Supplementary Information

Activating NRF2^{E79Q} mutation alters the differentiation of human non-small cell lung cancer

Samera H. Hamad[1,2,3,4,5*], Hansa Joshi[2], T. Hess[2], Stuart R Jefferys[4], Zena Saleh[2], Rani S. Sellers[4,6], Gord Zhu[1,2], Travis Shrank[4,7], Rayvon T. Moore[4], David Corcoran[4,8,9], Jeremy M. Simon[4,8,9], Francis R Spitz[1,2,3], David Shersher[1,2,3], Michael B. Major[4,10], and Bernard E. Weissman[4,5,6]

[1]Cooper Medical School of Rowan University, Surgery Department, Camden, New Jersey, United States.

[2]Cooper University Health Care, Surgery Department, Camden, New Jersey, United States.

[3]MD Anderson Cancer Center at Cooper, Camden, New Jersey, United States.

[4]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, United States.

[5]Curriculum in Toxicology and Environmental Medicine, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, United States.

[6]Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, United States.

[7]Department of Otolaryngology-Head and Neck Surgery, The University of North Carolina School of Medicine at Chapel Hill, Chapel Hill, North Carolina.

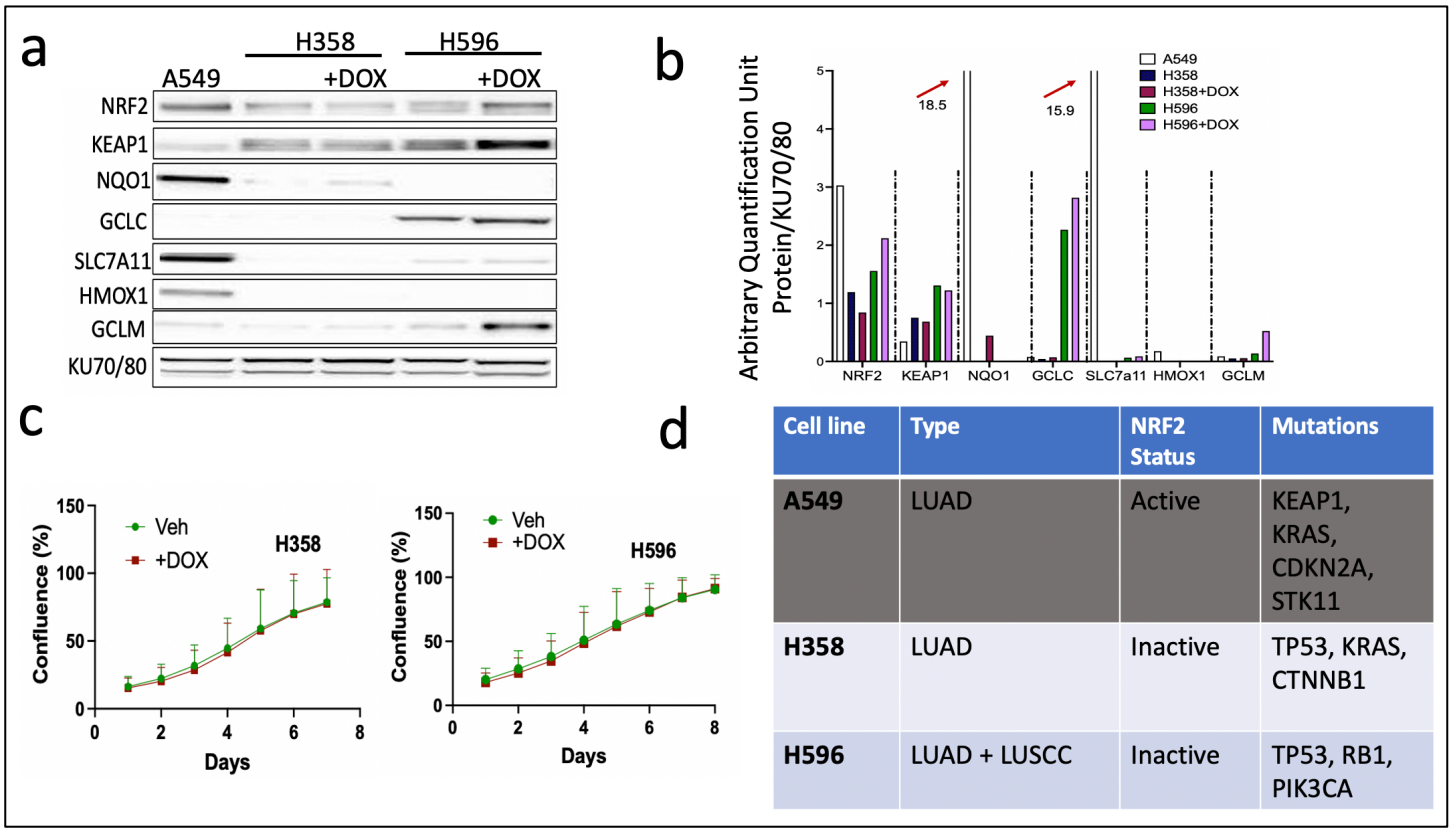
[8]Department of Genetics, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, United States.

