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In vivo evidence that RBM5 is a tumour suppressor in the lung

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Cigarette smoking is undoubtedly a risk factor for lung cancer. Moreover, smokers with genetic mutations on chromosome 3p21.3, a region frequently deleted in cancer and notably in lung cancer, have a dramatically higher risk of aggressive lung cancer. The RNA binding motif 5 (RBM5) is one of the component genes in the 3p21.3 tumour suppressor region. Studies using human cancer specimens and cell lines suggest a role for RBM5 as a tumour suppressor. Here we demonstrate, for the first time, an *in vivo* role for RBM5 as a tumour suppressor in the mouse lung. We generated *Rbm5* loss-of-function mice and exposed them to a tobacco carcinogen NNK. Upon exposure to NNK, *Rbm5* loss-of-function mice developed lung cancer at similar rates to wild type mice. As tumourigenesis progressed, however, reduced *Rbm5* expression lead to significantly more aggressive lung cancer i.e. increased adenocarcinoma nodule numbers and tumour size. Our data provide *in vivo* evidence that reduced RBM5 function, as occurs in a large number of patients, coupled with exposure to tobacco carcinogens is a risk factor for an aggressive lung cancer phenotype. These data suggest that RBM5 loss-of-function likely underpins at least part of the pro-tumourigenic consequences of 3p21.3 deletion in humans.

Late stage detection makes lung cancer one of the most fatal forms of cancer, with a five-year survival rate of 17% overall, or below 2% for those with stage IV disease at diagnosis¹. A 2012 World Health Organization report estimated that of the 1.8 million people diagnosed with lung cancer worldwide, 1.6 million would die from the disease². However, if diagnosed in its earliest stages, surgery, chemotherapy and radiation therapy present a likely cure.

RNA binding motif 5 (*RBM5*) is one of the genes located within the tumour suppressor region 3p21.3; a region containing 19 genes that is frequently deleted in lung cancer and other types of carcinomas³. Moreover, the 3p21.3 deletion is detected in pre-neoplastic lesions in smokers⁴, indicating that it is an early change in the multistep pathogenesis of lung cancer. Despite this, a role for 3p21.3 deletions, and its component genes, in *in vivo* tumour development is still a matter of conjecture.

Human lung cancers can be divided into two main histopathological subtypes: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC accounts for approximately 85% of lung cancers and can be divided into adenocarcinoma and squamous cell carcinoma (SCC). About 40% of human lung cancers are adenocarcinomas. Decreased *RBM5* expression, at the mRNA and protein levels, have been reported in primary NSCLC specimens compared to normal adjacent tissues⁵. Further, *RBM5* is one of nine genes down-regulated in metastases of primary tumours⁶. *RBM5* is also included in the 17 common gene signatures associated with metastasis identified in multiple solid tumour types. Solid tumours carrying this gene expression signature have higher rates of metastasis and poor clinical outcomes⁶. Decreased *RBM5* expression in primary lung tumours has been shown to correlate with lymph node metastasis⁷. In addition, *RBM5* has been implicated in breast cancer

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development^{8,9}, vestibular schwannomas¹⁰ and renal carcinomas¹¹. Collectively, these data suggest that reduced *RBM5* expression is associated with increased cancer risk and that *RBM5* is a tumour suppressor.

In support of this hypothesis, *in vitro* over-expression of *RBM5* was shown to inhibit the growth of human lung cancer cell lines by increasing apoptosis and inducing cell cycle arrest in G1¹². The inhibition of cell growth was associated with decreased cyclin A and phosphorylated retinoblastoma (RB) and an increase in the expression of the proapoptotic protein Bax¹². *RBM5* has been shown to suppress anchorage-dependent and anchorage-independent growth in A9 mouse fibrosarcoma cells and to inhibit their tumour forming activity in nude mice⁵.

RBM5 is an RNA binding protein that has previously been shown to regulate the splicing of apoptosis-related pre-mRNAs, including Caspase 2¹³, *FAS* receptor and *c-FLIP*¹⁴, B-lymphocyte cytidine deaminase enzyme *AID* (activation-induced cytidine deaminase)¹⁵, Notch pathway regulator *Numb* in HeLa cells¹⁶ and numerous transcripts required for male germ cell development¹⁷. Its role as a splicing regulator is conserved in *Arabidopsis*¹⁸.

