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NNAL, a major metabolite of tobacco-specific carcinogen NNK, promotes lung cancer progression through deactivating LKB1 in an isomer-dependent manner

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20 **Abstract**

21 Smoking is associated with worse clinical outcomes for lung cancer patients. Cell-based studies
22 suggest that NNK (a tobacco specific carcinogen) promotes lung cancer progression. Given its
23 short half-life, the physiological relevance of these *in vitro* results remains elusive. NNAL, a major
24 metabolite of NNK with a similar structure, a chiral center, and a longer half-life, has never been
25 evaluated in cancer cells. In this study, we characterized the effect of NNAL and its enantiomers
26 on cancer progression among a panel of NSCLC cell lines and explored the associated
27 mechanisms. We found that (R)-NNAL promotes cell proliferation, enhances migration, and
28 induces drug resistance while (S)-NNAL has much weaker effects. Mechanistically, (R)-NNAL
29 phosphorylates and deactivates LKB1 via the β -AR signaling in the LKB1 wild type NSCLC cell
30 lines, contributing to the enhanced proliferation, migration, and drug resistance. Of note, NNK
31 exposure also increases the phosphorylation of LKB1 in A/J mice. More importantly, human lung
32 cancer tissues appear to have elevated LKB1 phosphorylation. Our results reveal, for the first time,
33 that NNAL may promote lung cancer progression through LKB1 deactivation in an isomer-
34 dependent manner.

35 1. Introduction

36 Lung cancer has been the leading cause of cancer-related deaths for decades [1-3]. It accounts
37 for one in every five cancer deaths worldwide with about 160,000 deaths annually in the United
38 States. While there are several other factors that may increase lung cancer risk, tobacco smoke is
39 the main etiological factor associated with lung cancer development [4]. It is also associated with
40 worse clinical outcome, including reduced therapeutic efficacy and shorter survival [5, 6]. For
41 instance, the median survival for non-smokers, former smokers, and active smokers among
42 patients with non-small cell lung cancer (NSCLC) is 41.9, 22.6, and 14.7 months respectively [7].
43 However, 30 – 65% of NSCLC patients were active smokers at diagnosis and a significant portion
44 of them continued to smoke (Table 1) [7-13]. It is therefore imperative to understand how tobacco
45 use contributes to the worse clinical outcomes of lung cancer patients.

46 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (commonly known as “NNK”, Fig. 1A) is a
47 tobacco specific lung carcinogen [14]. As a carcinogen, NNK is bioactivated by cytochrome P450
48 enzymes to induce DNA damage followed by subsequent mutations and carcinogenesis [15, 16].
49 NNK also promotes cell proliferation, enhances cell migration, and suppresses apoptosis in various
50 cancer cell lines [17, 18]. Nicotinic acetylcholine receptors (nAChRs) [19] and β -adrenergic
51 receptors (β -ARs) [20] have been suggested as the potential upstream targets of NNK. One key
52 uncertainty of these *in vitro* results is their physiological relevance since NNK has a short-half life
53 *in vivo* [21-24]. Indeed, NNK has never been detected in human biospecimens. Because of this,
54 the metabolites of NNK have been used to investigate its human exposure and carcinogenic risk
55 [25-28].

56 The major metabolite of NNK is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (commonly
57 known as “NNAL”, Fig. 1A), which is formed via carbonyl reduction [29, 30]. Although
58 structurally similar, NNAL has two key differences from NNK. First, NNAL has a much longer
59 half-life *in vivo* relative to NNK [24]. In fact, NNAL is detectable in human urine samples weeks
60 after the last tobacco exposure [31-33]. NNAL is also readily detected in blood samples from
61 smokers [25]. For instance, Church *et al.* profiled serum levels of NNAL among 200 smokers
62 selected from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO). One
63 hundred of these participants eventually developed lung cancer while the rest did not. Although
64 the participants in these two groups were not rigorously matched in terms of age, gender and
65 smoking history, NNAL concentrations were 92.4 ± 40.7 pM in the lung cancer cases and $77.4 \pm$
66 39.3 pM in the control groups. Second, NNAL has a chiral center (Fig. 1A). Its formation from
67 NNK is catalyzed by a range of carbonyl reductases [26] and its elimination is mainly mediated
68 through glucuronidation via UDP-glucuronosyltransferases (UGT) [27, 34]. Because of germline
69 genetic variance in these metabolizing enzymes, smokers have a heterogeneous ratio of (R)-NNAL
70 and (S)-NNAL [35]. These two enantiomers could have distinct biological activities. For instance,
71 (S)-NNAL is much more carcinogenic than (R)-NNAL in A/J mice [34]. With the same dose
72 treatment, (S)-NNAL resulted in much higher levels of DNA damage in the lung tissues and
73 subsequently more lung adenoma formation than (R)-NNAL while (R)-NNAL was more
74 efficiently eliminated via glucuronidation. These data argue that NNAL enantiomers may induce
75 different biological effects and should be characterized as distinct individual entities. To date, the
76 effect of NNAL on transformed lung cancer cells has not been reported. Such knowledge is

77 important to help understand the reason for the worse outcome of lung cancer patients who
78 continue to smoke.

79 In this study, we evaluated the effect of NNAL enantiomers on five human NSCLC cell lines
80 at physiologically relevant concentrations. (R)-NNAL promoted cell proliferation, enhanced cell
81 migration, and induced drug resistance while (S)-NNAL was substantially less effective. The
82 effects of NNAL on cell migration and drug resistance required wild type liver kinase B1 (LKB1).
83 Mechanistically, NNAL exposure, particularly the R enantiomer, led to LKB1 phosphorylation
84 and deactivation through activating β -ARs. LKB1 phosphorylation was also observed in the lung
85 tissues of A/J mice upon NNK exposure. Human lung cancer tissues had substantially higher levels
86 of phosphorylated LKB1 relative to the paired normal lung tissues. In summary, NNAL,
87 particularly (R)-NNAL, deactivates LKB1 through β -ARs in NSCLC cancer cell lines. Such LKB1
88 deactivation confers drug resistance and promotes invasion. In addition, LKB1 loss-of-function
89 human lung cancers may be highly prevalent via phosphorylation due to common tobacco
90 exposure in addition to the mutational deactivation. Overall, our results depict a novel mechanism
91 through which active smoking may contribute to the worse outcome of lung cancer patients.

92

93 **2. Results**

94 **2.1. Detection and quantification of NNK and NNAL in human blood samples from smokers** 95 **and non-smokers**

96 To determine the physiologically relevant compound(s) and concentrations for our studies, we
97 quantified NNK and NNAL in the plasma from smokers (n = 46) and non-smokers (n = 3) via an

98 established liquid chromatography with tandem mass spectrometry (LC-MS/MS) method [36].
99 The smoking status of the plasma donors was confirmed by measuring their urinary total nicotine
100 equivalents (TNE). Some of the results have been published [37, 38]. Consistent with its short
101 half-life, NNK was not detected in any of these samples (Fig. 1B). NNAL was not detectable in
102 the plasma samples from non-smokers (not shown) while it was readily detected in the plasma
103 samples from smokers (Fig. 1B). The plasma concentration of NNAL ranged between 10.4 pM to
104 296.0 pM with a mean value of 59.7 ± 61.1 pM. NNAL was therefore evaluated in our *in vitro*
105 study instead of NNK.