[9]UNC Neuroscience Center, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, United States.

[10]Department of Cell Biology and Physiology, Washington University in St. Louis, St. Louis, Missouri, United States.

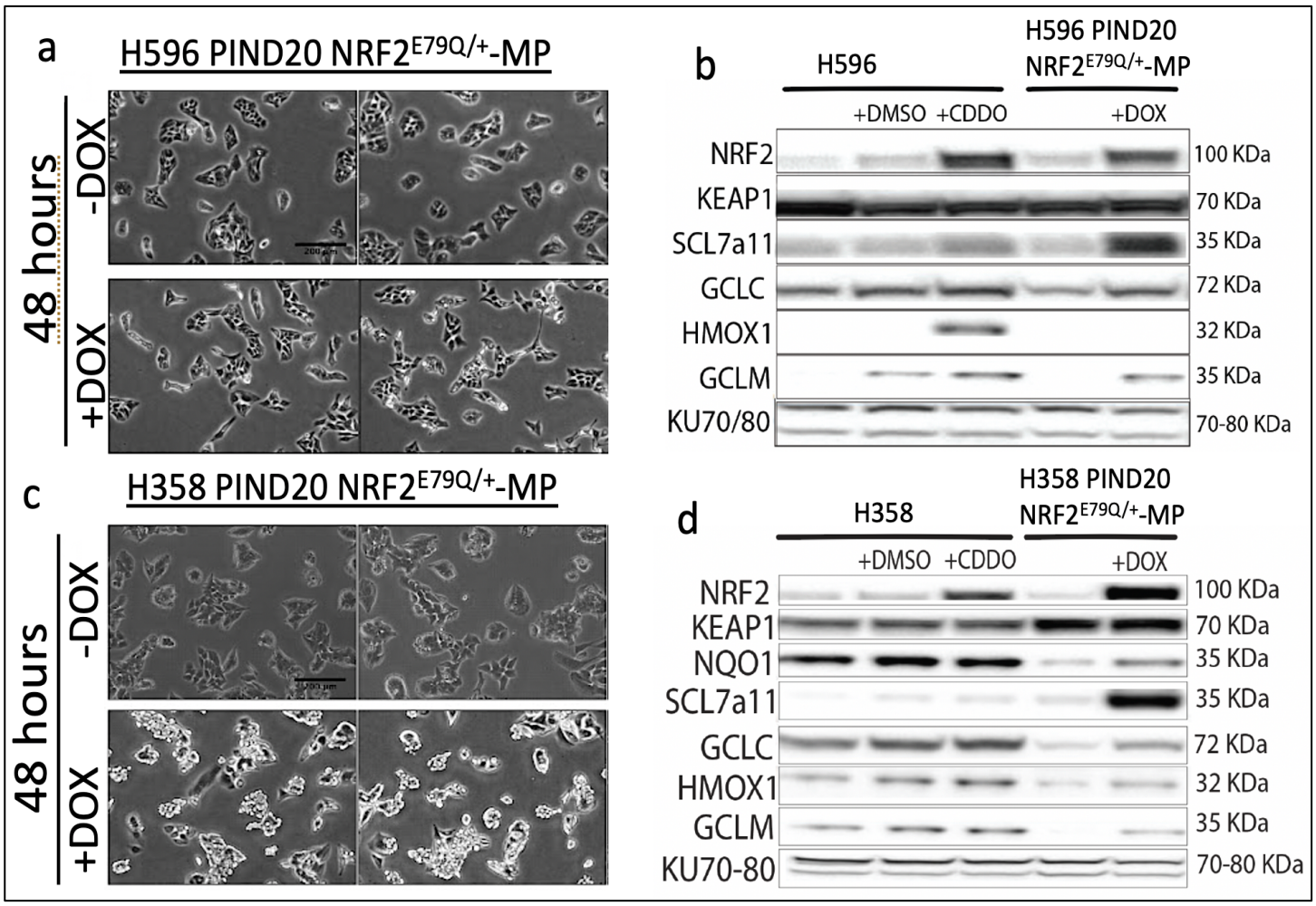
***To whom the correspondence should be addressed**

