Differential involvement of gp130 signalling pathways in modulating tobacco carcinogen-induced lung tumourigenesis

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Running title: gp130 signalling modulates NNK lung tumourigenesis

Financial support: This work was supported by the National Health and Medical Research Council (NHMRC) of Australia, as well as the Operational Infrastructure Support Program by the Victorian Government of Australia. S.M.R. is supported by a NHMRC Post-doctoral Training Fellowship. B.J.J. is supported by a Sylvia and Charles Viertel Senior Medical Research Fellowship.

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Word count: 4063 Figures: 6 Tables: 0
ABSTRACT
Interleukin (IL)-6 family cytokines signal exclusively via the gp130 co-receptor, and are implicated in smoking-associated lung cancer, the most lethal cancer worldwide. However, the role of gp130 signalling pathways in transducing the carcinogenic effects of tobacco-related compounds is ill-defined. Here, we report that lung tumourigenesis induced by the potent tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is suppressed in gp130F/F knock-in mice characterized by the contrasting gp130-dependent hypo-activation of ERK MAPK and PI3K/Akt, and hyper-activation of STAT3, signalling cascades. Specifically, in response to NNK, the absolute number and size of lung lesions in gp130F/F mice were significantly reduced compared to gp130+/+ littermate controls, and associated with lower cellular proliferation without any alteration to the level of apoptosis in gp130F/F lung tumours. At the molecular level, reduced activation of ERK MAPK, but not Akt, was observed in lung tumours of gp130F/F mice, and corresponded with impaired expression of several tumour suppressor genes (e.g. Trp53, Tsc2). Notably, STAT3 was not activated in the lungs of gp130+/+ mice by NNK, and genetic normalization of STAT3 activation in gp130F/F:Stat3+/+ mice had no effect on NNK-induced tumourigenesis. The expression of tumour suppressor genes was reduced in tumours from current versus never-smoking lung cancer patients, and in vitro pharmacological inhibition of ERK MAPK signalling in human lung cancer cells abrogated NNK-induced down-modulation of tumour suppressor gene expression. Among IL-6 cytokine family members, IL-6 gene expression was specifically up-regulated by NNK in vitro and in vivo, and inversely correlated with tumour suppressor gene expression. Collectively, our data reveal that a key molecular mechanism by which NNK promotes tumour cell proliferation during tobacco carcinogen-induced lung carcinogenesis is via up-regulation of IL-6 and the preferential usage of gp130-dependent ERK MAPK signalling to down-modulate tumour suppressor gene expression.
KEY WORDS
NNK, lung adenocarcinoma, gp130 signalling, IL-6, tumour suppressor genes
INTRODUCTION

Tobacco smoke is the greatest risk factor for lung cancer, the leading cause of cancer death worldwide, and accounts for 80-90% of all lung cancer cases.\textsuperscript{1,2} Lung adenocarcinoma (LAC) is the most common (~40%) sub-type of lung cancer, and diagnosis is often at an advanced stage, with treatment options primarily restricted to a combination of chemotherapy and/or radiation therapy. However, these and more recent targeted treatment strategies are associated with an unacceptably high risk of tumour re-occurrence and poor patient survival rates (5-year relative survival rate for lung cancer is ~15%).\textsuperscript{1-3} Accordingly, identification of the full spectrum of oncogenic signalling pathways and gene networks involved in the initiation and progression of smoking-associated lung tumourigenesis is an integral step in the design of therapeutics against smoke-induced lung carcinogenesis.

Among the many carcinogens identified in tobacco smoke, the most potent lung carcinogen is 4- (methylnitrosamino)-1-(3-pyridinyl)-1-butanone (Nicotine-derived Nitrosamine Ketone; NNK).\textsuperscript{4} A key aspect to the pro-tumourigenic activities of NNK is the activation of intracellular signalling pathways which promote cell survival and proliferation, in particular the extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt cascades. For instance, phosphorylation of ERK and Akt is observed in lung tumours of NNK-treated mice, and in vivo activation levels positively correlate with the extent of NNK-induced tumourigenesis.\textsuperscript{5-8} The clinical relevance of these findings is also supported by the frequent constitutive activation of ERK and Akt in human LAC cell lines,\textsuperscript{6,9-11} and the aberrant activation of these signalling molecules in tumour biopsies of LAC patients.\textsuperscript{9,10,12,13} Despite these observations, the molecular basis by which NNK binding to its cell surface nicotinic acetylcholine receptors leads to ERK MAPK and Akt signalling cascades is ill-defined.