106

107 **2.2. The effects of NNAL enantiomers on cell proliferation, migration and drug resistance in** 108 **NSCLC cancer cells with different LKB1 status**

109 The concentrations of NNAL detected in the plasma samples from smokers were in the
110 picomolar range, consistent with those reported in the literature [25, 39, 40]. NNAL concentrations
111 in the lung are expected to be much higher because of the direct exposure of lung to tobacco smoke.
112 We proposed that NNAL concentrations between 1-100 nM are physiologically relevant, and this
113 range was therefore used in our subsequent *in vitro* studies.

114 NNAL exposure in H1299 and A549 cells resulted in no detectable O^6 -mG (data not shown),
115 suggesting the lack of NNAL bioactivation and associated carcinogenesis. However, NNAL at 10
116 nM significantly increased cell proliferation (Fig. 2A). When the two enantiomers of NNAL were
117 evaluated, (R)-NNAL recapitulated this activity in both cell lines while (S)-NNAL had minimal
118 effects (Fig. 2B). Similarly, (R)-NNAL significantly increased colony formation in H1299 and

119 A549 cells while (S)-NNAL was not effective (Fig. 2C). The potential of NNAL enantiomers on
120 cell migration was evaluated via wound healing assay (Fig. 2D) and trans-well assays (Fig. 2E) in
121 H1299 and A549 cells. In both assays, (R)-NNAL substantially increased cell migration while (S)-
122 NNAL had little effects in H1299 cells. Intriguingly, NNAL treatment had no effects in A549 cells
123 (Fig. 2D and 2E). Lastly, the effect of NNAL on the cytotoxicity of gemcitabine and cisplatin was
124 evaluated via a cell viability assay. (R)-NNAL significantly reduced the cytotoxicity of
125 gemcitabine and cisplatin. (S)-NNAL conferred less resistance compared with (R)-NNAL (Fig.
126 2F). Both NNAL enantiomers failed to induce resistance in A549 cells (Fig. 2F).

127 Although there are many molecular and genetic differences between H1299 and A549 cell
128 lines that could account for the observed differences in cellular migration and drug resistance, we
129 focused on LKB1 because of its importance in lung cancer development [41-48] and its different
130 status in H1299 (wild-type) and A459 (mutational deactivation). Moreover, LKB1 is a potential
131 down-stream target for β -ARs, which NNK has been reported to activate [19, 20]. We therefore
132 evaluated the effect of (R)-NNAL in HCC827 (*LKB1* WT), H1975 (*LKB1* WT) and H460 (*LKB1*
133 mutant) cells. As have been observed in H1299 and A549, (R)-NNAL enhanced cell proliferation
134 in all of these cell lines (Fig. S1). While (R)-NNAL reduced the sensitivity of HCC827 and H1975
135 cells to cisplatin and gemcitabine, it failed to reduce the sensitivity of H460 to these therapies (Fig.
136 S2). Together, these data suggest that the status of *LKB1* in NSCLC cancer cell lines may be critical
137 to the detrimental effects of NNAL, particularly in cell migration and drug resistance.

138

139 **2.3. The role of LKB1 on NNAL-mediated cell migration and drug resistance in NSCLC cells**

140 To characterize the role of LKB1 in mediating the cellular effects of NNAL, an *LKB1*-
141 knockout H1299 cell line was generated using a CRISPR knockout approach (LKB1-KO H1299,
142 Fig. 3A). As expected, (R)-NNAL failed to facilitate cell migration in LKB1-KO H1299 cells (Fig.
143 3B) and did not induce resistance to gemcitabine nor cisplatin (Fig. 3C). Similarly, WT LKB1 was
144 knocked into A549 cells (Fig. 3D). (R)-NNAL was able to promote cell migration (Fig. 3E) and
145 confer drug resistance in A549 cells with LKB1 knock-in (Fig. 3F). Altogether, these data suggest
146 that LKB1 plays a key role in NNAL-mediated migration and drug resistance in *LKB1* WT lung
147 cancer cells and the loss of LKB1 significantly reduced the impact of (R)-NNAL exposure.

148

149 **2.4. The effect of NNAL enantiomers on LKB1 phosphorylation in lung cancer cells**

150 We next characterized the effect of NNAL enantiomers on LKB1 phosphorylation in H1299,
151 H1975, and HCC827 cells. Increased deactivating phosphorylation of LKB1 at Ser428 was
152 observed in all of these cells with greater increase upon the (R)-NNAL exposure than the (S)-
153 enantiomer (Fig. 4A). The time course of LKB1 phosphorylation by (R)-NNAL was characterized
154 in H1299 cells (Fig. 4B). (R)-NNAL treatment also led to a significant reduction in phosphorylated
155 AMPK, and increase in phosphorylated mTOR and 4E-B1 (Fig. 4C). Since AMPK, mTOR and
156 4EB-P1 are downstream proteins of LKB1, the reduction in AMPK phosphorylation and increase
157 in mTOR and 4E-BP1 phosphorylation suggest the deactivation of LKB1, consistent with its
158 increased phosphorylation. (R)-NNAL treatment also reduced cleaved PARP caused by cisplatin
159 treatment, had little effect on cisplatin-induced DNA damage, and may slightly reduce the level of
160 Bim protein (Fig. 4D and Fig. 4E), which may explain the reduced sensitivity of H1299 cells to

161 cisplatin treatment in the presence of (R)-NNAL. In addition, (R)-NNAL treatment resulted in a
162 slight increase in the level of PCNA (Fig. 4F), potentially accounting for its stimulation of cell
163 proliferation.

164

165 **2.5. The upstream signaling responsible for NNAL-mediated LKB1 phosphorylation**

166 Upon establishing the role of LKB1, we explored the potential upstream targets of NNAL
167 responsible for LKB1 phosphorylation and associated phenotypes. NNK has been reported to act
168 as an agonist for nAChRs [19] and β -ARs [20]. This could result in the activation of protein kinase
169 A (PKA) via intracellular calcium influx and cAMP synthesis, which would phosphorylate and
170 deactivate LKB1 [49]. First, we found (R)-NNAL could promote PKA-C α nucleus translocation
171 in H1299 (Fig. 5A). And then, we utilized a panel of pharmacological inhibitors to probe the
172 relevance of these potential upstream signaling processes, including propranolol (a β -AR
173 antagonist), nifedipine (a calcium channel blocker), H89 (a PKA inhibitor) and yohimbine (an α 2-
174 AR antagonist as a control). We evaluated their effects on NNAL-induced proliferation in H1299
175 cells (Fig. 5B). At non-cytotoxic concentrations, each pharmacological inhibitor, except
176 yohimbine, effectively blocked (R)-NNAL-induced enhanced proliferation. Similarly, these
177 pharmacological inhibitors, with the exception of yohimbine, effectively blocked (R)-NNAL
178 induced resistance against gemcitabine or cisplatin in H1299 cells (Fig. 5C). Consistently, each
179 pharmacological inhibitor, with the exception of yohimbine, reduced the phosphorylation of LKB1
180 (Ser428) induced by (R)-NNAL exposure (Fig. 5B). Overall, these data delineate the signaling
181 process of LKB1 deactivation by NNAL.