Samera H Hamad, PhD: Cooper Medical School of Rowan University, 401 Haddon Ave, E&R Building, Camden, NJ 08103, Tel: (608) 217-3839, United States hamad@rowan.edu or samera3h@gmail.com

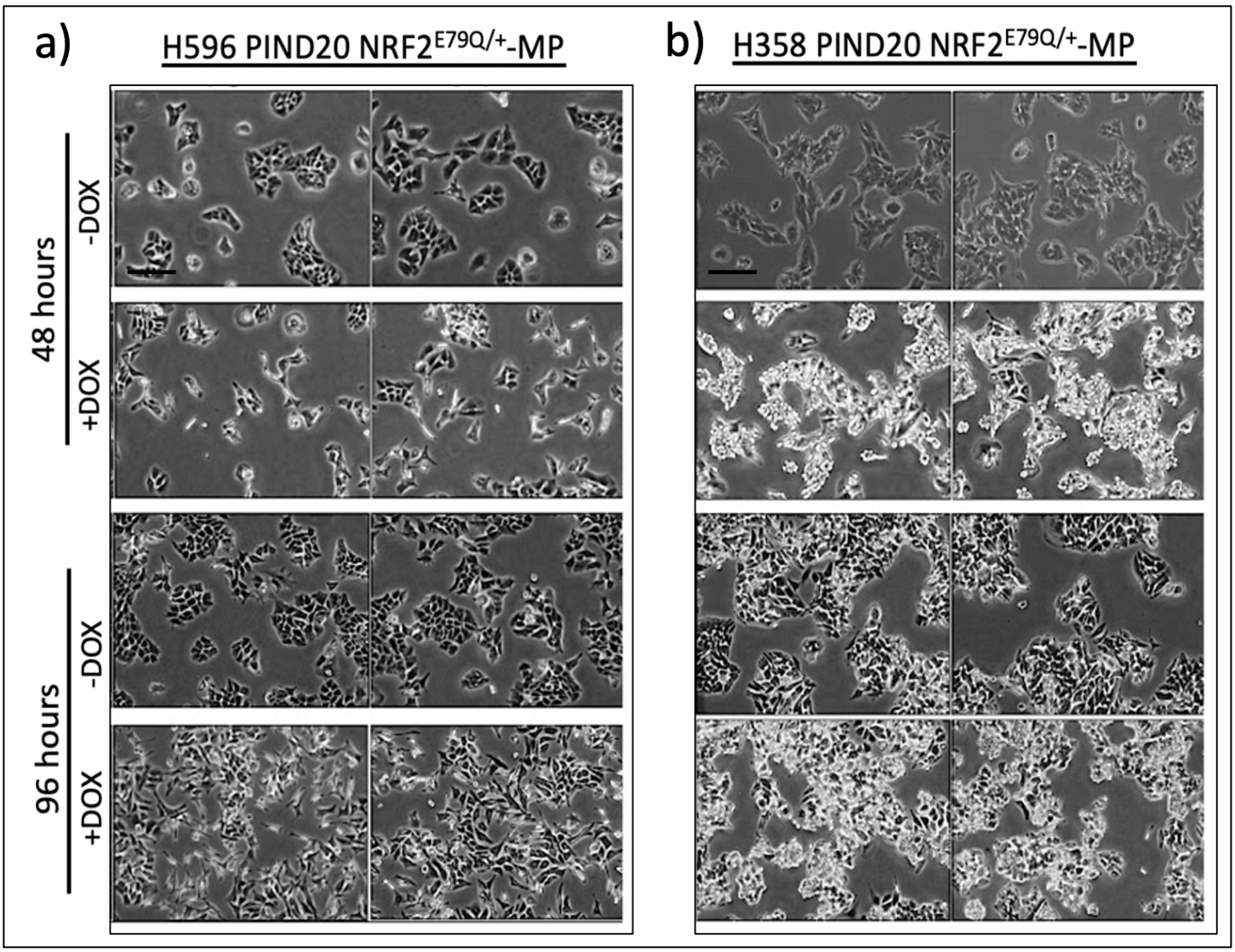


Cell line	Type	NRF2 Status	Mutations
A549	LUAD	Active	KEAP1, KRAS, CDKN2A, STK11
H358	LUAD	Inactive	TP53, KRAS, CTNNB1
H596	LUAD + LUSCC	Inactive	TP53, RB1, PIK3CA

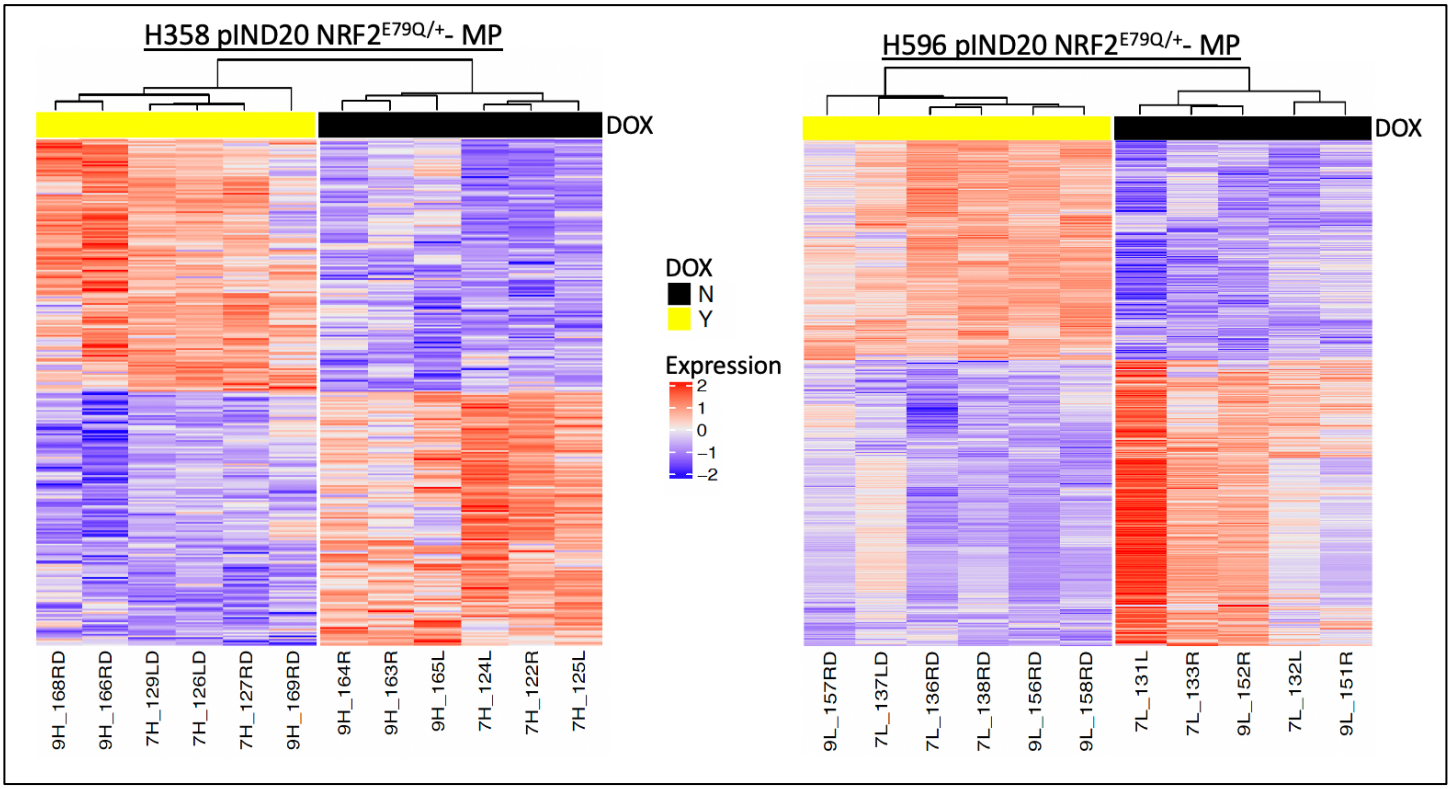
Supplementary Figure 1. Human NSCLC cell lines used in this study. **a** Western blotting of human cell lines with and without DOX, **b** protein quantification presented in **a**, **c** In vitro growth of H358 and H596 parent cell lines, error bars represent 3 technical replicates across 3 biological replicates, **d** Table shows A549 (positive control), H358 and H596 cell lines with NRF2 status and major mutations.



