

1 **NFATc2 enhances tumor-initiating phenotypes through the NFATc2/SOX2/ALDH axis in**  
2 **lung adenocarcinoma**

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12 **Abstract**

13 Cancers display intratumoral genetic and molecular heterogeneity with tumor initiating cells  
14 (TIC) showing enhanced tumor phenotypes. In this study, we show the calcium pathway  
15 transcription factor NFATc2 is a novel regulator of lung TIC through the  
16 NFATc2/SOX2/ALDH1A1 regulatory axis. *In vitro* and *in vivo* cancer cell modeling demonstrated  
17 supportive evidences including cell renewal, tumorigenicity at limiting dose, cell motility,  
18 resistance to cytotoxic chemotherapy and EGFR targeted therapy. In human lung cancers, high  
19 NFATc2 expression predicts poor tumor differentiation, adverse recurrence-free and overall  
20 patient survivals. Mechanistic investigations identified NFATc2 response elements in the SOX2  
21 3' enhancer region, and NFATc2/SOX2 coupling upregulates ALDH1A1 by binding to its 5'  
22 enhancer. Through this axis, oxidative stresses and reactive oxygen species induced by cancer drug  
23 treatment are attenuated, accounting for a mutation-independent mechanism of drug resistance.

24 Targeting this axis provides a novel approach for the long term treatment of lung cancer through  
25 TIC elimination.

## 26 **Introduction**

27 Lung cancer results from mutations induced by DNA adducts, free radicals and reactive  
28 oxygen species (ROS) generated during tobacco smoking and chronic inflammation (Acharya, Das  
29 et al. 2010, Okumura, Yoshida et al. 2012, Houghton 2013). Late presentation and the lack of  
30 effective long term therapy accounts for the high mortality necessitating new therapy. Recent  
31 research has shown the cellular landscape of a cancer is heterogeneous. Cells showing aberrant  
32 expressions of various molecules have enhanced propensities for survival, tumorigenicity, drug  
33 resistance and are designated as cancer stem cells or tumor-initiating cells (TIC). In some cancers,  
34 constitutive activities of inherent embryonic or developmental pathways for stem cell renewal are  
35 involved in TIC maintenance, such as the WNT/ $\beta$ -catenin pathway in colonic adenocarcinomas  
36 (AD). In tissues with slow cell turnover, mechanisms that elicit cell plasticity and stemness  
37 properties during tissue response to intracellular and extracellular stresses are involved (Valent,  
38 Bonnet et al. 2012, Visvader and Lindeman 2012, Beck and Blanpain 2013). For the adult lung,  
39 stem cell niches or their physiological regulatory mechanisms are ill-defined, and molecular  
40 programs sustaining lung TIC are still elusive.

41 Intracellular free calcium is at the hub of multiple interacting pathways activated by  
42 extracellular and/or intrinsic stimulations, e.g. EGFR, endoplasmic reticulum and mitochondrial  
43 stresses, etc. (Roderick and Cook 2008, Prevarskaya, Skryma et al. 2010, Zhao, Wang et al. 2013,  
44 Deliot and Constantin 2015), raising the possibility stress signals transduced by calcium pathway  
45 mediators could be involved in the induction of TIC phenotypes. In cancers of the breast, pancreas,  
46 colon and melanoma, the calcium signaling transcription factor Nuclear Factor of Activated T-

47 cells (NFAT) has been shown to contribute to malignant properties including cell invasion,  
48 migration, survival, proliferation, stromal modulation and angiogenesis (Werneck, Vieira-de-  
49 Abreu et al. 2011, Gerlach, Daniel et al. 2012, Qin, Nag et al. 2014). But information on its role in  
50 TIC phenotypes especially drug resistance is limited. Details of the molecular pathways linking  
51 calcium signaling to TIC induction and drug resistance have not been reported. In this study, we  
52 demonstrated the isoform NFATc2 supports tumorigenicity, cell survival, motility and drug  
53 resistance of human lung AD. Amongst essential factors of pluripotency, SOX2 is an NFATc2  
54 target upregulated through its 3' enhancer, while SOX2 couples to a 5' enhancer of ALDH1A1  
55 mediating overexpression. NFATc2 induces TIC that coexpress the markers ALDH and CD44  
56 (ALDH<sup>+</sup>/CD44<sup>+</sup>-TIC), where ROS scavenging by the NFATc2/SOX2/ALDH1A1 axis enhances  
57 drug resistance. Our study reports a novel mechanism of lung TIC maintenance pathway whereby  
58 micro-environmental stimulation is linked to induction of stemness phenotypes, evasion of cell  
59 death and enhancement of drug resistance in lung AD. NFATc2 could be an important target in  
60 treatment strategies aiming at disruption of TIC in lung cancer.

## 61 **Results:**

### 62 **NFATc2 expression correlated with adverse survivals of human NSCLC**

63 NFATc2 transcripts were significantly overexpressed in NSCLC compared to normal lung  
64 (Fig. 1A). In 102 excised primary human lung cancers expressing NFATc2 by IHC, 41 (40.2%)  
65 showed high level activated NFATc2 shown as intense nuclear staining in large sheets of tumor  
66 cells distributed over extensive areas, while 61 (59.8%) showed low expression featuring weak  
67 cytoplasmic with or without nuclear staining in scattered, isolated or small clusters of tumor cells  
68 (Fig. 1B, C). In normal lung epithelium, NFATc2 was expressed in the bronchiolar stem cell  
69 compartment of basal reserve cells while differentiated bronchiolar cells or alveolar pneumocytes

70 were negative (Fig. 1D). Using log rank test and Kaplan-Meier survival analysis, tumors with high  
71 level NFATc2 expression showed significantly shorter recurrence-free survival (RFS) and overall  
72 survival (OS) (Fig. 1C, D). High NFATc2 expression significantly predicted poor tumor  
73 differentiation, advanced tumor stage and TNM stage (Table. 1A). Furthermore, multivariate Cox  
74 regression analysis showed high NFATc2, late pathological stage, age and smoking history were  
75 independent prognostic indicators for shorter OS, while high NFATc2 and advanced pathological  
76 stage were associated with shorter RFS (Table. 1B, C). The results indicated NFATc2 expression  
77 is associated with repressed tumor differentiation and adverse patient outcomes.

### 78 **NFATc2 enhanced *in vitro* and *in vivo* tumorigenesis and cell motility**

79 If NFATc2 supports tumor initiating phenotypes, it is expected to be expressed at a higher  
80 level in TIC compared to non-TIC. Marker-independent TIC surrogates were first raised as  
81 tumorspheres from 4 lung AD cell lines cultured in non-adherent, stem cell conditions (Liu, Deng  
82 et al. 2007, Shi, Li et al. 2015, Sun, Hu et al. 2015). Compared to non-TIC harvested from the  
83 monolayers, tumorspheres expressed higher NFATc2 by Western blot (Fig. 2A), while transcripts  
84 of *NFATc2* and its target *FASL* were also significantly upregulated (Figure 2-figure supplement  
85 1A). Furthermore, luciferase reporter assays showed significantly higher NFAT activities in  
86 spheres isolated from H1299 and A549 cells (Figure 2-figure supplement 1B).

87 Next, two lung cancer cell lines with high basal NFATc2 (HCC827, PDCL#24) were silenced  
88 by 2 shRNA sequences (shNFATc2-A and -B), and two cell lines with relatively low *de novo*  
89 expression (A549, H1299) were used for NFATc2 ectopic expression (Fig. 2B). NFATc2 knockout  
90 by the CRISPR/CAS9 technique using gRNA targeting NFATc2 (gNFATc2) was also performed  
91 on HCC827 cells (Fig. 2B). Abrogation of NFATc2 significantly reduced 60-70% of tumorspheres  
92 in all the cell models and inhibited tumorspheres renewability for 2 consecutive generations (Fig.

93 2C-D, figure 2-figure supplement 2A), while overexpression significantly augmented  
94 tumorspheres in both A549 and H1299 (Fig. 2E, figure 2-figure supplement 2B). Transwell assays  
95 for cell motility showed silencing NFATc2 significantly reduced the migration and invasion ability  
96 of both HCC827 and PDCL#24 cells (Fig. 2F, figure 2-figure supplement 2C), while the opposite  
97 effects were rendered by NFATc2 overexpression in A549 and H1299 cells (Fig. 2G, figure 2-  
98 figure supplement 2D). Congruent with our hypothesis, NFATc2 inhibition suppressed anchorage  
99 independent growth (figure 2-figure supplement 2E), while ectopically expressed NFATc2  
100 facilitated colony formation (figure 2-figure supplement 2F). *In vivo* subcutaneous xenograft  
101 models showed NFATc2 knockdown significantly retarded tumor sizes and growth rates for  
102 HCC827 and PDCL#24, respectively (Fig. 2H, figure 2-figure supplement 3A), while NFATc2  
103 overexpression in A549 led to the reciprocal effects (Fig. 2I). To evaluate TIC frequencies *in vivo*,  
104 limiting dilution assays were performed by subcutaneous transplantation of decreasing numbers  
105 of tumor cells in nude mice. NFATc2 knockdown led to reduction in tumor incidence with  
106 significantly reduced TIC frequency of HCC827 xenografts (Fig. 2J, figure 2-figure supplement  
107 3B). Reciprocal effects were observed with NFATc2-overexpression, where the TIC frequency of  
108 A549 xenografts was significantly increased (Fig. 2K, figure 2-figure supplement 3C). Together,  
109 the data supported NFATc2 mediates *in vitro* and *in vivo* TIC properties.

#### 110 **NFATc2 promoted cancer resistance to cytotoxic and targeted therapy**

111 The effect of NFATc2 on treatment response was first investigated using cisplatin  
112 chemotherapy on PDCL#24, a *KRAS V12D* mutant lung AD cell line raised from a local male  
113 chronic smoker. Upon NFATc2 inhibition, the drug response was sensitized with significantly  
114 reduced IC<sub>50</sub> compared to control cells (Fig. 3A). Similarly for HCC827, NFATc2 knockout  
115 significantly reduced cisplatin IC<sub>50</sub> (Figure 3-figure supplement 1A). In contrast, overexpressing

116 NFATc2 in A549 increased cisplatin resistance with significantly elevated  $IC_{50}$  (Fig. 3B). *In vivo*,  
117 mice bearing PDCL#24 xenografts treated with cisplatin alone showed 1.68 fold tumor shrinkage  
118 compared with vehicle control. With additional stable NFATc2 knockdown, tumor shrinkage was  
119 significantly accentuated to 7 and 8.6 folds ( $p<0.01$ ), respectively (Fig. 3C), while tumor growth  
120 rate was also retarded (Figure 3-figure supplement 1B). Besides, NFATc2 knockdown  
121 significantly increased paclitaxel sensitivity of HCC827 cells (Figure 3-figure supplement 2A).  
122 Furthermore, chronic exposure to increasing doses of cisplatin was used to induce resistant A549  
123 cancer cells (A549CR). This led to NFATc2 upregulation with elevated  $IC_{50}$  compared to parental  
124 A549 cells (Fig. 3D-E). With NFATc2 suppression, A549CR was re-sensitized with return of  $IC_{50}$   
125 to around the pre-induction level ( $p<0.01$ ) (Fig. 3E). In line with this observation, induction of  
126 H1299 for paclitaxel resistance also caused NFATc2 upregulation (Figure 3-figure supplement  
127 2B).

128 HCC827 is a lung AD cell line known to harbor an activating *EGFR* exon19 deletion which  
129 sensitizes it to tyrosine kinase inhibitor (TKI) therapy. To investigate whether NFATc2 contributes  
130 to resistance to targeted therapy, NFATc2 was stably inhibited by shRNA knockdown or CRISPR  
131 knockout, which led to significantly reduced  $IC_{50}$  for gefitinib (Fig. 3F, G). *In vivo*, while either  
132 gefitinib treatment or NFATc2 knockdown alone was associated with smaller HCC827 xenografts  
133 and slower growth rates compared to their respective controls, the combined treatment accentuated  
134 the level of xenograft shrinkage (Fig. 3H and figure 3-figure supplement 3A). Further, HCC827  
135 induced for gefitinib resistance (HCC827GR) showed upregulated NFATc2 and increased NFAT  
136 promoter activities compared to parental cells (Figure 3-figure supplement 3B and C). With NFAT  
137 inhibition by the calcineurin/NFAT inhibitor cyclosporin A (CSA), drug sensitization with  
138 significantly reduced  $IC_{50}$  for gefitinib was effected (Figure 3-figure supplement 3D).

139 Integrating the *in vitro* and *in vivo* data of various combinations of multiple cell lines and  
140 cancer drug treatments, the enhancing effect of NFATc2 on drug resistance to cytotoxic and  
141 targeted therapy was demonstrated.

#### 142 **NFATc2 upregulated SOX2 expression through its 3' enhancer**

143 To understand the molecular mechanism through which NFATc2 mediates cancer cell  
144 stemness and drug resistance, we hypothesize NFATc2 might be linked to the pluripotency  
145 machinery through its regulatory action. Indeed, analysis of 4 lung AD cell lines showed  
146 transcripts of the major stemness factors *SOX2*, *OCT4* and *NANOG* were significantly elevated in  
147 tumorspheres compared to monolayers (Figure 4-figure supplement 1). Genetic inhibition of  
148 NFATc2 in HCC827 (Fig 4A) and PDCL#24 (Figure 4-figure supplement 2A) led to consistent  
149 *SOX2* repression with the highest magnitude of change compared to the other 2 factors ( $p < 0.01$ ),  
150 while all 3 were significantly upregulated on NFATc2 ectopic expression (Fig. 4B, Figure 4-figure  
151 supplement 2B). Corresponding changes were shown at the protein level (Fig. 3C, Figure 4-figure  
152 supplement 2C). Together, the data suggested *SOX2* is a major stemness target of NFATc2.

153 To further delineate the molecular mechanism of *SOX2* regulation by NFATc2, we screened,  
154 *in silico*, the genomic sequences spanning 5 kb up- and downstream of the *SOX2* transcription start  
155 site (TSS), which identified 4 regions encompassing multiple conserved NFAT binding sequences  
156 (Figure 4-figure supplement 3A). Alignment with ChIP-seq data of A549 cells retrieved from  
157 public databases showed significant overlap at loci of H3K27ac occupancy with regions 2 and 3,  
158 respectively, implicating these regions might harbor sites of active transcription, which was  
159 confirmed by luciferase assay (Fig. 4D). To evaluate this possibility, *SOX2* luciferase reporter  
160 assays of the putative sites were tested which revealed significant activities mediated by sites 1, 2,  
161 4, and 5 (Figure 4-figure supplement 3B, Fig. 4E). Using H441 lung cancer cell line with transient

162 NFATc2 overexpression, significant enhancement was observed for sites 1, 4 and 5 only (Fig. 4F).  
163 Further delineation by site directed mutagenesis of NFAT motifs (GGAAA to GACTA) prevented  
164 reporter activities of sites 4 and 5 only (Fig. 4G), and the findings were supported by data from  
165 A549 and H1299 cells bearing stable NFATc2 overexpression (Figure 4-figure supplement 3C,  
166 D). Notably, sequence homology analysis showed *SOX2* sites 4 and 5 are highly conserved across  
167 different mammalian species (Fig. 4H). Next, using NFATc2-overexpressing cell lines, NFATc2  
168 ChIP-qPCR assays yielded significant enrichment of sites 4 and 5 sequences compared to vector  
169 (Fig. 4I), or IgG control (Figure 4-figure supplement 3E), respectively, demonstrating physical  
170 binding of NFATc2 to *SOX2*. Together, the data showed NFATc2 upregulates *SOX2* by binding  
171 to its 3' enhancer region at around 3.2kb (site 4) and 3.6kb (site 5) from the TSS, respectively.

172 **NFATc2 and SOX2 expressions were significantly correlated in human lung AD and**  
173 **NFATc2/SOX2 coupling augmented tumor functions**

174 The clinical significance of NFATc2/SOX2 coupling was first assessed in human lung cancers.  
175 To avoid the confounding effect of *SOX2* gene amplification in squamous cell carcinoma (SCC)  
176 and focusing on tumors in which we demonstrated the role of NFATc2, IHC was performed on 92  
177 moderately to poorly differentiated AD which showed significant correlation between *SOX2* and  
178 NFATc2 expressions (Fig. 4J). In lung AD cell lines, *NFATc2* and *SOX2* transcripts expression  
179 were also positively correlated (Fig. 4K). Together, the data supported NFATc2 upregulates *SOX2*  
180 in clinical and cultured lung AD.

181 Next, we evaluated whether NFATc2-induced *SOX2* upregulation was functionally relevant  
182 for its role in sustaining TIC. Using A549 transduced for NFATc2 overexpression, *SOX2*  
183 suppression by 2 sh*SOX2* sequences led to significantly reduced tumorspheres formation (Figure  
184 4-figure supplement 4A, B), and cell motility (Figure 4-figure supplement 4C). *In vivo*, NFATc2-



185 mediated enhancement of xenograft size and tumor growth rate were also abrogated by SOX2  
186 knockdown (Fig. 4L).