The interleukin (IL)-6 cytokine family is a potent activator of both ERK MAPK and PI3K/Akt pathways via the common signal-transducing receptor subunit gp130, and is
strongly implicated in lung cancer. For instance, elevated levels of IL-6 are characteristic of many lung LAC patients, and are also associated with an increased risk of lung cancer.\textsuperscript{14-17} It is also noteworthy that IL-6 expression is induced in both human lung epithelial cells and the lungs of mice in response to cigarette smoke, and has been proposed to protect against apoptosis triggered by smoke-induced DNA damage.\textsuperscript{18,19} Interestingly, however, in the context of tobacco smoke and lung carcinogenesis, the role of IL-6 has been intimately linked to its activation of the potent signal transducer and activator of transcription (STAT)3 oncogenic latent transcription factor, whose aberrant expression and/or activation is frequently observed in tumour tissue from human LAC patients.\textsuperscript{15,18-21} Thus, while there is strong evidence supporting a causal role for the IL-6/gp130/STAT3 signalling axis in the molecular pathogenesis of LAC, the involvement of specific gp130-dependent signalling cascades, namely ERK MAPK, PI3K/Akt and STAT3, in facilitating NNK-driven lung carcinogenesis \textit{in vivo} is poorly understood.

In this current study, we have used \textit{gp130\textsuperscript{F/F}} knock-in mice displaying deregulated activation of gp130-dependent ERK MAPK, PI3K/Akt and STAT3 signalling pathways to elucidate their role in NNK-induced lung tumourigenesis. At the molecular level, these mice carry a phenylalanine (F) knock-in substitution of the cytoplasmic tyrosine (Y) 757 residue in gp130 which simultaneously abolishes binding of i) Src homology phosphatase (SHP)2 leading to abrogated gp130-mediated activation of ERK MAPK and PI3K/Akt signalling, and ii) suppressor of cytokine signalling (SOCS)3 resulting in hyper-activation of janus kinase (JAK)/STAT3 signalling.\textsuperscript{22} Chronic exposure of \textit{gp130\textsuperscript{F/F}} mice to NNK was associated with a significant suppression in the incidence and size of lung tumours compared to wild-type \textit{gp130\textsuperscript{+/+}} mice. The suppressed lung tumourigenesis in NNK-treated \textit{gp130\textsuperscript{F/F}} mice correlated with reduced activation of the ERK MAPK pathway, while activation of the PI3K/Akt pathway remained unchanged. Notably, the reduced tumour load in \textit{gp130\textsuperscript{F/F}} mice was also independent of STAT3, as the development of NNK-induced lung lesions was comparable in \textit{gp130\textsuperscript{F/F}} and compound mutant \textit{gp130\textsuperscript{F/F}:Stat3\textsuperscript{-/+}} mice, the latter displaying genetically
normalized STAT3 signalling.\textsuperscript{23} Furthermore, unlike the activation of ERK MAPK and Akt in response to NNK, no activation of STAT3 was detected \textit{in vitro} or \textit{in vivo} upon the exposure of a panel of human LAC cell lines or wild-type mice, respectively, to NNK. Collectively, our data reveal the differential role of specific gp130-dependent signalling pathways in promoting NNK-induced lung carcinogenesis, and identify the gp130 receptor as a key intermediary transducer of ERK MAPK pathway activation downstream of NNK.
RESULTS

**NNK-induced tumour formation is reduced in the lungs of gp130^{F/F} mice**

The \( gp130^{F/F} \) and control \( gp130^{+/+} \) mice were back-crossed 3 generations onto a pseudo-A/J background that confers susceptibility to NNK-induced lung carcinogenesis,\(^7,24 \) following which mice were administered with NNK or, as a control PBS, and tumourigenesis assessed at 16 weeks. While no sporadic lung tumours were seen in any PBS treated animals, all mice administered with NNK developed tumours. Strikingly, there was a significant (~5-fold) reduction in the number of tumours in \( gp130^{F/F} \) mice (5.22±1.29) compared to \( gp130^{+/+} \) mice (24.88±2.76, \( P<0.0001 \)) (Fig. 1A,B). In addition, the size of tumours was significantly smaller in the \( gp130^{F/F} \) mouse (0.70mm versus 0.93mm; \( P<0.0001 \)) (Fig. 1C). We note that the lower oncogenic responsiveness of \( gp130^{F/F} \) mouse lungs to NNK was not a consequence of reduced expression of the receptor for NNK, \( \alpha 7 \) nicotinic acetylcholine receptor (Supplementary Fig. S1A).