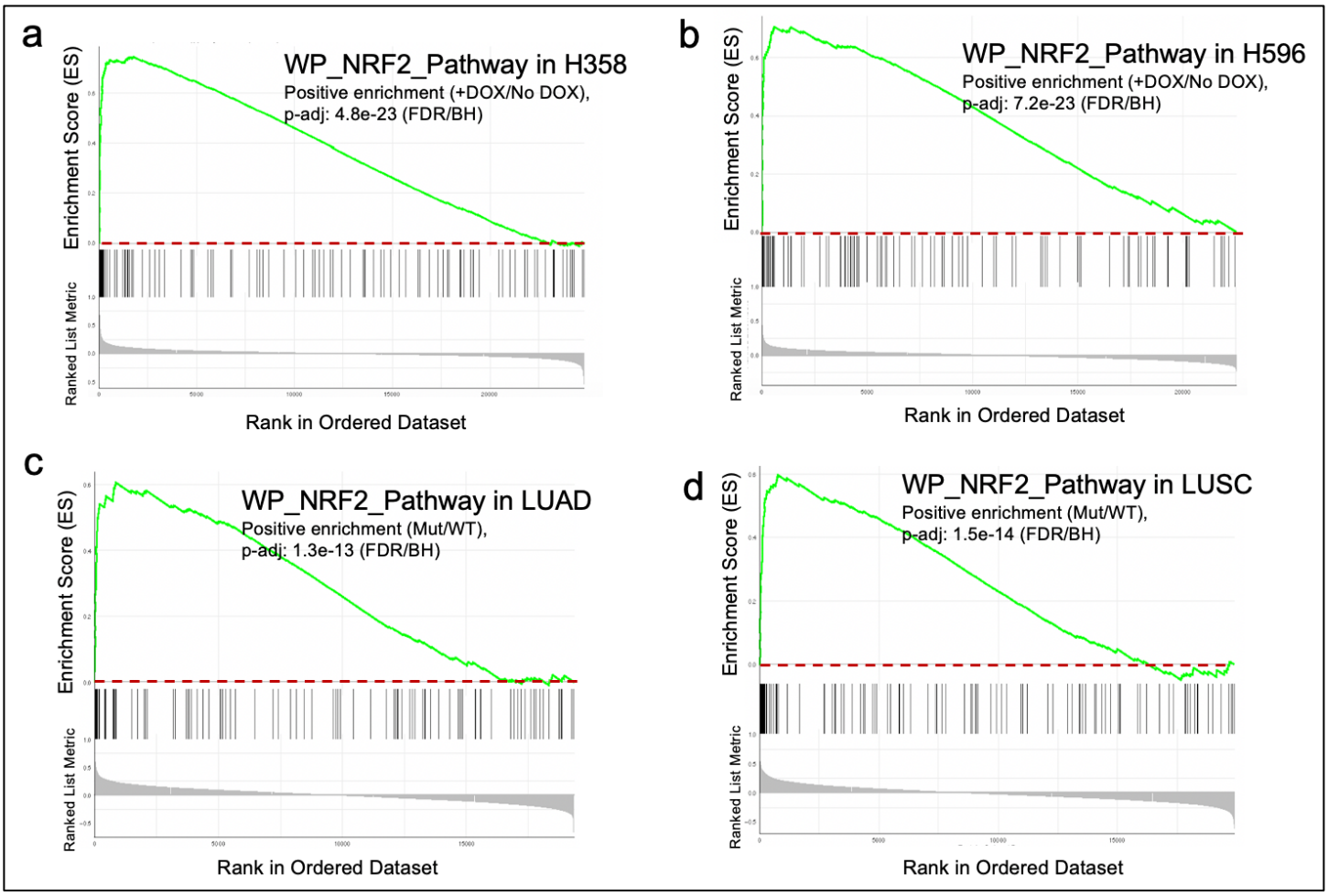
Supplementary Figure 2. Induction of NRF2^{E79Q} in human non-small cell lung cancer cell lines in vitro. **a** Changes in cell morphology observed (in vitro) after 48 hours-Doxycycline (DOX) treatment to activate NRF2^{E79Q} in H596 pIND20 NRF2$^{E79Q/+}$-MP (+DOX, 1μg/ml), with **b** Immunoblotting showing the NRF2^{E79Q} mutation and its downstream targets**; c** Changes in cell morphology observed (in vitro) after 48 hours of DOX treatment to activate NRF2^{E79Q} in H358 pIND20 NRF2$^{E79Q/+}$-MP (+DOX, 1μg/ml), with **d** western blotting showing the NRF2^{E79Q} mutation and its downstream targets; MP: polyclonal, scale bar=200μm.