187 Similar to NFATc2, SOX2 was upregulated in A549 induced for cisplatin resistance (A549CR)  
188 but on NFATc2 knockdown, SOX2 levels were repressed (Fig. 4M), suggesting NFATc2/SOX2  
189 coupling was functionally active in resistant cancer cells. In MTT assays of A549 cells, while  
190 NFATc2 overexpression induced cisplatin resistance, SOX2 silencing restored sensitivity to a  
191 level comparable to the control cells (Fig. 4N). Overall, the data indicated NFATc2 induces TIC,  
192 cancer initiating phenotypes and drug resistance through upregulating SOX2 expression.

### 193 **ALDH1A1 was a target of NFATc2/SOX2 regulation**

194 ALDH<sup>+</sup>/CD44<sup>+</sup>-TIC was investigated as a candidate cellular target of NFATc2. The  
195 ALDH<sup>+</sup>/CD44<sup>+</sup> subset isolated by flow cytometry from HCC827 and H1650 cell lines showed  
196 significantly higher levels of *NFATc2* and *FASL* transcripts compared to ALDH<sup>-</sup>/CD44<sup>-</sup> (Fig. 5A),  
197 suggesting NFATc2 played a role in regulating this cell population. When NFATc2 was knocked  
198 down or knocked out in HCC827, ALDH<sup>+</sup>/CD44<sup>+</sup>-TIC was significantly reduced (Fig. 5B, C).  
199 Consistent changes were observed for PDCL#24 with NFATc2 knockdown (Figure 5-figure  
200 supplement 1A). Conversely, in both A549 with NFATc2 overexpression and in A549CR cells,  
201 ALDH<sup>+</sup>/CD44<sup>+</sup>-TIC proportions were increased (Figure 5-figure supplement 1B-C). Breakdown  
202 analysis showed the trend of changes were consistent with those of the ALDH<sup>+</sup> but not CD44<sup>+</sup>  
203 population, suggesting ALDH might be the main target of NFATc2.

204 ALDH1A1 is the most frequent and important ALDH isozyme reported in lung cancer TIC  
205 (Ucar, Cogle et al. 2009, Tomita, Tanaka et al. 2016). Although ALDH1 is marketed as the major  
206 subtype contributing to ALDH activities detected by the ALDEFLUOR<sup>TM</sup> assay, cross-reactivity  
207 with other isoforms cannot be excluded. Hence, to explore the part contributed by ALDH1A1, we

208 abrogated ALDH1A1 in A549 engineered to overexpress NFATc2, which led to significantly  
209 suppressed aldefluor activities (Figure 5-figure supplement 2A). Furthermore, in cancer cells with  
210 NFATc2 up-or down-regulation (Fig. 5D, E), in A549CR (Fig. 5F), or upon NFATc2 knockout  
211 (Figure 5-figure supplement 2B), ALDH1A1 mRNA were correspondingly altered, indicating  
212 NFATc2 regulates ALDH1A1 expression which contributes to the majority of ALDH positivity in  
213 ALDH<sup>+</sup>/CD44<sup>+</sup>-TIC.

214 Further analyze of the role of NFATc2/SOX2 coupling in ALDH1A1 regulation showed  
215 silencing SOX2 in NFATc2-overexpressing A549 cells consistently prevented the increase of  
216 ALDH<sup>+</sup> and ALDH<sup>+</sup>/CD44<sup>+</sup> subpopulations only (Fig. 5G). Specifically, the expected  
217 upregulation of *ALDH1A1* was also abolished (Fig. 5H). In addition, as analyzed by IHC,  
218 xenografts derived from NFATc2-overexpressing A549 cells showed corresponding upregulation  
219 of SOX2 and ALDH1A1 (Fig. 5I), while NFATc2 suppression in PDCL#24 led to congruent  
220 changes with marked SOX2 and ALDH1A1 downregulation (Figure 5-figure supplement 2C),  
221 respectively. Together, the data strongly supported NFATc2/SOX2/ALDH1A1 form a regulatory  
222 axis in lung cancer.

223 To examine the mechanism of SOX2 in ALDH1A1 regulation, ChIP-seq analysis was  
224 performed to identify SOX2 binding sequences in PDCL#24 cell line. Two loci of peak signals  
225 encompassing the SOX2 consensus binding motif (ATTCA) were identified in the 5' region of the  
226 *ALDH1A1* gene at around 27 kb from the TSS (site 1 and 2, Fig. 5J). Bioinformatics analysis  
227 showed these loci were located at chr9:75,595,820-75,595,832 and chr9:75,602,860-75,602,872  
228 which overlap with H3K27ac occupancy deposited in public ChIP-seq database of A549 cells as  
229 well as mammalian conserved sequences (Fig 5J). On the other hand, no SOX2 binding motif was  
230 identified in regions flanking other *ALDH* isoform genes. Together, the findings suggested the

231 presence of an accessible and highly conserved chromatin region encompassing putative SOX2  
232 binding motifs 5' to the *ALDH1A1* gene. To confirm, ChIP-q-PCR assay using PDCL#24 cells  
233 was performed which showed anti-SOX2 antibodies significantly enriched both sequences 1 and  
234 2 (Fig. 5K). To study their transcriptional regulatory role, luciferase reporter constructs for sites  
235 1 and 2 were co-transfected with SOX2 expression plasmids into A549 cells which yielded  
236 significantly enhanced reporter activities compared to control cells (Fig. 5L). Compatible results  
237 were obtained for A549 with NFATc2 overexpression (Figure 5-figure supplement 2D).

238 To evaluate whether ALDH1A1 is a functionally relevant target, ALDH1A1 was knocked-  
239 down by siRNA in NFATc2-overexpressing A549 cells (Figure 5-figure supplement 3). This led  
240 to significant suppression of cell motility (Fig. 5M) and cisplatin sensitization (Fig. 5N).  
241 Furthermore, moderately to poorly differentiated human lung AD showed statistically significant  
242 positive correlation between SOX2 and ALDH1A1 expressions by IHC staining (Fig. 5O).  
243 Collectively, the data supported ALDH1A1 is a functional target of regulation through  
244 NFATc2/SOX2 coupling.

#### 245 **NFATc2/SOX2/ALDH1A1 coupling enhanced drug resistance and tumor properties** 246 **through ROS attenuation**

247 Alleviation of oxidative stress induced by chemotoxicity promotes cancer cell survival and  
248 mediates drug tolerance. Thus, in A549CR cells, intracellular ROS levels were significantly lower  
249 compared to parental A549 cells (Fig. 6A). To investigate for possible relation between NFATc2  
250 and ROS modulation, NFATc2 was silenced by knockdown or knockout which led to increased  
251 ROS levels (Fig. 6B-D). As shown in Fig. 6E, NFATc2 depletion sensitized PDCL#24 cells to  
252 cisplatin treatment, but the addition of the reducing agent NAC reversed cisplatin IC<sub>50</sub> to above  
253 the control level dose-dependently. Reciprocally, the enhanced resistance of A549 by NFATc2

254 overexpression was reversed by oxidative stress induced by the glutathione inhibitor BSO (Fig.  
255 6F), consistent with the suggestion that drug resistance by NFATc2 is effected through ROS  
256 attenuation. Similarly, ROS regulation also supported other tumor phenotypes mediated by  
257 NFATc2. For example, tumorspheres suppression by NFATc2 knockdown was restored by NAC  
258 dose-dependently but in control cells, no significant changes were induced even in the presence of  
259 additional NAC (Fig. 6G-H). Likewise, cell migration and invasion efficiencies inhibited by  
260 NFATc2 depletion were reversed by NAC (Fig. 6I). To further address the involvement of SOX2  
261 coupling and ALDH1A1, we showed suppression of ROS by NFATc2-overexpression in A549  
262 cells were reversed by silencing SOX2 or ALDH1A1, respectively (Fig. 6J-K). Together, the data  
263 suggested NFATc2/SOX2/ALDH1A1 form a functional axis in the homeostatic regulation of an  
264 optimal level of ROS for *in vitro* tumorigenicity, cell motility, and mediation of drug resistance.

## 265 **Discussion**

266 The elucidation and disruption of TIC maintenance pathways offer the opportunity to eliminate  
267 the most resilient cancer cells and improve treatment outcome. Many studies have demonstrated  
268 cell populations expressing high levels of specific markers such as ALDH, CD44, CD166, CD133,  
269 etc. are enhanced for a multitude of tumor phenotypes, with tumorigenicity and drug resistance  
270 being clinically the most important. Constitutive stem cell programs or stress-induced pathways  
271 are the main TIC sustaining mechanisms but details of their regulation are still elusive. NFAT is a  
272 family of transcription factors with the calcium-responsive isoforms NFATc1, -c2, -c3 and -c4  
273 being expressed in a tissue-dependent manner. In both clinical NSCLC and lung AD cell lines, we  
274 observed significantly upregulated transcripts of NFATc2, and -c4 compared to paired normal lung  
275 and immortalized bronchial epithelial cells BEAS-2B, respectively, while NFATc2 was the most  
276 frequently involved entity with the highest magnitude of change (data not shown). In this study,

277 using NFATc2 depletion and overexpression models of multiple lung cancer cell lines in TIC-  
278 defining functional assays (Pattabiraman and Weinberg 2014), as well as clinical evidence from  
279 excised human lung cancers, we showed NFATc2 mediates TIC phenotypes. *In vitro* cell  
280 renewability was demonstrated by tumorspheres passaged for consecutive generations and  
281 augmentation of tumorigenicity was illustrated by the limiting dilution assay. In clinical tumors,  
282 high level NFATc2 segregated with impaired tumor differentiation, advanced pathological stage,  
283 shorter recurrence-free and overall survivals in NFATc2-positive NSCLC, suggesting NFATc2  
284 mediates the more primitive and aggressive tumor phenotypes. In the literature, only one research  
285 group has reported NFATc2 expression in 52% of 159 lung cancers and similar to our findings,  
286 high expression was associated with late tumor stage and poor survival. Supportive evidences for  
287 cell proliferation, invasion and migration were demonstrated by cell models but the *in vivo* role of  
288 NFATc2 and, in particular, its effects on TIC, drug response or mechanisms of action were not  
289 addressed (Chen, Zhao et al. 2011, Liu, Zhao et al. 2013).

290 To identify the TIC sustaining mechanism of NFATc2, we hypothesized NFATc2 might be  
291 coupled to the core pluripotency factors SOX2, NANOG and/or OCT4, aberrant activities of which  
292 would be most suitable to orchestrate multifaceted cancer propensities through extensive  
293 transcriptional and epigenetic reprogramming. Using multiple analyses of cancer cells and  
294 tumorspheres, we observed SOX2 was the most consistently altered factor with the highest  
295 magnitude of change when NFATc2 expression was genetically manipulated. SOX2 is an  
296 important oncogene for squamous cell carcinomas (SCC) of the lung and other organs (Lu, Futtner  
297 et al. 2010, Boumahdi, Driessens et al. 2014), and aberrant expression is mostly due to  
298 amplification of the SOX2 locus at 3q26.3 (Hussenet, Dali et al. 2010). For lung AD, although  
299 SOX2 is often expressed at high levels and predicts adverse survivals (Chou, Lee et al. 2013),

300 distinct genetic mechanisms are not identified, indicating regulatory or signaling aberrations might  
301 be involved. To evaluate the effects of NFATc2 on SOX2 expression, we avoided the potentially  
302 confounding element of SCC and focused on moderate to poorly differentiated AD where NFATc2  
303 is shown to play an important prognostic role. Indeed, SOX2 expression is significantly correlated  
304 with that of NFATc2 in this group of human lung cancer. Functionally, NFATc2/SOX2 coupling  
305 contributes to tumor behavior as depletion of SOX2 in cell lines with NFATc2 overexpression led  
306 to significant suppression of TIC phenotypes. Hence, clinical and experimental evidences support  
307 NFATc2 impedes tumor differentiation and negatively affects patient outcome through coupling  
308 to SOX2 in lung AD.

309 We observed NFATc2 binds to the 3' enhancer region of *SOX2* at around 3.2 kb and 3.6 kb  
310 from TSS, effecting functional TIC enhancement and supporting the direct involvement of  
311 NFATc2 in stemness induction. In a recent study of a transgenic mouse model of pancreatic ductal  
312 adenocarcinoma with *KRAS*<sup>G12VD</sup> mutation and p53 heterozygous inactivation, Singh *et al* reported  
313 an analogous mechanism involving another NFAT family protein, NFATc1, which acts as a  
314 coactivator and transcriptional regulator of SOX2 (Singh, Chen et al. 2015). They showed  
315 NFATc1 played a permissive role for tumor dedifferentiation and expression of EMT genes, while  
316 p53 disruption was essential for tumorigenesis, suggesting under the appropriate genetic context,  
317 NFATc1 activation transduces EMT through SOX2 upregulation. The authors proposed since  
318 chronic inflammation is a known etiology of pancreatic AD, NFATc1 might be a crucial factor  
319 involved in the progression of this cancer. NFAT family members are distributed in a tissue-  
320 specific manner and we have observed NFATc2, rather than NFATc1, is the main factor highly  
321 expressed in lung cancers. Our data also distinguish a different SOX2 enhancer region accessible  
322 to NFATc2 which functions not only in the presence of *KRAS* mutation (A549, PDCL#24) but

323 also in *EGFR* mutant (HCC827) cancers. Chronic inflammation is an important etiological  
324 mechanism of lung cancer through release of ROS and free radicals from alveolar macrophages  
325 and neutrophils. While many studies modeling carcinogenetic mechanisms of tobacco toxicity or  
326 chronic obstructive pulmonary diseases have featured the NF- $\kappa$ B pathway as a major mediator,  
327 our findings on the effects of NFATc2 on TIC induction might add to this repertoire. In fact,  
328 NFATc2 and NF- $\kappa$ B share highly similar DNA binding domains but differ in the upstream  
329 activators, with NF- $\kappa$ B being stimulated by cytokine receptors and inflammatory molecules while  
330 NFATc2 is downstream of calcium signaling reputed as a stress response integrator, illustrating  
331 the multiple mechanisms through which inflammation-induced carcinogenesis might be initiated  
332 in the lung.

333 We have shown the ALDH<sup>+</sup>/CD44<sup>+</sup> fraction of lung cancer cells, despite being the smallest  
334 subset, demonstrates the highest tumorigenic capacity compared to their counterpart cell  
335 compartments (Liu, Xiao et al. 2013). In trying to understand the role of NFATc2/SOX2 coupling  
336 in inducing this cell fraction, we observed only ALDH but not CD44 showed consistent changes  
337 upon NFATc2 suppression or overexpression, respectively, and more specifically, ALDH1A1 was  
338 identified as a major functional target. Recent reports have shown  $\beta$ -catenin can directly regulate  
339 ALDH1A1 (Condello, Morgan et al. 2015), and since SOX2 can upregulate  $\beta$ -catenin (Yang, Hui  
340 et al. 2014), this could infer SOX2 might indirectly regulate ALDH1A1 through  $\beta$ -catenin. We  
341 thus assessed  $\beta$ -catenin activity by western blot in A549 cells with NFATc2 overexpression and  
342 SOX2 knockdown, but no significant alternation of total or activated  $\beta$ -catenin levels was observed  
343 in these cells and the possible mechanism of indirect ALDH1A1 upregulation through  $\beta$ -catenin  
344 was not supported (Figure 5-figure supplement 4). On the other hand, computational screening did  
345 not detect significant and conserved SOX2 binding motifs within the proximal promoter region of



346 ALDH1A1, but a more distant locus at 27 kb upstream from its TSS was shown to be a probable  
347 response region which was identified and confirmed by CHIP-seq and luciferase reporter assays,  
348 respectively. This illustrates a distal enhancer is involved in the trans-regulation of ALDH1A1  
349 expression, which has not been reported before. Current views on cancer stem cells suggest TIC  
350 is unlikely to be a single population with unique identifiers; instead, cell plasticity might induce  
351 variant TIC populations through dynamic response mechanisms, enabling cancer cells to meet the  
352 requirements of a complex micro-environment. In this connection, since we have only  
353 demonstrated the NFATc2/SOX2/ALDH1A1 axis, further investigation for the mechanisms  
354 regulating CD44 in ALDH<sup>+</sup>/CD44<sup>+</sup>-TIC is needed.

355 Acquired drug resistance is mediated through complex genetic and molecular mechanisms  
356 (Holohan, Van Schaeybroeck et al. 2013). We have shown NFATc2 augments resistance to both  
357 cytotoxic chemotherapy and targeted therapy. Adaptive antioxidant response for alleviating  
358 oxidative stress from ROS surge during systemic therapy is one of the most important mechanisms  
359 of drug resistance (Zhang, Du et al. 2011), suggested to be accentuated in cancer stem cells (Diehn,  
360 Cho et al. 2009, Achuthan, Santhoshkumar et al. 2011, Ishimoto, Nagano et al. 2011, Chang, Chen  
361 et al. 2014). Indeed, we have observed in multiple cell lines with induced resistance to  
362 chemotherapy or targeted therapy, NFATc2 was upregulated, while ROS was maintained at a  
363 lower level in A549CR compared to parental cells. Changes in TIC phenotypes induced by  
364 NFATc2 up- or down-regulation were correspondingly restored by the redox reagents BSO or  
365 NAC, respectively, suggesting ROS scavenging is an important mechanism of drug resistance and  
366 other TIC properties mediated by NFATc2. In line with this suggestion, it has been reported in  
367 adult immortalized bronchial epithelial cells, NFAT can be upregulated in response to  
368 inflammatory and carcinogenic stimulation such as those due to benzo-(a)-pyrene and heavy



369 metals stimulation (Huang, Li et al. 2001, Ding, Wu et al. 2007, Cai, Li et al. 2011), leading to  
370 ROS-induced COX2 pathway signaling and enhanced cell survival (Ding, Li et al. 2006).  
371 However, whether ROS is in turn suppressed by NFATc2 through a negative feedback mechanism  
372 has not been reported. Our findings supplement this information and show NFATc2 facilitates  
373 ROS scavenging, and further implicates this effect is mediated by ALDH1A1 through SOX2  
374 coupling, which is consistent with other studies showing that ALDH1A1 is involved in mediating  
375 drug resistance through repressing ROS level (Singh, Brocker et al. 2013, Raha, Wilson et al. 2014,  
376 Mizuno, Suzuki et al. 2015).