Since the cloning of the *RBM5* gene¹⁹, a growing body of literature strongly suggests a role for *RBM5* as a tumour suppressor^{12–14,16}. However, the *in vivo* tumour suppression activity of *RBM5* has not been tested. Within this study, we have generated *Rbm5* heterozygous knockout mice, i.e. analogous to the reduced expression seen in lung cancer patients, and have used them to demonstrate a role for *RBM5* in *in vivo* tumour suppression function in the lung.

Results and Discussion

To test the *in vivo* function of *RBM5*, we generated an *Rbm5* knockout mouse line using a gene trapped ES cell line. The gene-trapped cassette was inserted into intron 1 of the *Rbm5* gene. This produced a truncated *Rbm5* mRNA containing exon 1, resulting in no protein production (null allele) (Fig. 1a). Upon the establishment of a heterozygous knockout colony, the colony was backcrossed onto a C57BL6/J background for 10 generations. Mice heterozygous for the *Rbm5* knockout allele (referred to as HET, *Rbm5*^{+/-}) were viable, fertile and survived to adulthood with no detectable developmental defects. By contrast, and although a small number of homozygous *Rbm5* knockout mice (referred to as KO, *Rbm5*^{-/-}) were found, there was a pronounced deficit in *Rbm5*^{-/-} pups on the day of birth i.e. 8%, compared to an expected frequency of 25%. There was an absence of *Rbm5*^{-/-} mice at weaning age (0% at 3 weeks postnatal) (Fig. 1b). Indeed, virtually all *Rbm5*^{-/-} pups were dead by 3 days post-natal. These data show that *Rbm5* is essential for either embryonic or early post-natal development. To define the time point when the embryo/foetus loss occurred, we collected pups from heterozygous time matings at E18.5. At this age, the ratio of each genotype was as expected according to the Mendelian rule (Fig. 1b). This finding indicates that *Rbm5* is absolutely required for the survival of newborn pups. The pathology underlying *Rbm5*^{-/-} pup death is currently unknown.

In order to ascertain the degree of gene trapping efficiency, we collected lung tissues from wild type (WT, *Rbm5*^{+/+}) and *Rbm5*^{-/-} fetuses at E18.5 and performed RT-PCR using primers flanking exons 1 and 4 (Fig. 1c). No *Rbm5* transcript was detected in the *Rbm5*^{-/-} lungs compared to that of *Rbm5*^{+/+} lungs. Furthermore, using quantitative PCR (qPCR) we showed that there was a significant reduction in the levels of *Rbm5* mRNA in the *Rbm5*^{+/-} lung (64% reduction, Fig. 1d) and testis (76% reduction, Fig. 1e) collected from 8 week old mice. This data was mirrored using western blotting of adult lungs (Supplementary Fig. 1). This data indicate that the gene trap cassette was efficiently interrupting *Rbm5* gene expression as expected and that *Rbm5*^{+/-} mice contained reduced *Rbm5* expression, analogous to the situation observed in many lung cancer patients.

Based on mRNA expression pattern, *Rbm5* is a ubiquitously expressed gene with the highest expression level found in the testis¹⁷. Using immunofluorescence labelling, we showed that *RBM5* protein was widely localized in the adult mouse lung. *RBM5* was detected in almost all cells within the conducting airway epithelium, as shown by double-labelling with the secretory cell marker CC10 (also known as SCGB1A1) (Fig. 2a,b). *RBM5* expression in CC10-positive and CC10-negative cells indicated localisation in both secretory and ciliated cells, respectively. In the distal lung, *RBM5* expression was also detected in many cell lineages, including type II alveolar epithelial cells (AECs), as shown by double-labelling with the type-II AEC marker Pro-surfactant protein C (ProSPC) (Fig. 2c,d). A strong nuclear localisation was observed in both cell types. This result was consistent with previous studies demonstrating nuclear *RBM5* localisation in male germ cells¹⁷ and HeLa cells¹³.

To date, the cellular origin of most types of lung cancers remained largely unknown. However, studies using a knock-in mouse model carrying a codon 12 *K-Ras* mutant gene indicate that type II AECs are the cells of origin of *K-Ras*-induced adenocarcinomas²⁰. Given the localization of *RBM5* to type II cells, and previous studies in cell lines and human tissue suggesting reduced *RBM5* expression is associated with increased lung cancer risk^{7,12}, we decided to test the susceptibility of reduced *Rbm5* expression (*Rbm5*^{+/-}) on lung cancer progression i.e. is ascertain if *RBM5* is a tumour suppressor *in vivo*.