Histopathological evaluation of H&E-stained lung sections at high power indicated that the lesions in \( gp130^{F/F} \) and control \( gp130^{+/+} \) mice were histologically similar and represented well-circumscribed spherical adenomas (Fig. 2A). Further immunohistochemical evaluation of the adenomas from both genotypes also revealed that they were similarly positive throughout for the alveolar type II cell marker TTF-1 (Fig. 2B), which is used for the clinical diagnosis of pulmonary adenocarcinomas.\(^25 \) In addition, and consistent with a non-inflammatory component in NNK-induced lung carcinogenesis, sporadic CD45\(^+ \) cells at comparable numbers were observed within tumours from both genotypes (Fig. 2C). Collectively, these data indicate that deregulated gp130 signalling modulates NNK-induced tumourigenesis.

**Reduced NNK-induced lung tumourigenesis in gp130^{F/F} mice correlates with a lower proliferative potential of tumour cells**
Since a key oncogenic activity of NNK is to augment cellular proliferation, we next examined whether the reduced NNK-induced lung tumourigenesis in \( gp130^{F/F} \) mice correlated with a lower proliferative potential in the lung. Indeed, as shown in Fig. 3A, there was a significant reduction (2-fold) in the number of PCNA-positive cells in tumours from \( gp130^{F/F} \) compared to \( gp130^{+/+} \) mice. By contrast, the numbers of apoptotic TUNEL\(^+\) cells in the tumours of both genotypes was comparable (Fig. 3B). Since NNK-induced tumourigenesis is also associated with the up-regulated gene expression of angiogenic factors, namely vascular endothelial growth factor (VEGF) and the glutamic acid-leucine-arginine (ELR) motif-containing chemokines CXCL-1 and CXCL-2,\(^{26}\) we next assessed their gene expression levels in the lungs of mice. However, the expression of these genes was comparable between NNK-treated \( gp130^{F/F} \) and \( gp130^{+/+} \) mice (Fig. 3C). These data therefore suggest that gp130 signalling selectively modulates the proliferative responsiveness of tumour cells to NNK in the lung.

**Reduced ERK MAPK pathway activation in tumours from \( gp130^{F/F} \) mice**

A possible explanation for the reduced tumour load in NNK-treated \( gp130^{F/F} \) mice is abrogated activation of specific gp130 signalling pathways in tumour cells as a consequence of the gp130Y\( _{757} \)F mutation. In this regard, the \( gp130^{F/F} \) mouse lacks gp130/SHP2-dependent activation of PI3K/Akt and ERK MAPK signalling pathways,\(^{22}\) both of which are activated in response to NNK and widely implicated in the development of lung cancer.\(^{5-8,13}\) Immunohistochemistry revealed a high degree of phosphorylated (p) ERK1/2 immuno-reactive cells within the tumours of NNK-treated \( gp130^{+/+} \) mice which was significantly (~2-fold, \( P<0.05 \)) higher than the pERK1/2 staining in the tumours of NNK-treated \( gp130^{F/F} \) mice (Fig. 4A). By contrast, only a low and comparable number of pAkt immuno-reactive cells were observed within the tumours of both NNK-treated \( gp130^{+/+} \) and \( gp130^{F/F} \) mice (Fig. 4B). Gene expression profiling by qPCR of various IL-6 family cytokines and their receptors, including gp130, indicated that the suppressed tumour formation in NNK-treated \( gp130^{F/F} \) mice was not attributed merely to a reduction in their gene expression in \( gp130^{F/F} \) compared
to \(gp130^{+/+}\) tumours (Supplementary Fig. S1B). Therefore, these data suggest that \textit{in vivo} NNK activates a robust ERK MAPK signal which, at least in part, is transmitted via the gp130Y757 residue.

**NNK down-regulates tumour suppressor gene expression which is coincident with up-regulated expression of IL-6**

To further understand the molecular basis by which the \(gp130Y_{757}F\) mutation modulates NNK-induced tumourigenesis \textit{in vivo}, we assessed the expression levels of numerous oncogenes and tumour suppressor genes previously implicated in the pathogenesis of LAC.\textsuperscript{27-32} Among the various oncogenes examined (e.g. \textit{Kras}, \textit{Ccnd1}), we did not detect any significant gene expression changes in the lungs of either \(gp130^{+/+}\) or \(gp130^{F/F}\) mice administered with NNK compared to PBS treated control mice of the corresponding genotype (data not shown). However, the expression of numerous tumour suppressor genes, \textit{Trp53}, \textit{Tsc2}, \textit{Cdkn1b} and \textit{Myd88}, was significantly down-regulated in the lungs of \(gp130^{+/+}\) mice treated with NNK compared to their PBS-treated counterparts (Fig. 4C). Conversely, no such down-modulation of these genes was observed when comparing the lungs of \(gp130^{F/F}\) mice treated with NNK versus PBS (Fig. 4D).