377 In summary, this study demonstrates the calcium signaling molecule NFATc2 enhances  
378 functional characteristics associated with cancer stemness phenotype. Our data reveal a novel  
379 mechanism of SOX2 upregulation in lung cancers through enhancer binding by NFATc2. The  
380 NFATc2/SOX2/ALDH1A1 axis contributes to drug resistance by mediating a negative feedback  
381 mechanism for ROS scavenging and restoration of redox homeostasis. Together, the findings  
382 implicate NFATc2 is a potential therapeutic target for sequential or combination therapy of lung  
383 cancer that aims to eliminate TIC.

## 384 **Materials and Methods**

### 385 **Cell lines**

386 Established cell lines (H1993, HCC1833, H358, H1650, H2228, H1299, H1437, H1975, H23,  
387 H2122, HCC827, HCC78, A549, H441, and BEAS-2B) were obtained from ATCC. HCC366 and  
388 HCC78 were kindly provided by Dr. J. Minna (University of Texas Southwestern Medical Center,  
389 Dallas). All cell lines were cultured into individual aliquots and frozen upon receipt. Only first 20  
390 passage of cell lines were used in experiment. Patient derived cell lines (HKULC1, HKULC2,  
391 HKULC3, HKULC4, PDCL#24, and FA31) were raised from resected lung cancers or malignant

392 pleural effusions and only cells of the 1st to 10th passage were used for study. Cancer cells were  
393 maintained in RPMI-1640 (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen, Carlsbad, CA).  
394 BEAS-2B were cultured in Keratinocyte-SFM (Invitrogen, Carlsbad, CA). Gefitinib, paclitaxel or  
395 cisplatin-resistant (-GR, TR or -CR, respectively) cells were generated by chronic exposure of  
396 cancer cells to stepwise increasing doses of the respective drugs. All procured cell lines used in  
397 this study were authenticated using the AmpFISTR® Identifiler® PCR Amplification  
398 Kit for short tandem repeat profiling according to the manufacturer's instruction (Thermo Fisher  
399 Scientific, Waltham, MA).

#### 400 **SiRNA and Plasmids**

401 Small interfering RNA (siRNA) with pre-designed sequences targeting human PPP3R1,  
402 ALDH1A1 and scramble siRNA were from Sigma-Aldrich (St Louis, MO). GFP-VIVIT (11106),  
403 pGL3-NFAT luciferase (17870), two shRNA sequences targeting SOX2, pLKO.1 Sox2 3HM a  
404 (26353) and pLKO.1 Sox2 3H b (26352), the negative control vector pLKO.1-puro (1864), the  
405 envelope vector pMD2.G (12259) and packaging vector psPAX2 (12260) were purchased from  
406 Addgene (Cambridge, MA; <http://www.addgene.org>). The pLKO.1-lentiviral shRNA with different  
407 inserts specifically targeting NFATc2 were purchased from Sigma-Aldrich (TRCN0000016144,  
408 TRCN0000230218). Human full length NFATc2 were amplified by PCR, and the RFP-NFTAc2  
409 plasmids were generated by cloning the sequences into PCDH-CMV-MCS-EF1-COPRFP vector  
410 (SBI, Mountain View, CA). For luciferase reporter construction, SOX2 regulatory regions were  
411 amplified by PCR from human genomic DNA and cloned into pGL3 (Promega) to generate the  
412 SOX2-luc constructs. Primers used for genomic DNA amplification were listed in supplementary  
413 table 1. Site directed mutagenesis of the consensus NFAT binding site (GGAAA to GACTA) were  
414 performed using QuikChange (Stratagene).

415 **Lentiviral knockdown of NFATc2 and SOX2**

416 Lentiviral shRNA was produced by transfecting the shRNA, envelope and packaging vectors into  
417 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Viruses were harvested after 48  
418 hrs of transfection followed by infection of target cells for 72 hrs. Cells stably expressing shRNA  
419 were selected using puromycin (Sigma-Aldrich) for 14 days after 72 hrs of viral infection.

420 **Lentiviral over-expression of NFATc2**

421 RFP-NFATc2 lentiviral particles were produced and transduced into target cells using Lenti Starter  
422 kit (SBI, Mountain View, CA) according to manufacturer's instructions. RFP-positive cells stably  
423 over-expressing NFATc2 and SOX2 were selected by FACS using BD Aria (BD Biosciences).

424 **Lentiviral knock out of NFATc2 by CRISPR/Cas9**

425 LentiCas9-Blast and lentiGuide-Puro were purchased from Addgene (Cambridge, MA;  
426 <http://www.addgene.org>). The gRNA targeting NFATc2 was designed using Zifit  
427 (<http://zifit.partners.org/ZiFiT/>) and listed in supplementary table 1. The annealed gNFATc2  
428 oligonucleotides were cloned into lentiGuide-Puro. Lenti-viral cas9 and lenti-viral gNFATc2 were  
429 generated by transfecting lentiCas9-Blast or lenti-viral gNFATc2 together with pMD2.G and  
430 psPAX2, respectively, into 293FT cells by lipofectamine 2000 according to protocols as described  
431 (Sanjana, Shalem et al. 2014). After infection of lenti-viral cas9, cells stably expressing Cas9 were  
432 selected using Blasticidin (Sigma-Aldrich) for 10 days. HCC827-Cas9 cells were further infected  
433 with lenti-gNFATc2 virus for 72 hrs, and cells stably expressing gNFATc2 were selected using  
434 puromycin (Sigma-Aldrich) for 14 days.

435 **Flow cytometry and fluorescence activated cell sorting (FACS)**

436 ALDH activity was analyzed by the Aldefluor kit (Stem Cell Technologies) according to  
437 manufacturer's instructions. CD44 expression was stained by anti-CD44-APC (BD Pharmingen)

438 as previously described (Liu, Xiao et al. 2013). Flow cytometry was performed using FACS Canto  
439 II (BD Biosciences) and data were analyzed using FlowJo (Tree star). RFP positive cells with  
440 NFATc2 over-expression were isolated by FACS using BD Aria (BD Biosciences). Sorted cells  
441 were re-analyzed after collection to ensure a purity of > 95%. Non-viable cells were identified by  
442 propidium iodide inclusion.

#### 443 **Sphere formation and serial passage**

444 Five hundred cells were seeded in an ultra-low plate (Costar) and cultured in cancer stem cell  
445 medium (RPMI-1640 medium supplemented with 20 ng/mL FGF, 20 ng/mL EGF, 40 ng/mL IGF  
446 and 1X B27 (Invitrogen, Carlsbad, CA) for 14 days. Tumorspheres were harvested, dissociated  
447 with trypsin, re-suspended in RPMI-1640, and 500 cells were seeded again under previously  
448 described stem cell culture conditions for generation of 2<sup>nd</sup> passage.

#### 449 **Colony formation assay**

450 Cells were seeded into 6-well plates at a density of 500 cells per well. After culturing for 10 to 14  
451 days, cells were fixed with methanol and stained with crystal violet. Colonies comprising >50 cells  
452 were counted.

#### 453 **Anchorage - independent growth assay**

454 Six-well plates coated with a layer of 0.5% agar dissolved in RMPI-1640 medium with 10% FBS  
455 were used for plating of 2,500 cells suspended in RMPI-1640 with 0.35% agarose. After 4 weeks,  
456 the soft agar was stained with crystal violet, and colony numbers were determined under light  
457 microscopy.

#### 458 **Cell motility assessment by migration and invasion assay**

459 The migration and invasion assays were performed using Corning® Transwell. Both chambers  
460 were filled with RMPI-1640 medium, and the lower chamber was supplemented with 10% FBS.

461 For the migration assay,  $5 \times 10^4$  cells were seeded into the upper chamber and allowed to migrate  
462 for 24 hrs. For the invasion assay, the upper chamber was first coated with Matrigel (BD  
463 Pharmingen);  $1 \times 10^5$  cells were seeded and allowed to invade for 24 to 36 hrs. Cells that migrated  
464 or invaded to the lower surface of the transwells were fixed with methanol and stained with crystal  
465 violet. Cell densities were photographically captured in three random fields. The dye on the  
466 transwell membrane was dissolved by 10% acetic acid, transferred to a 96 well plate, and the dye  
467 intensity was measured by a plate spectrophotometer at 570 nm.

#### 468 **Drug sensitivity assays**

469 Drug sensitivity was tested by MTT assays. 6000 cells per well were seeded into 96-well plates  
470 and incubated for 24 hrs at 37°C, followed by exposure to gefitinib (Selleckchem Houston, TX),  
471 paclitaxel (Sigma-Aldrich, St Louis, MO), or cisplatin (Sigma-Aldrich, St Louis, MO) at various  
472 concentrations for 72 hrs with or without CSA (Selleckchem, Houston, TX), NAC or BSO (Sigma-  
473 Aldrich, St Louis, MO). Subsequently, Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-  
474 Aldrich, St Louis, MO) was added and the mixture was incubated at 37°C for 4 hrs. The absorbance  
475 was read at 570 nm using a plate spectrophotometer. The drug response curve was plotted and  $IC_{50}$   
476 was calculated using nonlinear regression model by GraphPad Prism 7.0.

#### 477 **Quantitative PCR (qPCR) analysis**

478 Total RNA was isolated using RNAiso Plus reagent (Takara, Mountain View, CA) and  
479 complementary DNA (cDNA) was generated using PrimeScript RT Reagent Kit (Takara,  
480 Mountain View, CA) according to the manufacturer's instructions. Gene mRNA levels were  
481 analyzed by quantitative RT-PCR (qPCR) (7900HT, Applied Biosystems, Carlsbad, CA) and  
482 SYBR green (Qiagen, Hilden, Germany) detection. Average expression levels of *RPL13A* and

483 *beta-2-microglobulin (B2M)* were used as internal controls. Primers were listed in supplementary  
484 table 2.

#### 485 **Western blot analysis**

486 Cells were harvested and lysed on ice by lysis buffer [50mM Tris HCl pH 7.4, 1% Triton X-100,  
487 1mM EDTA, 150 mM NaCl, 0.1% SDS, with freshly added 1:50 Phosphatase Inhibitor Cocktail  
488 2 (Sigma), 1:50 Protease Inhibitor Cocktail (Sigma)] for 30 min. The cell lysate was then  
489 centrifuged at 13k rpm for 20 min at 4°C to remove cell debris. The protein amount was quantified  
490 by the Dc Protein Assay (Bio-Rad). Cell lysates were resolved by 6-10% SDS-PAGE and then  
491 transferred onto PVDF membranes (Millipore). Primary antibodies including SOX2 (1:1000),  
492 NFATc2 (1:1000),  $\beta$ -catenin (1:1000), non p- $\beta$ -catenin (1:1000), p- $\beta$  catenin (1:1000) or  
493 ACTIN (1:1000) (Cell Signaling, Beverly, MA), respectively, where appropriate, were added.  
494 After overnight incubation, the membrane was washed with PBS and then incubated with the anti-  
495 rabbit secondary antibody. Target proteins on the membrane were visualized on X-ray films using  
496 ECL Plus Western Blotting Detection Reagents (Amersham, Buckinghamshire, UK).

#### 497 **Chromatin immunoprecipitation (ChIP)-qPCR assay**

498 ChIP assay was performed using the Magna ChIP™ A kit (Millipore, Billerica, MA) according to  
499 manufacturer's instructions. Briefly, cells were sonicated and lysed after protein/DNA cross-  
500 linking by 1% formaldehyde for 10 min. The crosslinked complex was immuno-precipitated by  
501 anti-NFATc2 antibody or normal rabbit IgG (Cell Signaling, Beverly, MA) bound to protein A  
502 magnetic beads. After overnight incubation at 4°C, the complex was eluted and DNA was purified.  
503 The immune-precipitated DNA was quantified by qPCR using primer sequences designed to detect  
504 specific regulatory regions listed in Supplementary Table S2.

#### 505 **ChIP-seq assay**

506 ChIP assay was performed using the EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit  
507 (Millipore, 17-10086) according to manufacturer's instructions. Cells were cultivated and treated  
508 with 1% formaldehyde to crosslink the protein and DNA. cell lysate was sonicated to reduce the  
509 DNA length from 100 to 500 bp. The DNA-protein fragments were then incubated with 10ug  
510 SOX2 antibodies (Abcam) and magnetic beads coated with protein A/G to form DNA-protein-  
511 antibody complex. The DNA was isolated and purified by Spin column and sent to the  
512 company(BGI) for library construction and sequencing using Illumina Hi-Seq platforms. Sequence  
513 reads were aligned to Human Reference Genome (hg19) using Bowtie (Langmead, Trapnell et al.  
514 2009). Model based analysis of ChIP-Seq(MACS) was used for peaks identification by comparing  
515 ChIP sample over input sample with default parameters (Zhang, Liu et al. 2008).

#### 516 **NFATc2 binding sites predication**

517 The 5'- and 3'- flanking regions (-5000 to +5000 bp) of SOX2 were scanned for NFAT binding  
518 sequences using PWMSCAN (Levy and Hannehalli 2002). The significance of the predicted sites  
519 was evaluated statistically using a permutation-based method and comparison with occurrence of  
520 the motif in background genomic sequences of intergenic regions. Phylogenetically non-conserved  
521 binding sites were filtered (Li, Sham et al. 2010).

#### 522 **Luciferase reporter assay**

523 Cells were transfected with luciferase reporters, expression plasmids and pRL-TK vector using  
524 lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase activity were measured by using the  
525 Dual-Luciferase Reporter Assay System (Promega).

#### 526 ***In vivo* tumorigenicity**

527 All animal experiments were performed after approval by the Animal Ethics Committee, the  
528 University of Hong Kong according to issued guidelines. Briefly, different numbers of cells mixed

529 with an equal volume of matrigel (BD Pharmingen) were injected subcutaneously at the back of  
530 6-week old severe combined immunodeficiency (SCID) mice or Ncr-nu/nu-nude mice. Tumors  
531 sizes were monitored every 3 days using digital vernier calipers, and tumor volumes were  
532 calculated using the formula [sagittal dimension (mm)  $\times$  cross dimension (mm)<sup>2</sup>] / 2 and expressed  
533 in mm<sup>3</sup>.

#### 534 **Reactive Oxygen species (ROS) measurement**

535 Cells with or without respective treatments were washed with PBS and stained with 1  $\mu$ M the ROS  
536 probe CellROX™ Deep Red (Lift Technologies) for 30 mins according to manufacturer's  
537 instructions. Fluorescence was measured by flow cytometry (FACSCanto II Analyzer, BD  
538 Biosciences) and data were analyzed using FlowJo (Tree star).

#### 539 **Human lung cancers**

540 Surgically resected primary human NSCLC and corresponding normal lung tissues were collected  
541 prospectively in the Queen Mary Hospital, University of Hong Kong. Tissue collection protocols  
542 were approved by the Joint Hospital and University Institutional Review Board and written  
543 informed consents from patients were obtained. Fresh tissues were snap-frozen within 45-60 min  
544 after vascular clamping and kept in -70°C until use. Adjacent tumor tissues were fixed in 4% neural  
545 buffered formalin for 24 hrs and processed into formalin fixed, paraffin embedded (FFPE) tissue  
546 blocks. Tumor classification and differentiation grading was according to the WHO classification  
547 of lung tumors, 2004. Tumor typing and pathological staging was performed by a qualified  
548 anatomical pathologist (MPW). Clinical parameters and outcomes were charted from hospital  
549 records in consultation with relevant clinicians.

#### 550 **Immunohistochemistry (IHC)**



551 Tissue microarrays were constructed using at least 5 cores of tissue from different representative  
552 tumor areas and 1 core of corresponding normal lung from each case. Tumor cores were randomly  
553 arranged in the microarray to prevent positional bias during recording of IHC results. 5um thick  
554 de-paraffinized tissue microarray sections were subjected to antigen retrieval using microwave  
555 heating at 95T°C in 1mM EDTA buffer, pH 8.0. Endogenous peroxidase was quenched with 3%  
556 hydrogen peroxide for 10 minutes. Blocked sections were labeled with primary antibodies against  
557 NFATc2 (1:50 dilution, Cell Signaling), SOX2 (1:200 dilution, Cell Signaling) and ALDH1A1  
558 (1:1000 dilution, Abcam) overnight at 4°C. Anti-rabbit HRP-labeled polymer (DAKO) was used  
559 as a secondary antibody. Color detection was performed by liquid DAB+ substrate chromogen  
560 system (DAKO). Primary antibodies were omitted in control reactions. Protein expression levels  
561 were semi-quantitatively analyzed using an automated image capturing and analysis system  
562 (Aperio).