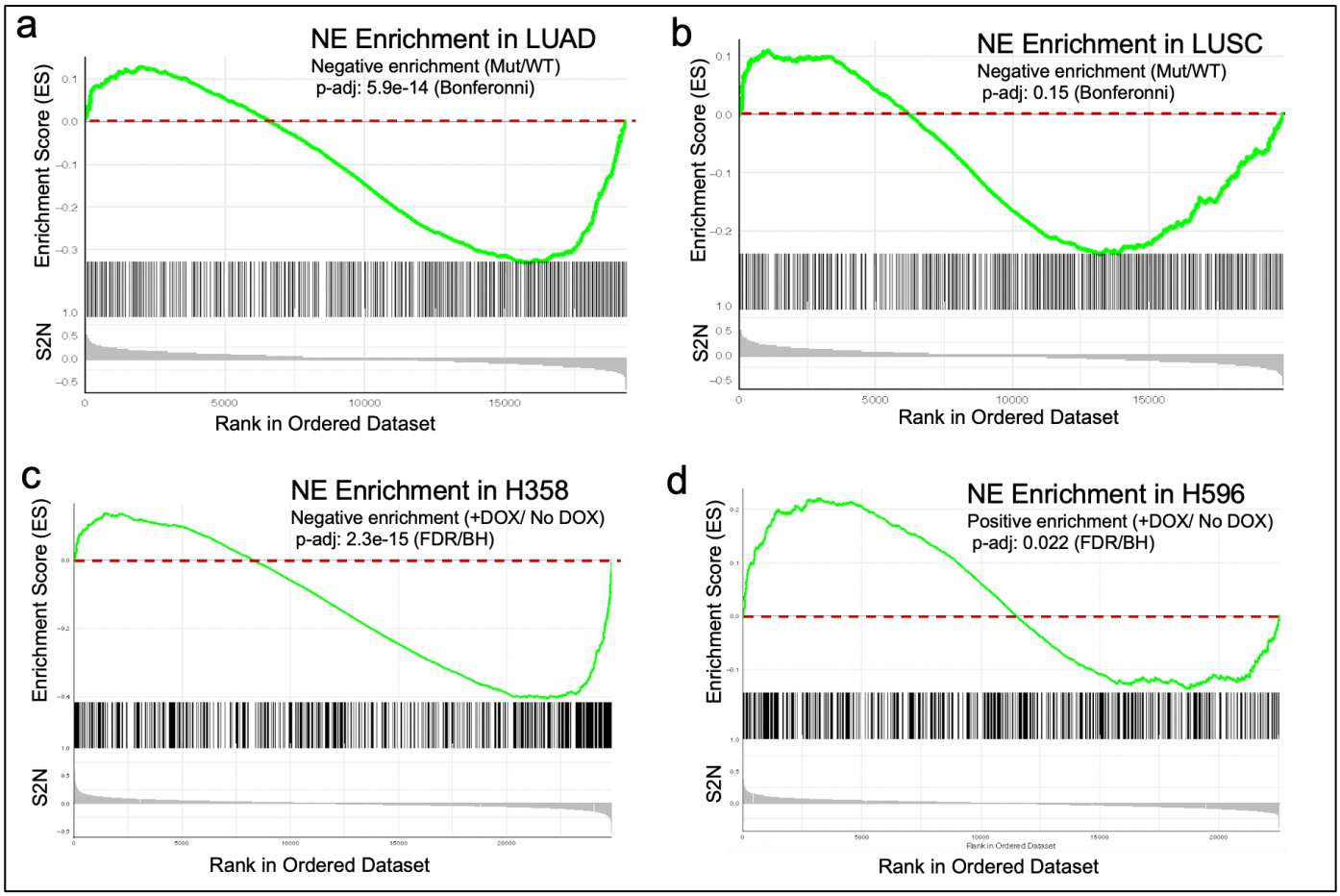
Supplementary Figure 3. Activating NRF2^{E79Q} in human non-small cell lung cancer cell lines in vitro. Changes in cell morphology observed (in vitro-in 2 different fields per cell line) after 48 and 96 hours of DOX treatment to activate NRF2^{E79Q} in **a** H596 pIND20 NRF2^{E79Q}-MP; and **b** H358 pIND20 NRF2$^{E79Q/+}$-MP, (+DOX, 1mg/ml), MP-Polyclonal, scale bar=200μm.



