Sex disparities in non-small cell lung cancer: mechanistic insights from a cRaf transgenic disease model

*Shen Zhong and Jürgen Borlak**

Centre for Pharmacology and Toxicology, Hannover Medical School, Carl-Neuberg-Str. 1, Hannover 30625, Germany

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Summary

Background Women are at greater risk of developing non-small cell lung cancer (NSCLC), yet the underlying causes remain unclear.

Methods We performed whole genome scans in lung tumours of cRaf transgenic mice and identified miRNA, transcription factor and hormone receptor dependent gene regulations. We confirmed hormone receptors by immunohistochemistry and constructed regulatory gene networks by considering experimentally validated miRNA-gene and transcription factor-miRNA/gene targets. Bioinformatics, genomic foot-printing and gene enrichment analysis established sex-specific circuits of lung tumour growth. Translational research involved a large cohort of NSCLC patients. We evaluated commonalities in sex-specific NSCLC gene regulations between mice and humans and determined their prognostic value in Kaplan–Meier survival statistics and COX proportional hazard regression analysis.

Findings Overexpression of the cRaf kinase elicited an extraordinary 8-fold increase in tumour growth among females, and nearly 70% of the 112 differentially expressed genes (DEGs) were female specific. We identified oncogenes, oncomirs, tumour suppressors, cell cycle regulators and MAPK/EGFR signalling molecules, which prompted sex-based differences in NSCLC, and we deciphered a regulatory gene-network, which protected males from accelerated tumour growth. Strikingly, 41% of DEGs are targets of hormone receptors, and the majority (85%) are oestrogen receptor (ER) dependent. We confirmed the role of ER in a large cohort of NSCLC patients and validated 40% of DEGs induced by cRaf in clinical tumour samples.

Interpretation We report the molecular wiring that prompted sex disparities in tumour growth. This allowed us to propose the development of molecular targeted therapies by jointly blocking ER, CDK1 and arginase 2 in NSCLC.

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Keywords: Lung cancer; cRaf; Hormone receptors; Regulatory gene network; Clinical validation

Introduction

According to the recent cancer statistics, lung cancer (LC) is the second most frequently diagnosed cancer worldwide and the primary cause of cancer mortality with an annual 2.2 million new cases (11.4% of total cancer cases) and 1.8 million death (18.0% of total cancer deaths). About 80%–85% of LC cases are non-small cell lung cancer (NSCLC).[1] There is conclusive evidence for tobacco product consumption to be the major risk factor for NSCLC with 80% of cases being linked to cigarette smoking. Strikingly, the incidence rate of NSCLC among women is increasing or even surpassing that of men to possibly suggest sex-related differences in the development of NSCLC.[2] Even more astonishingly is the fact that women who had never smoked are at higher risk of developing lung cancer, i.e., 20% of females as compared to 6% for males.[3,4] While the reasons remain uncertain, hormonal and genetic factors have been linked to sex disparities in NSCLC, notably mutations in the p53 tumour suppressor and KRAS kinase, regulation of growth factor and DNA repair enzymes.[5]

In regards to drug treatment responses there are also sex-related differences among NSCLC patients. While

*Corresponding author. Centre for Pharmacology and Toxicology, Hannover Medical School, Carl Neuberg Str. 1, Hannover 30625, Germany.
E-mail address: Borlak.Juergen@mh-hannover.de (J. Borlak).

Research in context

Evidence before this study

The incidence of lung cancer among young women is higher than in men and appears to be independent of sex differences in smoking behaviours. Moreover, the incidence of lung adenocarcinomas of female never smokers is significantly higher when compared to males. Together, there is cumulative evidence for sex disparities in lung cancer.

Added value of this study

We investigated lung tumour growth in a transgenic disease model and observed an 8-fold increased tumour burden in female mice. The genomic study revealed the critical role of the oestrogen receptor alpha in influencing immune cells of the tumour microenvironment and in the regulation of genes controlling tumour growth. Translational research confirmed clinical relevance of the animal data in a large cohort of lung cancer patients, and we identified major differences in the control of oestrogen receptor regulated genes among pre- and postmenopausal lung cancer patients.

Implications of all the available evidence

The genomic data provided strong evidence for the need to consider sex differences in the treatment of lung cancer patients. We propose a combination therapy consisting of an anti-oestrogen receptor alpha, cyclin-dependent kinase 1 and arginase 2 inhibition for the treatment of female lung cancer patients.

immune checkpoint inhibitors (ICIs) and molecular targeted therapy have become the mainstay of NSCLC therapy, the results vary between smokers and non-smokers, and male and female patients.[6–8] For example, the Keynote-024 study investigated the effectiveness of Pembrolizumab, i.e., a PD-1 antagonist as monotherapy in metastatic NSCLC patients. Patients with PD-L1 expression >50% and especially male patients benefitted from this treatment to a greater extend (hazard ratio (HR) 0.54 vs. 0.95 for male and females).[7] Conversely, the Impower130 study focused on the therapeutic efficacy of atezolizumab, i.e., a PD-L1 antagonist which was given in combination with chemotherapy and the median survival of female NSCLC patients exceeded that of males, i.e., 21.4 vs. 16 month (HR 0.66 vs. 0.87).[8]

Together, these and other observations are suggestive for sex-related differences in NSCLC and demanded mechanistic investigations. Among the genetic events in NSCLC, miRNAs are of critical importance in instructing cellular transformation and tumorigenesis. MiRNAs are small non-coding RNAs (~20–22 nucleotide) and typically repress gene expression. Recently, we published a systematic review on the predictive and prognostic roles of miRNA in NSCLC patients,[9] and oncomirs are of particular interest as they frequently target tumour suppressor genes. RNA-based therapeutics, i.e., antagomirs are therefore developed to block the activity of oncomirs and this requires in-depth knowledge on the complex interplay between miRNAs and their target genes.[10]

Furthermore, transcription factors (TFs) play a fundamental role in the control of gene expression by binding to cognate recognition sites in enhancers or core promoter elements of gene promoters, to either activate or repress transcription.[11] Aberrant transcriptional regulations in cancers is the focus of oncogenomics, especially as deregulated TFs are one of the hallmarks of cancer.[12] Adding to complexity are TF-miRNA-gene networks that function as feed-forward loops (FFLs) in the control of gene expression.[13] However, miRNAs-gene regulatory networks and sex-related differences in tumour growth have not been investigated so far.

In the past, we reported the genomics of NSCLC in a cRaf transgenic mouse model. We identified in laser dissected material differentially expressed genes (DEGs) specifically linked to atypical adenomatous hyperplasia (AAH), i.e., a precursor lesion with high risk of malignant transformation as well as genes specifically regulated in adenocarcinomas of the lung. The regulated genes code for multiple processes such as cellular growth and proliferation, cell death, and immune response.[14,15] Specifically, the Raf kinase family consists of a, b and cRaf proteins and these serine/threonine kinases are part of the MAPK/ERK signalling pathway.[16] Although similar, the signalling outputs of RAF paralogs can differ.[17] The sequential phosphorylation of MAP kinases stimulates MYC activity and endorses entry into the G1 phase of the cell cycle to initiate cell proliferation. Ablation of cRaf significantly reduced tumour burden in a $Kras^{G12V}$ oncogene-driven NSCLC model.[18] In addition, cRAF mutation have been identified in NSCLC patients[19] and the common $KRAS^{G12C}$ mutation appears to be more frequent among women even with a lesser smoking history.[20]

Our study aimed at investigating sex-based differences in tumour growth in a cRaf transgenic NSCLC disease model. We observed a highly significant disproportional 5 and 8-fold increase in tumour growth in the right and left lung of female transgenic mice. This prompted us to examine the complex interplay of miRNAs, TFs, hormone receptors and target genes in transgenic females. Next to histopathology, oncogenomic investigations and DNA-sequencing of tumour suppressor genes, we employed genomic footprinting to construct gene regulatory networks. Furthermore, we assessed clinical relevance by comparing DEGs and miRNAs of the cRaf lung cancer disease model with their regulation in a large cohort of lung

adenocarcinoma patients. Importantly, we identified genes mechanistically linked to tumour growth and determined the prognostic value of highly regulated genes in Kaplan–Meier survival plots.

Overall, we report new insight into sex differences in NSCLC and highlight the role of hormone receptors, miRNAs and TF in the control of cell cycle regulators, tumour suppressors, oncogenes and oncomirs.

Methods

Ethics

We performed the animal study in accordance with the American Association for Laboratory Animal Science Policy on the Human Care and Use of Laboratory Animals. Approval to carry out animal studies was granted by the ethical review board of the Lower Saxony State Office for Customer Protection and Food Safety (LAVES), Germany (Az: 33-42502-04/869 and 33-42502-06/1081).

Furthermore, we obtained FFPE tissue blocks and fresh frozen tissue of human NSCLC from the Pathology Department of Hannover Medical School. Ethical approval for the use of anonymized specimen was obtained from the local ethics committee (3381-2016) and the written consent from all participants was obtained. We collected samples from both male and female NSCLC patients and the data were self-reported by study participants. Further details are given in Supplementary Table S1.

SPC cRaf transgenic mice

The original cRaf transgenic mouse model stems from the laboratory of Prof. Ulf Rapp (University of Würzburg, Germany) and its targeted overexpression in respiratory epithelium induced tumour growth.[21] The cRaf-1 transgene lacks the regulatory NH2-terminal sequences of the cRaf-1 protein and therefore is constitutively active without interaction with upstream regulators such as RAS. By employing the surfactant protein SPC promoter, the transgene is specifically targeted to the respiratory epithelium of the lung. However, unlike the original animal model, which was bred in a C57/BL6 and 2 DBA hybrid background, we kept the transgenic mouse line in a C57/BL6 background.

Supplementary Table S2 gives an overview of the experimental groups.

Histopathology and immunohistochemistry

The left and right lung of N = 8 cRaf transgenic males and females and non-transgenic controls were fixed in 4% buffered formaldehyde in PBS for approximately 20 h, dehydrated and embedded in paraffin (Roti-Plast™, Roth, Karlsruhe, Germany). Tissue sections were obtained with a microtome and stained with hematoxylin and eosin according to standard protocols. We performed serial sectioning of tissue blocks and used the Mann–Whitney U test to compare the number of tumours sized >200 μm.

Additionally, we employed standard protocols to confirm the expression of the oestrogen alpha and androgen receptor. Briefly, 3 μm tumour sections were cut and mounted on coated slides. For the detection of ER alpha we used the clone SP1 (Roche-Ventana REF790-4325) on a BenchMark Ultra instrument (Ventana) according to the manufacturers recommendation. The staining protocol consisted of a deparaffinization, cell conditioning at pH9, primary antibody, biotin blocking and counterstain step. For the detection of the androgen receptor we used the monoclonal mouse anti-human androgen receptor clone AR441 at a dilution of 1:50 at pH9.

Whole genome miRNA profiling

We previously reported a cross-platform comparison of the Affymetrix and Agilent microarrays-based miRNA expression analysis in lung tumours of cRaf transgenic mice.[22]

Affymetrix microarray platform: We isolated from each lung 200 ng of total RNA and labelled nucleic acids with the FlashTag Biotin HSR labelling kit according to the manufacturer's instructions (Genisphere, Hatfield, PA, USA, http://media.affymetrix.com/support/downloads/manuals/mirna_flashtag_manual.pdf). We hybridized the samples onto the Affymetrix GeneChip® miRNA array 1.0, which contains 722 and 690 mouse mature and pre-miRNAs, respectively. All experimental procedures followed the manufacturer protocol.

Agilent microarray platform: We dephosphorylated 100 ng of total RNA and performed 3′ end labelling with the Cy3-pCp dye, purified the samples with Micro Bio-Spin columns, and hybridized the samples onto arrays with the miRNA Microarray System labelling kit V2 according to the manufacturer's instructions (https://www.agilent.com/store/en_US/Prod-5190-0456/5190-0456). The Agilent mouse miRNA microarray (Release 12.0, catalogue ID G4472B) contains 612 mouse mature miRNAs (https://www.agilent.com/cs/library/usermanuals/public/G4170-90011.pdf). We scanned the hybridized microarray slides with an Agilent DNA Microarray Scanner G2505C and analysed the data with the Agilent ScanControl version 8.1.3 software. We processed the scanned TIFF images numerically, applied QC tools and corrected for background and outlier pixels with the Agilent Feature Extraction Software version 10.7.7.1.

Whole genome gene expression profiling

We performed whole genome gene expression profiling as previously reported and disrupted the frozen lung tissues and homogenized it with a rotor-stator homogenizer and isolated RNA with the miRNeasy Mini Kit (QIAGEN, Germany) which included DNase treatment of the RNA extract.[14,15] We performed a second cleanup of isolated RNA with the miRNeasy Mini Kit and checked RNA for quantity, purity and integrity of the

18S and 28S ribosomal bands by capillary electrophoresis with the Agilent 2100 Bioanalyzer system and the NanoDrop ND-1000. We used 8 μg of RNA as starting material to prepare cDNA with the GeneChip® one-cycle cDNA Kit (Affymetrix) and achieved the cleanup of double-stranded cDNA with the GeneChip® Sample Cleanup module (Affymetrix).

We used 12 μl of cDNA solution for the in vitro transcription assay according to the manufacturers' recommendation (GeneChip® IVT Labelling Kit, Affymetrix) and purified the reaction product with the GeneChip® Sample Cleanup module (Affymetrix). We quantified the purified cRNA and checked the quality with the NanoDrop ND-1000 and the Agilent 2100 Bioanalyzer system. We prepared cleaved cRNA by metal-induced hydrolysis and determined the degree of fragmentation and the size of the fragmented biotinylated cRNA by capillary electrophoresis. Typically, we obtained fragments of the size of 35–200 bases.

We hybridized 10 μg of biotinylated fragmented cRNA to the GeneChip® Mouse Genome 430 2.0 array according to the manufacturer's recommendation. The hybridization was set to 16 h at 60 rpm and 45 °C in a GeneChip® Hybridization Oven 640 (Affymetrix) followed by a washing and staining step of the arrays in the GeneChip® Fluidics Station 400 (Affymetrix). We performed an antibody signal amplification with streptavidin R-phycoerythrin, followed by a washing and staining protocol (Affymetrix) (SAPE; Invitrogen, USA) according to the manufacturer's recommendation. To amplify signals, we added the SAPE solution twice with a biotinylated anti-streptavidin antibody (Vector Laboratories, CA) and a staining step in between.

We scanned the arrays on a GeneChip® Scanner 3000 and visually inspected scanned images for artifacts. We scaled each image to the same target value for comparison between chips. We used the GeneChip® Operating Software (GCOS) to control the fluidics station and the scanner, to capture probe array data and to analyse hybridization intensity data. Finally, we applied default parameters of the Affymetrix software package for analysis.

Data processing and statistics

Differentially expressed genes (DEGs)

We applied an unpaired t-test to compare the average signal values between cRaf transgenic and WT animals and to determine the significance of change in a transcript expression level.