Lung cancer, like many other cancers, is thought to conform to the 2-hit model i.e. genetic and environmental hits. Cigarette smoking is the greatest risk factor for lung cancer²¹ and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most potent carcinogen identified in cigarette smoke and has been widely used as accepted means of lung cancer induction²². As such, in this study, we utilized NNK to induce lung cancer in *Rbm5* heterozygous knockout (carrying one copy of the *Rbm5* gene) and wild type mice. The purpose of using NNK was to accelerate carcinogenesis into a time frame that would allow an in-depth analysis of lung cancer initiation and progression. Although there is a strong correlation between cigarette smoking and lung cancer, susceptibility to lung cancer among smokers is not uniform²³. Smokers who carry particular genetic mutations have a dramatically higher risk of developing lung cancer²³. Studies have shown that the *KRAS* activating mutation (G12V) is often associated with smoking-related NSCLC²⁴. Similarly, different strains of mice have been shown to have differential susceptibility to lung cancer induction upon exposure to NNK²⁵. The A/J mouse strain, that carries a naturally occurring *KRAS* G12V mutation, is one of the most susceptible strains for NNK-induced lung cancer. Importantly, *RBM5* has been found to be down-regulated in rat embryonic fibroblast cells that have

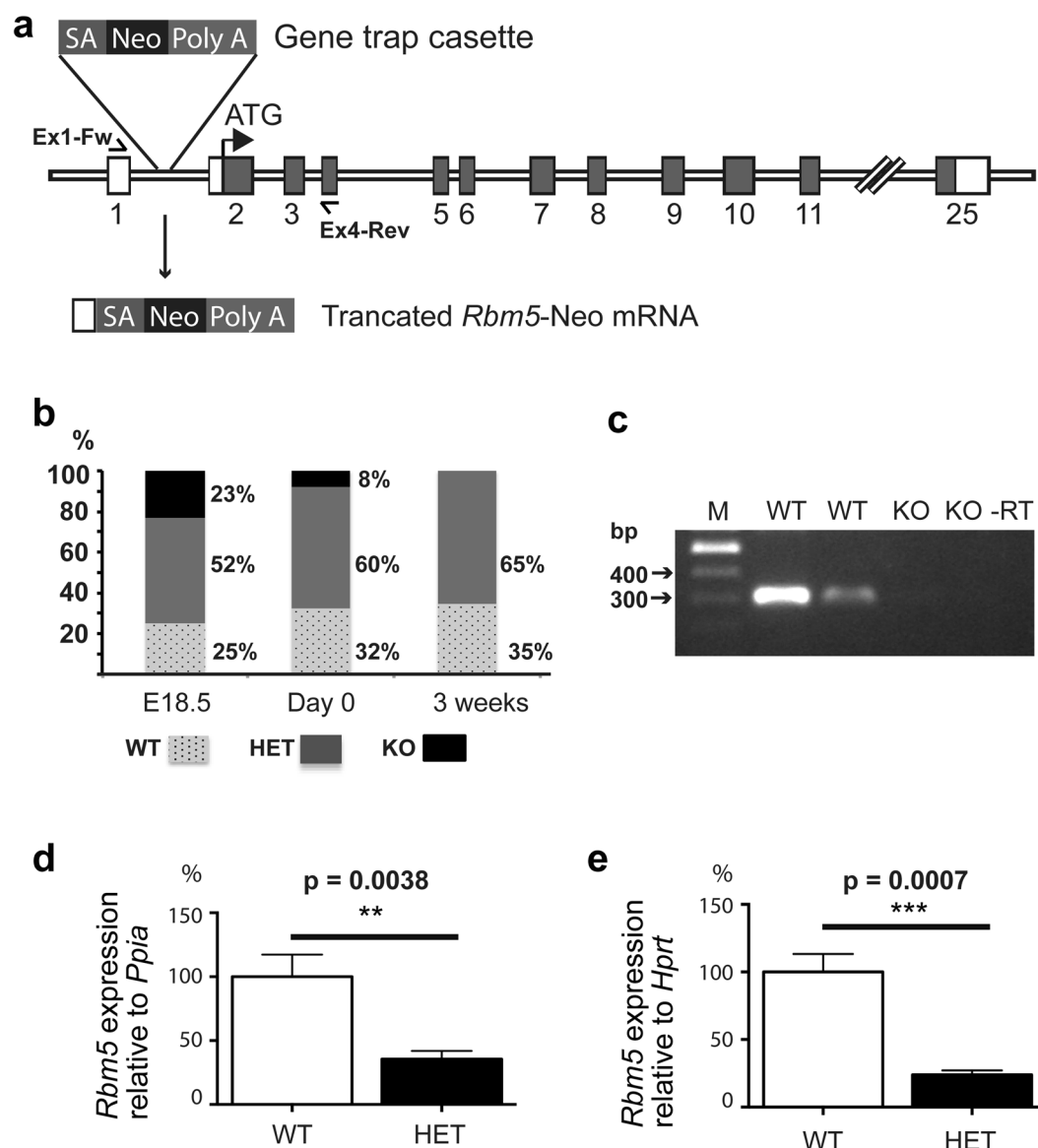


Figure 1. Generation and characterisation of the *Rbm5* gene trap mouse line. **(a)** The *Rbm5* gene trap mouse line: The U3neoSVFS gene-trapped cassette was inserted into intron 1 of the *Rbm5* gene (ENSMUSG0000032580). This produced a truncated *Rbm5* mRNA containing exon 1 (ENSMUSE00000371436), resulting