The notion that NNK can down-regulate the transcriptional induction of tumour suppressor genes was further confirmed in human LAC cells, whereby NNK stimulation of A549 cells significantly down-regulated the gene expression of \textit{TSC2} and \textit{TRP53} (Fig. 5A). Since the ability of NNK to down-regulate the expression of tumour suppressor genes in \(gp130^{+/+}\) mouse lungs was abrogated in the lungs of \(gp130^{F/F}\) mice displaying reduced ERK MAPK pathway activation, we further examined whether the ERK MAPK pathway downstream of NNK contributed to the down-regulated expression of these genes in human LAC cells. Indeed, pre-treatment of cells with the specific ERK MAPK pathway inhibitor U0126 significantly impaired the NNK-induced transcriptional suppression of these genes.
(Fig. 5B). Therefore, these data point to a crucial role for the gp130/ERK MAPK pathway in the transcriptional down-regulation of tumour suppressor genes.

To identify how NNK communicates with gp130 signalling, we next measured the gene expression levels of IL-6 family cytokine members upon stimulation with NNK, both in vitro and in vivo. Notably, only Il6 mRNA was significantly up-regulated in the lungs of wild-type mice in response to NNK (Fig. 5C), and the ability of NNK to up-regulate IL-6 gene expression was also confirmed in human A549 cells (Fig. 5D). Furthermore, IL-6 stimulation of A549 cells also significantly suppressed the expression of TSC2 and TRP53 tumour suppressor genes (Fig. 5E), comparable to that observed upon NNK stimulation of A549 cells. Importantly, augmented expression of IL6 was observed in lung biopsies from current versus never-smoking LAC patients, and inversely correlated with the reduced expression of TRP53, TSC2 and other tumour suppressor genes (CDKN1B, MYD88) in the lungs of never-smoking LAC patients (Fig. 5F), thus providing clinical evidence for a link between IL-6 up-regulation and tumour suppressor gene down-regulation upon exposure to tobacco carcinogens.

NNK-induced tumourigenesis is independent of STAT3 activation

Although increased activation of STAT3 is a common finding in human LAC,15,20,21 and STAT3 is activated in response to tobacco (cigarette) smoke,18,19 the role of STAT3 in NNK-induced lung carcinogenesis is ill-defined. We therefore initially compared the phosphorylation (i.e. activation) status of STAT3 and ERK1/2 signalling proteins in human LAC cell lines with and without NNK. While stimulation of the human LAC cell line NCI-H838 with 50µM NNK led to a strong activation of ERK1/2 within 5 minutes, there was no increased STAT3 activation (Fig. 6A). Rather, the high phospho-STAT3 basal level appeared to gradually decrease over time upon exposure to NNK, which was also observed in the NCI-2228 LAC cell line displaying a high basal level of phospho-STAT3 (data not shown). Considering the high STAT3 activation level in unstimulated NCI-H838 cells may limit the potential for any exogenous factor to further increase STAT3 activation, we also examined
the ability of NNK to activate STAT3 in the human LAC cell line A549 which displays a very low basal level of STAT3 activation (Fig. 6B). However, unlike the increased activation of the ERK MAPK pathway by NNK, there was no increase in phospho-STAT3 levels (Fig. 6B). The failure of NNK to activate STAT3 was not a consequence of any inherent defect in upstream pathways leading to STAT3 tyrosine phosphorylation, since IL-6 stimulation of cells induced the activation of STAT3, as well as ERK1/2 (Fig. 6B).

To further validate these findings in vivo, we initially examined the activation of STAT3 in the lungs of gp130+/+ mice exposed to NNK over 16 weeks. As shown in Fig. 6C, the levels of phospho-STAT3 were similar in the tumour-bearing lungs of NNK-treated gp130+/+ mice and tumour-free lungs of control PBS-treated gp130+/+ mice. Furthermore, despite the suppressed NNK-induced tumourigenesis in gp130F/F mice, the number of pSTAT3+ cells in the lung tumours of NNK-treated gp130+/+ and gp130F/F mice were similar (Fig. 6D). While these observations support the notion that NNK-induced tumourigenesis occurs independently of STAT3, we next undertook a genetic approach to formally exclude a role for gp130/STAT3 signalling in the development of NNK-induced tumours in gp130F/F mice by employing pseudo-A/J gp130F/F:Stat3−/+ mice displaying genetically-normalized activation of the gp130/STAT3 signalling axis.23,33 Notably, similar to the pseudo-A/J gp130F/F mice, pseudo-A/J gp130F/F:Stat3−/+ mice administered with NNK over 16 weeks also had significantly fewer and smaller tumours than gp130+/+ mice (Fig. 6E). Collectively, these in vitro and in vivo data strongly suggest that NNK-induced lung carcinogenesis occurs independently of (gp130-driven) STAT3 activation.
DISCUSSION

