claim **20**, wherein the cancer is selected from the group consisting of lung cancer, melanoma, esophageal squamous cancer (ESC), head and neck squamous cell carcinoma (HNSCC), and breast cancer.

23. The method of claim **22**, wherein the lung cancer is non-small-cell lung cancer (NSCLC).

24. The method of claim **23**, wherein the NSCLC is adenocarcinoma, squamous cell carcinoma, or large cell carcinoma.

25. The method of claim **20**, further comprising administering one or more chemotherapeutic agents to the subject.

26. The method of claim **25**, wherein the one or more chemotherapeutic agents are selected from the group consisting of cisplatin, vinorelbine, carboplatin, and a combination thereof.

27. A method of reducing resistance to a chemotherapeutic agent in a cancer comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a guide RNA (gRNA) comprising a DNA-binding domain and a Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated endonuclease protein-binding domain, wherein the DNA-binding domain is complementary to a target sequence in an NRF2 gene, and wherein the gRNA does not comprise the nucleic acid sequence of SEQ ID NO:4.

* * * * *