563 NFATc2 expression level was scored according to the extent and intensity of nuclear staining in  
564 the tumor cells only and expression in the cytoplasm, stromal or inflammatory cells was excluded  
565 from evaluation. The intensity was graded as 1, 2, or 3 according to whether nuclear staining was  
566 absent or weak, moderate, or strong, respectively. The staining extent was graded as 1, 2, or 3  
567 according to whether expression was observed in scattered individual cells, aggregates of 5 or  
568 more but <19 cells, or sheets of 20 or more cells. The products of the 2 grades were then computed,  
569 and cases with scores of 4 and above were counted as high level expression.

## 570 **Statistics**

571 Data were analyzed by SPSS (version 16.0; SPSS Inc., Chicago, IL, USA), GraphPad Prism 7.0  
572 or Excel (Microsoft, Redmond, WA, USA) software packages and shown as mean  $\pm$  standard  
573 deviations (s.d.). Differential expression between paired tumor/normal tissues were analyzed by

574 Wilcoxon test. Differences between groups were analyzed by *t* test for continuous variables.  
575 Differences between growth curve of xenograft model were analyzed by two-way ANOVA.  
576 Correlation between NFATc2 and SOX2 mRNA level were analyzed by Pearson correlation test.  
577 Correlation between NFATc2, SOX2, ALDH1A1 expressions and clinicopathological variables in  
578 lung cancers were analyzed by the  $\chi^2$ -test. Association between NFATc2 expression and overall  
579 survival and recurrence-free survival were analyzed by the Kaplan–Meier method with log-rank  
580 test. Multivariate survival analyses were performed by Cox regression model. Two-sided P values  
581 <0.05 were considered as being statistically significant.

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## 587 **Conflict of interest**

588 We declare there is no conflict of interest amongst any of the listed authors, or between any author  
589 and the sponsoring institutions or other parties.

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### 739 **Figure legends**

#### 740 **Figure 1 NFATc2 overexpressed in human NSCLC**

741 (A) NFATc2 expression analyzed by qPCR human NSCLC and corresponding normal lung. P:  
742 Wilcoxon test. P= 0.0003 (B-C) NFATc2 expression in lung cancers was analyzed by IHC, with  
743 representative views of high NFATc2 score featuring strong nuclear staining in the majority of  
744 cancer cells shown in (B), and low NFATc2 score featuring weak nuclear staining in scattered  
745 cancer cells shown in (C), respectively. Scale bars, 50  $\mu$ M. (D) NFATc2 expression in normal  
746 bronchial epithelium by IHC. Nuclear NFATc2 was expressed in the reserve cell layer of normal  
747 bronchial epithelium. Scale bars, 50  $\mu$ M. (E-F) Log-rank tests and Kaplan Meier survival curves  
748 for recurrence-free survival (RFS) (E) and overall survival (OS) (F) on 102 resected primary  
749 NSCLC stratified by NFATc2 expression score.

750 Figure1-source data 1: Statistical analyses for figure 1A.

#### 751 **Figure 2 NFATc2 regulated lung TIC phenotypes**

752 (A) Expression of NFATc2 analyzed by Western blot in TIC isolated by tumorspheres compared  
753 to the corresponding monolayer controls. (B) Expression of NFATc2 analyzed by Western blot in  
754 cells with stable NFATc2 knockdown, overexpression, or knockout, respectively. (C-E)



755 Tumorsphere formation and serial passage assays in HCC827 cells after stable NFATc2  
756 knockdown (C) or knockout (D), or in A549 cells with stable NFATc2 over-expression (E)  
757 compared to controls. (F-G) Cell migration and invasion assays in cells with stable NFATc2 knock  
758 down (F) or over-expression (G). C-G: \* $p < 0.05$  \*\* $p < 0.01$ , comparison with control by t-test. Error  
759 bar indicates the mean  $\pm$  SD for at least three independent replicates. (H-I)  $1 \times 10^4$  of HCC827 cells  
760 (H) and A549 cells (I) were subcutaneously inoculated into the flanks of SCID mice, and tumor  
761 volumes were monitored. Representative tumor images and tumor growth curves are shown.  
762 \*\* $p < 0.0001$ , comparison with respective control by two-way ANOVA. Error bar indicates the  
763 mean  $\pm$  SD of tumor volumes of mice as indicated. (J-K) Limiting dilution assay *in vivo*. Indicated  
764 numbers of HCC827 cells (J) and A549 cells (K) were subcutaneously inoculated into SCID mice,  
765 and the tumor incidence and latency were monitored for 3 months. The TIC frequency and P values  
766 were calculated using the L-Calc software (Stemcell Tech, Vancouver, Canada,  
767 <http://www.stemcell.com>).

768 The following figure supplements and source data are available for figure 2:

769 Figure 2-figure supplement 1: NFATc2 was up-regulated in tumorspheres.

770 Figure 2-figure supplement 2: NFATc2 regulated *in vitro* TIC properties.

771 Figure 2-figure supplement 3: NFATc2 regulated *in vivo* tumorigenesis.

772 Figure 2-source data 1: Statistical analyses for figure 2H and I, and figure supplement 3A.

### 773 **Figure 3 NFATc2 promoted cancer resistance to cytotoxic and targeted therapy**

774 (A-B) Cisplatin response analyzed by MTT assay in PDCL#24 cells with NFATc2 knockdown  
775 (A) and A549 cells with NFATc2 overexpression (B). \* $p < 0.05$ , \*\* $p < 0.01$  versus control by t-test.  
776 Error bar indicates the mean  $\pm$  SD for at least three independent replicates. (C) *In vivo* cisplatin  
777 response analysis of PDCL#24 xenografts. Nude mice bearing subcutaneous xenografts were

778 randomly separated into two groups and treated with intraperitoneal injections of cisplatin (4mg/kg  
779 every three days) or saline control, respectively. Xenografts were photographed and histograms of  
780 tumor volumes were compared to vector and no-treatment controls.  $**p < 0.0001$  versus control  
781 vehicle by two-way ANOVA. Error bar indicates the mean  $\pm$  SD of tumor volumes of five mice  
782 for Sh-Ctrl vehicle and Sh-NFATc2-A vehicle groups (1 mouse from each group failed to develop  
783 tumor) and six mice for other groups. **(D-E)** Expression of NFATc2 analyzed by Western blot (D),  
784 and cisplatin sensitivity analyzed by MTT assay (E) in A549 and A549 CR cells with or without  
785 NFATc2 knockdown. **(F-G)** MTT assay for HCC827 cells with NFATc2 knockdown (F), or  
786 knockout (G), treated with gefitinib for 72 hrs. E-G:  $**p < 0.01$  versus control by t-test. Error bar  
787 indicates the mean  $\pm$  SD for at least three independent replicates. **(H)** Effects of NFATc2 stable  
788 knockdown on *in vivo* response of HCC827 xenografts to gefitinib. Nude mice bearing subcutaneous  
789 xenografts were randomly separated into two groups and treated with gefitinib (25mg/kg/day by  
790 oral gavage) or 1% Tween 80 as control. Xenografts were harvested.  $**p < 0.0001$  versus control  
791 vehicle by two-way ANOVA. Error bar indicates the mean  $\pm$  SD of tumor volumes of six mice.

792 The following figure supplements and source data are available for figure 3:

793 Figure 3-figure supplement 1: NFATc2 promoted cancer resistance to cisplatin treatment.

794 Figure 3-figure supplement 2: NFATc2 promoted cancer resistance to paclitaxel treatment.

795 Figure 3-figure supplement 3: NFATc2 promoted cancer resistance to gefitinib treatment

796 Figure 3-source data 1: Statistical analyses for figure 3C and H.

#### 797 **Figure 4 NFATc2 regulated tumor function through trans-activating SOX2 expression**

798 **(A-B)** Pluripotency genes expressions analyzed by qPCR indicated cells. **(C)** Effects of stable  
799 NFATc2 knock-down, overexpression, or knockout on SOX2 expression in lung cancer cells by  
800 Western blot analysis. **(D)** Genome browser view of NFAT binding sites (site 1 to 5 shown as



801 black curve) on SOX2 regulatory region 2 and 3 with H3K27Ac mark in A549 cells (shown in  
802 black). **(E)** Promoter activities of site 1-5 by dual luciferase reporter assays in H441 cells. **(F)**  
803 Effect of transient NFATc2 over-expression on transcriptional activities of indicated reporters in  
804 H441 cells. **(G)** Site-directed mutagenesis of NFAT binding sequences in indicated NFAT  
805 reporters was performed. Reporter activity of wild type and the corresponding mutant reporters  
806 were studied in H441 cells with transient NFATc2 overexpression. **(H)** Sequence alignment of  
807 site 4 and site 5 sequences in different species. Putative NFAT binding sites are highlighted in  
808 rectangle. Identical sequences were highlighted in gray. **(I)** Confirmation of NFATc2 binding to  
809 SOX2 sites by ChIP-qPCR analysis in A549 cells with stable NFATc2 overexpression. **(J)**  
810 Correlation of immunohistochemical expressions of NFATc2 and SOX2 in 92 moderately to  
811 poorly differentiated human lung adenocarcinoma by  $\chi^2$ -test. Pearson r: Pearson correlation  
812 coefficient. **(L)** *In vivo* tumorigenicity of A549 cells with NFATc2 overexpression and SOX2  
813 knockdown was assessed by subcutaneous inoculation of  $1 \times 10^4$  cells into SCID. Xenograft  
814 formation was monitored by tumor growth curves and tumor sizes.  $**p < 0.0001$  versus control  
815 vehicle by two-way ANOVA. Error bar indicates the mean  $\pm$  SD of tumor volumes of six mice.  
816 **(M)** Effect of NFATc2 knockdown on SOX2 expression in A549 CR cells analyzed by  
817 immunoblot. **(N)** MTT assay of cisplatin sensitivity for NFATc2-overexpressing A549 cells with  
818 stable knockdown of SOX2.

819 For A, B, E-G, I, N  $*p < 0.05$ ,  $**p < 0.01$  versus control by t-test. Error bar indicates the mean  $\pm$  S.D.  
820 for at least three independent replicates.

821 The following figure supplements and source data are available for figure 4:

822 Figure 4-figure supplement 1: Expression of pluripotency factors in tumorspheres.

823 Figure 4-figure supplement 2: NFATc2 regulated SOX2 expression

824 Figure 4-figure supplement 3: NFATc2 regulated SOX2 expression through binding to 3'  
825 regulatory regions.

826 Figure 4-figure supplement 4: NFATc2 regulated tumor function through SOX2.

827 Figure 4-source data 1: Statistical analyses for figure 4K.

828 **Figure 5 ALDH1A1 was a target of NFATc2/SOX2**

829 **(A)** Expression of *NFATc2* and its target *FASL* analyzed by qPCR in ALDH<sup>+</sup>/CD44<sup>+</sup> TIC  
830 population compared to ALDH<sup>-</sup>/CD44<sup>-</sup> population. **(B-C)** Flow cytometry analysis of TIC  
831 proportions by ALDH<sup>+</sup>/CD44<sup>+</sup> markers, in HCC827 cells with NFATc2 knockdown (B), or  
832 knockout (C). **(D-F)** mRNA levels of *ALDH1A1* analyzed by qPCR in cells with NFATc2 up- (D)  
833 or down-regulation (E), or A549 CR cells (F). **(G)** Effects of SOX2 knockdown in NFATc2-  
834 overexpressing cells on ALDH/CD44 distribution by flow cytometry. **(H)** Expression of *SOX2* and  
835 *ALDH1A1* in A549 cells with NFATc2 overexpression and SOX2 knockdown. **(I)** Representative  
836 image of immunohistochemical expression of NFATc2, SOX2 and ALDH1A1 in xenografts  
837 derived from indicated A549 cells. Scale bars, 50  $\mu$ M. **(J)** ChIP-seq genome browser view of  
838 SOX2 peak with SOX2 binding motif (curved arrow) on ALDH1A1 enhancer. The SOX2 peaks  
839 were co-localized with mammalian conservation peaks (blue) and H3K27Ac peak in A549 cells  
840 (black). **(K)** Confirmation of SOX2 binding to ALDH1A1 sites by ChIP-qPCR analysis in  
841 PDCL#24 cells. **(L)** Site 1 and 2 reporters were co-transfected with SOX2 overexpressing vectors  
842 and reporter activity was analyzed by dual luciferase reporter assay in A549 cells. **(M-N)** Effects  
843 of transient ALDH1A1 suppression on invasion and migration abilities (M), and cisplatin  
844 sensitivity (N) of indicated cells. **(O)** Correlation between ALDH1A1 and SOX2 expressions by  
845 IHC in human lung adenocarcinomas by  $\chi^2$ -test. \*P<0.05, \*\*p<0.01 versus control by t-test. Error  
846 bar indicates the mean  $\pm$  S.D. for at least three independent replicates.

847 The following figure supplements and source data are available for figure 5:

848 Figure 5-figure supplement 1: NFATc2 regulated the ALDH+ population.

849 Figure 5-figure supplement 2: NFATc2 regulated ALDH1A1 expression.

850 Figure 5-figure supplement 3: Effect of siALDH1A1 on ALDH1A1 expression.

851 Figure 5-figure supplement 4: Effect of NFATc2/SOX2 on  $\beta$ -catenin activity.

852 Figure 5-source data 1: Statistical analyses for figure 5B, C and G.

### 853 **Figure 6 NFATc2 regulated TIC properties through ROS suppression**

854 **(A)** ROS levels detected by flow cytometry in A549 and A549 CR cells. **(B-C)** ROS levels in  
855 HCC827 cells **(B)** and PDCL#24 cells **(C)** with or without NFATc2 stable knockdown. **(D)** ROS  
856 levels in HCC827 cells with or without NFATc2 knockout. **(E-F)** Cisplatin sensitivity expressed  
857 as IC<sub>50</sub> by MTT assays of NFATc2-silenced PDCL#24 cells treated with increasing doses of NAC  
858 **(E)**, or NFATc2-overexpressing A549 cells treated with the oxidizing agent (BSO) **(F)**,  
859 respectively. \* p<0.05, \*\* p<0.01 versus vector control without REDOX reagents; ### p<0.01 versus  
860 the corresponding treatment control; t-test. Error bar indicates the mean  $\pm$  S.D. for three  
861 independent replicates. **(G-H)** Effects of increasing doses of NAC on tumorsphere formation  
862 ability of HCC827 **(G)** cells and PDCL#24 cells **(H)**. \* p<0.05, \*\* p<0.01 versus corresponding  
863 treatment controls, ### p<0.01 versus vector control, by t-test. Error bar indicates the mean  $\pm$  S.D.  
864 for three independent replicates. **(I)** Effects of increasing doses of NAC on cell migration and  
865 invasion ability of HCC827 cells with NFATc2 knockdown. **(J-K)** ROS levels in NFATc2  
866 overexpressing A549 cells with stable SOX2 **(J)** or transient ALDH1A1 **(K)** knockdown.  
867 For A-D, I-K \*p<0.05, \*\*p<0.01 versus respective control by t-test. Error bar indicates the mean  
868  $\pm$  S.D. for at least three independent replicates.