Despite the large amount of experimental and clinical data reported over the last 2 decades linking gp130-activating cytokines to the pathogenesis of LAC, the role of gp130 cytokines and their signalling cascades in the promotion of LAC in response to known tobacco smoke carcinogens is poorly understood. Here, we report the use of the genetically-defined \( \text{gp130}^{+/+} \) mouse model displaying hypo- or hyper-activation of discrete gp130 signalling pathways to reveal the differential involvement of gp130-dependent ERK MAPK versus PI3K/Akt and STAT3 signalling during lung tumourigenesis induced by the potent tobacco smoke carcinogen NNK. Specifically, a key finding of our current study is that gp130 transduces, at least in part, the net signal output of the oncogenic ERK MAPK pathway in response to NNK. Furthermore, we observed that NNK-driven lung tumourigenesis is coincident with the down-modulation of tumour suppressor gene expression which correlates with the gp130/ERK MAPK signalling cascade. The gp130-activating cytokine which most likely facilitates the bridge between NNK and gp130 signalling is IL-6, since we identified here that IL-6 i) was significantly up-regulated by NNK, ii) was able to down-modulate tumour suppressor gene expression comparable to that observed by NNK, and iii) was over-expressed in LAC patients with a history of smoking compared to non-smokers. We also note these data are consistent with the previous report of IL-6 expression in human LAC tumours.\(^{17}\)

The persistent activation of ERK MAPK is a common feature of human NSCLC.\(^{13}\) In addition, ERK MAPK signalling has been implicated in the molecular pathogenesis of LAC from numerous mouse LAC models characterized by hyper-phosphorylation of ERK MAPK,\(^{34-36}\) as well as studies demonstrating the efficacy of suppressing lung tumourigenesis \textit{in vivo} upon pharmacological blockade of ERK MAPK signalling.\(^{34-36}\) Furthermore, increased ERK MAPK activation is associated with the exacerbated NNK-induced lung tumourigenesis observed in \( \text{CC10}^{-/-} \)-deficient mice.\(^{37}\) Although the mechanisms by which NNK (or other tobacco-related lung carcinogens for that matter) transduces ERK MAPK signals in the lung are largely unknown, our data presented here suggest gp130 acts as key upstream transducer
of ERK MAPK signalling via NNK during lung tumourigenesis. It is also worth noting that, consistent with the well documented pro-proliferative role of the ERK MAPK pathway in cancer, we observed that the reduced ERK MAPK activity observed in smaller tumours of gp130^{F/F} mice (unable to elicit gp130/SHP2-dependent ERK MAPK signalling) correlated with a lower proliferative, but not survival/anti-apoptotic, index. While the *in vitro* activation of ERK MAPK in human LAC cells induced by various stimuli, including NNK, promotes both cellular proliferation and survival via upregulation of proto-oncogenes (e.g. *Bcl2*, *c-myc*, *Ccnd1*),\(^{38,39}\) we did not observe any alterations to the expression of such proto-oncogenes *in vivo* in response to NNK. However, we revealed that NNK suppressed the *in vivo* expression of several tumour suppressor genes which was coincident with intact ERK MAPK signalling via gp130. Considering the expression of several of these tumour suppressor genes (e.g. *TRP53*, *TSC2*) was also reduced in LAC patient biopsies from smokers versus non-smokers, these data suggest a hitherto unknown effect of cigarette carcinogens, such as NNK, on tumour suppressor gene expression and cell proliferation during lung tumourigenesis.

Our data indicating that gp130 contributes substantially to the robust net signal output of ERK MAPK via NNK contrasts the relatively low level of NNK-induced Akt activation that was similarly observed in tumours of both gp130^{+/+} and gp130^{F/F} mice. Akt is a potent cell survival stimulant that is over-activated in human LAC from smokers, and its activation both *in vitro* and *in vivo* in response to NNK suggests a role in promoting tobacco-related lung carcinogenesis.\(^{5,6}\) Although the intermediary factors linking NNK with PI3K/Akt pathway activation in the lung are ill-defined, our study here suggests that gp130 does not contribute to NNK-induced Akt signalling during lung tumourigenesis, and it is likely that other upstream regulators of Akt, for instance thromboxane A2, are involved.\(^{40}\)