in the production of truncated *Rbm5* mRNA and no protein production (null allele). SA: Splicing acceptor site; Neo: Neomycin resistance gene; Poly A: Polyadenylation signal. **(b)** Distribution of genotypes of progeny from heterozygous knockout breeding pairs at embryonic day 18.5 (E18.5), day of birth (Day 0) and at 3 weeks. WT: wild type; HET: heterozygous knockout; KO: homozygous knockout. **(c)** Verification of gene trapping efficient by RT-PCR on E18.5 lung using primers Ex1-Fw (5'-CTCCTGCTTTGTTCCCTCTG-3') and Ex4-Rev (5'-CCATCTTCAGACCGGTCACCT-3'). The WT allele expected PCR product is 298 bp and no products for the competed gene trap KO allele. -RT: negative control (no reverse transcriptase). **(d,e)** Quantitative PCR (qPCR) was performed to measure *Rbm5* mRNA expression levels in adult lung (D) and adult testis (E) samples. $n = 3$ per genotype, 8 weeks old. Data is expressed as mean $\pm$ SD. Statistical significance for all analyses was determined using a two-tailed student t-test.

been constitutively over-expressed RAS G12V protein²⁶. These studies suggest a correlation between RBM5 and KRAS activating mutation in the pathogenesis of lung cancer.

Given this association, to investigate the role of RBM5 in lung cancer, *Rbm5*^{+/-} mice were backcrossed onto the A/J mouse strain for 10 generations. Six week-old *Rbm5*^{+/-} (HET) and *Rbm5*^{+/+} (WT) littermates were injected with NNK to induce lung cancer as previously described²⁷. The use of *Rbm5*^{+/-} mice (rather than *Rbm5*^{-/-} mice) is reflective of the situation in patients wherein chromosome 3p21.3 loss occurs in heterozygosity. Lung tissues were analysis for the presence of tumours and the progression of tumour formation at 16, 20 and 48 weeks post-NNK injection.