Differentially expressed miRNA (DEMs)

We uploaded raw signal intensity data of the Agilent and Affymetrix microarrays onto the geneXplain platform and normalized the data with the LIMMA and the Robust Multi-array Average algorithm. We computed the principal component analysis based on the "prcomp" function in R[23] to identify sources that contribute by large to the variance of the data, and removed individual animals who grossly differed in their genomic responses by comparing whole genome data among different treatment groups (Supplementary File S1).

We used the hypergeometric test to calculate statistical significance of DEMs. For each miRNA, fold change and standard deviation were calculated by comparing the signal intensity of each sample in the treatment group to the average signal intensity of control group. Furthermore, we used Wilcoxon signed-rank test to compare the expression of significantly regulated miRNAs that act as tumour suppressors and oncomirs in female and male cRaf animals.

Only genes and miRNAs with a false discovery rate (FDR) < 0.05 and fold change ($|FC| \geq 2$ were considered statistically significant. We compile the data in Supplementary Table S3.

Gene ontology (GO) enrichment analysis and immune cell marker identification

We searched for enriched GO terms by considering up- and downregulated DEGs. We analysed the data with Metascape (https://metascape.org/)[24] and GeneXplain (https://genexplain.com/) software and considered significantly enriched GO terms based on the criteria p-value < 0.05 (Supplementary Table S4). We visualized the results with ggplot2 package[25] in R.[23]

We queried the CellMarker database (http://biocc.hrbmu.edu.cn/CellMarker/index.jsp) and searched literature to search for markers of immune cells and explored other repositories as summarized in Supplementary Table S5.

MiRNA-gene regulatory networks

We used the miRNet 2.0 database to search for target genes of DEMs. The database provides comprehensive information on experimentally validated miRNA targets (https://www.mirnet.ca/miRNet/home.xhtml).[26] Specifically, we compared DEMs that were identified in lung tumuors of cRaf transgenic mice to database entries of miRNet 2.0. This allowed us to identify validated DEG targets and to construct miRNA-gene regulatory networks which we visualized with the software Cytoscape 3.9.1.[27]

In-silico genomic foot printing of transcription factor binding sites

To identify transcription factor binding sites (TFBSs), we performed promoter analysis of DEGs. First, we converted DEGs to Ensembl IDs; second, promoter regions were defined as sequences from –2000 to +100 bp relative to the transcription start sites. We used positional weight matrices of the TRANSFAC® database to search for TFBSs within the promoter regions of the selected genes. The frequency of TFBSs in DEMs or DEGs (='Yes set') were compared to miRNAs and genes

which did not change their expression (='No set'). We considered enriched TFBSs of the Yes set data to be statistically significant based on the criteria fold enrichment ratio ≥1.5 and adj. p-value < 0.05. Furthermore, we utilized the GSEA database (https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp?collection=TFT), Transmir v2.0 database (https://www.cuilab.cn/transmir) and hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget#!/) to identify ChIP-seq validated TF target genes and miRNAs. Together, we only considered DEGs with proven evidence for the binding of TF proteins to recognition sites in promoters of regulated genes.

Translational research

We queried the Xena database[28] to obtain miRNA and mRNA sequencing data of TCGA-LUAD patients as well as other clinical information. For the DEG analysis, we considered a total of 510 tumour samples and 58 adjacent non-cancerous samples. For the DEM analysis, we considered 513 tumour samples and 45 adjacent non-cancerous samples. To determine sex-specific regulations, we compared female tumour samples to female adjacent non-cancerous controls, and male tumour samples to male adjacent non-cancerous controls. To further explore the differences between pre- and postmenopausal lung adenocarcinoma patients, we compared pre- and postmenopausal female lung adenocarcinoma patients to female adjacent non-cancerous controls. DEGs and DEMs were identified using Deseq2 package[29] in R.[23] Genes and miRNAs with $|FC| \geq 2$, FDR < 0.05 were considered significantly regulated. We summarize the grouping information and the results in Supplementary Fig. S1.

For the survival analysis, we included 491 lung adenocarcinoma patients with OS information (https://xenabrowser.net/datapages/?dataset=survival%2FLUAD_survival.txt&host=https%3A%2F%2Ftcga.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443). The start of the survival analysis is defined as definitive diagnosis of lung adenocarcinoma, the earliest year was 1991, and the end time for survival analysis is 2015. The median of the follow-up time is 21.9 month, and the IQR is 23.5 month. For sex-dependent genes/miRNA regulations, we considered 265 females and 226 male lung adenocarcinoma patients. We divided the patients into high and low expression individuals according to the median value of the gene/miRNA expression, and constructed Kaplan–Meier curves to determine OS. We performed log-rank test and univariate Cox proportional hazards regression analysis to determine statistical significance and HR estimate with 95% CIs. To evaluate the proportional hazard assumption, we computed in R the Schoenfeld residuals, and assessed linearity of the Cox proportional hazard regression analysis by calculating fractional polynomials (Supplementary Table S6 and File S2).

Statistics

We used an online tool of the institutional animal care and use committee of Boston University to calculate the sample size (https://www.bu.edu/research/ethics-compliance/animal-subjects/animal-care/research/sample-size-calculations-iacuc/). Based on the assumption of a two-fold difference between sexes, the power analysis suggested a sample size of at least 4 animals at an alpha level 0.05. We prepared independent pools of four mice/pool, thus totalling 16 animals per study group and used 8 animals to identify sex dependent differences in tumour burden based on histopathology. Equally, for the miRNA expression studies, we used 6 animals per sex. Further details are given in Supplementary Table S2.

We used R and the geneXplain (https://genexplain.com/) software to perform statistical analysis. If not otherwise specified, all tests were two-tailed, and a FDR adjusted p-value < 0.05 was considered to be statistically significant.

Role of funders

The funders had no role in study design, data collection, data analysis, interpretation, or writing of the manuscript.

Results

Fig. 1 depicts the workflow and Supplementary Table S2 provides details of the experimental groups. We performed whole genome scans to identify DEGs and miRNAs (DEMs) and searched for sex-specific responses. Furthermore, we searched for gene targets of regulated miRNAs by querying the miRNet 2.0 repository, and considered experimentally proven entries only. We constructed TF-miRNA-gene networks by identifying TF binding sites in promotors of regulated genes. To define molecular circuitries in NSCLC, we examined experimentally validated TF-miRNA and TF-gene interactions and investigated the role of hormone receptors in tumour-related gene regulations. Finally we established clinical relevance by comparing the genomic data from cRAF transgenic mice to publicly available human lung adenocarcinoma cases.

Lung tumour burden in cRaf transgenic mice

We previously reported the histopathology of cRaf transgenic mice and its progression from epithelial dysplasia to NSCLC.[14,15] Depicted in Fig. 2a is the lung section of a healthy wild type (WT) animal, whereas panel b–d show typical examples of progressive lung disease starting from AAH (Fig. 2b) and multifocal tumour growth (Fig. 2c) to adenocarcinomas that consume the entire lobe (Fig. 2d). By employing the cRaf genetic disease model, we were able to follow the time-dependent sequence of events, and we demonstrate high tumour multiplicity and tumour collisions

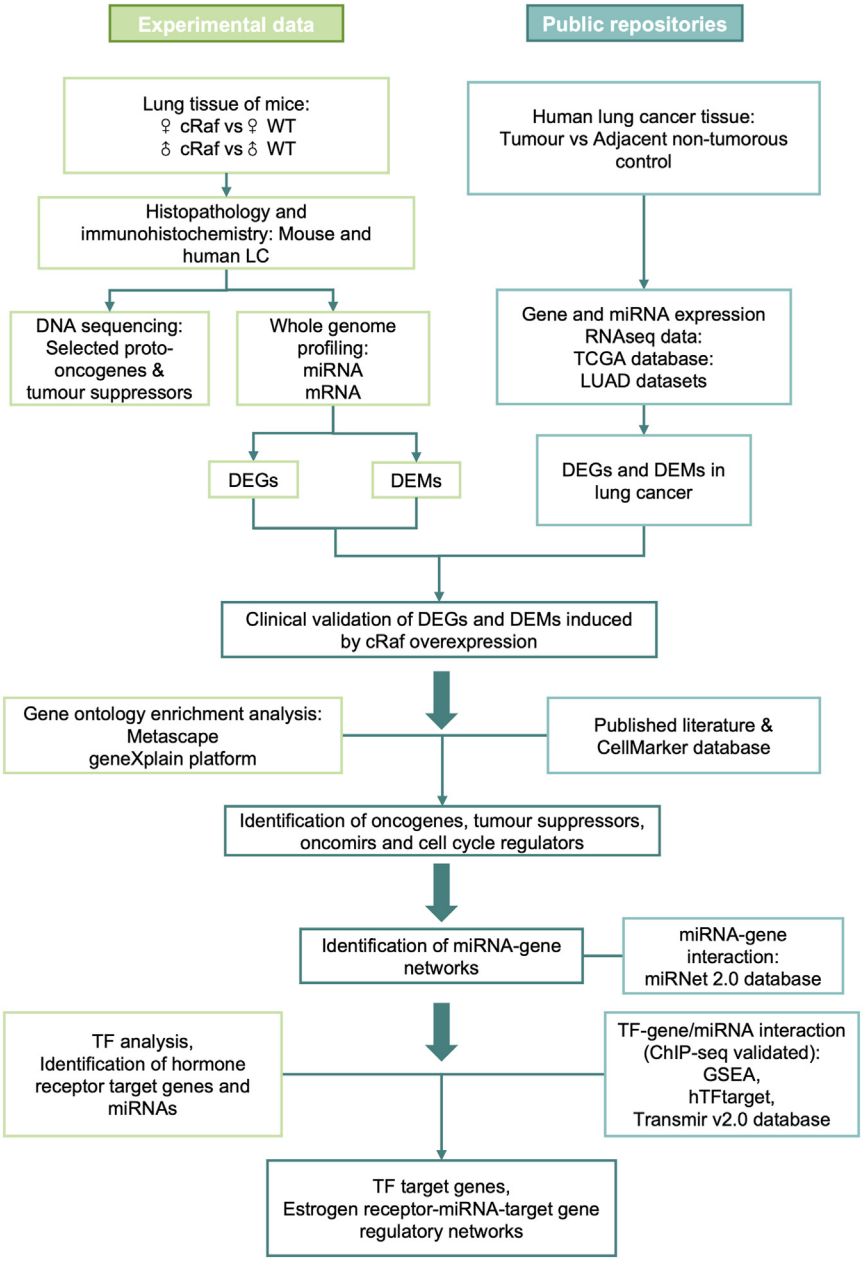

Fig. 1: Workflow of the experimental works, the genomic data analysis and data retrieval from public repositories.

(Fig. 2b and d). Therefore, overexpression of the cRaf kinase domain resulted in distinct morphological changes of the respiratory epithelium. However, none are triggered by mutational events of common oncogenes or tumour suppressors, i.e., Lmyc1, p53, Tslc1 and Kras as evidenced by DNA sequencing (data not shown). Depicted in Fig. 2e and f are sections of lung tumours and infiltration of tumour-associated macrophages (TAMs). Their role in tumour associated immune responses will be discussed below.

To determine the effects of cRaf on lung tumour growth, we performed serial sectioning of lung tissue and counted the number of tumours sized >200 μm (Fig. 2b and c). Note the mouse lung is anatomically composed of a single left lobe and four lobes of the right lung (superior, middle, inferior and post-caval lobe). Depicted in Fig. 2g are the tumour counts for the left lung, and for transgenic females, we observed a significant increase in tumour multiplicity when compared to males ($p < 0.0001$). We also determined the number of

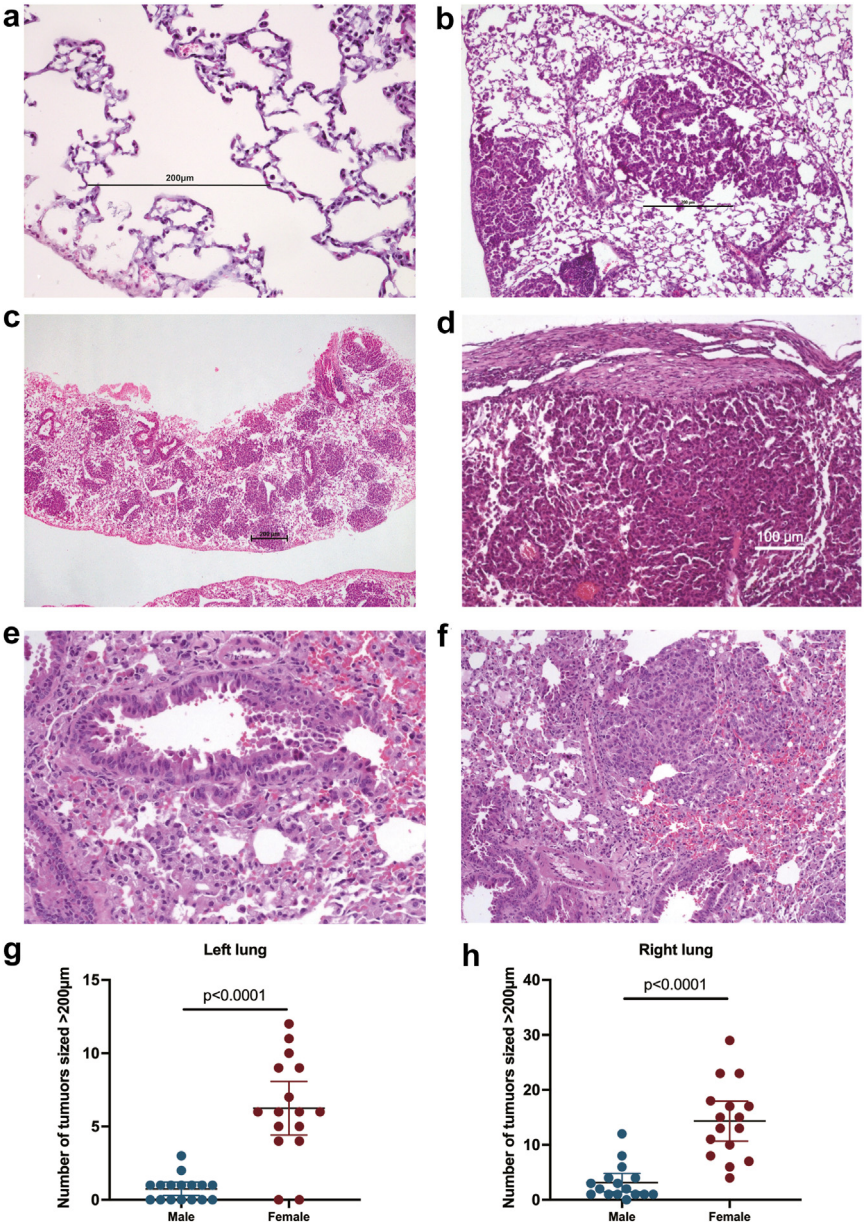

Fig. 2: Histopathology of the lung of healthy and cRaf transgenic mice. All images are H&E stained section of lung tissue. Panel a: Lung section of non-transgenic control animals. Panel b: H&E staining of the lung of cRaf transgenic mice. Note the focal proliferation of atypical cuboidal or columnar epithelial cells along the alveoli which defines atypical adenomatous hyperplasia. Panel c: Multifocal tumour growth in the left lung of cRaf transgenic mice. Panel d: Lung adenocarcinoma of cRaf transgenic mice. Lung section of a collision tumour with pleural fibrosis and desmoplastic reaction. Tumour cells infiltrate the pleura. Panel e and f: Lung sections of cRaf transgenic mice highlighting the tumour microenvironment and tumour infiltrating macrophages. Panel g and h: Multiplicity of >200 µm tumours in the left and right lung of male and female transgenic mice. The data represents N = 8 individual animals and from each animal two sections were analysed. The error bars represent 95% CI, and significance testing is based on a two-tailed Mann–Whitney U test.

tumours for the right lung of transgenic females and observed an increase in tumour multiplicity when compared to males (Fig. 2h). We therefore demonstrate sex disparity in tumour growth.