From an historical viewpoint, the gp130-mediated simultaneous activation of both ERK MAPK and PI3K/Akt cascades has been attributed to the SHP2 adaptor and phosphatase which acts as a molecular bridge by binding to pY\(^{757/759}\) of mouse/human gp130, whereby tyrosine phosphorylation of SHP2 leads to its association with the multi-adaptor protein.
Gab1.41 The association between Gab1 and SHP2 then links the receptor to the PI3K and ERK MAPK cascades via the binding of Gab1 to the p85 subunit of PI3K and the adaptor Grb2, respectively. However, our data revealing impaired activation of ERK MAPK, but not Akt, in the lung tumours of \(gp130^{F/F}\) mice lacking the interaction between gp130 and SHP2 suggest an uncoupling of PI3K/Akt and ERK MAPK activation via the gp130/SHP2 interaction. In this regard, it is of note that an alternate mode of gp130-driven PI3K/Akt pathway activation independent of the gp130/SHP2 interaction, but rather dependent on the gp130/JAK interaction, has recently been proposed during gastrointestinal tumourigenesis.42 Such a scenario is consistent with our own observations here which imply, at least in the context of NNK-driven lung tumourigenesis, the preferential utilization of the gp130/SHP2 interaction to facilitate gp130-dependent ERK MAPK signalling.

Another key finding of our current study is that STAT3 does not contribute \textit{in vivo} to NNK-induced carcinogenesis. While STAT3 is constitutively activated in approximately 50% of human LAC,43,44 there is little experimental evidence examining the interaction of STAT3 with \textit{bona fide} tobacco smoke carcinogens. A recent \textit{in vitro} study indicated that STAT3 is activated by NNK in human A549 LAC cells,44 which contrasts our findings that NNK does not activate STAT3 in multiple human LAC cell lines, including A549 cells, nor in lung tumours of wild-type mice. While this discrepancy in A549 cells is unknown, it is most likely attributed to variations including genetic drift which can impact on the responsiveness of transformed cell lines passaged over time. Conversely, in a carcinogen urethane-induced lung tumourigenesis model, epithelial-specific STAT3 has been assigned an anti-tumourigenic role by supporting anti-tumour immunity,45 an observation which most likely reflects the complex pro- and anti-tumourigenic activities of STAT3.46

In summary, our current study has delineated a specific role for IL-6/gp130-dependent ERK/MAPK signalling in NNK-induced lung tumours \textit{in vivo}, whereby the gp130/ERK MAPK signalling axis is associated with the down-regulation of tumour suppressor genes and increased tumour cell proliferation. Furthermore, we provide additional \textit{in vivo} evidence
indicating that STAT3 does not promote lung tumourigenesis in response to NNK. Accordingly, this study advances our current knowledge of the molecular basis by which NNK promotes lung carcinogenesis, and thus assists in the refinement of new molecular targets for therapy, with a particular focus on modulation of IL-6/gp130 signalling.
MATERIALS AND METHODS

Human biopsies

Lung tissue from resection surgery for the treatment of lung cancer (NSCLC) was collected from patients who were either smokers (n = 14, 23-143 packs per year) or never smokers (n = 5), upon formal written informed consent. Tissues were then snap-frozen in liquid nitrogen, prior to molecular analyses. Studies were approved by the Southern Health Human Research Ethics Committee.

Mice

To produce mice susceptible to the carcinogenic effects of NNK, \( gp130^{+/+} \), \( gp130^{F/F} \) and \( gp130^{F/F}:Stat3^{+/+} \) mice on a 129Sv × C57BL/6 background\(^{22,23} \) were back-crossed with A/J mice for 3 generations to produce mice on a “pseudo-A/J” background.\(^{24} \) Mice homozygous for the NNK tumour susceptible A/J \( Kras2_{37} \) allele were used for breeding and experimentation. Male and female mice were housed under specific pathogen-free conditions, and experiments were approved by the Monash University Animal Ethics committee.

Administration of NNK

NNK (Toronto Research Chemicals, North York, Canada) at 20mg/ml was dissolved in sterile Dulbecco's Phosphate-Buffered Saline (PBS) (Life Technologies, Carlsbad, CA, USA). Mice aged 6 to 8 weeks of age received 3 intra-peritoneal (i.p.) injections on alternate days of 100mg/kg NNK\(^{47} \) or, as a control, equivalent volume of PBS, and were observed over 16 weeks.

Cells

Human LAC cell lines A549 and NCI-H838 were serum starved (18 hours) prior to the addition of 100ng/ml recombinant human IL-6 (PeproTech, Rocky Hill, NJ, USA) or 1\( \mu M \) NNK for the indicated time points. For experiments involving the ERK MAPK inhibitor
U0126 (Sigma-Aldrich, St Louis, MO, USA), cells were pre-treated for 30 min with either dimethyl sulfoxide (DMSO) vehicle or U0126 (10µM) prior to stimulation with NNK.