869 Figure 6-source data 1: Statistical analyses for figure 5A-D, J and K.

**Table 1.**

**A. Clinico-Pathological correlation of NFATc2 in NSCLC patients**

Clinico-Pathological Variables	NFATc2		P value
	Low	High	
<b>Gender</b>			
Female	22	11	0.328
Male	39	30	
<b>Age (Years)</b>			
≤65	36	24	0.961
>65	25	17	
<b>Smoking History</b>			
Non-smoker	32	24	0.545
Smoker	29	17	
<b>Differentiation</b>			
Well to moderate	45	21	0.019*
Poor	16	20	
<b>Histologic type</b>			
Adenocarcinoma	42	24	0.357
Squamous cell carcinoma	11	10	
Others	8	7	
<b>Tumor Stage</b>			
T1-T2	53	24	0.001*
T3-T4	8	17	
<b>Lymph node metastasis</b>			
Absent	43	25	0.318
Present	18	16	
<b>Pathological (TNM) stage</b>			
Stage I	36	14	0.014*
Stage II-IV	25	27	

Statistical tests:  $\chi^2$ ; \*: P<0.05

**B. Multivariate COX regression analysis for RFS**

Variables	P value	Hazard Ratio (HR)	95.0% CI <sup>a</sup> of HR
NFATc2	0.037	1.905	1.039 - 3.494
TNM stage	0.001	2.035	1.347 - 3.075

a: Confidence Interval

Statistics: COX regression analysis

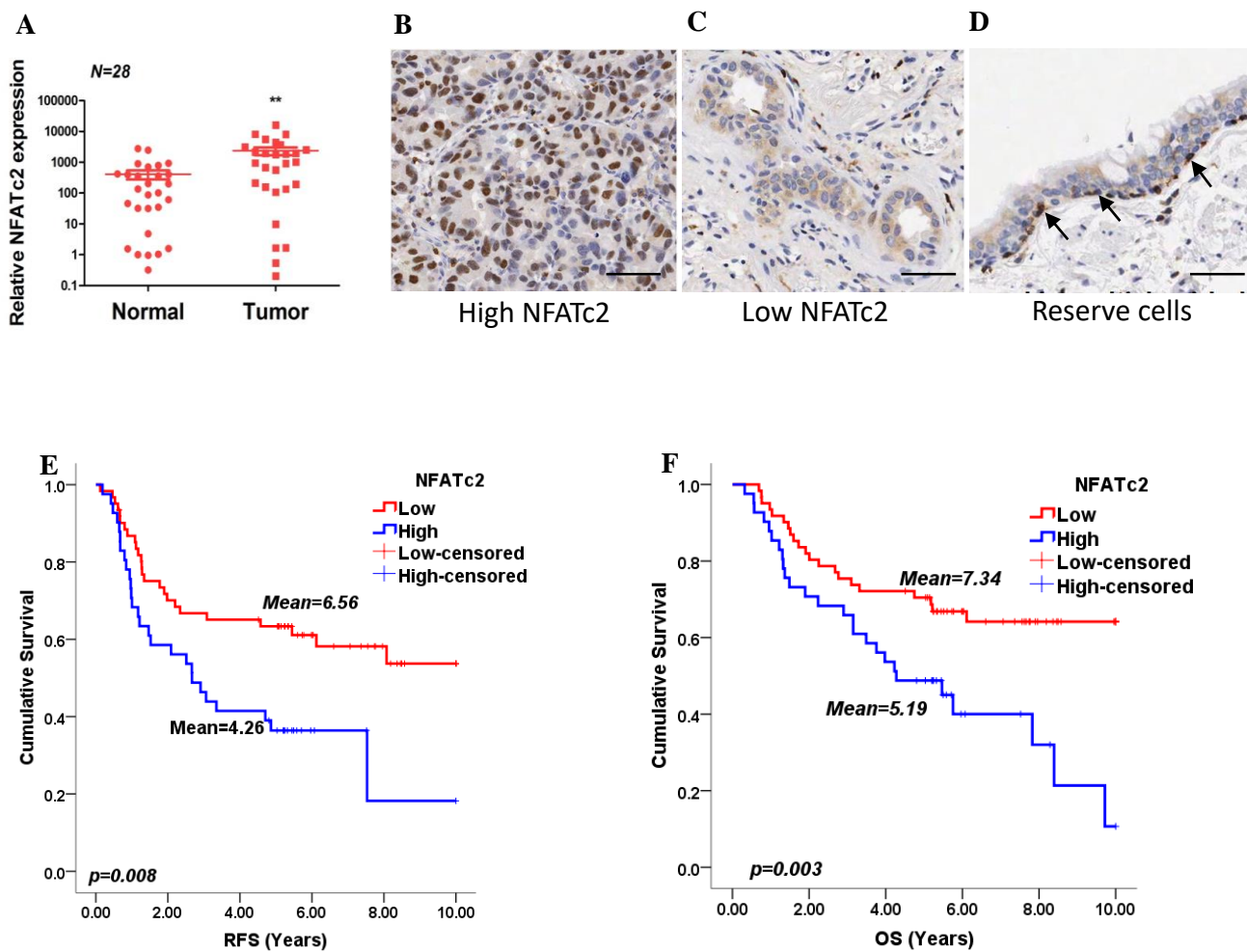
### C. Multivariate COX regression analysis for OS

<b>Variables</b>	<b>P value</b>	<b>Hazard Ratio (HR)</b>	<b>95.0% CI<sup>a</sup> of HR</b>
NFATc2	0.002	2.824	1.462 - 5.457
TNM stage	0.012	1.827	1.140 - 2.927
Age	0.01	2.33	1.224 - 4.432
Smoking history	0.009	2.416	1.251 - 4.665