The human TNM-staging of lung tumours cannot directly be applied to tumours of cRaf transgenic mice. Nonetheless, in human pathology, the size, grade and staging of tumour is considered in relation to prognosis

and given this paradigm, a proximate staging of transgenic lung tumours would be pT2, graded as polymorphic G2-G3.

Identification of DEGs and DEMs

We employed the Affymetrix and Agilent platform to identify differentially expressed genes (DEGs) and miRNAs (DEMs) and applied the criteria false discovery rate (FDR) ≤ 0.05 and fold change (FC) $\geq |2|$. Depicted in Fig. 3a and b are heatmaps to visualize gene expression changes and the algorithm segregated the genomic responses of male and female transgenic mice to cRaf hyperactivity.

Together, we identified 112 DEGs, of which 72 were up- and 40 downregulated (Supplementary Table S3, Fig. 3c). Likewise, we identified 57 DEMs of which 30 were up-, 27 were downregulated (Supplementary Table S3, Fig. 3d).

We performed gene enrichment analysis and shown in Fig. 3e and f are the consensus of the Metascape and geneXplain software. For upregulated genes, highly enriched terms are cell–cell adhesion, epithelial cell proliferation, inflammation and immune response, as well as regulation of hormone levels (Fig. 3e). Conversely, for downregulated genes, enriched terms are cellular response to growth factor stimulus, regulation of epithelial cell migration, cellular response to DNA damage stimulus, and immune response (Fig. 3f). A summary of the enriched terms were compiled in Supplementary Table S4.

Given that inflammation and immune response are highly enriched terms, we interrogated the CellMarker database. This defined 46 DEGs or 41% of total DEGs to code for immune responses (Supplementary Table S5). Depicted in Fig. 4 are significantly regulated marker genes for different immune cells and Supplementary Fig. S2 shows their general distribution among lymphoid and myeloid cells. We will discuss the importance of immune cells in the tumour microenvironment further below.

Sex differences in genomic responses to cRaf overexpression

First, we explored sex-specific responses among male and female WT mice. Based on the criteria FC $\geq |2|$, there are 4 genes significantly upregulated in females, i.e., the alkaline ceramidase 2 (*Acer2*), *Dusp10*, limb-bud and heart (*Lbh*), and zinc finger and BTB domain containing 16 (*Zbtb16*), and these code for tumour suppressors. These are upregulated in WT female but repressed in transgenic females as discussed below. Likewise, there are 15 DEMs (6 up- and 9 downregulated) in female WT mice (Supplementary Table S3).

Second, we searched for genes and miRNAs regulated in cRaf transgenic mice by comparing the genomes to WT mice. Strikingly, of the 112 DEGs 77 are female specific. Conversely, 6 DEGs were specifically regulated in male transgenic mice and 29 are commonly regulated among both sexes (Fig. 5a). Therefore, we observed a highly significant sex disproportional regulation of genes. Similar, we identified 57 differentially expressed miRNAs and discuss their regulation below (Fig. 5b). Initially, we performed a gene enrichment analysis irrespective of sex and this defined EGFR signalling, epithelial cell proliferation and immune response as significantly enriched terms (Fig. 5c). Next we considered female-specific DEGs, and enriched GO terms are cellular response to growth factor stimulus, regulation of epithelial cell migration, regulation of cell–cell adhesion, regulation of apoptotic signalling pathway and regulation of steroid metabolic process (Fig. 5d).

EGFR and MAPK signalling

cRaf transgenicity influenced EGFR signalling with an extraordinary upregulation of its ligands amphiregulin and epiregulin, i.e., 4 and 24-fold, respectively in males and 4 and 8-fold in females (Supplementary Table S3). Likewise, we observed >5 fold induced expression of rhomboid veinlet-like 2 (*Rhbdl2*) and this endopeptidase cleaves the EGF precursor and facilitates its secretion to promote autocrine EGFR stimulation. Moreover, we found claudin 2 (*Cldn2*) expression >10-fold induced. Previous research demonstrated EGF to stimulate *Cldn2* expression and cyclin D1 nuclear retention in an EGFR dependent manner.[30,31] The results infer a regulatory loop whereby cRaf activates EGFR signalling through induced expression of its ligands. Meanwhile, epiregulin serves as a marker of advanced disease in NSCLC patients and confers invasive properties on EGFR-mutant cells.[32]

Although *EGFR* itself was not regulated at the transcript level, we identified key molecules of the MAPK signalling pathway as highly regulated in cRaf transgenic mice. However their regulation differed between male and females. In fact, we identified 16 DEGs coding for MAPK signalling molecules of which 12 are specifically regulated in females and 4 are common between both sexes (Supplementary Table S3); however none are male specific. We show in Fig. 5e the signalling pathways in lung tumours of cRaf transgenic mice and highlight the cross-talk between the EGFR and MAPK signalling pathway and the oestrogen receptor (ER).

With females, we observed a 5-fold induced expression of the ras-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*). This protein stimulates the dissociation of GDP from KRAS to enable its activation. Although *Kras* itself was not regulated at the transcript level, the upregulation of *Rasgrf1* suggests an activated RAS/RAF/MAPK signalling pathway among females. Additionally, the ral gunanine nucleotide dissociation stimulator 1 (*Rgl1*), which uncouples Ras from activation of Raf-1,[33] was down regulated. Likewise, the lysine demethylase 2A (*Kdm2a*) was repressed, and this

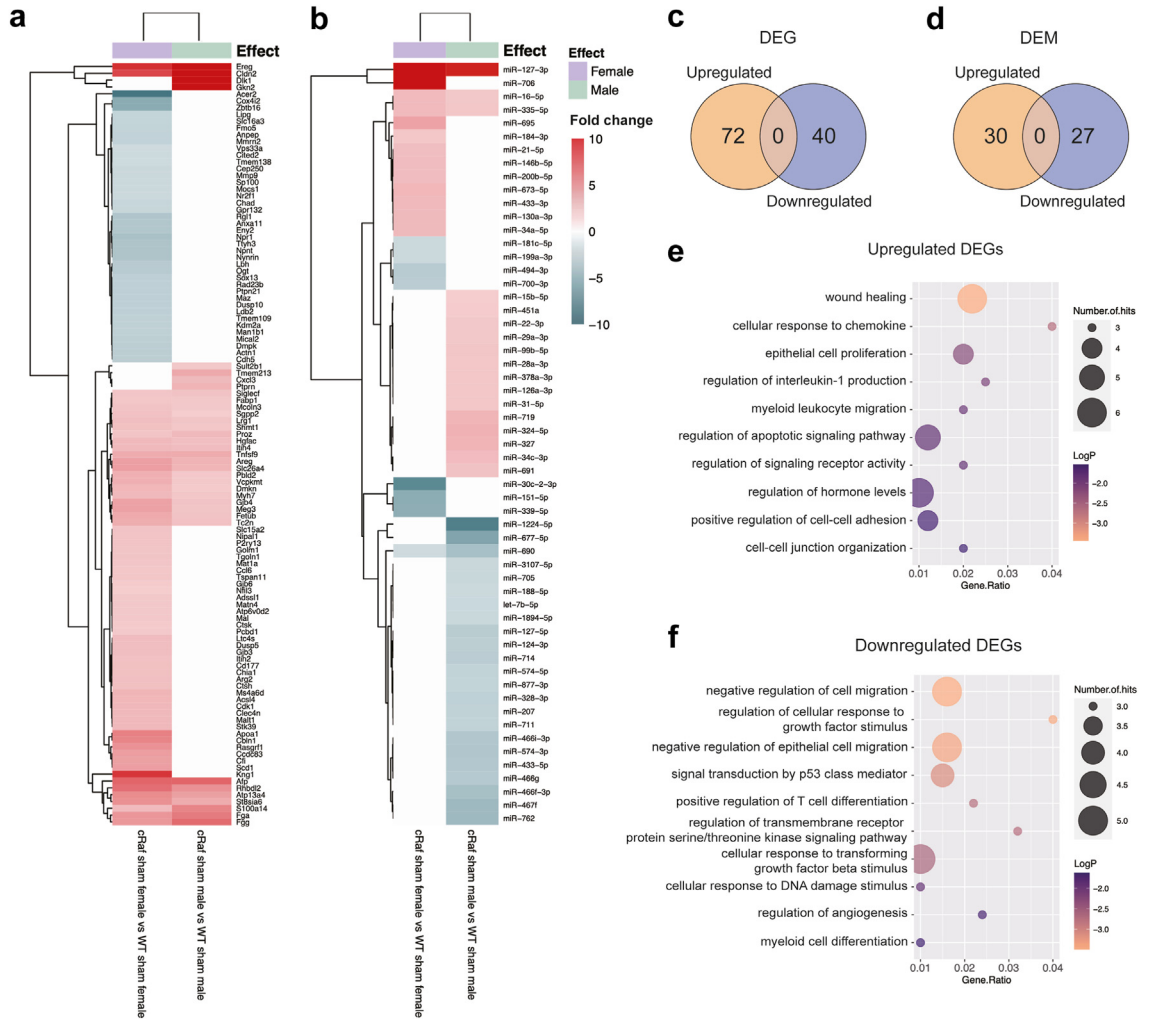

Fig. 3: Identification of genes and miRNAs in lung tumours cRaf transgenic mice. Panel: a and b Heatmaps of the DEGs (a) and DEMs (b). The Euclidian distance algorithm segregates DEGs and DEMs by sex. Panel c and d: Venn diagrams of up- and downregulated DEGs (c) and DEMs (d) in lung tumours of cRaf transgenic mice. Panel e: Bubble-chart highlighting enriched gene ontology terms for upregulated DEGs. Panel f: Bubble-chart highlighting gene ontology terms for downregulated DEGs. DEMs: differentially expressed miRNAs; DEGs: differentially expressed genes.

histone demethylase activates ERK1/2 signalling through epigenetic repression of the DUSP3.[34] Together, the findings imply upstream effector-loop regulations for the sustained Raf-1 activation. Indeed, failure of Kdm2a to repress DUSP3 allows for continuous MAPK signalling. Outstandingly, cyclin 1 dependent kinase (*Cdk1*) was uniquely induced in female transgenic mice and this kinase forms a complex with cyclin B to stimulate cell proliferation. Moreover, the 3-fold induced expression of the serine–threonine kinase *Stk39* in females influenced cell cycle progression (Fig. 5e). Indeed, independent research demonstrated *STK39* knockdown in NSCLC cells to inhibit cell proliferation, to repress cell migration and invasion and to suppress tumour growth in a xenograft mouse model.[35] Another example relates to the 4 fold induced stearoyl-coenzyme A desaturase 1 (*Scd1*). This enzyme plays a key role in ferroptosis, autophagy and immune response,[36] and is phosphorylated by EGFR at tyrosine residue 55.[37] As a result, the SCD protein is stabilized for sustained monosaturated fatty acid production. Together, the EGFR/SCD axis promotes NSCLC tumour growth[37] and further examples are highlighted in Fig. 5e.

The expression of MAPK signalling molecules is also influenced by the oestrogen receptor and the importance of steroid hormone receptor in the sex-dependent genomic responses in cRaf transgenic mice will be discussed below. Supplementary Fig. S3 informs on the protein–protein interaction networks of MAPK

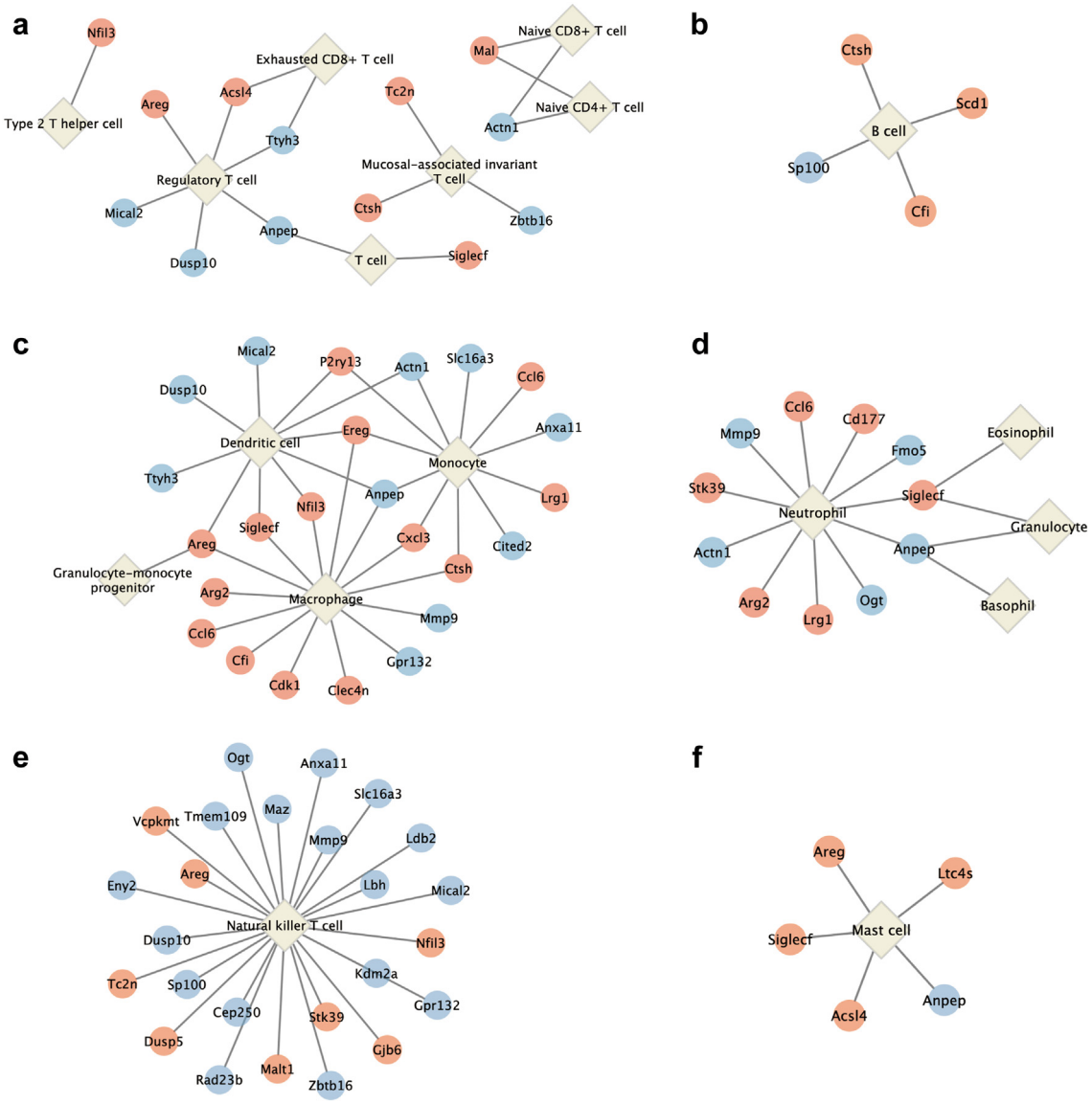

Fig. 4: Regulation of immune cell marker genes in lung tumours of cRaf transgenic mice. 41% of DEGs code for immune response genes. Shown are significantly regulated marker genes and their co-expression among different immune cell populations. Panel a: T cells. Panel b: B cell. Panel c: Monocyte, dendritic cell and macrophage. Panel d: Granulocytes. Panel e: Natural killer T cell. Panel f: Mast cell.