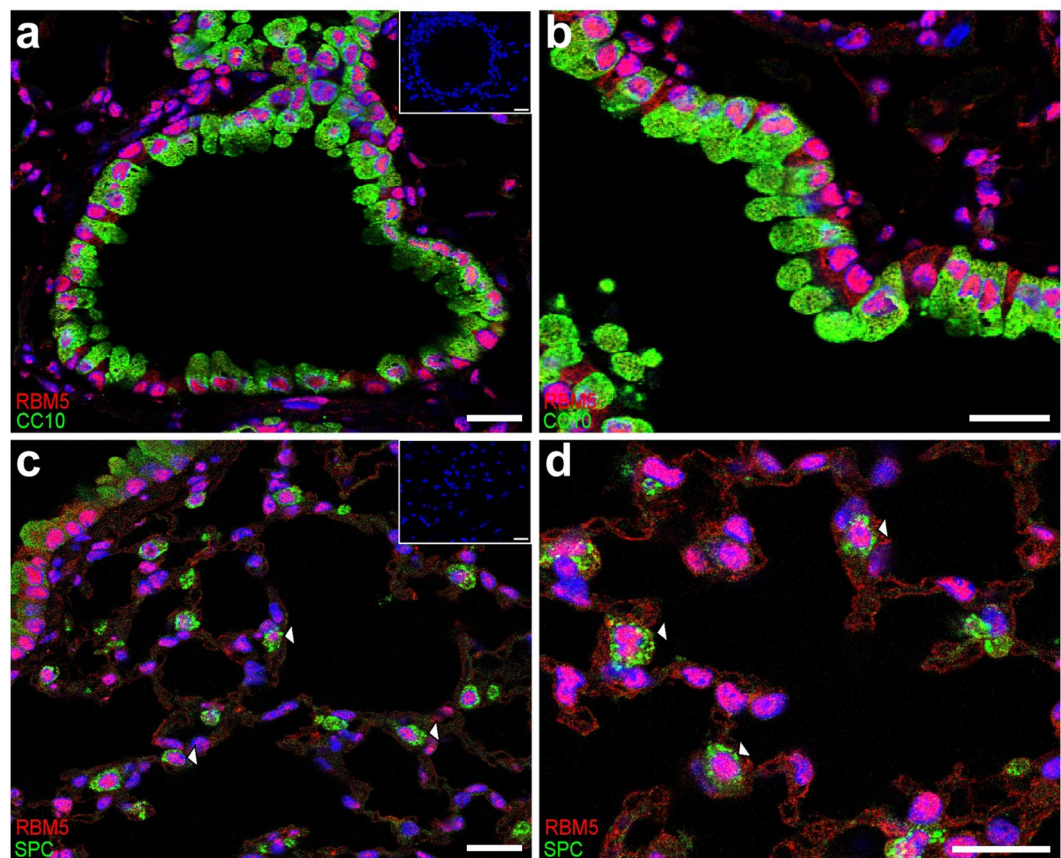


Figure 2. RBM5 localised to type II alveolar epithelial cells (AECs) and Clara cells. The localisation of RBM5 in the adult lung (8 weeks old) was determined by immunofluorescence using a RBM5 mouse monoclonal antibody as described previously¹⁷. (**a,b**) RBM5 localised to Clara cells as indicated by double staining for RBM5 (red) and CC10 (green). (**c,d**) RBM5 localised to type II alveolar epithelial cells (AECs) as indicated by double staining of RBM5 (red) and Pro-surfactant protein C (SPC, in green). Insets = negative controls (no primary antibody). Scale bars = 20 μ m.

At 16 and 20 weeks post-NNK injection, a small number of lung tumours were observed in both *Rbm5*^{+/+} and *Rbm5*^{+/-} mice, however no significant difference in tumour number or tumour area was observed (Supplementary Fig. 2a–d). The control group, which received saline injection, showed no sign of lung tumours ($n = 10$). This finding suggested that RBM5 dosage does not affect the initiation of NNK-induced lung cancer and is consistent with previously published data showing that NNK in isolation can induce lung cancer²⁸. While not examined at molecular detail here, the histopathological progression of NNK-induced lung cancer progression was consistent with previous publications²⁸.

At 48 weeks post-NNK injection, however, *Rbm5*^{+/-} mice displayed a significant increase in both the number of individual tumours, and tumour area, compared to *Rbm5*^{+/+} mice (Fig. 3). An independent, and blinded, analysis by a pathologist confirmed that the *Rbm5*^{+/-} lungs contained an increased numbers of adenocarcinoma nodules with increased tumour nodule sizes compared to that in *Rbm5*^{+/+} lungs (148% and 153% increase on control wild type tissue respectively) (Fig. 3, Supplementary Table 1). These data indicate that reduced *Rbm5* expression leads to more aggressive progression of lung adenocarcinomas in the *Rbm5*^{+/-} mice.