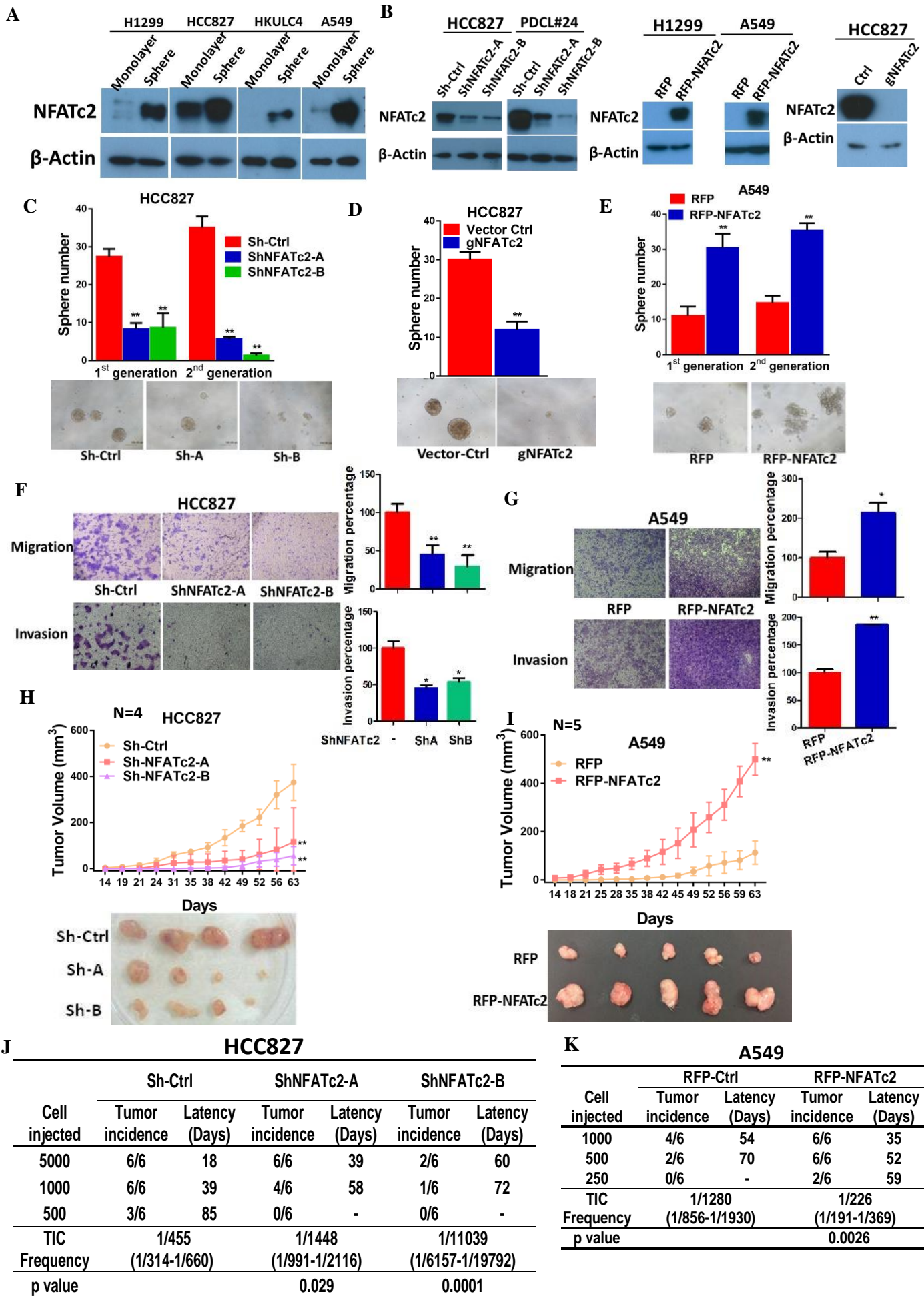
a: Confidence Interval

Statistics: COX regression analysis

**Figure 1.**

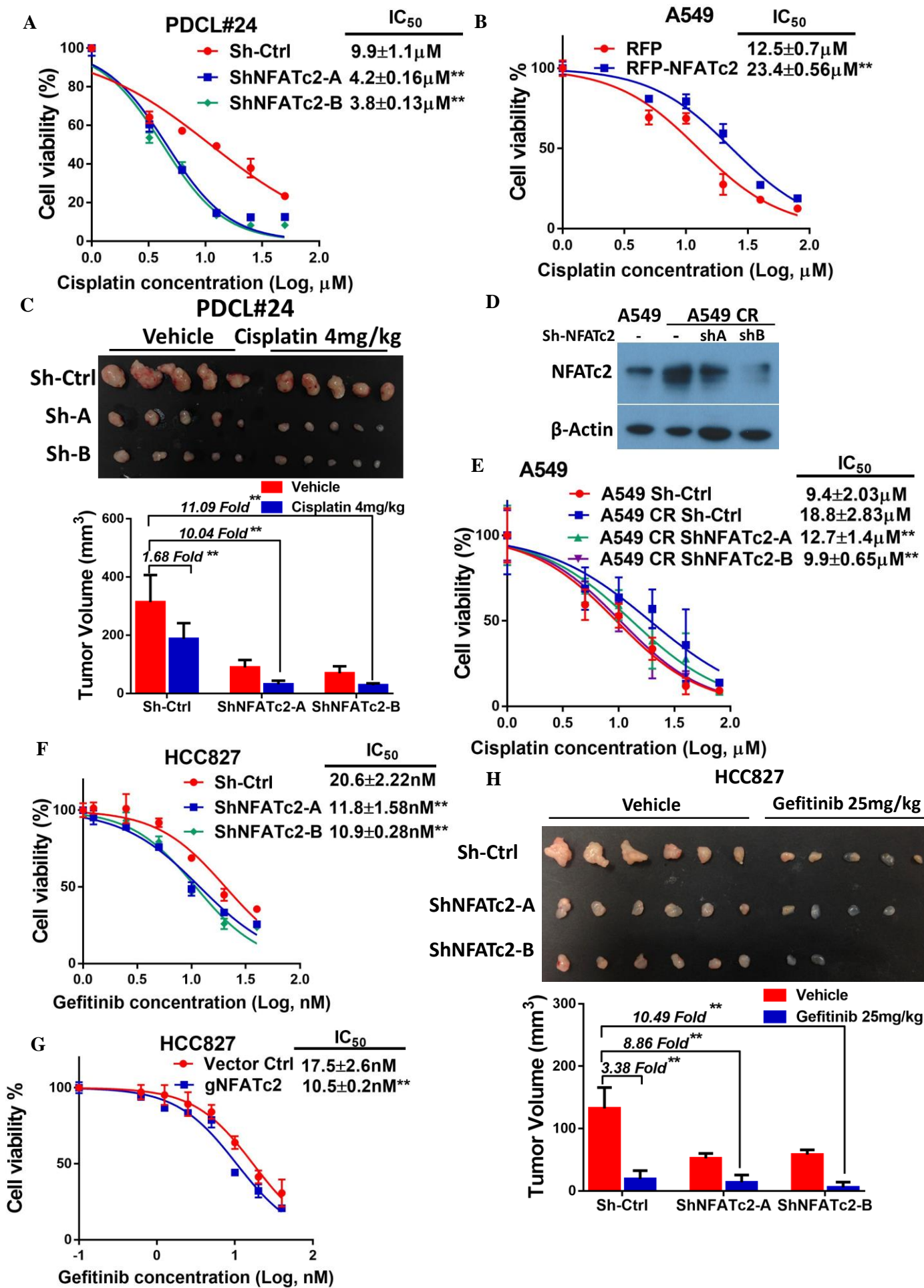


**Figure 2.**

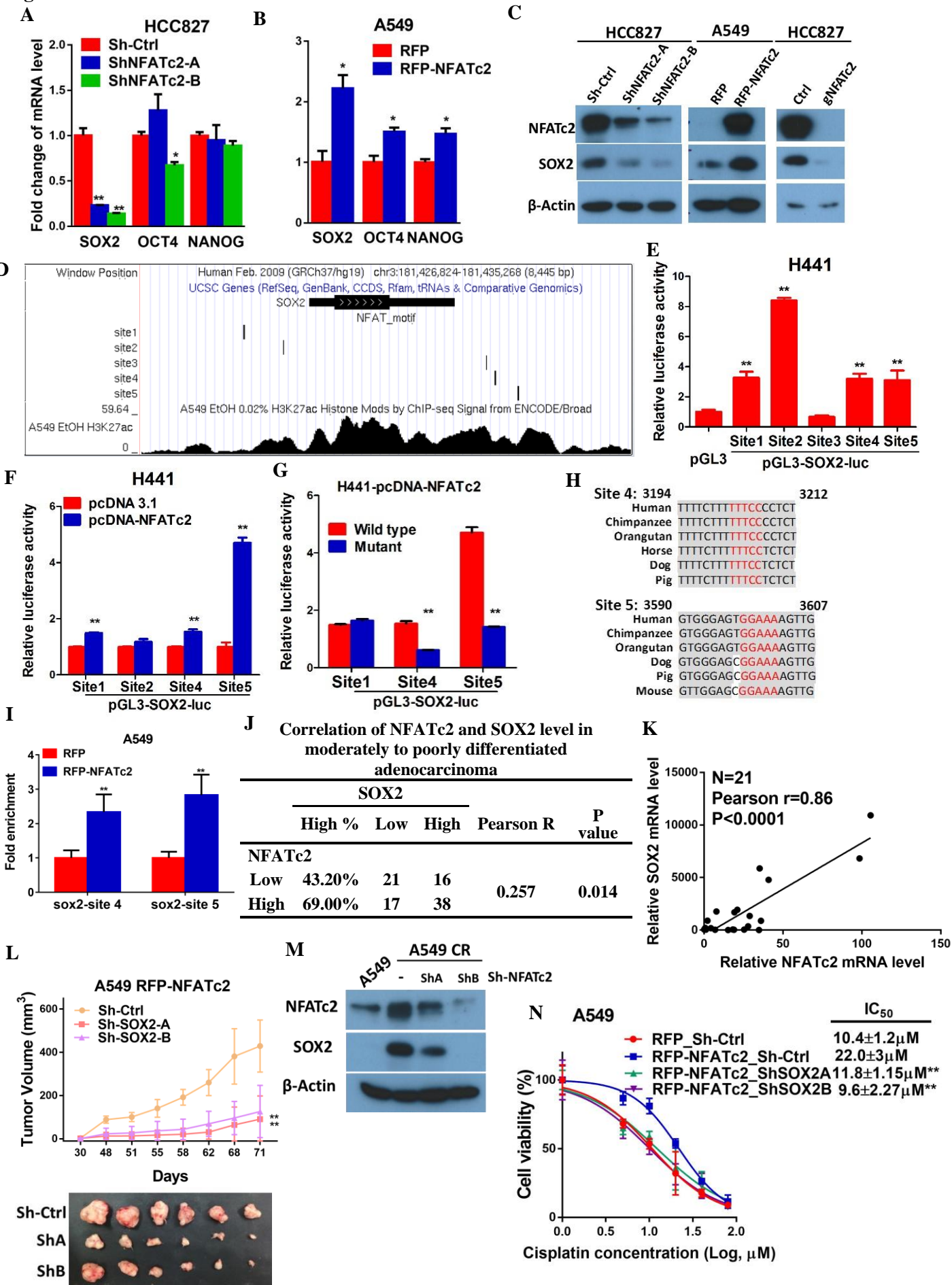




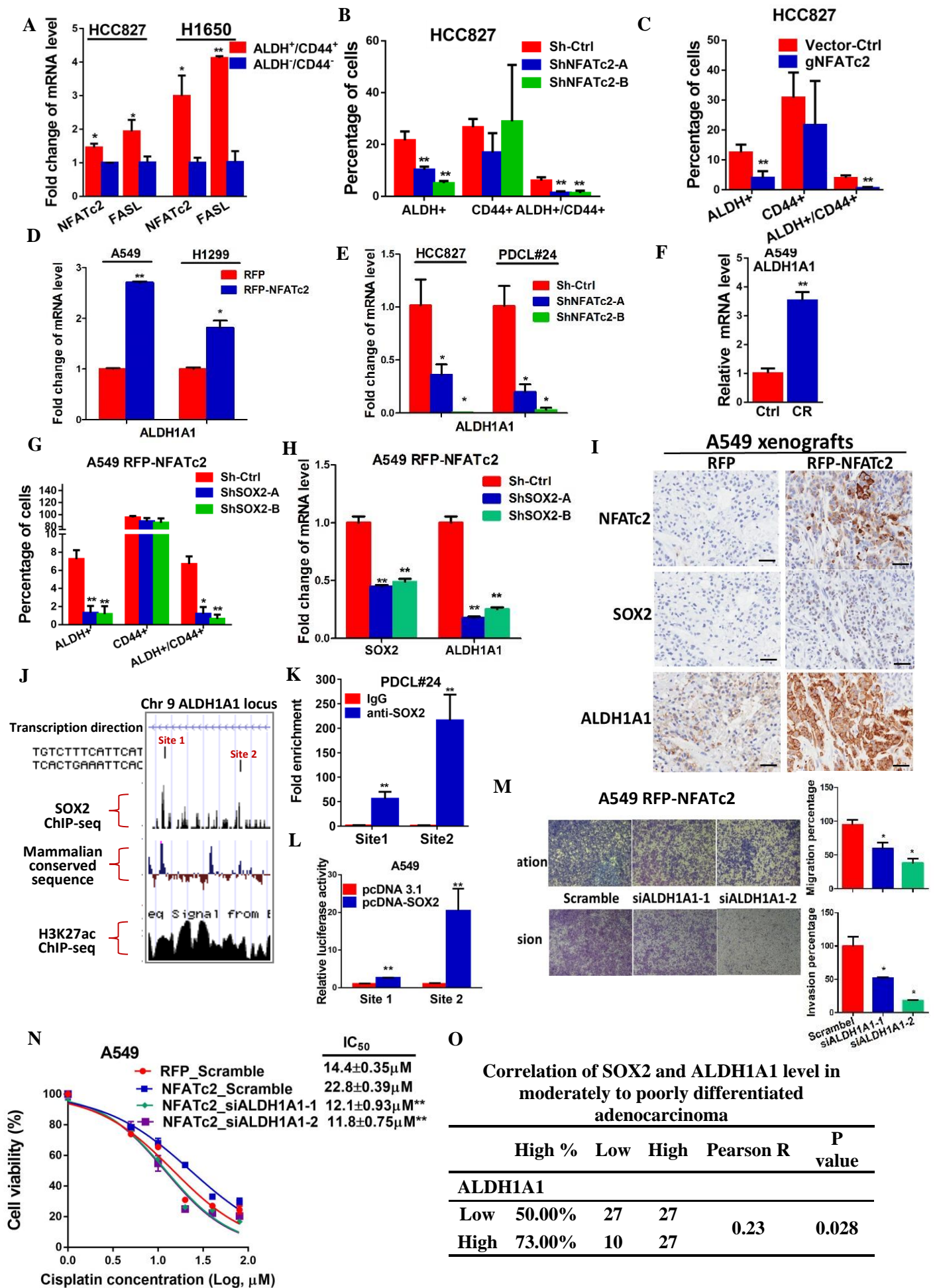
**Figure 3.**



**Figure 4.**

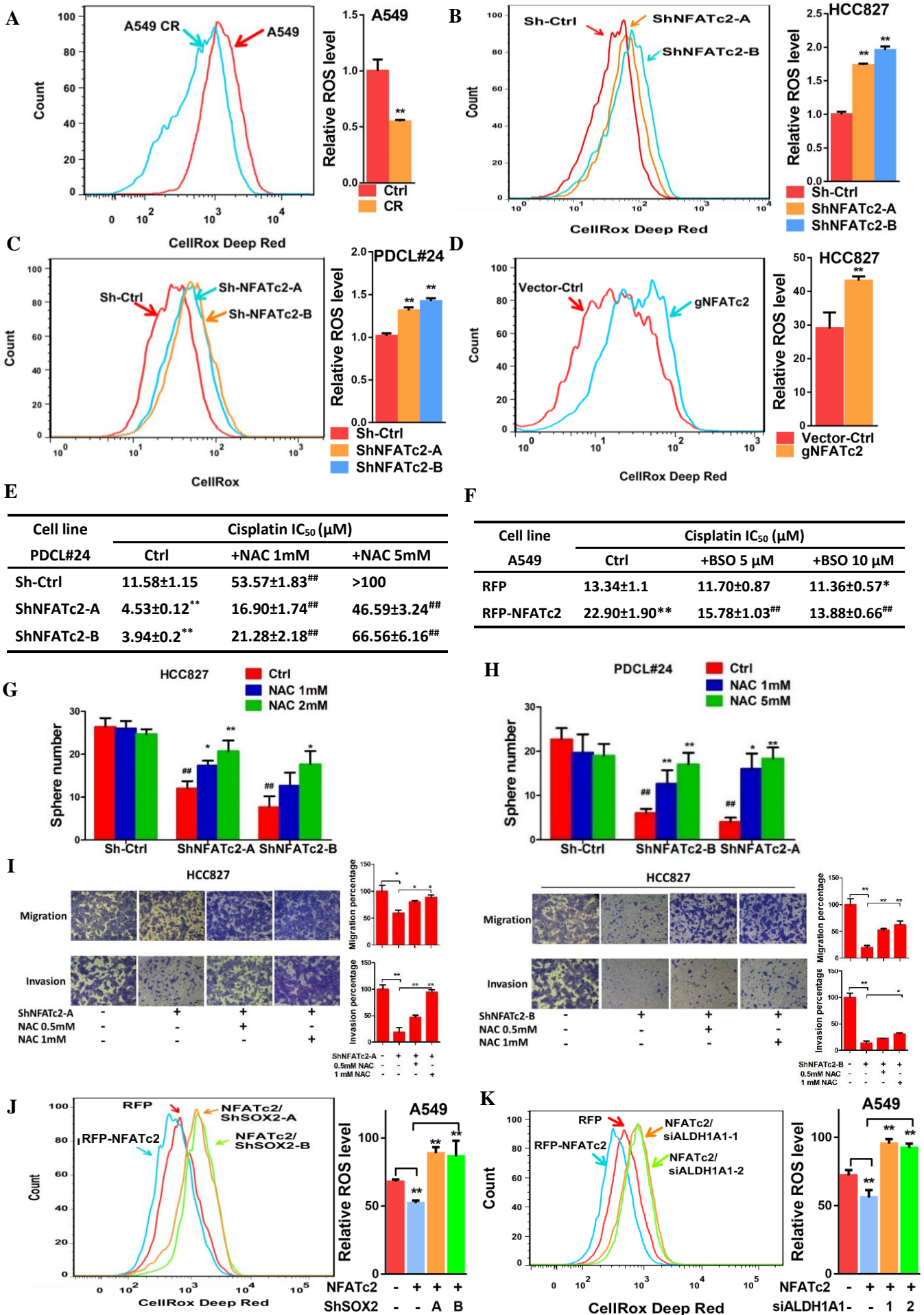


**Figure 5.**





**Figure 6.**



**Figure 2-figure supplement 1.**

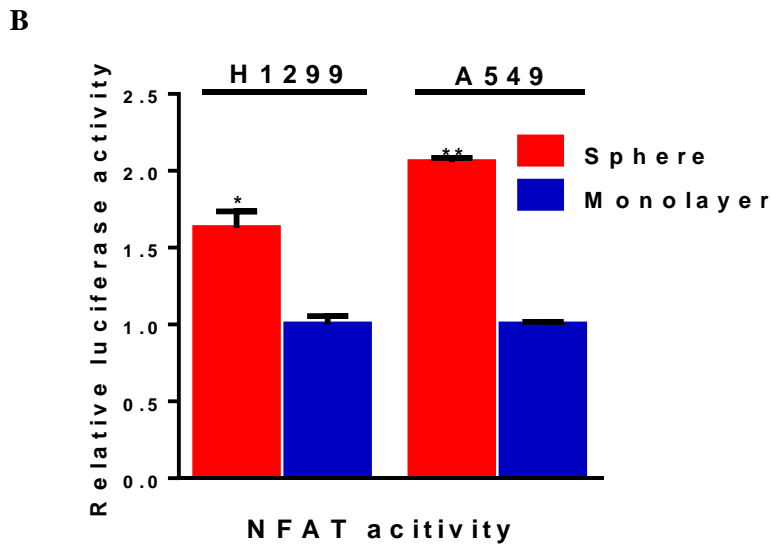
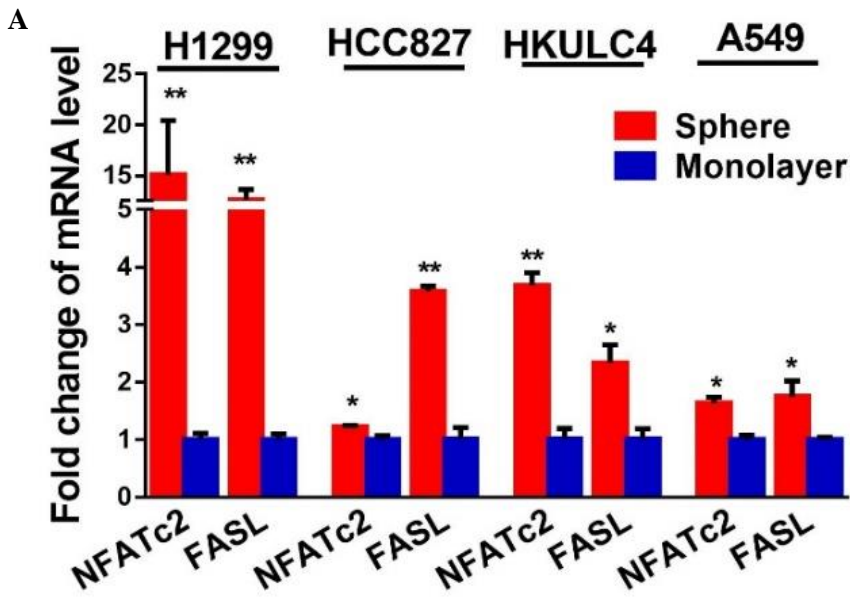


Figure 2-figure supplement 2.

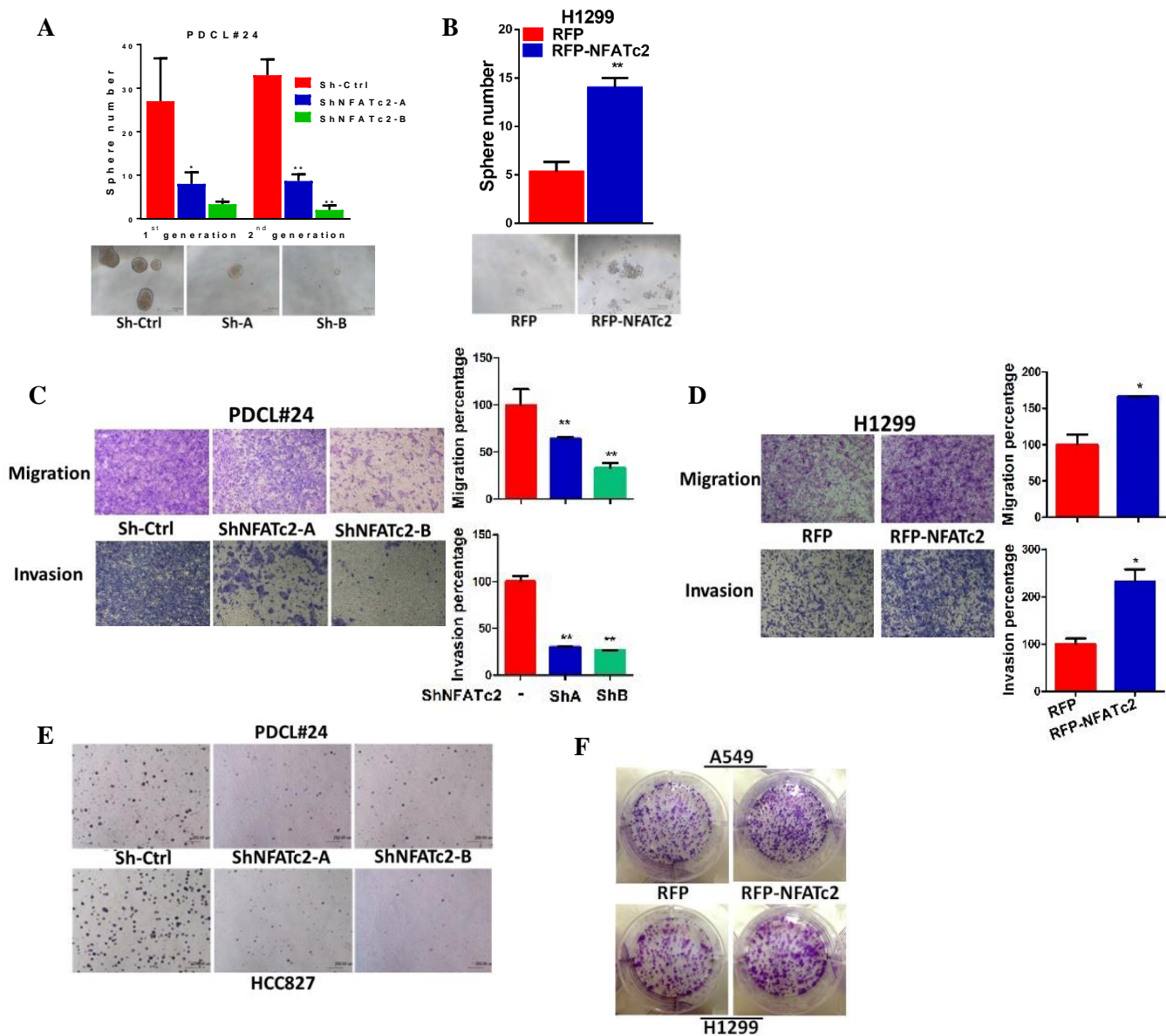


Figure 2-Figure supplement 3.

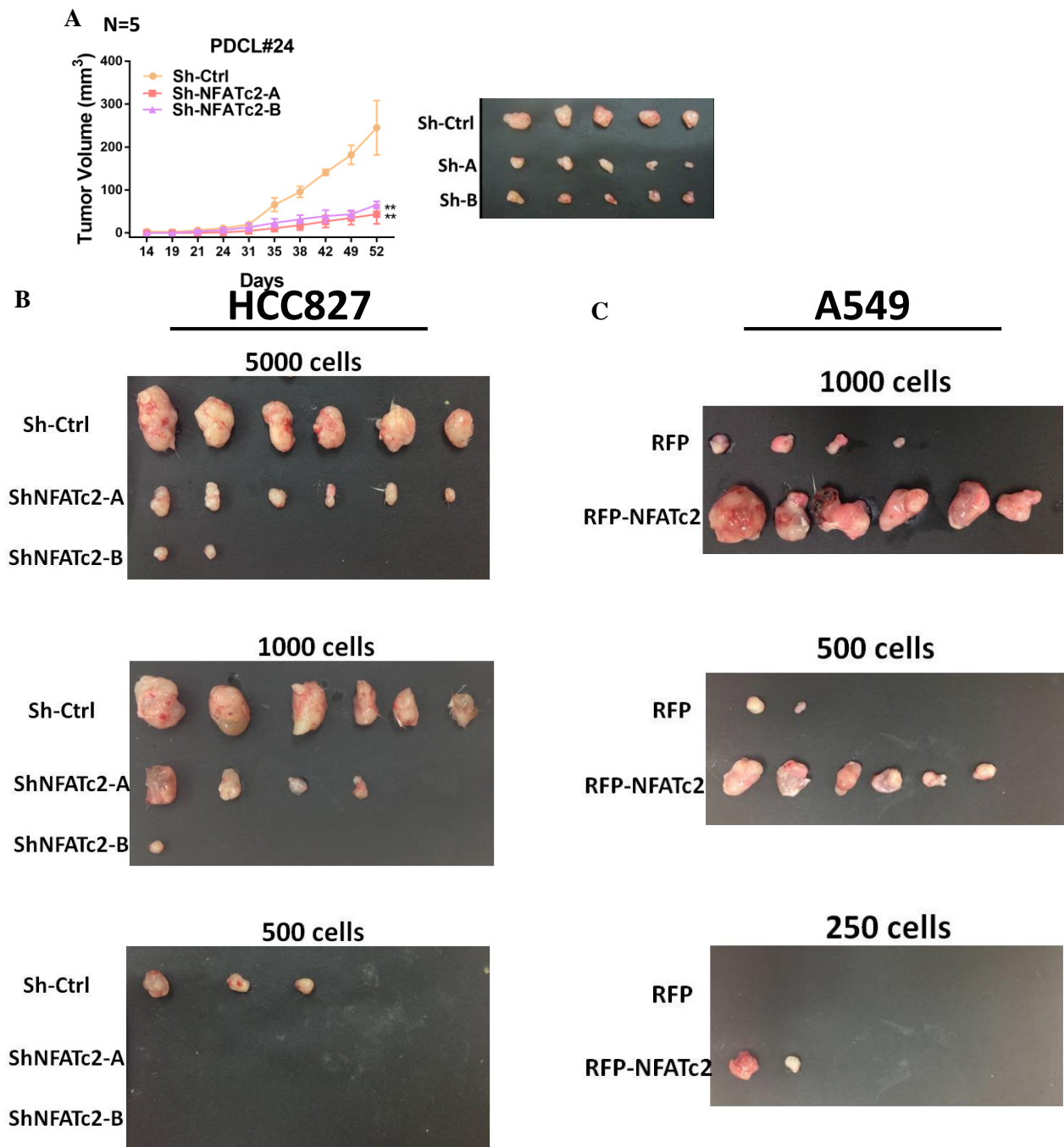
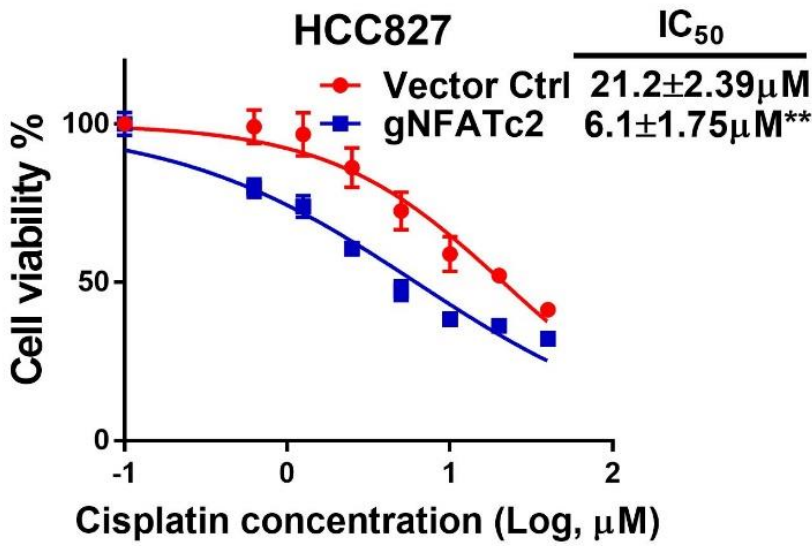




Figure 3-figure supplement 1.