182

183 **2.6. The effect of prolonged (R)-NNAL exposure on H1299 cells**

184 The lung tissue of smokers may be chronically exposed to NNAL due to the habitual use of
185 tobacco and the slow elimination of NNAL. We therefore evaluated the effect of long-term (R)-
186 NNAL exposure on H1299 cells. Specifically, H1299 cells were cultured with (R)-NNAL (1 nM)
187 for 60 days. Then, in the absence of NNAL, the phosphorylation status of LKB1 was characterized
188 and cell proliferation, colony formation, cell migration and the sensitivity of such cells to
189 gemcitabine and cisplatin treatment was evaluated. Long-term NNAL exposure resulted in a
190 substantial increase in LKB1 (Ser428) phosphorylation even in the absence of NNAL (Fig. 6A).
191 These H1299 cells proliferated faster, supported by the cell proliferation data (Fig. 6B) and colony
192 formation data (Fig. 6C). And these H1299 cells were also significantly less sensitive to cisplatin
193 and gemcitabine treatment in the absence of NNAL (Fig. 6D). These data suggest that the effect
194 of long-term NNAL exposure on LKB1 deactivation and drug resistance could be long-lasting.
195 Interestingly, the addition of NNAL to such cells failed to further enhance drug resistance (data
196 not shown). Mechanically 60 days exposure to 1 nM (R)-NNAL has little effect on cisplatin
197 induced DNA damage indicated by the level of γ H2A.X, and significantly reduced PARP cleavage
198 (Fig. 6E.). In addition, H1299 cell migration was also enhanced upon 60 days exposure to (R)-
199 NNAL (Fig. 6F. and Fig. 6G).

200

201 **2.7. The effect of NNK exposure in A/J mice on LKB1 phosphorylation**

202 To explore whether NNK induces LKB1 phosphorylation *in vivo*, a pilot study in A/J mice was
203 performed. In this study, NNK was administered in drinking water at a concentration of 40 ppm.
204 This treatment regimen mimics the chronic exposure of NNK among smokers although liver
205 instead of lung is the tissue of main exposure. The dose of NNK (40 ppm) in mice is comparable
206 to the level of NNK exposure among heavy smokers [50]. A similar treatment regimen has been
207 demonstrated to induce lung adenoma formation in A/J mice [51-53]. The lung and liver tissues
208 were collected after a 4-week NNK exposure. Again, NNK was not detectable in any serum
209 samples while NNAL was detected all (Fig. 6H), consistent with human data. The serum
210 concentration of NNAL in the mice ranged between 0.83 – 3.55 nM, similar to the concentration
211 used in our *in vitro* studies. NNK treatment substantially increased LKB1 phosphorylation in the
212 liver tissues with a slight increase in the lung tissues (Fig. 6H), indicating the deactivation of LKB1
213 in A/J mice upon NNK exposure. The higher levels of LKB1 phosphorylation in the liver tissues
214 than the lung tissues in this model may be caused by the NNK drinking water regimen that the
215 liver tissues have higher exposure to NNK than the lung tissues. In human smokers, the lung tissues
216 have higher exposure to NNK that may favor LKB1 phosphorylation in the lung tissues.

217

218 **2.8. LKB1 status in lung cancer tissues**

219 To explore the potential clinical significance of LKB1 phosphorylation by NNAL, we
220 characterized the phosphorylation status of LKB1 protein in five lung cancer tissues in comparison
221 to the normal tissues from the same patients (Fig. 6I). Although there are variations and no obvious
222 patterns in the total protein levels of LKB1 between the normal and cancer tissues, p-LKB1

223 (Ser428) were substantially higher in the cancer tissues relative to the normal tissues irrespective
224 of the lung cancer pathology.

225

226 3. Discussion

227 Clinical management of lung cancer has not been very successful and the overall survival from
228 lung cancer remains frustratingly low [1-3]. There are many contributing factors, including late
229 diagnosis and higher risk of drug resistance and metastasis [54-56]. At the same time, many lung
230 cancer patients are active smokers at the time of diagnosis and a significant portion of them
231 continue to smoke, which is associated with worse outcomes [5, 6]. NNK has been proposed as a
232 contributing factor because it could enhance lung cancer proliferation and survival, and promote
233 metastasis *in vitro* and *in vivo* [17, 18, 57, 58]. Potential mechanisms have been characterized *in*
234 *vitro*, including the activation of CREB, ERK1/2, and Akt with nAChRs and β -ARs as the
235 upstream targets [19, 20, 58, 59]. These mechanistic studies, however, may have limited
236 physiological relevance because NNK is not detectable in human plasma samples. Its major
237 metabolite, NNAL, on the other hand, is detected in the plasma samples from all smokers in our
238 study with a concentration approaching 300 pM. Given that lung tissue has the highest exposure
239 to tobacco smoke, NNAL between 1 and 100 nM *in vitro* is likely physiologically relevant. Within
240 this concentration range, NNAL enhanced cell proliferation in all five NSCLC cancer cell lines
241 tested, with (R)-NNAL being more potent than (S)-NNAL. NNAL also promoted cell migration
242 and induced drug resistance in NSCLC cell lines that have wild-type LKB1. Such effects were
243 also more pronounced with (R)-NNAL than (S)-NNAL. These results suggest that the detrimental

244 effects of NNAL may vary among smokers because of genetic polymorphisms in NNAL
245 metabolizing enzymes, such as carbonyl reductases and UGTs. It should also be noted that
246 although the stimulating effects by NNAL on proliferation, migration and resistance are not very
247 strong under our experimental conditions, the cumulative impact should not be underestimated
248 given the chronic exposure of the lungs to NNAL among smokers. Indeed, a 60-day exposure of
249 H1299 cells to (R)-NNAL (1 nM) resulted in significant enhancing of cell proliferation, migration
250 and drug resistance in combination with LKB1 phosphorylation even in the absence of NNAL.

251 LKB1 mutational deactivation has been observed in 10 – 30% of lung cancer patients of
252 different pathological subtypes [41-44]. Results from a number of genetic mouse models strongly
253 indicate that LKB1 inactivation plays an important role in lung cancer initiation, development, and
254 progression [46-48, 60]. Indeed, lower levels of LKB1 expression has been reported to be
255 associated with higher recurrence in NSCLC [61] and loss of LKB1 has been discussed beyond
256 just mutations [62]. Upon analyzing a limited number of lung cancer tissues, we observed
257 enhanced LKB1 phosphorylation in lung cancer tissues compared with paired normal lung tissues.
258 These data suggest that the function of wild-type LKB1 protein in lung cancers may be
259 compromised and the potential contribution of LKB1 deactivation to human lung cancer could be
260 substantially higher than its mutational frequency, something that warrants future investigation. It
261 is therefore of great importance to understand how wild-type LKB1 is phosphorylated in lung
262 cancer patients. Our results showed for the first time that NNAL, particularly (R)-NNAL, induces
263 LKB1 phosphorylation (Ser428) in NSCLC cancer cells. Since a 60-day NNAL exposure resulted
264 in phosphorylated LKB1 even upon NNAL removal, prior or active tobacco use among former
265 and current smokers, respectively, could contribute to the phosphorylation and deactivation of

266 LKB1 in human lung cancer tissues. This was further supported by our A/J mouse data that a 4-
267 week NNK exposure resulted in LKB1 phosphorylation in the lung tissues. Based on our results
268 with pharmacological inhibitors, β -ARs are the potential up-stream target(s) for NNAL that then
269 activate PKA, leading to LKB1 phosphorylation (Fig. 7). Other agonists for β -ARs, such as mental
270 stress-related stress hormones norepinephrine and epinephrine, may also deactivate LKB1 in
271 humans, which again warrants future investigation. Indeed, nicotine exposure and mental stress
272 have also been documented as potential factors contributing to the worse outcome of lung cancer
273 patients [63, 64].