Consistent with previous publications suggesting that AEC cells are the origin of many lung cancers²⁰, tumours in both *Rbm5*^{+/+} and *Rbm5*^{+/-} mice were Pro-SPC positive, and CC10 negative (Supplementary Fig. 3). Somewhat surprisingly, however, there was no significant difference in either the rates of apoptosis (TUNEL staining, data not shown) or Ki67 labelling with tumours between genotypes (Ki67: 186.5 $\pm$ 59.92 in wild type versus 149.0 $\pm$ 51.56 positive cells per mm² in *Rbm5*^{+/-}). The rates of apoptosis per tumour area were too low to reliably quantitate in both genotypes. This data is perhaps, however, consistent with the long progression time required to see a difference in tumour mass between genotypes.

Lung cancer, like many other types of cancer, is believed to be initiated by over-activation of oncogenes and/or down-regulation of tumour suppressor genes. Although several studies using cell lines have provided evidence for RBM5 being a regulator of apoptosis and the cell cycle, direct *in vivo* evidence for its tumour suppressor activity remained elusive. Our data is the first to demonstrate the physiological role for RBM5 as a tumour suppressor in the lung. At 16–20 weeks post-exposure to NNK, we showed that reduced *Rbm5* expression had no effect of the initiation of lung adenocarcinomas. As tumourigenesis progressed, however, reduced *Rbm5* expression resulted in more aggressive tumour growth in *Rbm5*^{+/-} lungs. This data is consistent with previous studies showing that