A



B

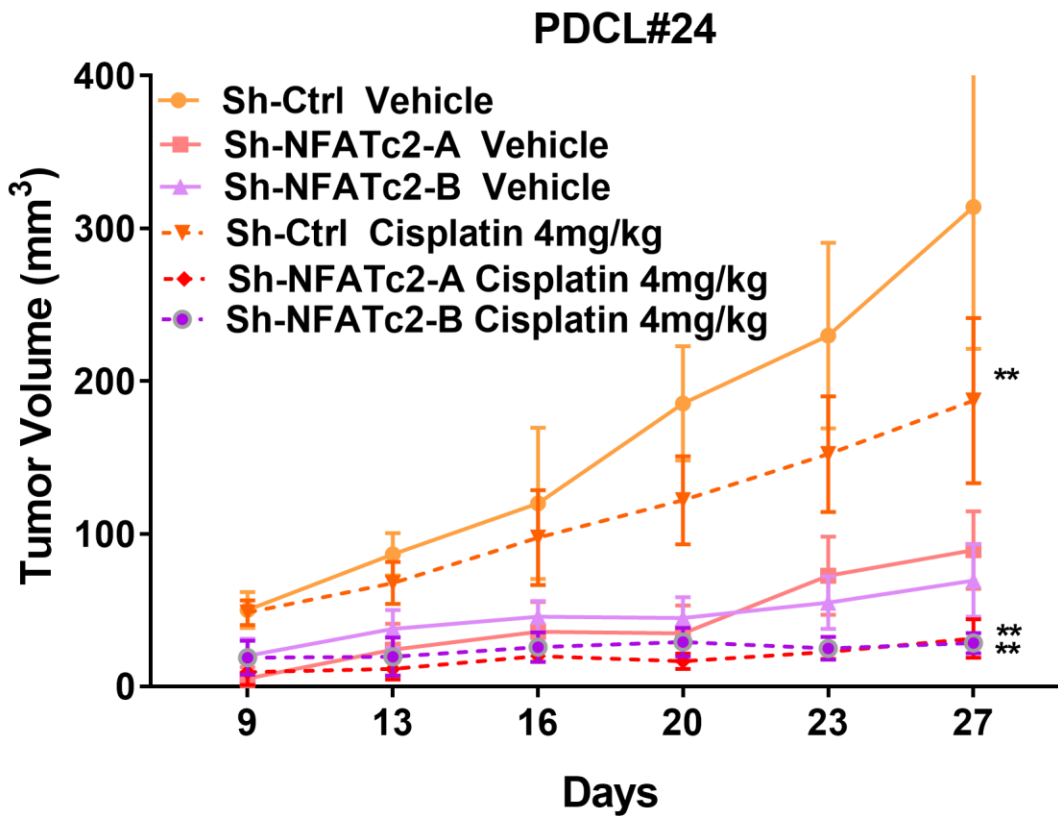


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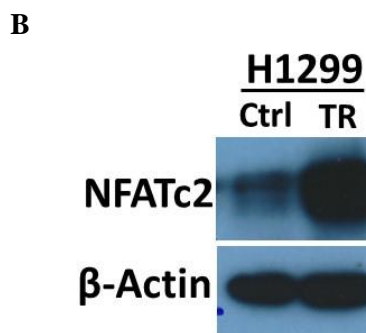
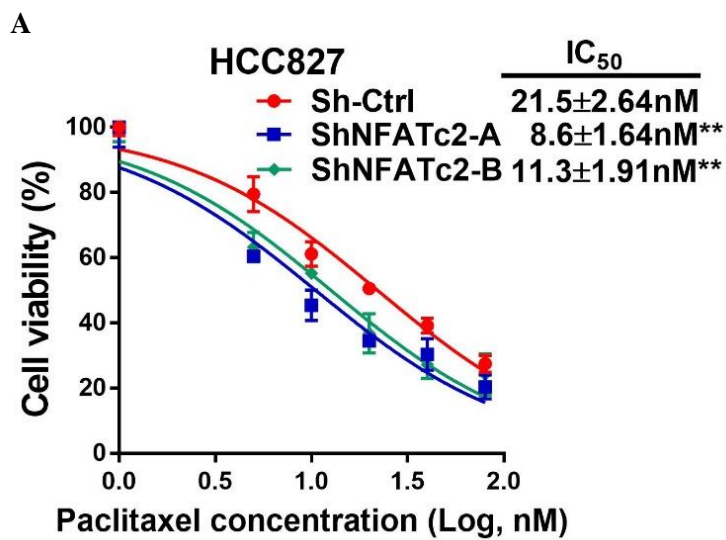


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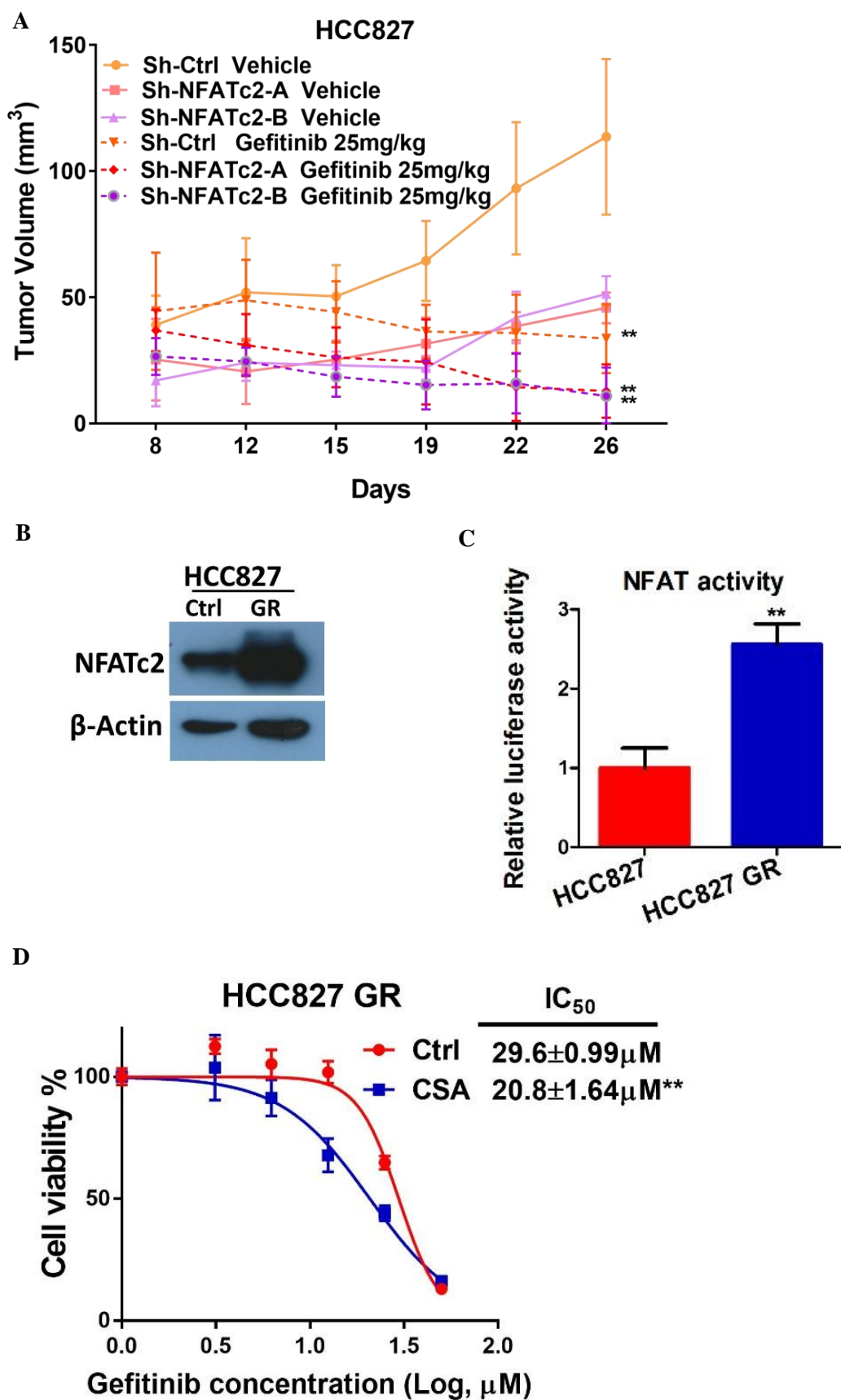
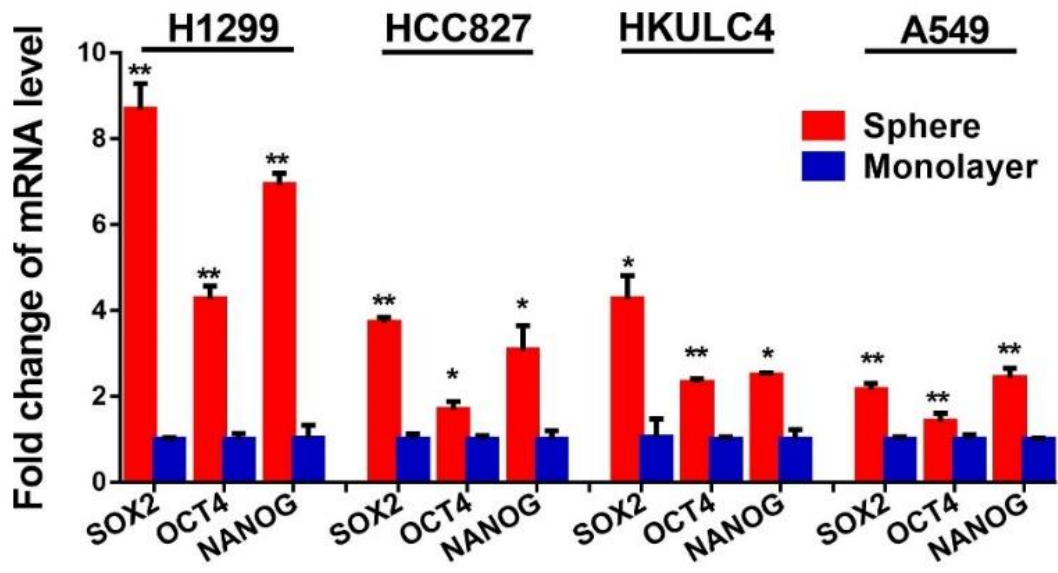
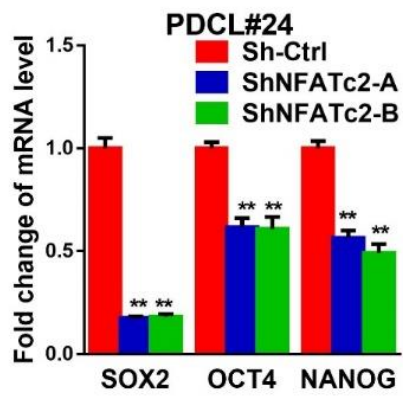


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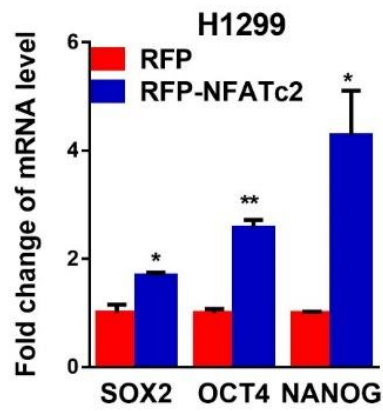


### Figure 4-figure supplement 2.

A



B



C

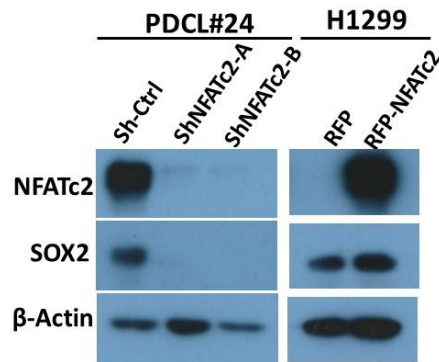
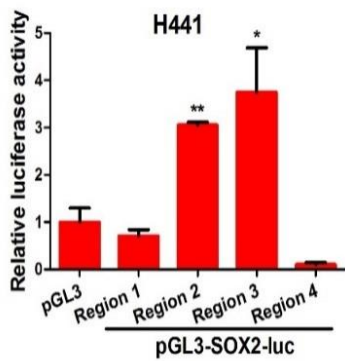


Figure 4-figure supplement 3.

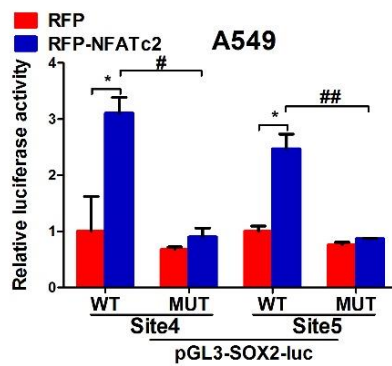
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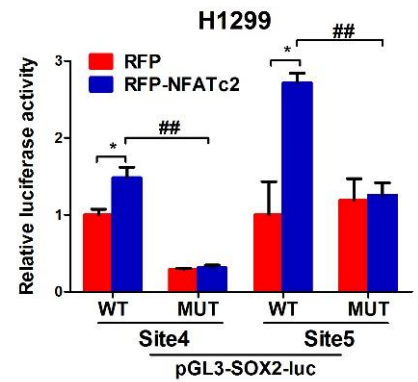
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D



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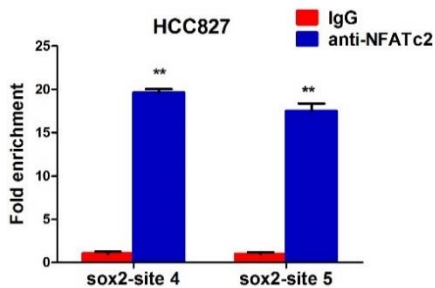
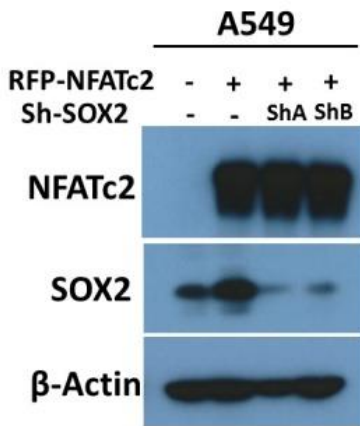
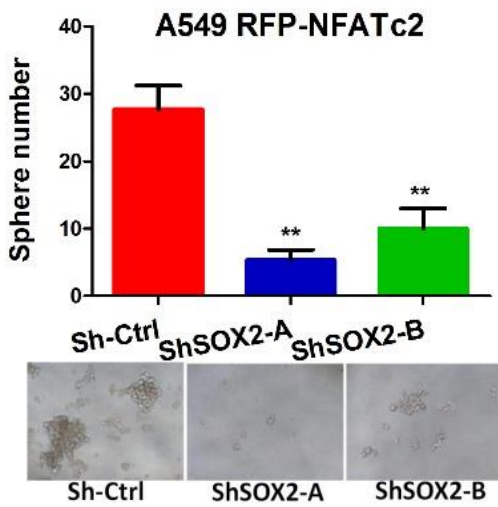


Figure 4-figure supplement 4.