signalling molecules, and we obtained evidence for their physical interaction based on information retrieved from the String database.[38]

In tumours of male transgenic mice, we observed a 15-fold induced expression of the delta-like 1 homolog (*Dlk1*) and this non-canonical Notch ligand contains EGF-like repeats in its extracellular domain. We noted a similar >18-fold induced expression of *Dlk1* in human NSCLC. Nonetheless, its expression did not differ between male and female NSCLC patients (Supplementary Table S11). DLK1 promotes NSCLC cell invasion through upregulation of matrix metallopeptidase 9 (*MMP9*) in a NOTCH dependent manner.[39] However, in transgenic females *Mmp9* was repressed. Currently DLK1 is explored as a therapeutic target for radio-immunotherapy in NSCLC.[40]

Oncogenes and tumour suppressors

As described above, we identified 112 DEGs in tumours of cRaf transgenic mice (Fig. 5a), of which 47 code for oncogenes and tumour suppressors. We observed profound differences in their regulation between male and female transgenic mice. With females, 20 and 11 genes, respectively code for oncogenes (13 up- and 7

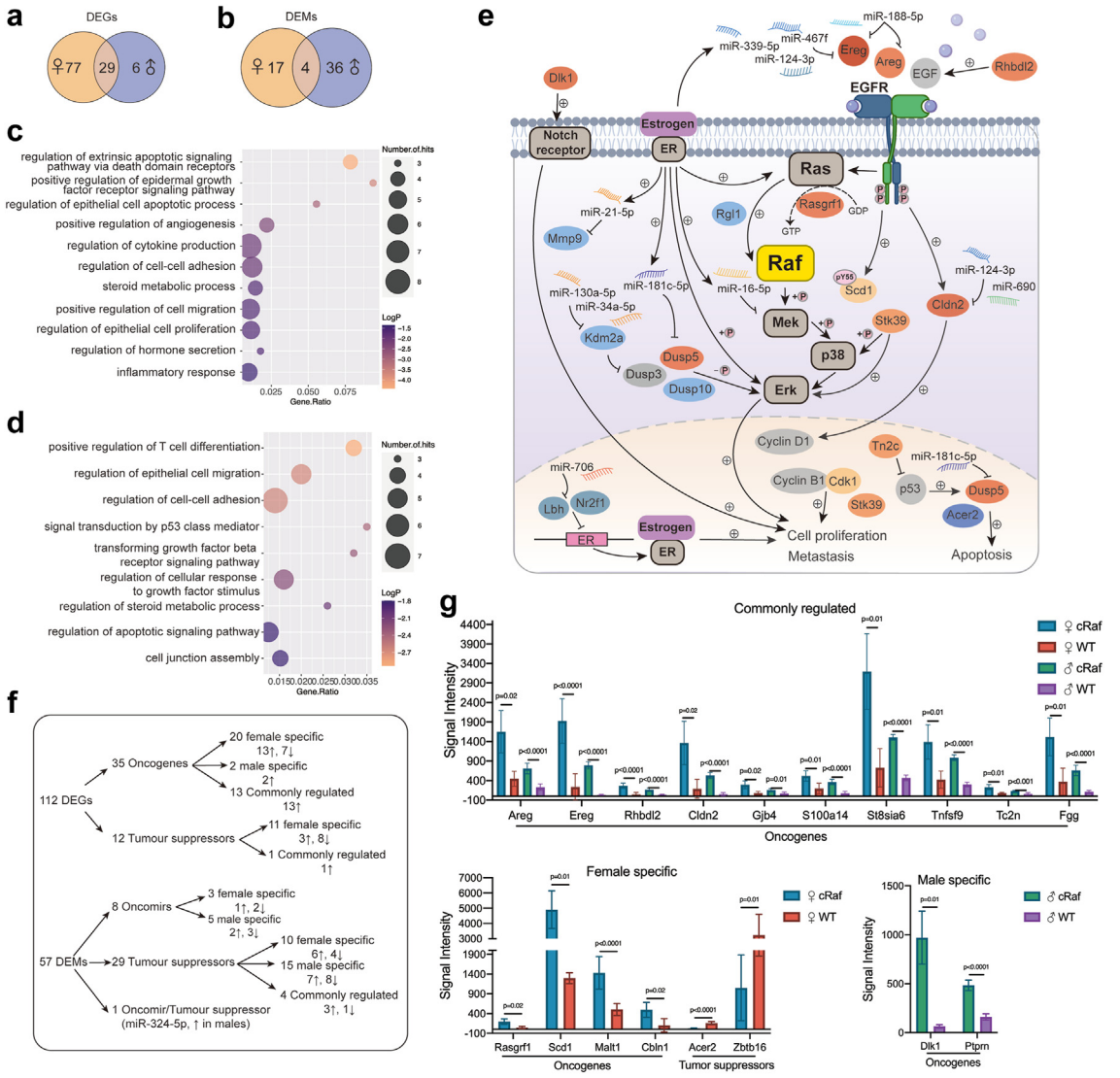

Fig. 5: Sex-dependent genomic responses in lung tumours of cRaf transgenic animals. Panel a and b. Venn diagrams showing sex specific regulations of DEGs (a) and DEMs (b) in female and male cRaf transgenic mice. Panel c: Bubble-chart of enriched gene ontology terms for all 112 cRaf responsive genes. Panel d: Bubble-chart of enriched gene ontology terms for all 77 female-specific DEGs. Panel e: The scheme depicts the various signalling pathways regulated in lung tumours of cRaf transgenic mice. Together 18 DEGs are regulated in the cross-talk between the MAPK/EGFR signalling cascade and the oestrogen receptor. We observed an extraordinary upregulation of EGFR ligands and downstream signalling molecules which stimulate cell proliferation and blocked p53 dependent cell death. Additionally, we highlight the functions of 11 miRNAs which target the various signalling molecules. Note the male specific regulation of Dlk1, i.e., a non-canonical NOTCH ligand that stimulates cell proliferation. Genes and miRNAs marked in red and blue refer to up- and down-regulation. Panel f: The flow diagram shows the number of oncogenes, oncomirs and tumour suppressors regulated in a sex-dependent manner in lung tumours of cRaf transgenic mice. Panel g: Histograms of highly regulated oncogenes and tumour suppressors. Shown are genes which were >3-fold regulated. All oncogenes are upregulated. However, in females the tumour suppressors are downregulated. The error bars represent 95% CI.

downregulated) and tumour suppressors (3 up- and 8 downregulated) while for males only 2 oncogenes were upregulated. Furthermore, of the 29 common DEGs (Fig. 5a) 13 and 1 code for oncogenes and tumour suppressors (Fig. 5f, Supplementary Table S7).

Of the 13 oncogenes specifically upregulated in females, we wish to highlight the >4-fold upregulation of cerebellin 1 precursor (*Cbln1*). Note, this protein is a Stat3 downstream target gene and is overexpressed in NSCLC.[41] Moreover, the mucosa-associated lymphoid tissue 1 (*Malt1*) was nearly 3-fold induced in cRaf females and promoted the progression of EGFR-induced NSCLC by activating NF-kappa B.[42] Other examples included the 2–3 fold upregulation of golgi membrane

protein 1 (*Golm1*), acyl-CoA synthetase long-chain family member 4 (*Acsl4*) and cathepsin k (*Ctsk*), which stimulate cell proliferation and metastasis.[43,44] Furthermore, we identified upregulation of Cd177 and this surface protein is expressed in tumour infiltrating Treg's and suppresses immune response.[45] Additionally, in female transgenic mice we observed 2-fold upregulated methionine adenosyltransferase I. The enzyme confers chemoresistance in NSCLC and bladder cancer.[46,47] Further upregulated oncogenes are *Cdk1*, *Rasgrf1*, *Cldn2*, *Areg*, *Ereg* and *Rhbdl2* and we already described their functions.

The only 2 oncogenes regulated in males are *Dlk1* (15-fold upregulated) which promotes cell invasion,[40] as well as protein tyrosine phosphatase receptor type N (*Ptprn*) which promotes transformation[48] (3-fold upregulated).

Among the 11 tumour suppressors specifically regulated in females, 8 are repressed and 3 are upregulated. For instance, the tumour suppressor *Acer2* was highly repressed in cRaf females to about 12% of its expression in non-transgenic controls and this enzyme induces apoptosis and autophagy in a p53 dependent manner.[49] Likewise, the TFs *Lbh* and Zbtb16 were 3- and 5-fold repressed in female cRaf transgenic mice and function as a negative regulator of ER signalling and MAPK signalling,[50] and programmed cell death.[51] Their repression is suggestive for sustained ER and survival signalling in cRaf induced tumorigenesis. Likewise, in cRaf females the tumour suppressor chondroadherin (Chad) was >2-fold repressed and through ECM receptor interactions supports migration of tumour cells.[52]

Of the commonly regulated DEGs (Fig. 5a), 13 code for oncogenes and all were upregulated (range 2-24-fold). These code for EGFR/MAPK signalling, cell proliferation, metastasis and inhibition of cell death. We already discussed the importance of Areg, Ereg, Cldn2, and Rhbdl2. Now we wish to highlight the regulation of fibrinogen alpha (*Fga*) and gamma (*Fgg*) which were 4- and 6-fold upregulated in males and females. Note, *FGG* is significantly elevated in NSCLC tissue and is a determinant of the metastatic potential of circulating tumour cells.[53] Similarly, we observed 4-fold induced expression of gap junction protein beta 4 (*Gjb4*) and this protein promotes metastasis and chemo-resistance through Src kinase activation and serves as a biomarker for NSCLC.[54] Further examples included induced expression of *S100a14* and this calcium binding protein stimulates cell migration and invasion,[55] while induced expression of sialyltransferase *St8sia6*, i.e., a siglec molecule elicits immune response, macrophage polarization and augments arginase 2 expression.[56] We also observed upregulation of tandem C2 domain, and this oncogene inhibits p53 signalling in lung cancer.[57] Conversely, induced *Tnfsf9* expression promotes immunosuppressive activity of regulatory T-cells in NSCLC.[58] Further information can be found in Supplementary Table S7, and we show highly regulated oncogenes and tumour suppressors in Fig. 5g.

MiRNA-gene networks in lung tumuors of cRaf transgenic mice

We identified 57 differentially expressed miRNAs in tumours of cRaf transgenic mice of which 27 and 30 were up- and downregulated (Supplementary Table S3). The regulation of DEMs differed, i.e., 36 and 17, respectively were male- and female-specific, and the findings are opposite to the sex specific regulations of DEGs. In fact, there are only 4 DEMs regulated in common (Fig. 5b). For instance, miR-127-3p was 10 and 17-fold upregulated whereas miR-690 was 3- and 2.0-fold downregulated in male and female transgenic mice. Likewise, miR-16-5p and miR-335-5p were 2 and 3-fold upregulations among both sexes.

With females, we identified 17 regulated miRNAs (Fig. 5b) of which 10 and 3, respectively act as tumour suppressors (6 up-, 4 downregulated) and oncomirs (1 up-, 2 downregulated). For instance, miR-30c-2-3p was reduced to 15% of WT controls. This miRNA functions as a tumour suppressor and is commonly repressed in various cancers and inhibits EMT in NSCLC.[59] A further example relates to the 2-fold repressed tumour suppressor miR-199a-3p which targets *ARG2*.[60] As described above, ARG2 is highly expressed in tumour associated macrophages and its induced expression is unique to cRaf females (Supplementary Table S3).

Conversely, with males 36 DEMs are regulated (Fig. 5b) of which 15 and 5, respectively act as tumour suppressors (7 up-, 8 downregulated) and oncomirs (2 up-, 3 downregulated). For instance, miR-124-3p, miR-127-5p, miR-328-3p, miR-433-5p, miR-466f-3p, miR-711, miR-877-3p and let-7-5p were 2–3 fold repressed and these tumour suppressors inhibit cell proliferation,[61–65] migration and invasion,[66,67] as well as immune response.[68]

To construct miRNA-gene networks, we considered experimental proven targets, i.e., cross-linked-immunoprecipitated miRNAs on targets as well as other experimental data. Eventually, the network consisted of 39 DEGs and 19 DEMs and the target genes code for positive regulation of EGFR signalling pathway, regulation of epithelial cell proliferation, positive regulation of cell–cell adhesion, as well as negative regulation of apoptotic signalling pathway (Fig. 6a).