274

275 Of note, (R)-NNAL stimulated cell proliferation in all NSCLC cancer cells irrespective of
276 LKB1 status, suggesting that NNAL modulates signaling mechanisms independent of LKB1.
277 NNK has been reported to activate CREB, a master oncoprotein [57, 65, 66], to promote
278 progression in established tumors. CREB activation is dominantly mediated via PKA as well. We
279 therefore evaluated the effect of NNAL on CREB phosphorylation in all five NSCLC cells. NNAL
280 rapidly activated CREB in these cell lines independent of LKB1 status with (R)-NNAL being more
281 potent than (S)-NNAL (Fig. S3). Thus, increased CREB phosphorylation and activation may
282 contribute to the increased proliferation of NSCLC cancer cells induced by NNAL.

283 In summary, our results show that NNAL can deactivate LKB1 in lung cancer cells at
284 physiologically relevant concentrations in an isomeric dependent manner. Such deactivation may
285 be of great clinical relevance given the tumor suppressive functions of LKB1 in lung cancer
286 initiation, development and progression and the high prevalence of tobacco exposure among lung

287 cancer patients. Further *in vivo* and clinical studies are warranted to validate NNAL's tumor
288 promoting effects, its contribution to LKB1 deactivation and the worse clinical outcome of lung
289 cancer patients who continue to smoke.

290

291 **4. Materials and Methods**

292 **Caution:** Both NNK and NNAL are highly carcinogenic. They should be handled in a well-
293 ventilated hood with extreme care, and with proper personal protective equipment.

294

295 **4.1. Chemicals and Reagents**

296 NNK, [¹³C₆]NNK, [¹³C₆]NNAL, [CD₃]O⁶-mG were purchased from Toronto Research
297 Chemicals (Toronto, Ontario, Canada). (±)-Propranolol hydrochloride, bupropion and H89 were
298 purchased from Sigma-Aldrich (St. Louis, MO, USA). Nifedipine was purchased from Alfa Aesar
299 (Ward Hill, MA, USA). Yohimbine hydrochloride was purchased from Acros Organics (Morris,
300 NJ, USA). All reagents were used without further purification.

301

302 **4.2. Human samples**

303 Plasma samples from active smokers were collected from a clinical trial previously conducted
304 at the University of Minnesota [36] and from active smoking population controls in the NCI-MD
305 Lung cancer Case Control Study [67]. Plasma samples from non-smokers were purchased from
306 Bioreclamation IVT (Baltimore, MD). Demographic information of these donors has been

307 published before [37, 68]. Paired normal and cancerous lung tissues from five patients were
308 acquired from University of Florida CTSI Biorepository. The protocols for human sample use were
309 reviewed and approved by Institutional Review Boards (IRB) at the University of Florida.

310

311 **4.3. NNAL synthesis, chiral resolution, and characterization**

312 Racemic NNAL was synthesized from NNK via sodium borohydride reduction. (R)- and (S)-
313 NNAL were separated from the racemic mixture via chiral chromatography by a contract service
314 from Kermanda Biotech Co Ltd. (Shanghai, China). Racemic, (R)- and (S)-NNAL were
315 characterized by ¹H-NMR and HPLC with > 95% purity. The chirality of the two enantiomers,
316 (R)- and (S)-NNAL, was assigned on the basis of reported optical rotations of NNAL [69].

317

318 **4.4. NNK and NNAL quantification in human plasma samples and mouse serum samples**

319 The concentrations of NNK and NNAL in human plasma and mouse serum samples were
320 quantified following a previously reported mass spectrometry method [70].

321

322 **4.5. Cell lines and culturing conditions**

323 H1299, H1975, HCC827, H460 and A549 cells were purchased from ATCC (Manassas, VA).
324 H1299, A549 and H460 were authenticated via the Cell Line Authentication Service provided by
325 Genetica DNA Laboratories (Burlington, NC). H1975 and HCC827 were authenticated by ATCC.
326 All of these cell lines were confirmed to be free from mycoplasma infection. H1299, H1975,

327 HCC827 and H460 cells were maintained in RPMI 1640 medium supplemented with 10% FBS
328 (Gibco). A549 cells were maintained in DMEM medium supplemented with 10% FBS. All cells
329 were cultured in a 37 °C, 5% CO₂ atmosphere. H1299 LKB1 knockout was reported before [71].
330 For A549 LKB1 knock-in, STK11(LKB1) gene was subcloned into PLX-304 vector. Lentivirus
331 production was performed using psPAX2 (Addgene#12260) and pMD2.G (Addgene#12259) as
332 previously described [72]. Single clones of cells expressing LKB1 were selected using blasticidin
333 (5ug/ml) and LKB1 expression was confirmed by western blot.

334

335 **4.6. Analysis of *O*⁶-methylguanine (*O*⁶-mG) in H1299 cells upon NNAL treatment**

336 Among the various forms of DNA damages caused by NNK and NNAL, *O*⁶-mG was the most
337 abundant in A/J mouse lung tissues [73] and of comparable abundance to other types of DNA
338 damage in F344 rat lungs [74] although such DNA damage has not been detected in human lung
339 tissues. We therefore quantified *O*⁶-mG in H1299 cells upon NNAL exposure (100 nM) using an
340 established mass spectrometry method [75].

341

342 **4.7. Cell counting assay**

343 Cell proliferation was determined using a cell count assay. Briefly, 5,000 cells/well were
344 seeded in a 24-well plate with 10% FBS medium. After overnight incubation, medium was
345 replaced with 0.5% FBS medium containing NNAL. After a 6-day incubation, cells were
346 trypsinized and cell numbers were determined using the Bio-Rad Automated Cell Counter.

347

348 **4.8. Colony formation assay**

349 Cell proliferation was also determined using colony formation assay. Briefly, cells were plated
350 in a 24-well plate (500 cells/well) in 0.5% FBS medium with or without NNAL. The number of
351 colonies was counted after a 7-day incubation under the microscope.

352

353 **4.9. Wound healing assay**

354 Cell migration was measured using the wound healing assay. Briefly, cells were seeded into a
355 six-well plate and allowed to grow to ~90% confluency. After starvation with FBS-free medium
356 for 48 h, cell monolayers were wounded with a 200- μ L pipette tip. Wounded monolayers were
357 washed three times with PBS and incubated in serum-free medium with different concentrations
358 of NNAL for 24 h. Cells were monitored under a microscope equipped with a camera. The wound
359 area was quantified using Image J software.

360

361 **4.10. Transwell assay**

362 Cell migration was also evaluated with the transwell migration assay using 6.5 mm diameter
363 inserts (Corning) with 8 μ m pore size. The inserts were plated in a 24-well plate with 600 μ L 10%
364 FBS medium. Briefly, 30,000 cells in 200 μ L serum free medium with 100 nM (R)- or (S)-NNAL
365 were seeded into each insert. After incubation at 37 °C for 24 h, the cells in the upper surface of
366 the membrane were removed with a cotton swab. Cells in the lower chamber were fixed with 70%

367 ethanol and stained with 0.2% crystal violet (Sigma-Aldrich in St. Lewis, MO, USA). Images were
368 taken with an inverted microscope and the number of cells was quantified using ImageJ.