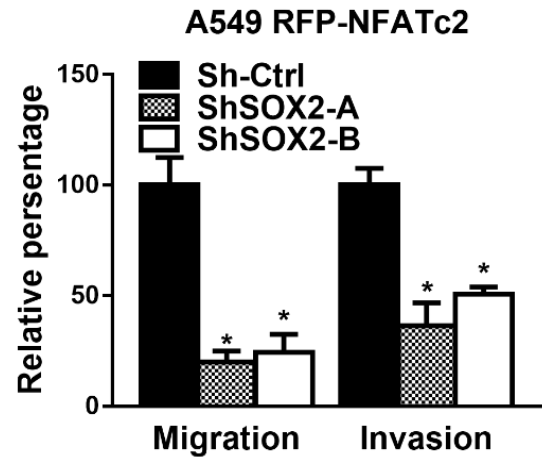
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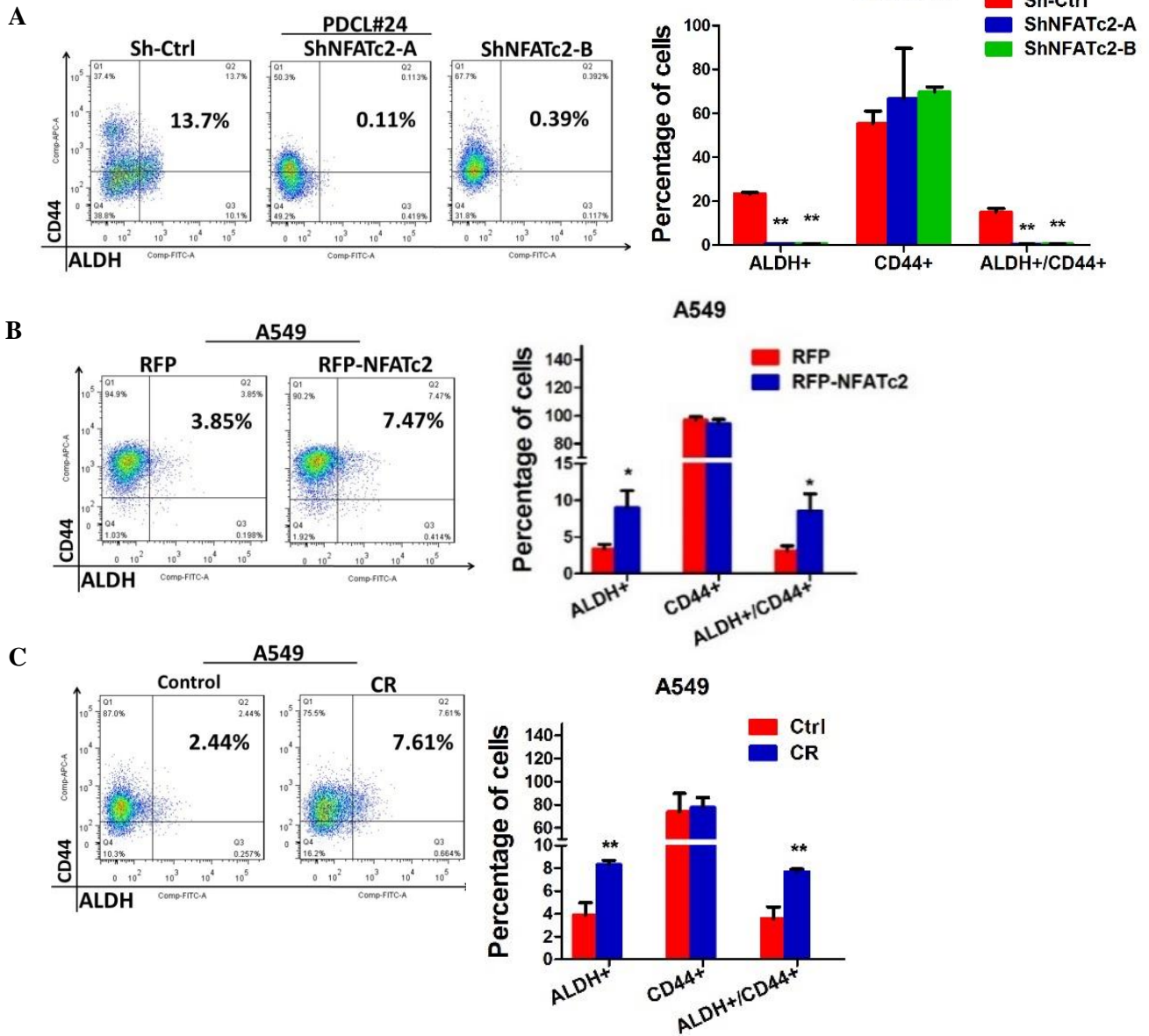


C





**Figure 5-figure supplement 1.**



**Figure 5-figure supplement 2.**

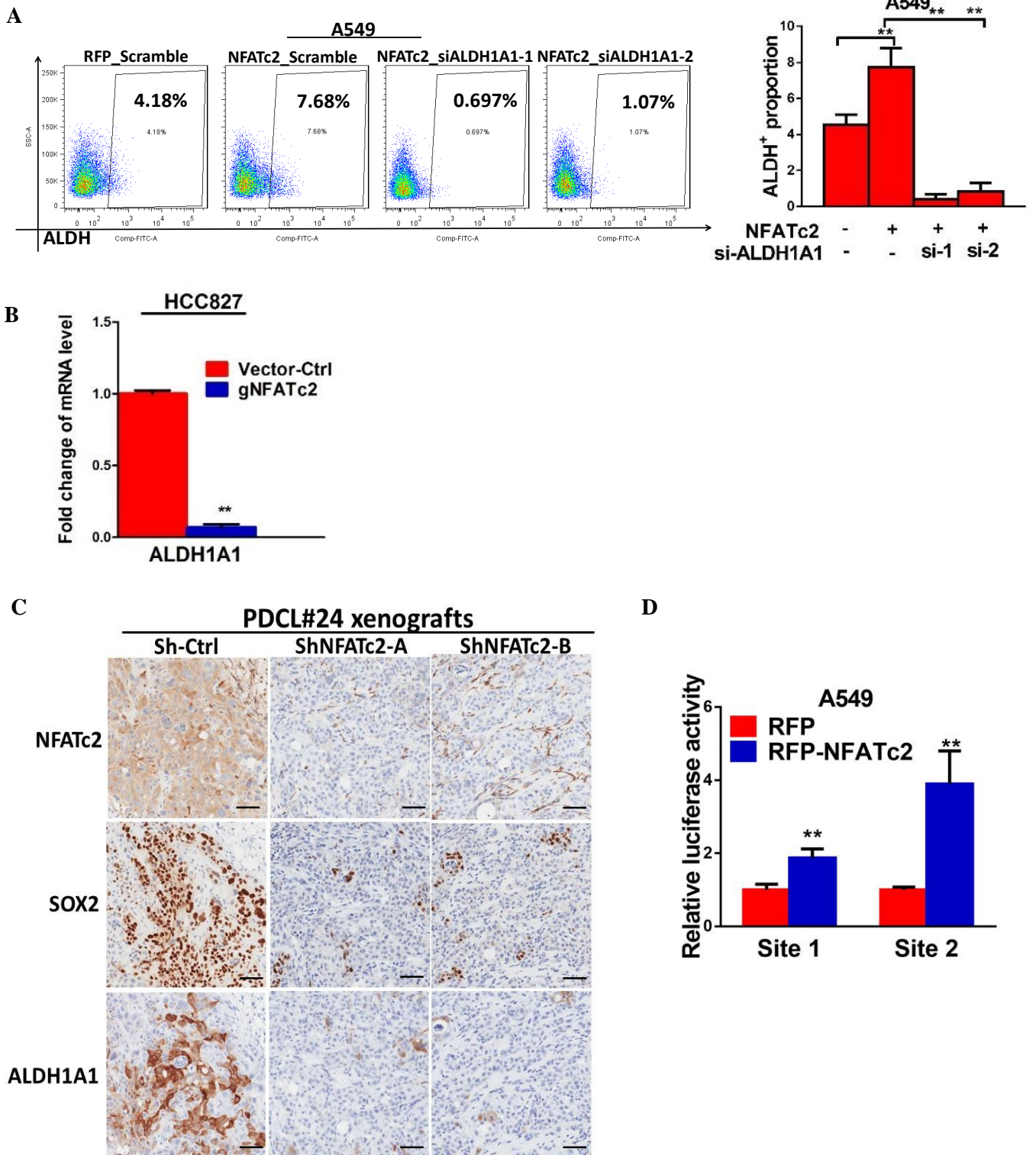


Figure 5-figure supplement 3.

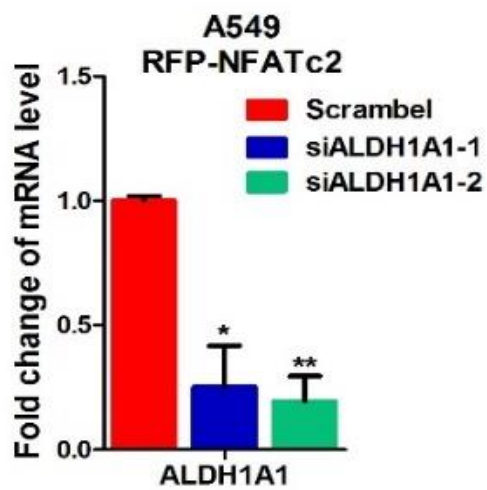
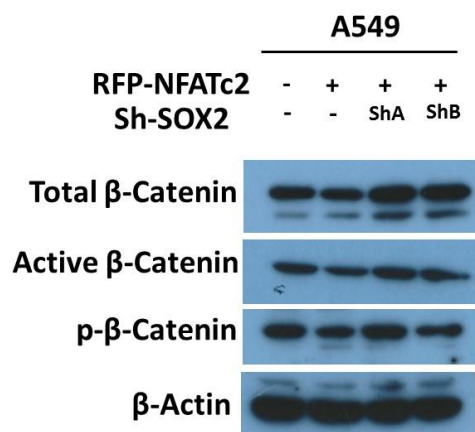


Figure 5-figure supplement 4.



1 **Figure 2-figure supplement 1. NFATc2 was up-regulated in tumorspheres**

2 (A) Expression of *NFATc2* and its target *FASL* analyzed by qPCR in TIC isolated by tumorspheres  
3 compared to controls. (B) NFAT luciferase reporter activity in TIC isolated by tumorspheres  
4 compared to the corresponding monolayer controls. \* $p < 0.05$  \*\* $p < 0.01$ , comparison with control  
5 by t-test. Error bar indicates the mean  $\pm$  SD for three independent replicates.

6

7 **Figure 2-figure supplement 2. NFATc2 regulated *in vitro* TIC properties**

8 (A-B) Tumorsphere formation assays in PDCL#24 cells after stable NFATc2 knockdown (A), or  
9 in H1299 cells with stable NFATc2 over-expression (B) compared to controls. (C-D) Cell  
10 migration and invasion assays in cells with stable NFATc2 knock down (C) or over-expression  
11 (D). (E) Anchorage independent growth assays of PDCL#24 and HCC827 cells with NFATc2  
12 knockdown. (F) Colony formation assay in A549 and H1299 cells with or without NFATc2  
13 overexpression. \* $p < 0.05$  \*\* $p < 0.01$ , comparison with control by t-test. Error bar indicates the mean  
14  $\pm$  SD for at least three independent replicates.

15

16 **Figure 2-figure supplement 3. NFATc2 regulated *in vivo* tumorigenesis**

17 (A) PDCL#24 cells with or without NFATc2 knockdown were subcutaneously inoculated into the  
18 flanks of SCID mice, and tumor volumes were monitored. Representative tumor images and tumor  
19 growth curves are shown. Error bars indicate the means  $\pm$  SD of average tumor volumes of 5 mice.  
20 \*\* $p < 0.0001$ , comparison with control by two-way ANOVA. Error bar indicates the mean  $\pm$  SD for  
21 average tumor volumes of 5 mice. (B) Xenografts of limiting dilution assays of HCC827 cells with  
22 stable NFATc2 knockdown. (C) Xenografts of limiting dilution assays of A549 cells with  
23 NFATc2 over-expression.

24 **Figure 3-figure supplement 1. NFATc2 promoted cancer resistance to cisplatin treatment**

25 (A) Cisplatin sensitivity analyzed by MTT assay in HCC827 cells with NFATc2 knockout.  
26 \*\*p<0.01, comparison with control by t-test. Error bar indicates the mean  $\pm$  SD for three  
27 independent replicates. (B) Growth curve of *in vivo* tumor response to cisplatin of PDCL#24  
28 xenografts with or without NFATc2 knockdown. \*\*p<0.0001 versus control vehicle by two-way  
29 ANOVA. Error bar indicates the mean  $\pm$  SD of tumor volumes five mice for Sh-Ctrl vehicle and  
30 Sh-NFATc2-A vehicle groups (1 mouse from each group failed to develop tumor) and six mice  
31 for other groups.

32

33 **Figure 3-figure supplement 2. NFATc2 promoted cancer resistance to paclitaxel treatment**

34 (A) Paclitaxel sensitivity analyzed by MTT assay in HCC827 cells with NFATc2 knockdown.  
35 \*\*p<0.01, comparison with control by t-test. Error bar indicates the mean  $\pm$  SD for three  
36 independent replicates. (B) NFATc2 expression analyzed by Western blot in H1299 parental and  
37 paclitaxel resistant (TR) cells.

38

39 **Figure 3-figure supplement 3. NFATc2 promoted cancer resistance to gefitinib treatment**

40 (A). Growth curve of *in vivo* tumor response to gefitinib of HCC827 xenografts with or without  
41 NFATc2 knockdown. \*\*p<0.0001 versus control vehicle by two-way ANOVA. Error bar indicates  
42 the mean  $\pm$  SD of tumor volumes of six mice. (B-C) NFATc2 expression by Western blot (B) and  
43 NFAT activity by luciferase reporter assay (C) in HCC827 parental and gefitinib-resistant (GR)  
44 cells. (D) Gefitinib sensitivity with or without CSA treatment analyzed by MTT assay in  
45 HCC827GR cells. \*\*p<0.01 versus control by Student's t-test. Error bar indicates mean  $\pm$  SD for  
46 at least three replicates.

47

48 **Figure 4-figure supplement 1. Expression of pluripotency factors in tumorspheres**

49 Expression of pluripotency factors in tumorspheres was analyzed by qPCR and normalized to  
50 monolayers of indicated cell lines. \* $p < 0.05$ , \*\* $p < 0.01$  versus control by t-test. Error bar indicates  
51 the mean  $\pm$  S.D. for three replicates.

52

53 **Figure 4-figure supplement 2. NFATc2 regulated SOX2 expression**

54 **(A-B)** Expressions of *SOX2*, *OCT4*, *NANOG* analyzed by qPCR in PDCL#24 cells with NFATc2  
55 knockdown (A), or H1299 cells with NFATc2 overexpression (B). \* $p < 0.05$ , \*\* $p < 0.01$  versus  
56 control by t-test. Error bar indicates the mean  $\pm$  S.D. for three replicates. **(C)** Effects of stable  
57 NFATc2 knockdown or upregulation on SOX2 expression in lung cancer cells by Western blot  
58 analysis.

59

60 **Figure 4-figure supplement 3. NFATc2 regulated SOX2 expression through binding to 3'**  
61 **regulatory regions**

62 **(A)** Computational prediction of NFAT binding sites (marked as red curve) on 5' and 3' SOX2  
63 regulatory regions (Regions 1 to 4). TSS: transcription start site. **(B)** Transcriptional activity of  
64 region 1-4 were studied in H441 cells using luciferase reporter assay. **(C-D)** Luciferase reporter  
65 activities of mutant or wild-type SOX2 reporters were analyzed in A549 (C) or H1299 (D) cells  
66 with or without NFATc2 stable overexpression. \* $p < 0.05$ , \*\* $p < 0.01$ , comparison with RFP; #  
67  $p < 0.05$ , ##  $p < 0.01$ , wild type versus mutant in RFP-NFATc2 cells by t-test. Error bars indicate the  
68 mean  $\pm$  SD for at least three independent replicates. **(E)** Confirmation of NFATc2 binding to  
69 candidate SOX2 sites by ChIP-qPCR analysis in HCC827 cells. For B and E, \* $p < 0.05$ , \*\* $p < 0.01$

70 versus control by Student's t-test. Error bars indicate the mean  $\pm$  SD for at least three independent  
71 replicates.

72

#### 73 **Figure 4-figure supplement 4. NFATc2 regulated tumor function through SOX2**

74 (A) Expression of NFATc2 and SOX2 analyzed by Western blot in A549 cells with or without  
75 NFATc2 overexpression and SOX2 stable knockdown. (B-C) Effect of SOX2 knockdown on  
76 tumorsphere formation (B) and cell migration and invasion ability (C) of NFATc2 overexpressing  
77 A549 cells. \* $p < 0.05$ , \*\* $p < 0.01$  versus control by t-test. Error bars indicate the mean  $\pm$  SD for at  
78 least three independent replicates.

79

#### 80 **Figure 5-figure supplement 1. NFATc2 regulated the ALDH<sup>+</sup> population**

81 A-C. Flow cytometry analysis of ALDH/CD44 distribution in PDCL#24 cells with stable NFATc2  
82 knockdown (A), A549 cells with NFATc2 overexpression (B), or A549CR compared to parental  
83 cells (C). \* $p < 0.05$ , \*\* $p < 0.01$  versus control by t-test. Error bars indicate the mean  $\pm$  SD for at least  
84 three independent replicates.

85

#### 86 **Figure 5-figure supplement 2. NFATc2 regulated ALDH1A1 expression**

87 (A) ALDH<sup>+</sup> proportions analyzed by flow cytometry in A549 cells with NFATc2 overexpression  
88 and transient ALDH1A1 knockdown. (B) mRNA level of *ALDH1A1* analyzed by qPCR in  
89 HCC827 cells with NFATc2 knockout. (C) Representative images of immunohistochemical  
90 expression of NFATc2, SOX2 and ALDH1A1 in xenografts derived from PDCL#24 cells with or  
91 without NFATc2 knockdown. Scale bars, 50  $\mu$ M. (D) Luciferase reporter assay for site1 and 2 in



92 A549 cells with or without NFATc2 overexpression. \*\* $p < 0.01$  versus control by t-test. Error bars  
93 indicate the mean  $\pm$  SD for at least three independent replicates.

94

95 **Figure 5-figure supplement 3. Effect of siALDH1A1 on ALDH1A1 expression**

96 Analysis of *ALDH1A1* expression by qPCR in NFATc2-overexpressing A549 cells with  
97 ALDH1A1 knockdown. \*\* $p < 0.01$  versus control by t-test. Error bar indicates the mean  $\pm$  S.D. for  
98 3 replicates.

99

100

101 **Figure 5-figure supplement 4. Effect of NFATc2/SOX2 on  $\beta$ -catenin activity.**

102 Expressions of total  $\beta$ -catenin, active  $\beta$ -catenin (non-phosphorylated), and phosphorylated  $\beta$ -  
103 catenin (p- $\beta$ -catenin) analyzed by immunoblot in A549 with or without NFATc2 overexpression  
104 and SOX2 knockdown.

105