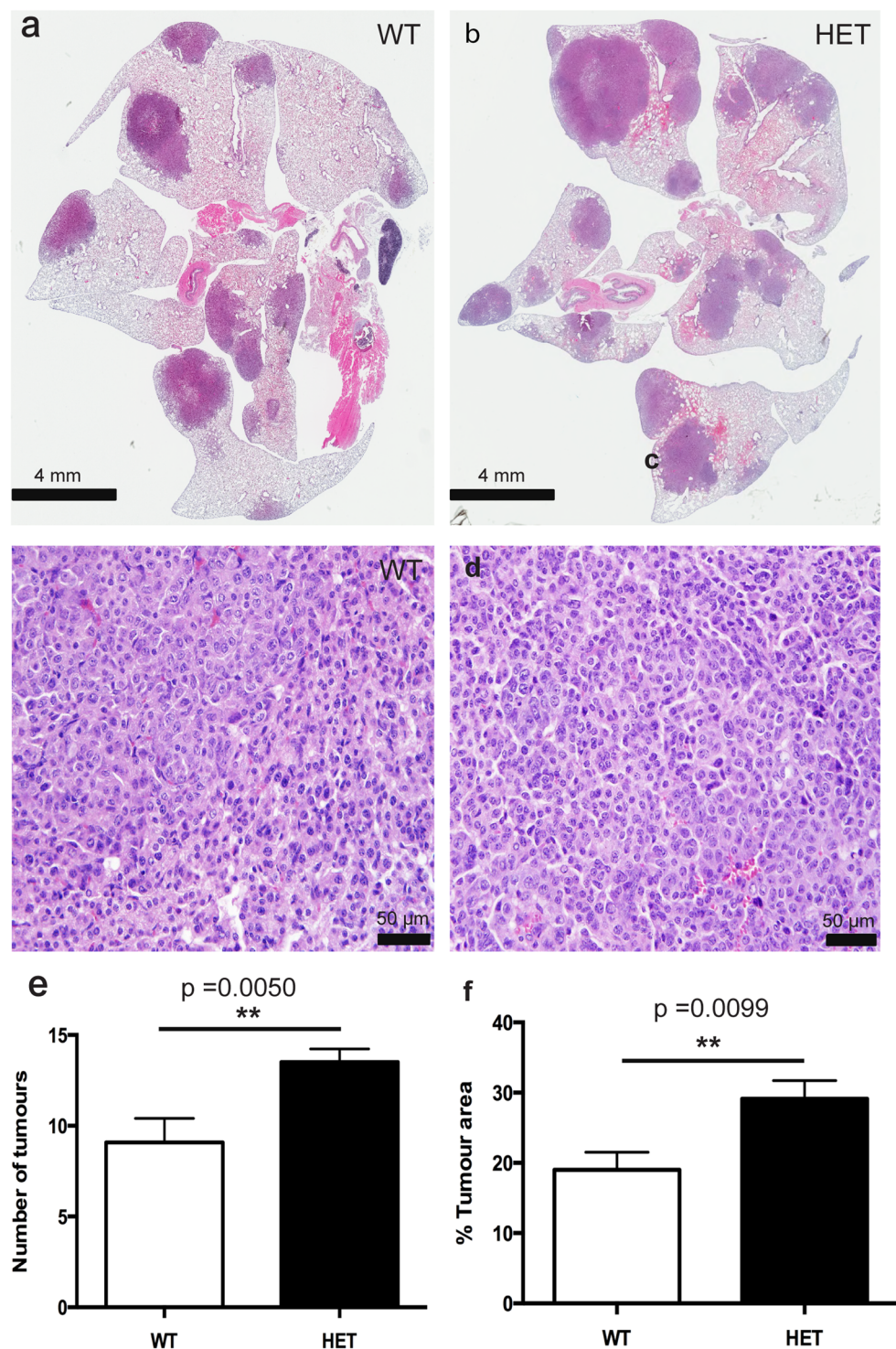


Figure 3. *Rbm5* haploinsufficiency leads to accelerated lung cancer progression. (a–d) H&E staining of lungs collected from mice 48 weeks post-NNK injection. Number of tumours (e) and tumour area (f) in mice 48 weeks post-NNK injection. Data are expressed as mean $\pm$ S.E.M. (standard error of mean). $n = 13$ WT and $n = 16$ HET. $p < 0.05$ was considered statistically significance. Statistical significance for all analyses was determined using a two-tailed student t-test.

decreased *RBM5* expression in human lung tumours is correlated with lymph node metastasis⁷, and ectopic over-expression of *RBM5* inhibited tumour forming activity in nude mice⁵.

Metastatic lung cancer is a leading cause of mortality. Surgery, chemotherapy, and radiation therapy present a likely cure if the cancer is diagnosed in its earliest stages. The data presented herein suggests that a quantitative

assessment of RBM5 expression in lung cancer biopsies, for example, may be a valuable prognostic marker for the prediction of high-risk cases for whom more aggressive post-surgical treatments may be warranted. Moreover, our data reveal that RBM5 is required for early post-natal survival and acts *in vivo* as a tumour suppressor that likely underpins at least part of the pro-tumourigenic outcomes resulting from 3p21.3 deletion in humans.

Methods

Mouse line production and genotyping. Animal procedures were conducted in accordance with the Australian National Health and Medical Research Council's Guidelines on Ethics in Animal Experimentation and were approved by the Monash University Animal Experimentation Ethics Committee. The *Rbm5* gene trap mouse line was generated at the Australian Phenomics Network (APN) Monash University Node using standard methods²⁹ and a gene-trapped ES cell (PST20293-NR, on a 129Sv x C57BL/6J background) obtained from the Toronto Centre for Phenogenomics Centre for Modelling Human Disease. The U3neoSVFS gene-trapped cassette was inserted into intron 1 of the *Rbm5* gene (ENSMUSG00000032580). This produced a truncated *Rbm5* mRNA containing exon 1 (ENSMUSE00000371436), resulting in the production of truncated *Rbm5* mRNA and no protein production (null allele).

Mouse genotypes were determined from tail biopsies using real time PCR with specific probes designed for each allele (Transnetyx, Cordova, TN). The *Rbm5* KO allele was detected using the Neomycin probe and primers set which included: Forward primer: GGGCGCCCGGTTCTT; Reporter: ACCTGTCCGGTGCCC; and Reverse primer: CCTCGTCCTGCAGTTTCATTCA. The *Rbm5* WT allele was detected using the *Rbm5* WT probe set which included: Forward primer: CATTACACCCAGTGATTTTGCA; reporter: TTGGTGCTGTCCCTTAAGTC; and Reverse primer: CCTCTGGCGGCTGACA.

Verification of gene trapping efficiency was performed by RT-PCR on E18.5 lung using primers Ex1-Fw (5'-CTCCTGCTTTGTTCCCTCTG-3') and Ex4-Rev (5'-CCATCTTCAGACCGGTCAC-3'). The WT allele expected PCR product is 298 bp and no products for the competed gene trap KO allele.