369

370 **4.11. Cell viability assay**

371 Drug resistance was evaluated via a cell viability assay. Briefly cells were plated in 96-well
372 plates (5,000 cells/well) with 10% FBS medium. After attachment, cells were treated with the test
373 compounds at the specified concentrations or combinations in triplicate with 0.5% FBS medium.
374 The relative cell viability in each well was determined after 72 h treatment using the MTT assay
375 (Life Technologies).

376

377 **4.12. NNK exposure in A/J mice**

378 Female A/J mice (5-6 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor,
379 ME) and maintained in specific pathogen-free facilities, according to animal welfare protocols
380 approved by Institutional Animal Care and Use Committee at the University of Florida. After 1-
381 week acclimation, mice were weighed, randomized into two groups (n = 5) and switched to AIN-
382 93G powdered diet, defined as Day 1. From Day 1, mice in the control group were given regular
383 drinking water while the NNK group was given NNK in drinking water (40 ppm). Mice were
384 euthanized 4 weeks after NNK exposure. The lung and liver tissues were harvested, snap-frozen
385 in liquid N₂ and stored at -80 °C until protein analysis. Serum was collected for NNK and NNAL
386 detection.

387

388 **4.13. Western blotting**

389 Whole cell lysates from H1299, H1975, HCC827, A549 and H460 cells were prepared in RIPA
390 lysis buffer. Protein lysates from human and mouse lung tissues were prepared similarly. Briefly,
391 20 mg tumor or normal tissue was homogenized in 250 μ L RIPA buffer and the supernatant was
392 collected after centrifugation at 13,000 g for 15 min at 4 °C. The concentration of protein in each
393 sample was quantified using BCA assay. Forty - sixty μ g of protein from each sample was
394 denatured in SDS-PAGE sample buffer and resolved on 4-12% Bis-Tris PAGE gels. The separated
395 proteins were transferred to Polyvinylidene difluoride (PVDF) membrane followed by blocking
396 with 5% non-fat milk powder (w/v) in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 100 mM
397 NaCl, 0.1% Tween-20) for 1 h at room temperature. After blocking, the membranes were probed
398 with desired primary antibodies overnight at 4 °C followed by appropriate peroxidase-conjugated
399 secondary antibody for 2 h at room temperature and visualized by the Bio-Rad ChemiDoc Imaging
400 system. To ensure equal protein loading, each membrane was stripped with Restore Western Blot
401 stripping buffer (Thermo Scientific) and re-probed with β -actin antibody. Detailed information on
402 antibodies is in Table S1.

403

404 **4.14. Immunofluorescence staining**

405 Treated cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 1%
406 Triton X-100 in PBS for 10 min, followed by blocking with 5% BSA in PBS for 1 h. After
407 blocking, cells were incubated with PKA-C α antibody overnight at 4 °C and secondary antibody

408 for 1 h at room temperature. Nucleus were stained with Dapi for 15 min. Cells were imaged using
409 Fluorescence microscopy (Nikon Ti2, Japan).

410

411 **4.15. Flow cytometry**

412 Detection of γ H2A.X and cleaved PARP protein level in cisplatin treated H1299 with/without
413 (R)-NNAL were performed using Apoptosis, DNA Damage and Cell Proliferation Kit (BD
414 Pharmingen), following the manufacturer's instructions. Briefly, H1299 cells were plated in 6-
415 well plates with 10% FBS medium. After attachment, cells were starved with 0.5% FBS medium
416 overnight and treated with test compounds. Treated cells were harvested, stained with Alexa
417 Fluor® 647 Mouse Anti-H2AX (pS139) antibody and PE Mouse Anti-Cleaved PARP (Asp214)
418 Antibody. The signals were assessed with a CytoFlex flow cytometer (Beckman Coulter Life
419 Sciences).

420 **4.16. Statistical analysis**

421 Two tailed Student's *t* tests were used for data analysis with two groups. One-way analysis of
422 variance (ANOVA) was used for data analysis with no less than three groups followed by
423 Dunnett's test for comparison between different groups. A P value ≤ 0.05 was considered
424 statistically significant. All analyses were conducted in GraphPad Prism4 (GraphPad Software,
425 Inc.)

426

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440

441 **Conflicts of Interest**

442 No potential conflicts of interest were disclosed.

443

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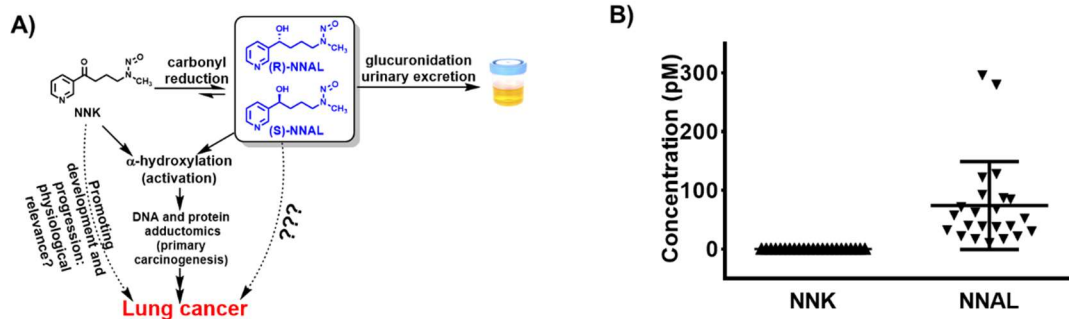
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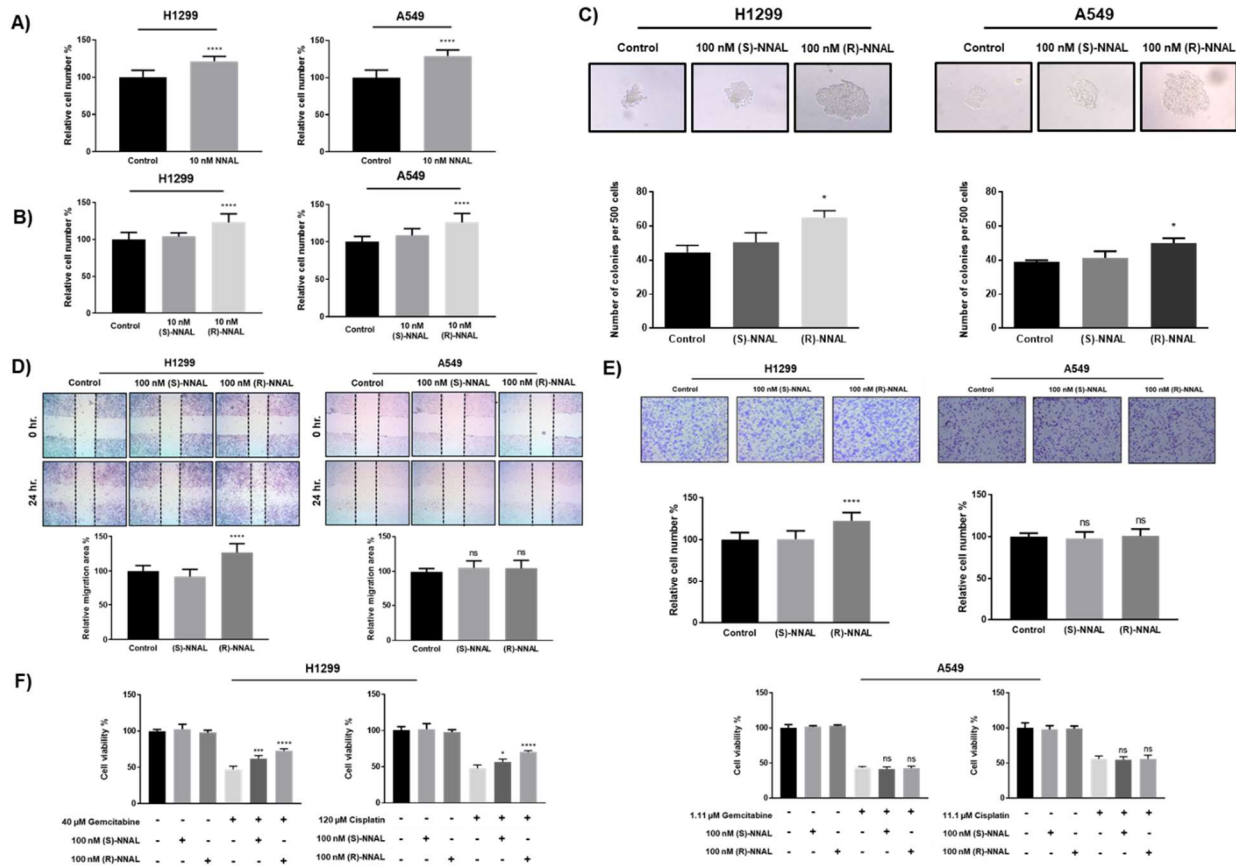
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734