Within the network (Fig. 6a), 12 miRNAs act as tumour suppressors of which 5 were up- and 6 downregulated. We observed repressed expression (range 2-5-fold) of let-7b-5p, miR-124-3p, miR-181c-5p, miR-199a-3p, miR-339-5p, miR-466f-3p and miR-711. These miRNAs suppress NSCLC tumorigenesis by regulating the tumour immune microenvironment,[60,68] induce apoptosis, inhibit cell proliferation,[69] EM transitions,[66,70] and inhibit cell invasion.[61,71] On the other hand, miR-130a-3p, miR-16-5p, miR-146b-5p, miR-335-5p and

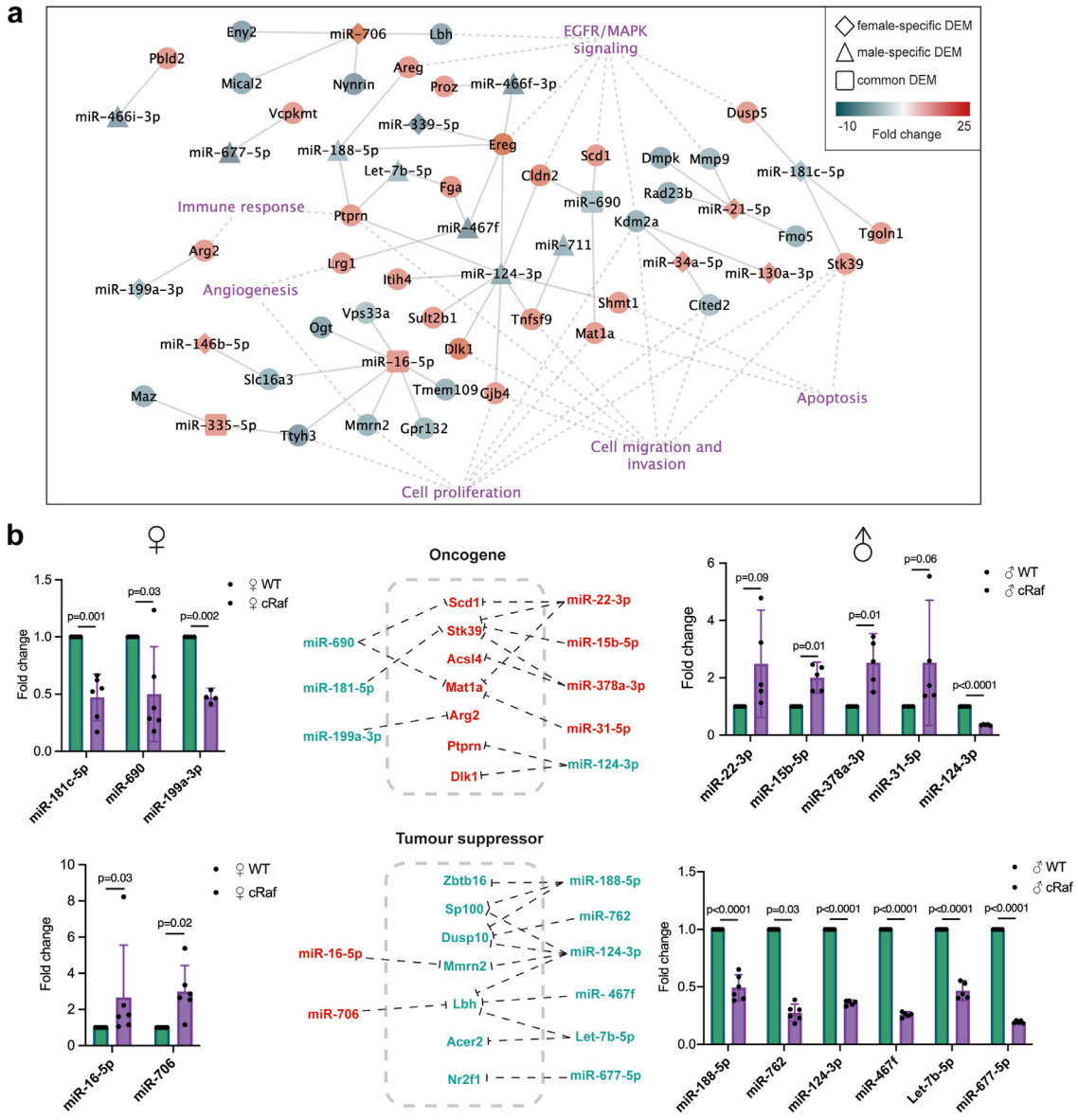

Fig. 6: MiRNA-gene regulations. Panel a: MiRNA-gene regulatory network in lung tumours of cRaf transgenic mice. The network consisted of 39 DEGs and 19 miRNA and we highlight their biological function. Panel b: Sex differences in the regulation of oncogenes and tumour suppressor genes in tumours of cRaf transgenic mice. In females, 5 oncogenes are upregulated and 7 tumour suppressors are downregulated. Conversely, in males 2 oncogenes are upregulated but, none of the tumour suppressors are regulated. Apart from the sex dependent regulation of oncogenes and tumour suppressors, we identified 13 oncogenes and 1 tumour suppressor regulated in common between both sexes. The DEGs and DEMs are marked in red and turquoise and significance testing is based on a signed-rank Wilcoxon test for N = 5 or 6 individual animals. The error bars represent 95% CI.

miR-34a-5p were upregulated (range 2-3-fold) and these miRNAs block tumour growth by regulating cell cycle.[72–74] Furthermore, miR-21-5p was about 2-fold upregulated and this oncomir promotes cell proliferation, enhances cell migration and invasion, and confers chemo- and radio-resistance in NSCLC.[75]

The sex disparities in the regulation of miRNAs are of critical importance and provided a molecular rationale for sex difference in tumour growth. Fig. 6b shows miRNAs upregulated in male transgenic mice which target oncogenes and tumour suppressor specifically regulated in females. For instance, miR-22-3p, miR-15b-

5p, miR-378a-3p and miR-31-5p were upregulated in cRaf males and these miRNAs block expression of the oncogenes *Scd1*, *Stk39*, *Acsl4* and *Mat1a*. Hence, upregulation of these miRNAs protected males from the expression of these oncogenes. Likewise, miR-188-5p, miR-762, miR-124-3p, miR-467f, let-7b-5p, and miR-677-5p were downregulated in males and these miRNAs target the tumour suppressors *Zbtb16*, *Sp100*, *Dusp10*, *Mmrn2*, *Lbh*, *Acer2* and *Nr2f1*. Thus, repression of these miRNAs protected males from dysfunction of these tumour suppressors as seen in females (Fig. 6b).

Additionally, with cRaf females, we observed repression of the miRNAs miR-181c-5p and miR-199a-3p which control expression of the oncogenes *Stk39* and *Arg2*. Their repression supported the upregulation of these oncogenes. Conversely, miR-706 was upregulated and this miRNA targets the tumour suppressor *Lbh*. Therefore, repression of this tumour suppressor can be linked to the female specific upregulation of miR-706. Moreover, miR-690 was downregulated in females which blocks expression of *Scd1* and *Mat1a*. Conversely, upregulation of miR-16-5p inhibits expression of the tumour suppressor *Mmrn2*.

Together, our findings are of critical importance in defining a molecular rationale for the accelerated tumour growth seen in female transgenic mice (Fig. 6b).

The role of the oestrogen and androgen receptor in sex-specific genomic responses

To understand sex-specific regulations of DEGs and DEMs, we searched for targets regulated by the oestrogen (Erα and Erβ) and androgen receptor (Ar). We queried the GSEA, Transmir v2.0 and hTFtarget databases and only considered chromatin-IP proven binding sites in promoters of DEGs and DEMs. Additional evidence stems from published gene expression studies of ERα ko mice, gene reporter and gene silencing studies in the MCF7 breast cancer cell line and ER positive breast cancers (Supplementary Table S8). As shown in Fig. 5a, cRaf transgenicity caused the regulation of 112 genes (Supplementary Table S3), of which 41% or 46 DEGs are targets of sex hormone receptors (Supplementary Table S8). We identified 26 and 7 DEGs, respectively as targets of the Erα and Ar, while an additional 13 DEGs contained binding sites for both hormone receptors (Supplementary Table S8, Fig. 7a). However, *Esr1*, i.e., the gene that codes for Erα and *Ar* were not regulated at the transcript level.

Based on the genomic foot printing data (Supplementary Table S8), we confirm all oestrogen receptor targets to be Erα specific. In fact, none of the promoters of tumour-regulated genes are enriched for Erβ binding sites, and we provide strong experimental evidence for an activated ER/EGFR axis in lung cancer (see Fig. 5e). There are 39 DEGs with binding sites for Erα of which 32 are specifically regulated in females while 6 are common for both sexes (Fig. 7b). Strikingly, of the 20 DEGs targeted by the androgen receptor, 18 are specifically regulated in females while the remaining two are common to both sexes (Fig. 7c).

Furthermore, we searched for hormone receptor binding sites in promoters of DEMs and this revealed one and 24 miRNAs, respectively which are targets of the androgen and oestrogen receptors, while three were regulated by both hormone receptors (Fig. 7d). Note, with the exception of miR-29a-3p and miR-34a-5p, the tumour regulated miRNAs are also predominantly ERα targets (Supplementary Table S8).

Depicted in Fig. 7e is the complex interplay between miRNAs and genes targeted by sex hormone receptors, and the network consisted of 46 DEGs and 28 DEMs. Note 39 out of 46 DEGs are targets of the ERα receptor and 32 are specifically regulated in females. In fact, only 6 DEGs are regulated in common between male and female transgenic mice. Moreover, sulfotransferase 2B is the only gene regulated in males, and this gene is also a target of ERα (Fig. 7e). Even more astonishingly is the fact that of the 20 DEGs targeted by the androgen receptor 18 are specifically regulated in females while the remaining 2 are common to both sexes (Fig. 7e). Therefore, the hormone receptor dependent regulation of genes provided a molecular rationale for the disproportional tumour growth in females.

Indeed, there is evidence for oestrogen to function as a potential mediator of immunosuppression with E2 stimulating macrophage M2 polarization and tumour infiltration.[76] Thus, apart from TAMs and other drivers of an immunosuppressive tumour microenvironment (see above, Fig. 4), oestrogen and oestrogen receptor regulated tumour suppressors likely contribute to sex differences in tumour growth. In fact, of the 8 downregulated tumour suppressors in cRaf females (Supplementary Table S7), 5 are targets of Erα and two are common with human lung cancer, i.e., MMRN2, an extracellular matrix glycoprotein that interferes with VEGF signalling pathway and the zinc finger TF ZBTB16, i.e., a regulator of cell cycle progression that interacts with histone deacetylase (see below for discussion).

Fig. 7f highlights the effects of the oestrogen receptor on immune cells in the tumour microenvironment. We show the complex relationship and cross talk between immunosurveillance and immunosuppressive cells and genes regulated by the oestrogen receptor.

Search for enriched transcription factor binding sites in promoters of regulated genes

Transcription factors are important regulators of gene expression. Initially, we searched for enriched transcription factor binding sites (TFBSs) in promoters of cRaf regulated genes and this revealed 99 significantly enriched TFBSs (Supplementary Table S8). Subsequently, we searched for genes coding for transcription factor and this defined the MYC-associated zinc finger

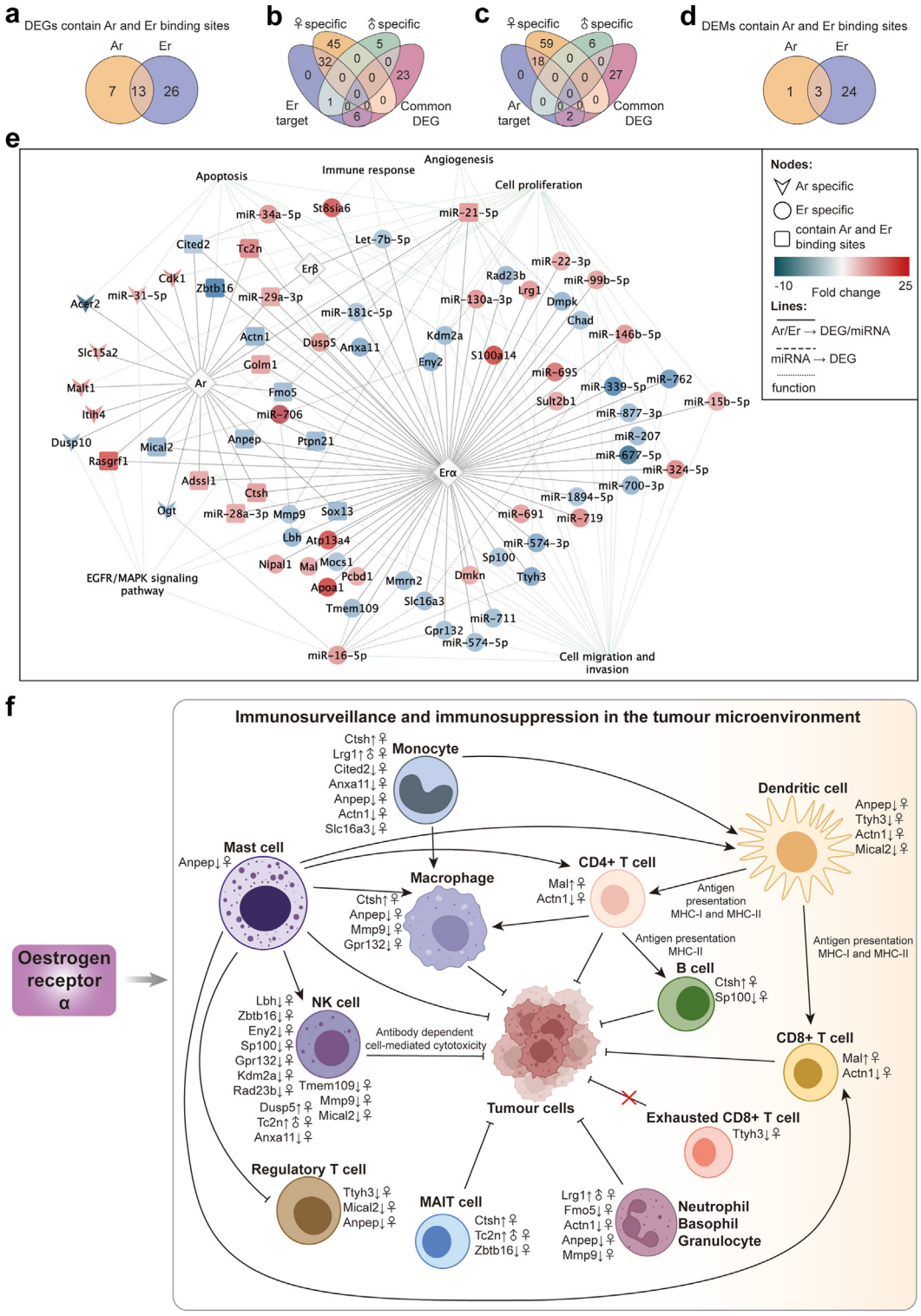

Fig. 7: The role of sex hormone receptors in tumour specific gene regulations. Panel a: Venn diagram showing the number of DEGs containing Ar and Erα binding sites. Panel b and c: Venn diagrams showing the number of DEGs targeted by Ar and Erα in lung tumours of female and male transgenic mice. Panel d: Venn diagram showing the number of DEMs containing Ar and Erα&β binding sites. Panel e: The importance of the androgen and oestrogen receptor in the control of gene expression. The network consisted of 46 DEGs and 27 DEMs. The hormone

protein (*Maz*) and nuclear receptor subfamily 2 group F member 1 (*Nr2f1* alias *Coup-tf1*) as > 2-fold regulated.