Quantitative PCR (qPCR) was performed to measure *Rbm5* mRNA expression levels in adult lung and adult testis samples ($n = 3$ per genotype, 8 weeks old) using TaqMan assays (*Rbm5* exons 4–5: Mm00455721, Thermo Scientific). The primers used in the assay were as follows: The levels of *Rbm5* mRNA expression between *Rbm5*^{+/+} and *Rbm5*^{+/-} lung and testis samples were normalised against mRNA levels of *Ppia* (Mm02342429) and *Hprt* (Mm00446968), respectively.

At the time of writing this manuscript, data contained within Ensembl indicated that the mouse *Rbm5* gene (entry ENSMUSG00000032580) produced 24 transcripts, 7 of which are predicted to be protein coding and another 4 which are predicted to be subject to nonsense-mediated RNA decay. The remaining 13 splice variants contain retained introns and are of undefined significance. Primers against exons 4–5 will detect 5 of the 7 predicted protein coding isoforms and an additional 6 of the transcripts which are thought to undergo non-sense mediated decay.

Western blotting. In order to assess the degree of protein reduction in *Rbm5*^{+/-} mice, lung tissue was from wild type and *Rbm5*^{+/-} adult mice then processed for western blotting as described previously³⁰. Blots were probed using the RBM5 monoclonal antibody A9 described in¹⁷. The A9 antibody is predicted to be able to bind to all 7 of the protein coding isoforms, and would also bind to 3 of the 4 transcripts/proteins predicted to be subject to nonsense-mediated RNA decay if protein was to be produced.

Immunochemical labelling of cells. The localisation of RBM5 in the adult lung (8 weeks old) was determined by immunofluorescence using an RBM5 mouse monoclonal antibody as described previously¹⁷. To distinguish different cell lineages in the lung, we co-labelled sections with antibodies against Pro-surfactant protein C (AB3786, Merck Millipore), as a marker for type II alveolar epithelial cells, and CC10 (CC10 (T-18), SC-9772, Santa Cruz Biotechnology), as a marker for secretory cells. Protein localisation was determined through confocal microscopy using an SP-8 microscope (Leica Microsystems).

Immunochemical labelling of cells. In order to label cells undergoing apoptosis sections were stained using the TUNEL kit (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, Merck, S71000) as per the manufacturer's instructions. In order to label proliferating cells, additional sections were labelled for Ki67 (NCL-Ki67p, Novocastra, at a dilution of 1 in 1000) using immunohistochemistry. Bound antibody was detected using the Dako polymer, anti-rabbit, HRP kit as per the manufacturer's instructions. The number of proliferating cells was subsequently quantitated from scanned slides using an Aperio ePathology scanner (Leica Biosystems) and Imagescope software. The number of apoptotic cells per tumour was too low to reliably quantitate. $N = 6$ samples per genotype were analysed. For both sets of labelling wild type tissue was used as a positive control.

Lung cancer induction. NNK (Sapphire Biosciences, Cat. No. 000–01622) was used to induce lung tumours. Six-week old *Rbm5*^{+/-} (HET) and *Rbm5*^{+/+} (wild type, WT) mice were administered three i.p. injections over one week (Monday, Wednesday, Friday) at a dose of 50 mg/kg body weight. Mice were separated into two groups and were either injected with NNK (in saline, 0.1 ml volume) or vehicle (saline, 0.1 ml volume). Mice were humanely killed at 16, 20 and 48 weeks after the final NNK injection. Mice were anaesthetised, an incision was made in the trachea then lungs were perfused fixed via a tracheal cannula with 4% formaldehyde at exactly 200 mm H₂O pressure. Fixed tissues were paraffin embedded, sectioned and used for histological analysis (hematoxylin and eosin (H&E) staining). Total tumour area was measured using Imagescope software (Aperio).

Data analysis was performed using GraphPad Prism 6 software and $p < 0.05$ was considered statistically significant. Histopathology was independently assessed in a blinded manner by a qualified pathologist (BK).

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Author Contributions

D.J., D.N.W., B.J.J. and M.K.O.B. - designed the experiments. D.J., D.N.W., A.E.O.C., D.J.M., S.G., A.D.B., B.K., A.M., T.J.C., B.J.J. and M.K.O.B. - conducted the experiments and analyzed data. D.J., T.J.C. and M.K.O.B. - wrote the manuscript. D.J. and A.E.O.C. - prepared the figures. All authors reviewed the manuscript.

Additional Information

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