735 **Figure 1. A.** Simplified major pathways of NNK metabolism, carcinogenesis, and potential effects
736 of NNK and NNAL on transformed lung cancer cells. **B.** Concentrations of NNK and NNAL in
737 the plasma samples from human smokers.

738



739

740 **Figure 2.** Effect of NNAL enantiomers on malignant phenotypes in H1299 and A549 lung cancer

741 cells.

742 **A.** Effect of NNAL (10 nM) on cell proliferation. **B.** Effect of NNAL enantiomer (10 nM) on cell

743 proliferation. **C.** Effect of NNAL enantiomers (100 nM) on colony formation. **D.** Effect of NNAL

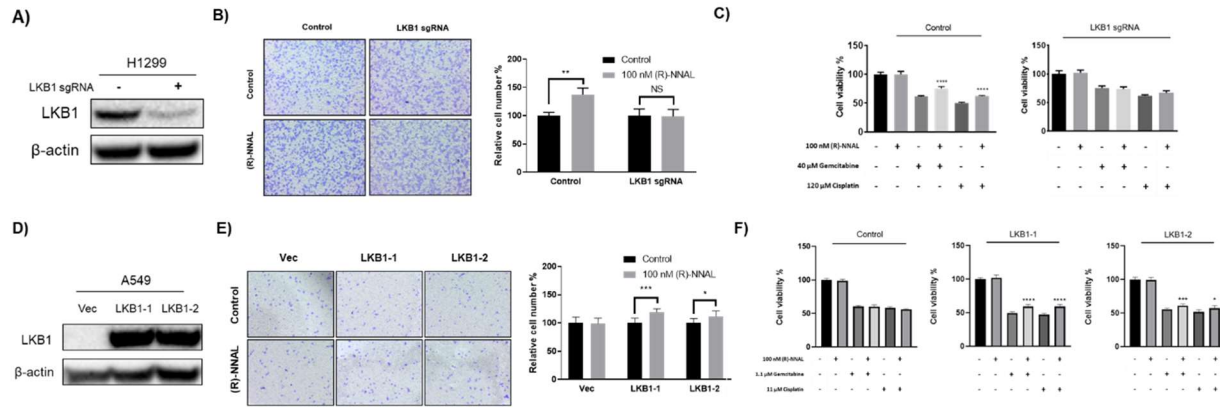
744 enantiomers (100 nM) on cell migration via wound healing assay. **E.** Effect of NNAL enantiomers

745 (100 nM) on cell migration via transwell assay. **F.** Effect of NNAL enantiomers (100 nM) on

746 conferring drug resistance to gemcitabine (40 μ M) or cisplatin (120 μ M) treatment. *, $P < 0.05$; **, $P < 0.01$;

747 $P < 0.001$.

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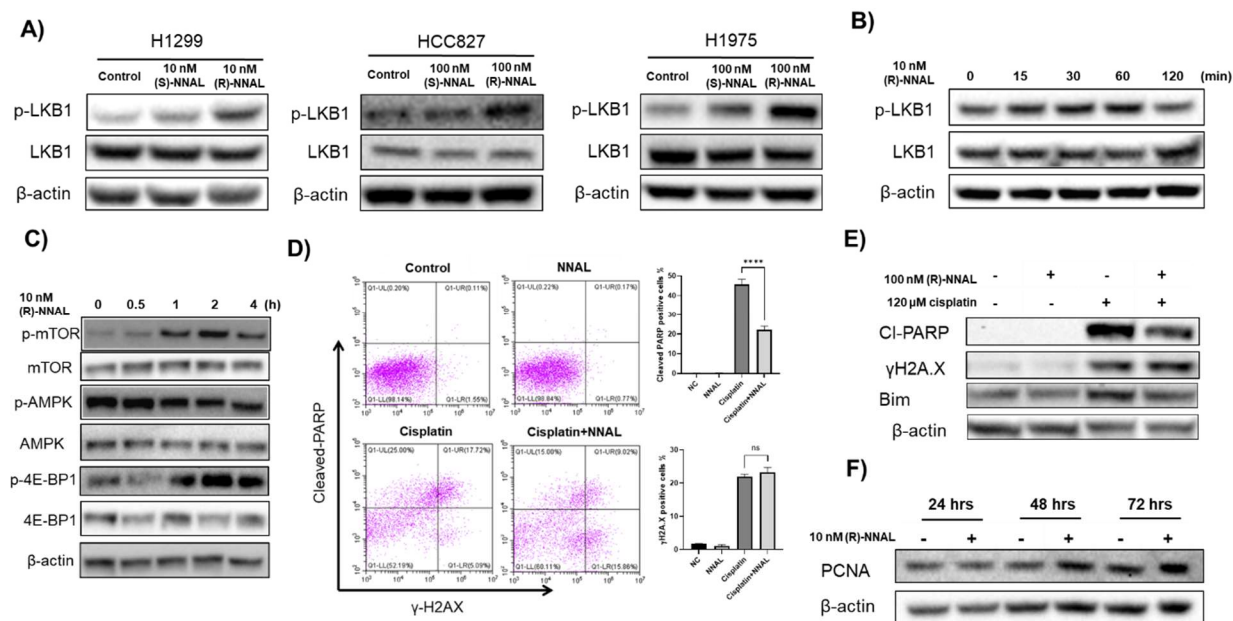
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750 **Figure 3.** The function of LKB1 on malignant phenotypes promoted by NNAL exposure.