Shown in Fig. 8a is the network of *Maz* and *Nr2f1* and their experimentally validated target genes. Maz is a TF that binds to cMyc and exerts dual functions as transcriptional activator and repressor.[77] Although *cMyc* itself was not regulated, we found several Maz target genes as highly regulated in cRaf transgenic mice (Fig. 8a). Specifically, Maz is a repressor of Myc activity[78] and was significantly repressed (3-fold) in cRaf females (Supplementary Table S3). A recent review summarizes the functions of Maz in various cancers though its role in NSCLC remains elusive.[79] Specifically, repression of *Maz* in cRaf females may be one of the reason for its increased tumour burden (Fig. 2g and h).

The downregulation of *Nr2f1* is of great importance. This TF is a nuclear hormone receptor and transcriptional regulator of cell differentiation and metabolism, and may function as a tumour suppressor.[80] In cRaf females, *Nr2f1* was >2-fold repressed. Importantly, through binding to its TFBS, Nr2f1 functions as a transcriptional repressor of the oestrogen receptor.[81,82] Although *Esr1* itself was not regulated in cRaf transgenic mice, the female-specific increase in tumour burden (Fig. 2g and h) tends to suggest the deliberate repression of the Nr2f1 tumour suppressor. Indeed, NR2F1 activation causes growth arrest in various cancer cell lines and suppresses metastasis in vivo.[80]

MiRNA-TF-gene regulatory network

To better understand sex dependent gene regulations in tumours of cRaf mice, we queried the GSEA, Transmir v2.0 and hTFtarget databases and searched for ChIP-seq validated targets which are regulated by sex hormone receptors.

The rules laid down by us were as follow: The hormone receptors must target both the promoters of miRNAs and target genes. Therefore, the hormone receptor functions as master regulator. We show in Fig. 8b the network which consisted of 7 miRNAs and 15 target genes all of which were significantly regulated in cRaf animals (Supplementary Table S3). Remarkably, all miRNA-gene targets are female-specific (Fig. 8b), and we highlight their functions. Of the 7 miRNAs, 5 act as tumour suppressor, and miR-181c-5p was downregulated while miR-130a-3p, miR-146b-5p, miR-16-5p, miR-34a-5p were upregulated. As described above, miR-21-5p functions as oncomir, and was specifically upregulated in cRaf females. The network underscores the complex regulation of genes and miRNAs in transgenic females.

As depicted in Fig. 8b, the oncomir miR-21-5p is a target of the Ar and Erα, and this miRNA regulates 4 genes in cRaf females, all of which contained oestrogen receptor binding sites. For example, Rad23 homolog B was 2-fold downregulated and this nucleotide excision repair protein plays an essential role in DNA repair.[83] Similar, the serine/threonine kinase *Dmpk* was 3 fold repressed and this kinase protects cells from oxidative stress[84]; and is a target of p53 signalling.[85] Another example relates to the 2.5-fold repressed flavin-containg monooxygenase *Fmo5*, and this xenobiotic defence enzyme is regulated by the circadian rhythm.[86] Moreover, upregulation of miR-21-5p regulates ROS and suppresses the antioxidant response.[87] Together, the data were suggestive for a regulatory loop whereby hormone receptors and miR-21-5p impair the detoxification of oxidative stress and DNA repair in cRaf females.

Another example relates to the network consisting of the oestrogen receptor, miR-181c-5p and dual specificity phosphatase 5 (Dusp5). In female transgenic mice, *Dusp5* is significantly upregulated and this phosphatase dephosphorylates ERK which abrogates ERK signalling. Furthermore, miR-181c-5p controls translation of Dusp5[88] and in tumours of cRaf females, this tumour suppressor was significantly repressed. While the results agree, i.e., repression of miR-181c-5p and upregulation of *DUSP5*, there is also evidence for DUSP5 to promote cytoplasmic ERK activation by releasing feedback inhibitors of upstream kinases.[89] Therefore, DUSP5 takes on a dual role in the control of ERK signalling. Adding to complexity is the fact that the histone lysine-specific demethylase Kdm2a regulates Erk1/2 signalling epigenetically,[34] and in cRaf females, *Kdm2a* was repressed. Together, we obtained evidence for a complex interplay involving Dusp5, miR-181c-5p, the oestrogen receptor and miR-130a-3p, which we found 2 fold upregulated in cRaf females and the latter miRNA targets Kdm2a.

In the network (Fig. 8b), we show DEGs coding for angiogenesis. For instance, *Mmrn2* was 2-fold repressed in cRaf females and this carrier protein for platelet factor V is a target of miR-16-5p. Research demonstrated *Mmrn2* to suppress neo-angiogenesis by inhibiting VEGFR2.[90] *Mmrn2* repression therefore supports angiogenesis in cRaf females.

receptors bind to the promoters of miRNAs and target genes and the majority of the target genes are regulated by the oestrogen receptor. Panel f: ERα supports an immunosuppressive tumour microenvironment. ERα influences the expression of immune cell marker genes to evade immunosurveillance. The gene regulations are depicted by the arrow and most genes are down regulated in females. Ar: androgen receptor; Erα: oestrogen receptor α; Erβ: oestrogen receptor β.

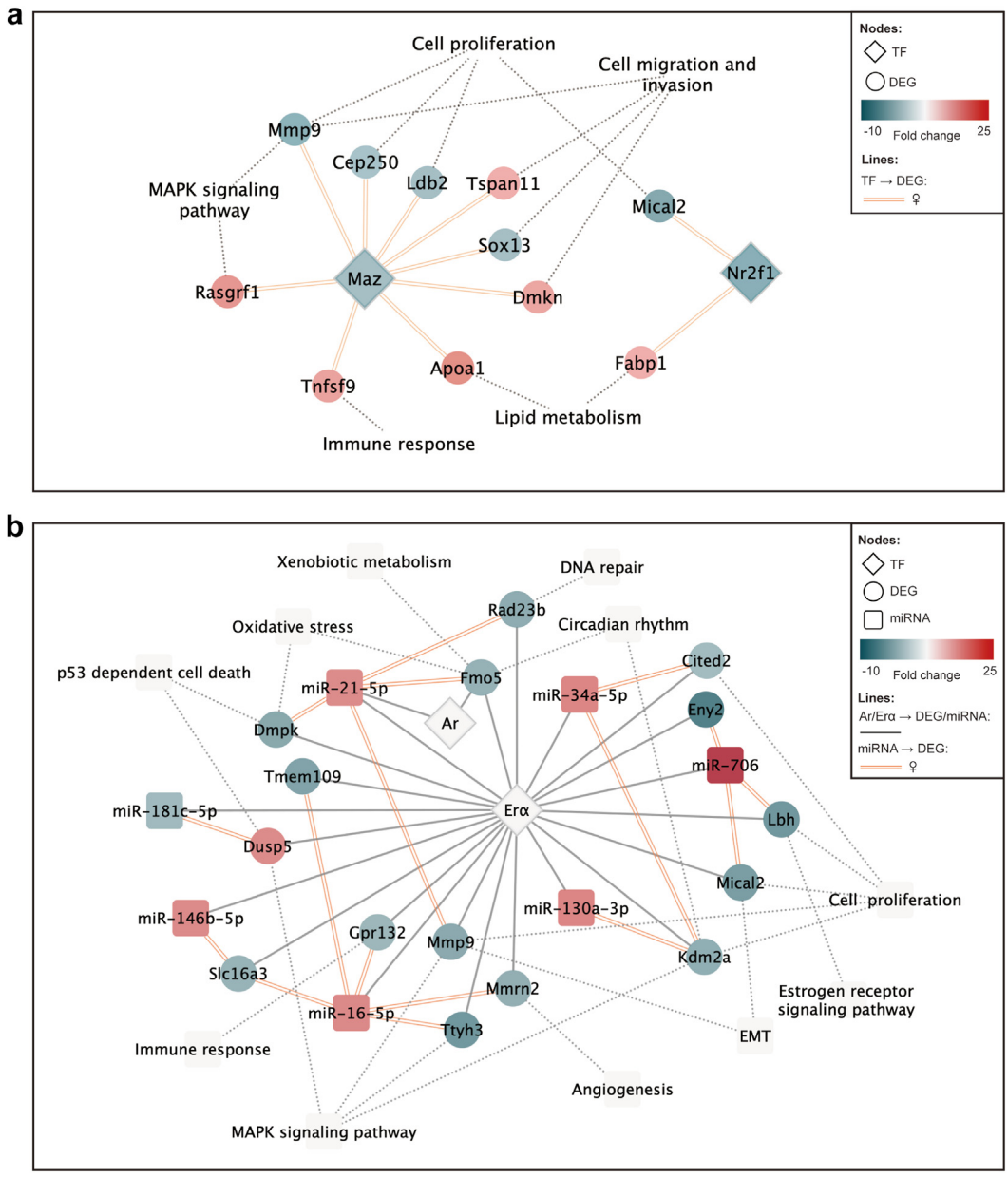

Fig. 8: TF-miRNA-gene regulatory network in lung tumours of cRaf transgenic mice. Panel a. TF-gene regulatory networks. The transcription factor Maz and Nr2f1 are repressed in lung tumours of cRaf transgenic mice. Shown are the target gens and we highlight their functions. Panel b. TF-miRNA-gene regulatory network whereby hormone receptors function as master regulator. The network consisted of 15 DEGs and 7 DEMs and the hormone receptors control gene expression of DEGs and miRNA at the same time.

Clinical validation–hormone receptor expression in human NSCLC tumour samples

We already discussed the complex interplay between sex hormone receptors and miRNAs in the regulation of DEGs in cRaf transgenic animals. As shown in Fig. 2g and h the tumour growth is strongly influenced by sex and the tumour multiplicity increased significantly in females.

Depicted in Fig. 9a and b are H&E stained sections of lung tumour and peritumoral tissue of two female (case I and II) and one male (case III) patient. The enlarged air spaces of alveoli in the peritumoral lung tissue signifies mild to moderate emphysema. Specifically, the tumours exhibited an acinar growth pattern with invasive glands and poorly formed glandular spaces (bI&bII), as well as invasive nests of tumour cells that

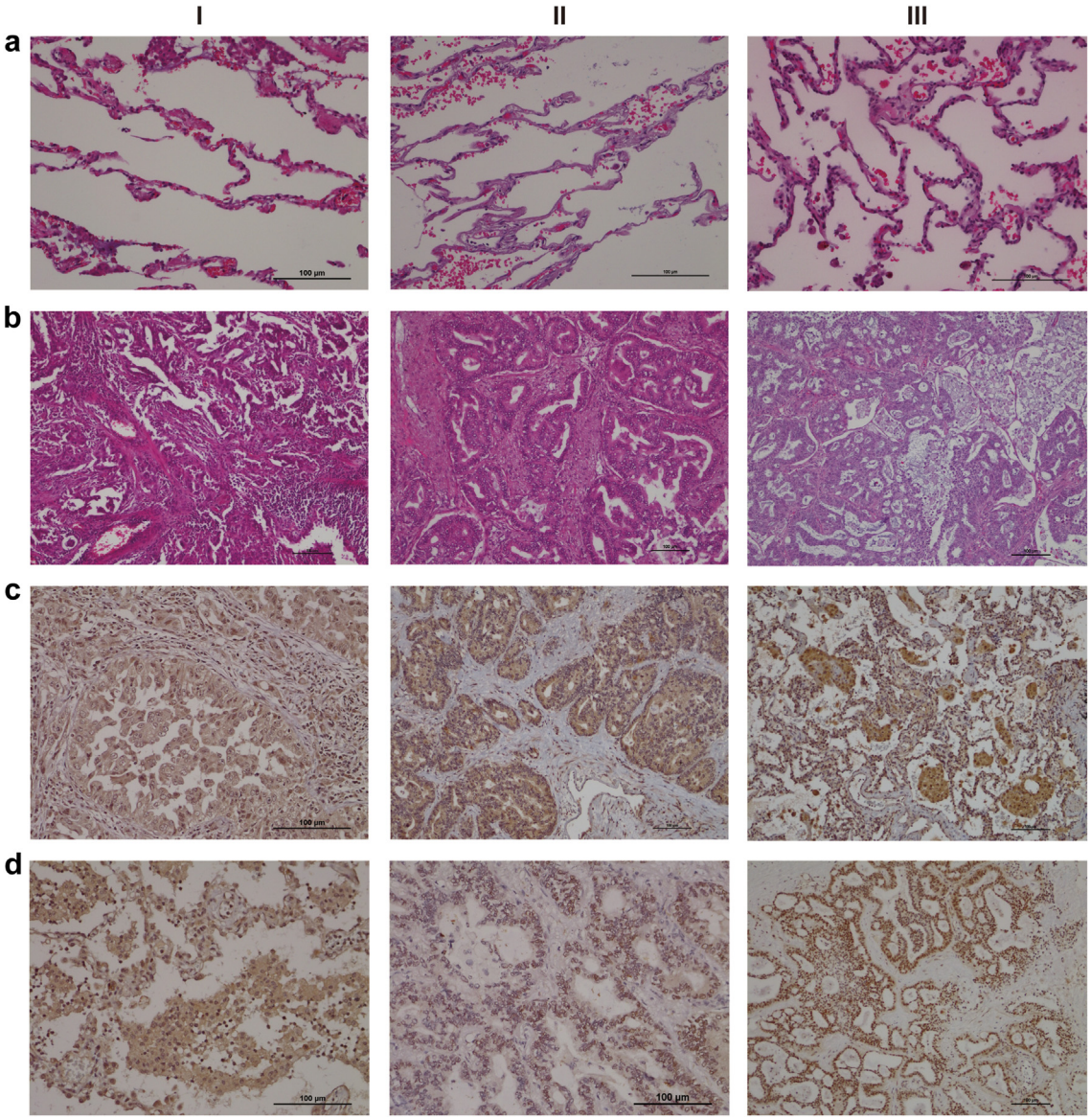

Fig. 9: Histopathology and hormone receptor expression in human lung adenocarcinoma cases. Panel a: H&E staining of peritumoral tissues. Shown are lung sections of peritumoral tissue of two female (case I and II) and one male (case III) patient with mild to moderate emphysema. Panel b: H&E staining of lung adenocarcinomas. The tumours exhibited an acinar growth pattern (BI & BII), as well as invasive nests of tumour cells that produced glandular lumina without solid components (BIII). Panel c: Immunohistochemistry staining of the oestrogen receptor in lung adenocarcinoma patients. The tumour cells display marked expression of the oestrogen receptor. Panel d: Immunohistochemistry staining of the androgen receptor in lung adenocarcinoma patients. Unlike case I and III, the expression of the androgen receptor in tumour cells of female case II is very slight to none.

produced glandular lumina without solid components (bIII). The immunohistochemistry evidenced marked expression of the ERα and androgen receptor. Notwithstanding the expression of the androgen receptor is less in case II (Fig. 9dII, see also Figure captions for more details). Therefore, we and others provide evidence for sex hormone receptors to be significantly regulated in lung adenocarcinoma.