**Antibodies**

Antibodies against pTyr705-STAT3, total STAT3 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Proliferating Cell Nuclear Antigen (PCNA), pSer473-Akt, total Akt, pThr202/pTyr204-ERK1/2 and total ERK1/2 from Cell Signaling Technology (Beverly, MA, USA), CD45 from BD Biosciences (Bedford, MA, USA), and Thyroid transcription factor-1 (TTF)-1 from Novus Biologicals (Littleton, CO, USA).

**Mouse histology and immunohistochemistry**

The collection of mouse lungs for histological evaluation (H&E), immunohistochemistry to detect CD45, TTF-1, pThr202/pTyr204-ERK1/2, pSer473-Akt, PCNA and pTyr705-STAT3, and terminal deoxynucleotidyl transferase (tdT)-mediated dUDP nick-end labeling (TUNEL) assay using an ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore, Billerica, MA, USA), was performed as before. To quantify cellular staining within mouse lung tumours, photomicrographs were taken of tumours at 20× magnification, and digital images viewed using Image J software (National Institutes of Health, Bethesda, MD). Positive staining cells (> 100 cells per tumour) were counted manually (n = 20 fields) within a grid that was placed over photomicrographs with a random offset.

**Protein extraction and immunoblotting**

Total protein lysates were prepared from snap-frozen lung tissue and subjected to immunoblotting with the indicated antibodies, following which proteins were visualized using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and quantified as before.

48-49
RNA isolation and gene expression analysis

Total RNA was isolated from snap-frozen mouse lung tissue and human LAC cell lines, and quantitative RT-PCR (qPCR) was performed on cDNA with SYBR Green (Life Technologies) on the 7900HT Fast RT-PCR System (Applied Biosystems, Forster City, CA, USA). Gene expression data acquisition and analyses were performed using the Sequence Detection System Version 2.4 software (Applied Biosystems) as previously described.

Mouse forward and reverse primer sequences for 18S, Cxcl1, Cxcl2, Il6, Il11 and Vegfa have been previously published, and forward and reverse primer sequences for other mouse and human genes are listed in Supplementary Table S1.

Statistical analyses

Statistics were generated using GraphPad Prism for Windows version 5.0, and where appropriate parametric (one-way ANOVA, student t-test) or nonparametric (Kruskal Wallis, Mann-Whitney) tests were used. P<0.05 was considered statistically significant. Data are expressed as the mean ± standard error of the mean (SEM).

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).
CONFLICT OF INTEREST

The authors have no conflicts to declare.
REFERENCES


**FIGURE LEGENDS**

**Figure 1**
Suppressed NNK-induced tumourigenesis in $gp130^{F/F}$ mice. A, representative photomicrographs showing H&E-stained lung cross-sections from $gp130^{+/+}$ (+/+) and $gp130^{F/F}$ (F/F) mice administered with NNK for 16 weeks. Arrows point to discrete tumours. B, number and C, size of NNK-induced lung tumours in +/+ and F/F mice. Scale bar = 500µm. Data from 8 mice per genotype are expressed as mean±SEM. ****$P<0.0001$.

**Figure 2**
Comparable histology of NNK-induced lung lesions in $gp130^{F/F}$ and $gp130^{+/+}$ mice. A-C, representative photomicrographs of lung cross-sections from NNK-treated $gp130^{+/+}$ (+/+) and $gp130^{F/F}$ (F/F) mice that were stained with A, H&E or antibodies against B, TTF-1 or C, CD45. In A, insets (scale bars = 500µm) depict high power images of the highlighted regions (black squares) in the low power main images (scale bars = 50µm), and arrows indicate tumours. In B, the graph depicts semi-quantitative enumeration of TTF-1 positive tumour cells per 20x field, and are presented as the mean ± SEM. n = 4 mice per genotype. Scale bars = 100µm. In C, the graph depicts semi-quantitative enumeration of CD45 positive cells (circles) per 20x field, and data are presented as the mean±SEM. n = 4 mice per genotype. Scale bars = 50µm.

**Figure 3**
Reduced tumour cellular proliferation, but not apoptosis, in lungs of NNK-treated $gp130^{F/F}$ mice. A and B, representative photomicrographs of lung cross-sections from $gp130^{+/+}$ (+/+) and $gp130^{F/F}$ (F/F) mice administered with NNK for 16 weeks that were A, stained with PCNA antibody, or B, subjected to TUNEL assay. Scale bars = 100µm. In A and B, the graphs depict semi-quantitative enumeration of PCNA and TUNEL positive tumour cells,
respectively, per 20x field, and data are presented as the mean±SEM. n = 4 mice per genotype. *P<0.05. C, qPCR expression analyses of the indicated genes were performed on lung cDNA from +/+ and F/F mice administered with NNK for 16 weeks. Expression data are normalized against 18S, and are presented from n = 4 mice per genotype as the mean±SEM.