Supplementary Figure 4. RNA-seq data analysis of lung adeno-squamous cell carcinoma (H596 pIND20 NRF2^{E79Q}-MP) and lung adenocarcinoma (H358 pIND20 NRF2^{E79Q}-MP) cell line. a heatmap showing the significantly and differently expressed genes (FDR < 0.05) with activating NRF2^{E79Q} mutation (after DOX treatment) versus no NRF2 activation in **a** H596 pIND20 NRF2^{E79Q}-MP, and **b** H358 pIND20 NRF2^{E79Q}-MP tumors.



Supplementary Figure 5. Enrichment score of NRF2 gene signatures in primary human tumors and human NSCLC cell lines used in this study. a NRF2 signaling pathway is also significantly enriched after $NRF2^{E79Q}$ activation, by doxycycline (DOX, 1μg/ml) treatment, in human lung adenocarcinoma (H358) cell line tumors, and **b** human lung adeno-squamous cell carcinoma (H596) cell line tumors; and **c** NRF2 signaling pathway is significantly enriched in mutant (Mut)-NRF2 human lung adenocarcinoma (LUAD) tumors, and **d** Mut-NRF2 human lung squamous cell carcinoma (LUSC) tumors compared to wild type (WT) tumors.



Supplementary Figure 6. Enrichment scores of neuroendocrine (NE) genes signature in primary human tumors (from TCGA) and human NSCLC cell lines used in this study. a. and **c.** Highly significant negative enrichment of NE in the NRF2 activated (mutant) primary lung adenocarcinoma (LUAD) tumors (a) and in the activated $NRF2^{E79Q}$ lung adenocarcinoma (H358) cell line (c). **b.** and **d.** No significant enrichment of NE in NRF2 activated (mutant) primary lung squamous cell carcinoma (LUSC) tumors (b) and significant positive enrichment in the activated $NRF2^{E79Q}$ lung adeno-squamous cell carcinoma (H596) cell line (d).

Supplementary Table 5. Antibodies used in this study with their primary and secondary dilutions.

Antibody	Company/Catalog number	1° Antibody dilution	2° Antibody dilution
NRF2	Abcam/ab137550	1:1000	1:2000
KEAP1	Proteintech/10503-2-AP	1:1000	1:3000
SLC7A11	Cell Signaling/12691	1:1000	1:2000
P63	Abcam/ab124762	1:1000	1:1000
NKX2-1	Abcam/ab76013	1:1000	1:1000
Synaptophysin (SYP)	Cell Signaling/ D8F6H	1:200	1:500

Supplementary Table 6. Scoring criteria for immunohistochemistry (IHC).

Positively Labelled Cells		Labelling Intensity	
Percent	**Score**	**Intensity**	**Score**
≤ 5%	0	Absent	0
6%-25%	1	Weak	1
26%-50%	2	Moderate	2
51%-75%	3	Strong	3
76%-100%	4		