Clinical genomics of NSCLC tumour samples

To demonstrate clinical relevance, we performed a comparative genomic analysis of human lung adenocarcinoma cases. We interrogated the TCGA database (https://portal.gdc.cancer.gov/) and compared 510 tumour vs. 58 adjacent, histologically proven non-tumorous samples of NSCLC patients. This revealed 5395 DEGs (3373 up-, 2022 downregulated). In the

same way we compared tumour associated miRNAs of NSCLC patients (513 tumour, 45 controls) and identified 414 DEMs as significantly regulated (251 up-, 163 downregulated) (Supplementary Fig. S1 and Table S9). The Venn diagram in Fig. 10a shows the commonly regulated DEGs and DEMs between human and mouse lung tumour samples. We identified 27 up-, 9 downregulated DEGs, as well as 4 up- and 3 downregulated DEMs. To evaluate the prognostic value of common DEGs and DEMs between mice and humans, we computed Kaplan–Meier survival plots by considering their high and low expression among NSCLC patients.

Based on Kaplan Meier plots, we found high expression of *ARG2*, *CLDN2* and molybdenum cofactor synthesis 1 to be associated with HR < 1 and therefore better overall survival (OS). Conversely, high expression of *CDK1*, *EREG*, fetuin B (*FETUB*), gap junction protein beta 3 (*GJB3*), *GJB4*, *GOLM1*, NIPA like domain containing 1, PTPRN was associated with HR > 1 and therefore reduced survival (Fig. 10b). To assess time dependency, we computed Schoenfeld residuals (Supplementary Table S6), and for the PTPRN gene the result reached statistical significance ($p = 0.025$). This means that the HR is time dependent and does not fit the proportional hazard assumption. Likewise, high expression of the tumour suppressor let-7b-5p and miR-127-5p was associated with better OS, while higher expression of the oncomir miR-21-5p was associated with worse outcome (Fig. 10c).

Additionally we evaluated commonly regulated genes in lung tumours of cRaf transgenic mice and lung adenocarcinoma patients in a COX proportional hazard model and assessed linearity of the model by computing fractional polynomials. The results are summarized in Supplementary Table S6 and for the genes NIPAL1, PTPRN, hsa-let-7b-5p, and hsa-miR-127-5p the computations confirmed the Null model. Therefore, we reject the linearity assumption for these genes in regards to survival of lung cancer patients.

Although for most genes a plausible association to OS could be ascertained, the result for arginase 2 is perplexing. Arginase supports an immunosuppressive microenvironment and there is strong evidence for high tumour arginase expression and activity across different tumours.[91] In fact, we observed opposite regulation of arginase 1 and 2 in human lung adenocarcinoma samples, i.e., arginase 1 was down regulated but arginase 2 was upregulated (Supplementary Table S9) whereas in cRaf females arginase 2 was uniquely upregulated. Given that arginase plays an essential role in cancer-specific immune responses and in the regulation of tumour associated macrophages, we investigated expression of TAM marker genes in lung adenocarcinoma patients based on TAM genes reported by Ma et al.[92] (Supplementary Table S10). Together we identified 68 regulated TAM marker genes (range 2-25-fold). For instance, we observed secreted phosphoprotein 1 (=osteopontin) 25-fold induced in lung adenocarcinoma patients and this macrophage derived cytokine confers drug resistance in human NSCLC.[93] Similar, matrix metalloproteinase 12 was highly induced (13- and 14-fold in female and male lung adenocarcinoma patients) and promotes angiogenesis 8.[94] The regulation of arginase 1 and 2 and TAM marker genes was sex-independent. However, unlike NSCLC patients, arginase 1 was not regulated in cRaf mice (Supplementary Table S3). Despite its important function in immune evasion, the prognostic value of arginase 2 for human NSCLC is less clear and controversial. We found high expression of arginase 2 to be associated with better survival (Fig. 10b).

Sex-specific gene regulations of NSCLC tumour samples

We compared lung adenocarcinoma tumour samples of female patients to adjacent non-tumorous tissues. This defined 5070 DEGs (3097 up- and 1973 downregulated) and 392 DEMs (211 up- and 181 downregulated). In the same way, we analysed male lung adenocarcinoma tumour samples, and obtained 5237 DEGs (3207 up- and 2030 downregulated) and 315 DEMs (241 up- and 74 downregulated) (Supplementary Fig. S1). To probe for sex-specific regulations, we constructed Venn diagrams and found 376 and 486 genes specifically upregulated in female and male lung adenocarcinoma patients (Supplementary Fig. S4a and Table S11). We show the Metascape enriched GO terms in Supplementary Fig. S4b, and with female lung adenocarcinoma patients, innate immune response, peptide-cross linking and cell–cell recognition were significantly enriched terms. Conversely, for males, specific annotations were cell surface receptor signalling, cell morphogenesis involved in differentiation, Notch signalling and epidermis morphogenesis. Similar, for the 273 and 330 downregulated genes the Metascape annotations are given in Supplementary Fig. S4d. With females, enriched GO terms were cell junction assembly, negative regulation of canonical Wnt signalling pathway, cellular response to hormone stimulus and cell–cell adhesion via plasma membrane adhesion molecules. Equally, for males enriched terms were regulation of cell activation, inflammatory response and immune effector process, positive regulation of immune response, phagocytosis, positive regulation of cytokine production, regulation of kinase activity and positive regulation of MAPK cascades. Lastly, there are 170 (56 up-, 114 downregulated) and 93 (86 up-, 7 downregulated) miRNAs regulated in female and male lung adenocarcinoma patients (Supplementary Fig. S4e).

Furthermore, we compared DEGs of cRaf transgenic lung tumours with human lung adenocarcinoma samples. This revealed 31 commonly regulated DEGs (23 up-, 8 downregulated) between female lung

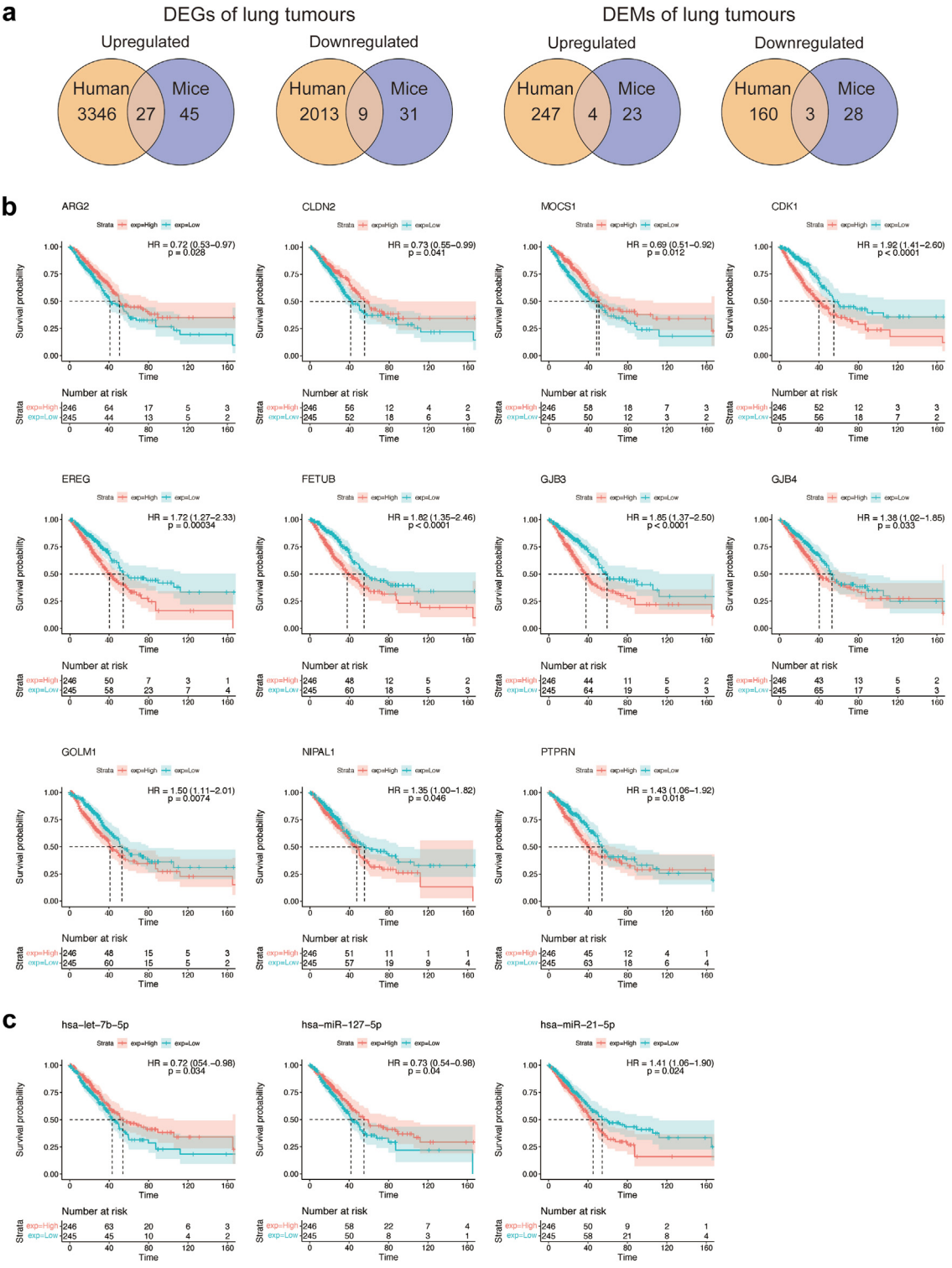

Fig. 10: Genomics of human lung adenocarcinoma. Panel a: Clinical validation of DEGs and DEMs which we identified in lung tumours of cRaf transgenic mice. Depicted are Venn diagrams of commonly regulated DEGs and DEMs between lung adenocarcinoma patients and cRaf transgenic mice. Panel b and c: Kaplan–Meier survival curves for 11 DEGs and 3 DEMs commonly regulated between lung adenocarcinoma patients and cRaf transgenic mice. HR: hazard ratio, brackets: 95% CI of the HR.

adenocarcinoma patients and female mice, while for males, 14 DEGs (14 upregulated) were commonly regulated (Fig. 11a). Furthermore, 3 upregulated DEMs were common between female lung adenocarcinoma patients and female mice, and 2 (1 up-, 1 downregulated) were common between male lung adenocarcinoma patients and male mice (Fig. 11b). The results imply that 40% of DEGs regulated in lung adenocarcinoma tumours of cRaf transgenic mice are likewise regulated in human lung adenocarcinoma. With DEMs, 14% and 5%, respectively of female and male cRaf mice were in common with human lung adenocarcinoma samples.

To determine the prognostic value of commonly regulated genes, we constructed Kaplan–Meier survival plots and compared the OS for 265 female and 226 male lung adenocarcinoma patients. We summarizes the results for the COX proportional hazard model in Supplementary Table S6 and although all reported proportional HR are statistically significant, the linearity assessment yielded the Null model for the gene hsa-miR-21-5p in females and EREG in males. Therefore, we reject the hypothesis of sex dependence for these two genes in lung adenocarcinoma patients.

Notwithstanding, higher expression of *CDK1* and *GJB3* was associated with poor OS among female lung adenocarcinoma patients, and higher expression of *CDK1* was also associated with worse outcome in male patients (Fig. 11c). Collectively, we confirmed clinical relevance of cRaf tumour associated genes with implication for OS.

To examine sex specific gene regulations in lung adenocarcinoma cases, we considered the hormonal status and therefore the age of lung cancer patients (Supplementary Fig. S1). In common are 2301 up- and 1747 downregulated genes (Fig. 11d). Conversely, 612 and 652 DEGs are uniquely up and 597 and 149 DEGs are uniquely downregulated among pre- and postmenopausal women (Fig. 11d). Based on solid experimental evidence, i.e., chromatin-IP, we searched for ERα and ERβ binding sites among uniquely regulated genes (Fig. 11d). This suggested that about 29% and 15% of DEGs, respectively are targets of the oestrogen receptor among pre- and postmenopausal women.

Furthermore, we compared ER target genes among male and female patients. This defined 482 and 538 upregulated, and 324 and 340 downregulated DEGs, among female and male LC patients (Supplementary Fig. S4f). Furthermore, 90% and 82% of upregulated ER target genes and 88% and 84% of the downregulated genes are common ER targets between female and male LC patients (Supplementary Fig. S4f).

Outstandingly, in premenopausal women, most genes are targets of ERα, and the difference is 7-fold when compared to ERβ targets, i.e., 173 over 25 genes (Fig. 11d). We observed a similar 8-fold difference for ERα regulated genes in lung tumours of postmenopausal women. Lastly, there are twice as many ERα target genes in premenopausal women, i.e., 173 vs. 94 DEGs when compared to postmenopausal women. Together, the data suggest the hormonal status to influence the gene regulation in lung adenocarcinomas and the gene targets are fundamentally different between pre- and postmenopausal women. However, the predilection for ERα regulated genes remains the same irrespective of the hormonal status. Shown in Fig. 11e and f are ontology terms based on hallmark gene sets, and for ERα upregulated target genes enriched terms are glycolysis and MYC, whereas for ERα down regulated genes response to oestrogen receptor and inflammatory response are prominent findings. Given the small number of ERβ targets, we were unable to define enriched ontology terms.

Likewise, among the downregulated genes, the difference between ERα and ERβ targets for premenopausal women is 9.5-fold and for postmenopausal women 22-fold and the results underscore important differences in ERα and ERβ regulated target genes. Together, we show the hormonal status to significantly influence the regulation of ER responsive genes in lung tumours with premenopausal women expressing a larger number of ERα target genes and this has important implications for cancer therapy. Finally, we provide independent experimental evidence for 16 ERα target genes and confirm their regulation among twenty male and female patients (N = 20, Fig. 11g and h). The data agree with the findings obtained from the TCGA database (Supplementary Table S12).

Discussion

Our study aimed at identification of sex-specific differences in lung tumour growth, and we show that overexpression of the kinase domain of cRaf causes complex genomic responses in lung epithelial cells of transgenic mice. Strikingly, cRaf overexpression elicited an extraordinary 8-fold increase in tumour growth among females, and nearly 70% of the 112 DEGs were female specific. Our study provides mechanistic insight into sex disparities in lung tumour growth, and we provide strong evidence for hormone receptors and especially ERα to target immune cells of the tumour microenvironment. Furthermore, ERα plays a crucial role in the regulation of genes/miRNAs act as tumour suppressors, oncogenes and oncomirs. Our findings allowed us to construct gene-regulatory networks consisting of hormone receptors, miRNAs and target genes in the control of tumour growth and we report crucial components of the molecular wiring that prompted sex disparities in tumour burden. Moreover, we confirm our findings in a large cohort of human lung adenocarcinoma cases and show the detrimental effects of oestrogen action on lung cancer cells by comparing pre- and postmenopausal lung adenocarcinoma patients.