751 **A.** Expression of LKB1 in H1299 LKB1 knockout cells. **B.** Effect of (R)-NNAL on cell migration
 752 in H1299 LKB1 knockout cells. **C.** Effect of (R)-NNAL on the cytotoxicity of gemcitabine and
 753 cisplatin in H1299 LKB1 knockout cells. **D.** Expression of LKB1 in A549 LKB1-knockin cells.
 754 **E.** Effect of (R)-NNAL on cell migration in A549 LKB1-knockin cells. **F.** Effect of (R)-NNAL on
 755 the cytotoxicity of gemcitabine and cisplatin in A549 LKB1-knockin cells. *, P<0.05; **, P<0.01;
 756 ***, P<0.001.

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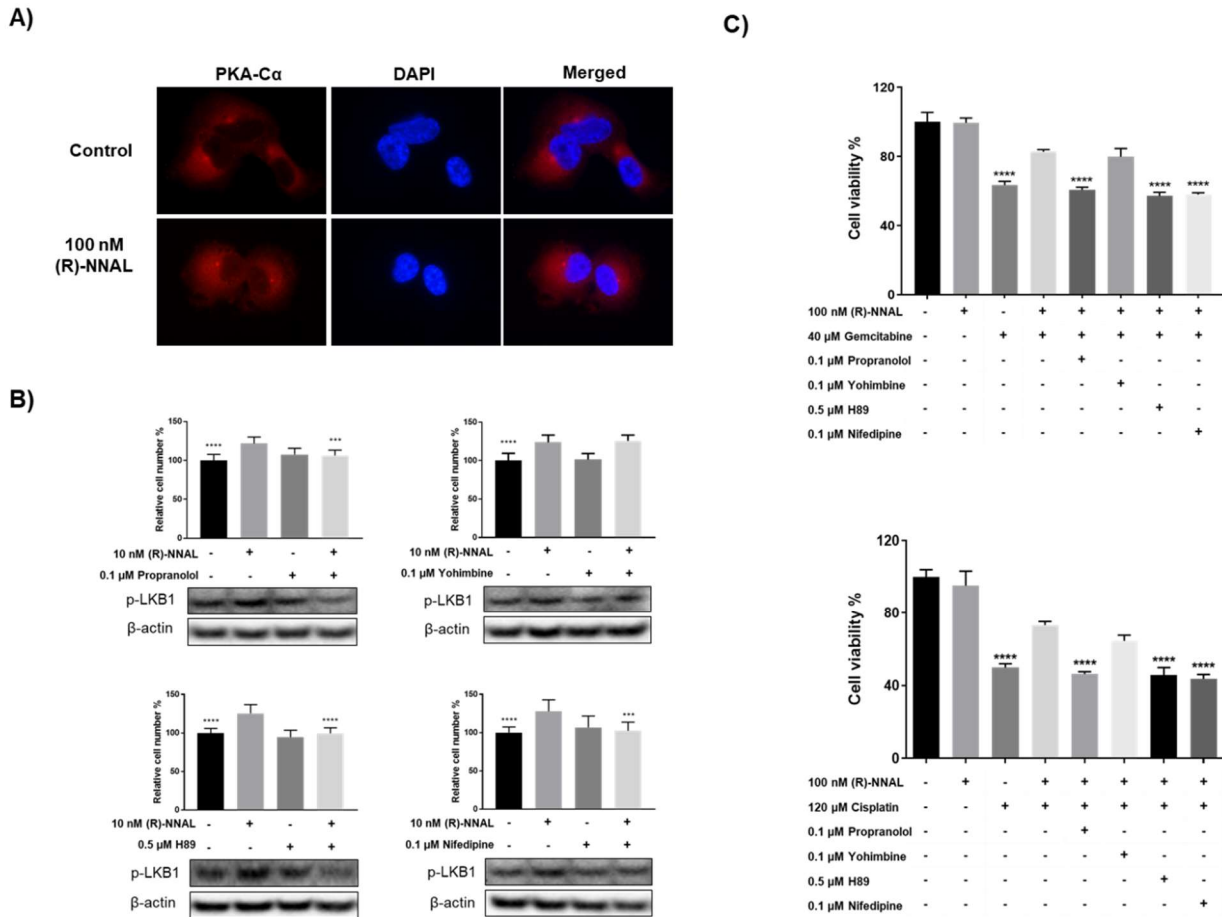


759

760 **Figure 4.** Effects of NNAL on LKB1 phosphorylation and associated signaling in *LKB1* WT lung
 761 cancer cells.

762 **A.** The effects of (R)- and (S)-NNAL on LKB1 deactivating phosphorylation at Ser428. Cells were
 763 treated with 10 nM NNAL for 30 min. **B.** Time course effect of (R)-NNAL on the phosphorylation
 764 of LKB1 at Ser428 (H1299). **C.** Time course effect of (R)-NNAL on AMPK, mTOR and 4EBP1
 765 phosphorylation in H1299. **D.** and **E.** Effect of (R)-NNAL on cisplatin induced DNA damage and
 766 PARP cleavage. **F.** Time course effect of (R)-NNAL exposure on PCNA levels in H1299.

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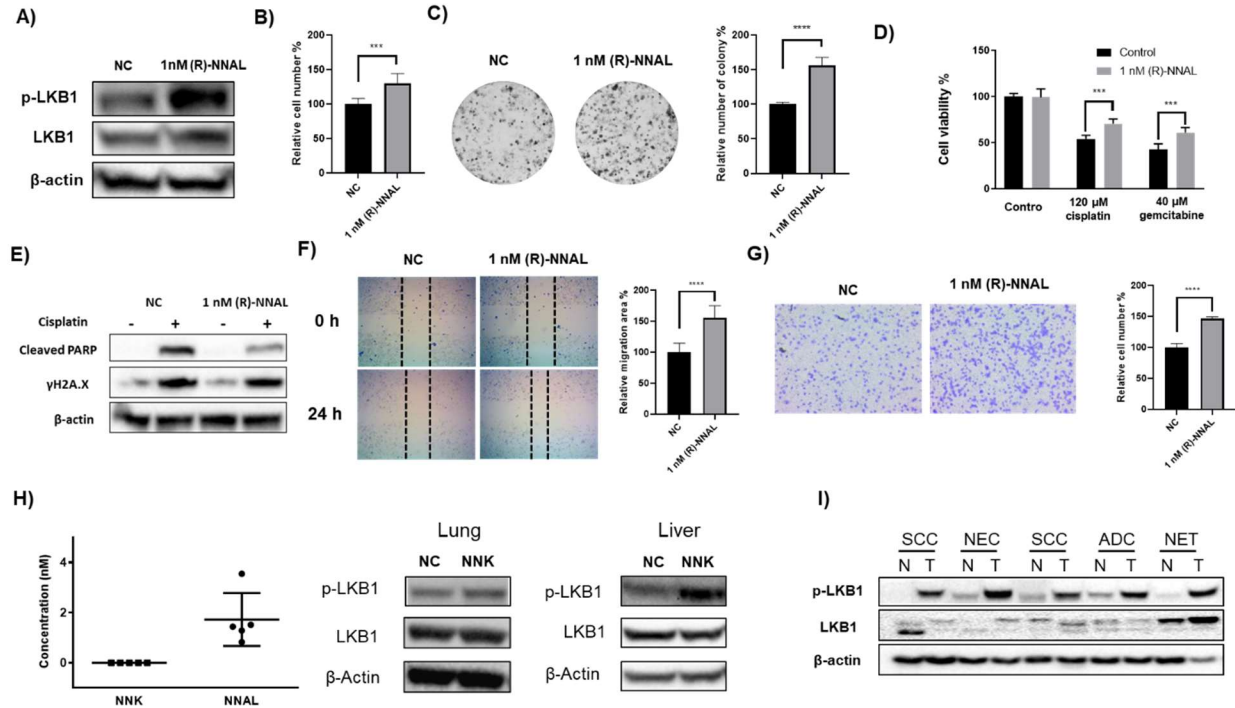
768

769 **Figure 5.** The potential upstream signaling events governing NNAL-promoted phenotypes with
 770 pharmacological inhibitors.