Figure 4
Reduced activation of ERK MAPK, but not PI3K/Akt, signaling in lung tumours of NNK-treated gp130FF mice. A and B, representative photomicrographs of lung cross-sections from NNK-treated gp130+/+ (+/+) and gp130FF (F/F) mice that were stained with A, pERK1/2 or B, pAkt antibodies. Scale bars = 100µm. Graphs depict semi-quantitative enumeration of pERK1/2 and pAkt positive tumour cells per 20x field, and are presented as the mean±SEM. n = 4 mice per genotype. C and D, qPCR expression analyses of the indicated genes were performed on lung cDNA from C, +/+ and D, F/F mice 16 weeks post-NNK or -PBS administration. Expression data are normalized against 18S, and are presented from n = 4 mice per genotype as the mean±SEM. *P<0.05, **P<0.001.

Figure 5
NNK-induced gene regulation of tumour suppressors and IL-6 family cytokines. A, qPCR expression analyses of the indicated genes were performed on cDNA from A549 cells either unstimulated (0) or stimulated with NNK for 6 hours. Expression data normalized against 18S from 3 independent experiments are presented as the mean±SEM. *P<0.05. B, qPCR of the indicated genes from A549 cells either unstimulated (-) or pre-treated with either DMSO or U0126 inhibitor prior to stimulation with NNK for 6 hours. Data normalized against 18S from 3 independent experiments are presented as the mean±SEM. *P<0.05, **P<0.01. C, qPCR of the indicated genes was performed on lung cDNA from wild-type mice treated with either PBS (-) or NNK (+) over 16 weeks. Data are normalized against 18S, and are presented from n = 4 mice per group as the mean±SEM. *P<0.05. D, qPCR of IL6 in A549 cells either
unstimulated (0) or stimulated with NNK for the indicated times. Expression data normalized against 18S from 3 independent experiments are presented as the mean±SEM. *P<0.05. E, qPCR of the indicated genes in A549 cells either unstimulated (0) or stimulated with IL-6 for 6 hrs. Expression data normalized against 18S from 3 independent experiments are presented as the mean±SEM. *P<0.05. F, qPCR of the indicated genes on lung cDNA from current smoker (S, n = 14) and never smoker (NS, n = 5) LAC patients. Expression data are normalized against 18S, and are presented as the mean±SEM. *P<0.05, **P<0.01. ns, not significant (P<0.08).

Figure 6

NNK-induced lung tumourigenesis occurs independently of increased activation of STAT3 in mice. A, immunoblots of NCI-H838 cells stimulated with NNK using the indicated antibodies. Densitometry quantification for pSTAT3 and pERK1/2 was performed for each time point, and relative expression was determined against Actin. B, immunoblots of A549 cells either unstimulated or stimulated with NNK or IL-6 using the indicated antibodies. C, immunoblots on whole lung lysates of gp130+/+ mice 16 weeks after administration with PBS or NNK using the indicated antibodies. Densitometry quantification for pSTAT3 was performed for each sample, and relative expression was determined against Actin. n = 4 mice per treatment. D, representative photomicrographs of lung cross-sections from NNK-treated gp130+/+ (+/+), and gp130F/F (F/F) mice stained with pSTAT3 antibody. Scale bars = 100µm. Graph depicts semi-quantitative enumeration of pSTAT3 positive tumour cells per 20x field (n = 20 fields), and are presented as the mean±SEM. n = 4 mice per genotype. E, number of NNK-induced tumours in the lungs of +/+, F/F and gp130F/F:Stat3+/+ (F/F:St3) mice. Data from 6 mice per genotype are expressed as mean±SEM. ***P<0.001.
Supplementary Information

Differential involvement of gp130 signalling pathways in modulating tobacco carcinogen-induced lung tumourigenesis

Alistair Miller, Louise McLeod, Gavin Brooks, Saleela Ruwanpura, and Brendan J. Jenkins.

Inventory of Supplemental Data

1) Supplementary Table S1
2) Supplementary Figure S1
## Supplementary Table S1: Primers used in quantitative RT-PCR assays

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Supplementary Figure S1.

Gene expression analyses in the lungs of NNK-treated $gp130^{+/+}$ and $gp130^{F/F}$ mice. A and B, qPCR expression analyses of the indicated genes were performed on lung cDNA from $gp130^{+/+}$ (+/+) and $gp130^{F/F}$ (F/F) mice 16 weeks post-NNK administration. Expression data are normalized against $18S$, and are presented from $n = 4$ mice per genotype as the mean ± SEM.