771 **A.** Effect of (R)-NNAL on PKA-C α nucleus translocation in H1299. Cells were treated with 100
 772 nM (R)-NNAL for 60 min. DAPI was used to stain the nucleus. **B.** Effect of inhibition of β -ARs
 773 (propranolol), Ca²⁺ channels (nifedipine), PKA (H89) and α -ARs (yohimbine) on NNAL-
 774 promoted cell proliferation and LKB1 phosphorylation (Ser428). H1299 cells were co-treated with
 775 10 nM (R)-NNAL and 0.1 μ M nifedipine, 0.1 μ M propranolol, 2.5 μ M bupropion, 0.5 μ M H89 or
 776 0.1 μ M yohimbine for 6 days. **C.** Effect of inhibition of β -ARs (propranolol), Ca²⁺ channels

777 (nifedipine), PKA (H89) an α -ARs (yohimbine) on NNAL-promoted resistance to gemcitabine and
778 cisplatin. H1299 cells were co-treated with 40 μ M gemcitabine or 120 μ M cisplatin along
779 inhibitors. *, P<0.05; **, P<0.01; ***, P<0.001.

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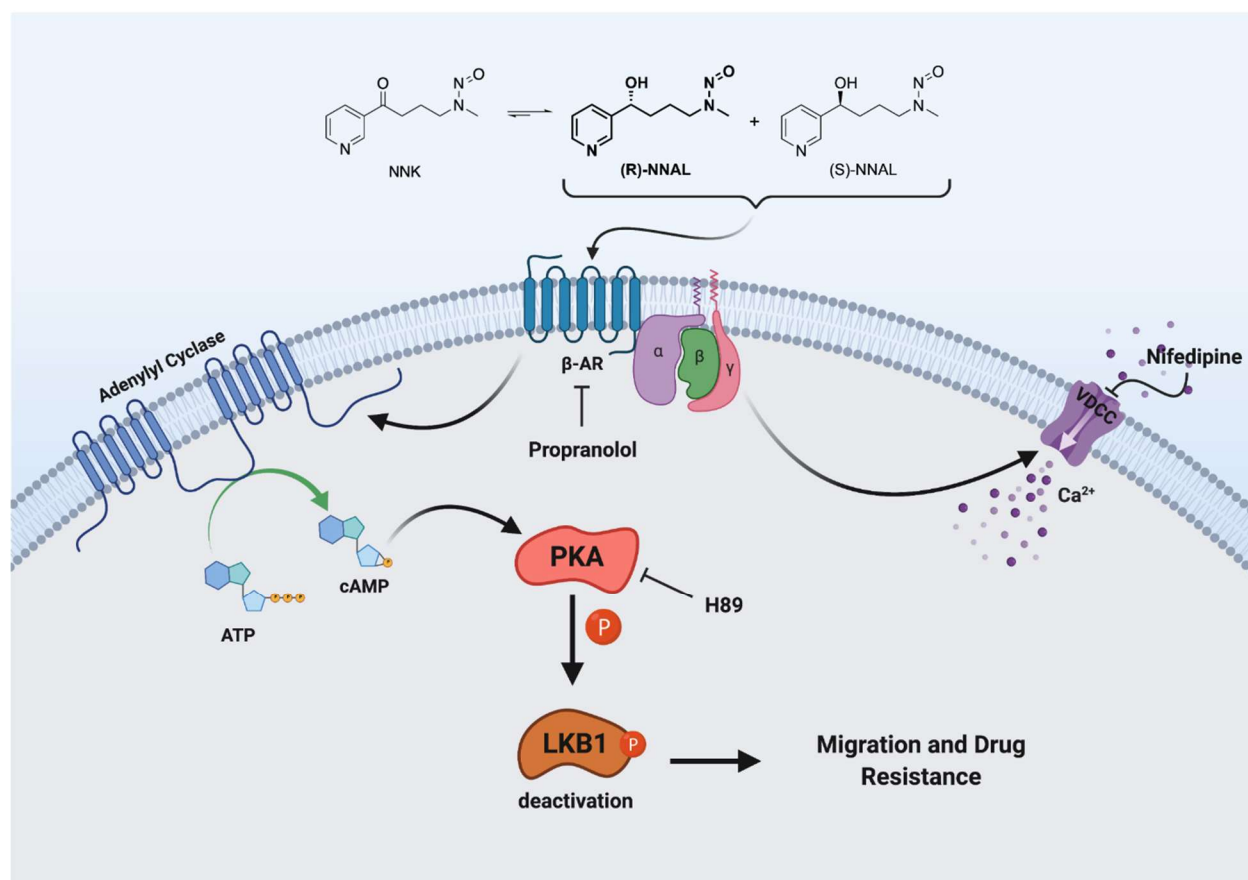


781

782 **Figure 6.** LKB1 phosphorylation status *in vitro*, *in vivo* and in clinical samples with potential
 783 chronic NNAL exposure.

784 Effect of 60-day (R)-NNAL exposure on cell proliferation (A), colony formation (B), sensitivity
 785 to gemcitabine and cisplatin treatment (C), cell migration (D and E), and LKB1 phosphorylation
 786 (F) in H1299 cells. H1299 cells was treated with (R)-NNAL (1 nM) for 60 days and no additional
 787 (R)-NNAL was added when running these assay. G. Concentrations of NNK and NNAL in mouse
 788 serum (n = 5) and LKB1 status in the lung and liver tissues of A/J mice upon 4-week NNK
 789 exposure. A/J mice were given NNK in drinking water (40 ppm) for 4 weeks. H. Status of LKB1
 790 in normal (N) or tumor (T) tissues of five lung cancer patients (SCC: squamous cell carcinoma;
 791 ADC: adenocarcinoma; NEC: neuroendocrine carcinoma; NET: neuroendocrine carcinoid).

792



793

794 **Figure 7.** Proposed mechanisms of NNAL in promoting progression of lung cancer cells with wild
795 type LKB1.

796 **Table 1.** Smoking status among NSCLC patients.

Number of Patients	Non-smokers	Former smokers	Continue smoking after diagnosis	Quit smoking after diagnosis	No information	Reference
1124	64 (5.7%)	696 (61.9%)	293 (26.1%)	71 (6.3%)		[8]
206	15 (7.3%)	98 (47.6%)	47 (22.8%)	46 (22.3%)		[9]
388	191 (49.2%)	79 (20.4%)	82 (21.1%)	36 (9.3%)		[10]
311	25 (8.0%)	82 (26.4%)	169 (54.3%)	35 (11.3%)		[11]
313	92 (29.4%)	125 (39.9%)	96 (30.7%)			[7]
4200	618 (14.7%)	2099 (50.0%)	1483 (35.3%)			[12]
3212	266 (8.3%)	1603 (49.9%)	1232 (38.4%)		111 (3.4%)	[13]

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