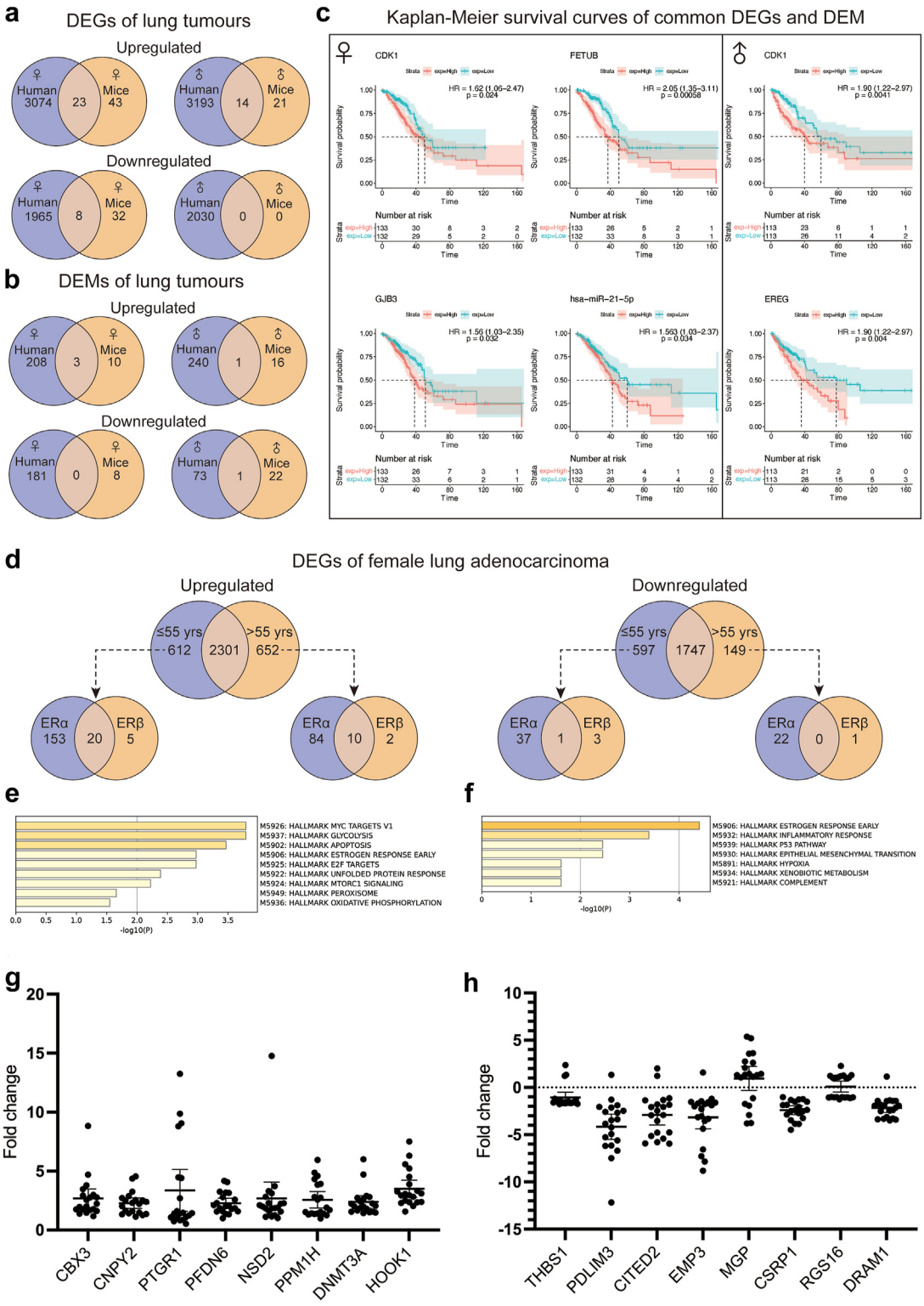

Fig. 11: Sex specific gene regulations in human lung adenocarcinoma. Panel a: Clinical validation of sex-specific DEGs identified in lung tumours of cRaf transgenic mice. Venn diagrams of commonly regulated DEGs between lung adenocarcinoma patients and cRaf transgenic mice. Panel b: Clinical validation of sex-specific DEMs identified in lung tumours of cRaf transgenic mice. Venn diagrams of commonly regulated DEMs between lung adenocarcinoma patients and cRaf transgenic mice. Panel c: Kaplan–Meier survival plots for sex-specific gene regulations. Shown

We identified 112 DEGs (Fig. 3c) of which 10% coded for MAPK signalling molecules (Fig. 5d). In general, the MAPK signalling pathway stimulates cell proliferation and blocks apoptosis, and has been the focus of targeted therapies for NSCLC.[95] Apart from an exaggerated MAPK signalling, the genomic data are highly suggestive for an impaired p53 activity, and we noted repression of tumour suppressors and upregulation of oncogenes and oncomirs. In fact 42% of the 112 DEGs act as oncogenes and tumour suppressors and of these, 20 and 11, respectively were uniquely regulated in females. Their regulation provided a molecular rationale for an increased lung tumour burden among female transgenic mice. On the contrary, there are only 2 oncogenes specifically regulated in cRaf males (Supplementary Table S7). Furthermore, we identified miRNAs specifically regulated in males which protected animals from the upregulation of oncogenes and repression of tumour suppressor as was seen in females. Therefore, with females, a larger number of tumour suppressors were repressed while oncomirs and oncogenes were significantly upregulated.

The incidence of *CRAF* mutations in human NSCLC is similar to that of *BRAF*,[19,96,97] and mutations cause uncontrolled serine/threonine kinase activity, and therefore exaggerated MAPK signalling. Interestingly, some studies suggested the *BRAF* V600E mutation to be more prevalent in female NSCLC patients[98–100] and one study reported even an incidence of 8.6% vs 0.9% between female and male NSCLC cases (HR = 11.29; $p < 0.001$).[98] However, the OS for advanced-stage patients with *BRAF* mutations did not differ when compared to other driver mutations.[101] In human LC, we identified the gene coding for Q motif containing GTPase activating protein 3 as >10-fold induced and this scaffold protein affects MAPK signalling. Importantly, inhibition of *IQGAP1* disrupts RAF signalling and tumour growth in a mouse lung cancer disease model.[102]

Although the cRaf transgene differs in its mode of action, i.e., overexpression of the non-mutated kinase domain as compared to driver mutations, both conditions result in hyperactivity of the kinase and therefore recapitulate important aspects of the molecular pathology of *RAF* mutated lung cancers. The use of BRAF inhibitors in molecular stratified NSCLC patients is the subject of a recent review.[103] Unfortunately, only patients with the *BRAF* V600E mutation benefit from such treatment; however BRAF inhibitors are ineffective against other BRAF mutants and do not inhibit other oncogenic drivers such as RAF1. Indeed, the complexities surrounding RAF paralogs and their signalling outputs was summarized by Desideri and colleagues.[17]

Our study highlights the genomic responses to cRaf hyperactivity and the regulation of oncogenes (13 up-, 7 downregulated) and tumour suppressors (3 up-, 8 downregulated). These function in cell signalling, proliferation, apoptosis, metastasis, angiogenesis and immune response.

We observed upregulation of the oncogenes *Rasgrf1*, *Scd1* and repression of the tumour suppressor *Dusp10*. Their aberrant expression is linked to an activated MAPK signalling. Furthermore, MAPK signalling promotes cell proliferation, and the oncogenes *Cdk1*, *Stk39*, *Acsl4*, *Cbln1*, *Ctsk*, *Golm1*, *Malt1*, *Mat1a* were significantly upregulated to enhance cell proliferation. Conversely, *Acer2*, *Chad* and *Zbtb16* were repressed, and these tumour suppressors inhibit cell proliferation. Correspondingly, their repression promoted cell proliferation.

Among cyclin dependent kinases, we identified *Cdk1* as significantly upregulated in tumours of female cRaf transgenic mice, and we confirmed the result in human lung adenocarcinoma. Importantly, Cdk1 functions as an essential cell cycle regulator and forms complexes with cyclin A and B to drive the cell cycle from G2 to the mitosis-phase.[104] So far there are approximately 75 known protein targets of CDK1,[105,106] and we identified 8 as significantly regulated in human lung adenocarcinoma (range 2-15-fold upregulated in both sexes, Supplementary Table S13).

We and others report poor outcome for high CDK1 expression in LC patients (Fig. 10b and 11c). Its upregulation promotes cancer stemness.[107] Note CDK1 inhibitors are currently developed to treat breast, colon and pancreatic cancers[108–110]; yet, the clinical efficacy of CDK1 inhibitors await confirmation. As part of an ongoing study, we determined CDK1 activity in surgical resection material and adjacent non-tumours tissue of NSCLC patients. We found CDK1 to be highly upregulated (data not shown, manuscript in preparation), and therefore, our data provides a molecular ration for CDK1 inhibition in NSCLC. We also observed a sex disproportional OS for epiregulin (Fig. 10b). Its high expression is associated with worse outcome in males (Fig. 11c); however did not pass the linearity assumption in the COX proportional hazard model (Supplementary Table S6). A further example relates to miR-21-5p. This miRNA is upregulated in all lung adenocarcinoma

are survival plots for DEGs and DEMs commonly regulated among cRaf transgenic mice and human lung adenocarcinoma. HR: hazard ratio, brackets: 95% CI of the HR. Panel d: DEGs of pre- and post-menopausal female lung adenocarcinoma patients. The Venn diagrams showing the number of ER target genes among the specific DEGs in pre- and post-menopausal female lung adenocarcinoma patients. Panel e–f: Bar plots depict the significantly enriched hallmark gene sets for the up- (e) and downregulated (f) ERα target genes specifically regulated in female lung adenocarcinoma patients aged ≤55 years. Panel g and h: Dot plots show the fold changes of the commonly up- (g) and downregulated (h) genes between 20 lung adenocarcinoma patients and the TCGA database. The error bars represent 95% CI.

patients (Fig. 10c), but only high expression in females is associated with poor outcome (Fig. 11c). Nonetheless, the linearity assumption failed in the COX proportional hazard model (Supplementary Table S6).

An intriguing finding of our study is the importance of the oestrogen receptor in lung cancer and the predilection for ERα regulated genes. We evidence expression of ERα in human lung neoplasms by immunohistochemistry (Fig. 9) and therefore provide independent confirmation for its tumour associated regulation.[111] One study suggests a "yin-yang" relationship between ERα and ERβ in the control of gene expression, and as outlined by Lindberg and colleagues, ERβ might inhibit ERα gene regulations but also triggers ERα responses in its absence.[112] Considering ER tissue distribution, it is even more astonishing that ERβ is predominantly expressed in the lung of mice.[113] Nonetheless, E2 (=17β estradiol) binds to both receptors with similar affinity[114] and targeting oestrogens and various oestrogen-related receptors in NSCLC was the subject of a recent perspective and a review.[115,116]

While most of the ER regulated genes are common between female and male LC patients (Supplementary Fig. S4f) there are extraordinary differences in the targets between pre- and postmenopausal women. With premenopausal women most genes are ERα targets, and the difference is 7-fold when compared to ERβ targets (Fig. 11d). We observed a similar 8-fold difference for ERα regulated genes in lung tumours of postmenopausal women and there are twice as many ERα target genes regulated in premenopausal women, i.e., 173 vs. 94 DEGs. Thus, the hormonal status influenced tumour associated gene regulations and the gene targets are fundamentally different between pre- and postmenopausal women (Fig. 11d).

To better comprehend the sex differences in lung tumour growth, we constructed gene networks and assessed the role of hormone receptors on tumour specific gene regulations in cRaf transgenic mice. This revealed a complex interplay between the oestrogen receptor, miRNAs and genes in the control of tumour growth.

Fig. 12a depicts the complex interplay between miRNA, ER and target genes, and Fig. 12b provides an overview of the genes strongly associated with sex disparities in lung tumour growth. Finally, we identified sex dependent gene regulations in human lung adenocarcinomas and demonstrate clinical relevance of the findings. The genes validated in human lung adenocarcinoma are highlighted as circled in Fig. 12b.

Given the primary objective of our study, we explored the role of hormone receptors in lung tumour growth. We identified 39 DEGs as targets of the ERα of which 32 are specifically regulated in females while 6 are common for both sexes (Fig. 7c). Furthermore, of the 20 DEGs targeted by the androgen receptor, 18 were specifically regulated in females while the reaming 2 are common to both sexes (Fig. 7d). Together, we provide strong evidence for the hormone receptor dependent regulation of genes in lung tumours of cRaf transgenic mice.

Moreover, we constructed a gene regulatory network consisting of the oestrogen receptor, TF, miRNAs and tumour associated gene regulations in cRaf transgenic animals and identified *Lbh* and *Nr2f1* as repressed in cRaf females. Both TFs are repressors of the oestrogen receptor, and therefore the data are suggestive for an active ER complex to stimulate cell proliferation and cell cycle progression.[117]

Furthermore, we show 12.6% of DEGs are targets of the ER in female lung adenocarcinoma patients (Supplementary Table S12) even though the ER itself was not transcriptionally regulated. Currently, an anti-oestrogen therapy in NSCLC is being evaluated.[118] Intriguingly, and as a result of its oxidative metabolism, oestrogen itself may cause DNA damage and therefore become mutagenic.[119]

There are conflicting reports on the role of ER in pulmonary neoplasms, with some studies suggesting a prominent role for ERβ,[120] while recent research documents a primary role of ERα in the control of lung tumour associated gene regulations.[121,122] An earlier study investigated the sex-dependent expression of ERα and ERβ receptors in human non-tumour and tumour lung tissue in a small cohort of patients.[123] Essentially, ERα is predominantly expressed in lung tumours of female patients but rarely expressed in males. Additionally, about 1/3 of female cases express ERα in adjacent non-tumour tissue but none of the male cases are positive. Noteworthy, the mitogenic activity of E2 appears to be sex specific; yet, only female derived lung cancer cells responded to such treatments.[124]

In regards to miRNAs and among female transgenic mice, we observed repressed expression of miR-30c-2-3p, miR-339-5p, miR-181c-5p and miR-151-5p. These tumour suppressors regulate cell proliferation, apoptosis and EMT.[59,70,71,125] Altogether, there are 57 DEMs (Fig. 5b) of which 26 and 4 are regulated by the oestrogen and the androgen receptor. While there are more DEMs regulated in males most are targets of the oestrogen receptor.

Specifically, miR-31-5p was upregulated in cRaf males and this miRNA is regulated by the androgen receptor. It functions as an oncomir by stimulating MEK, ERK activity and by inhibiting p53.[126,127] Of the 24 *Er*-specific miRNA targets, 8 and 15, respectively were uniquely regulated in females and males. The fact that 15 *Er* responsive miRNAs were uniquely regulated in males was a surprise finding. Nonetheless, oestrogens do play an important role in male physiology as well.[128] In regards to female-specific and *Er* responsive miRNAs, we identified 4 which code for tumour suppressors. Specifically, miR-130a-3p and miR-34a-5p were 2-3-fold upregulated and both miRNAs target *Kdm2a* which was down regulated. Likewise, miR-339-5p and

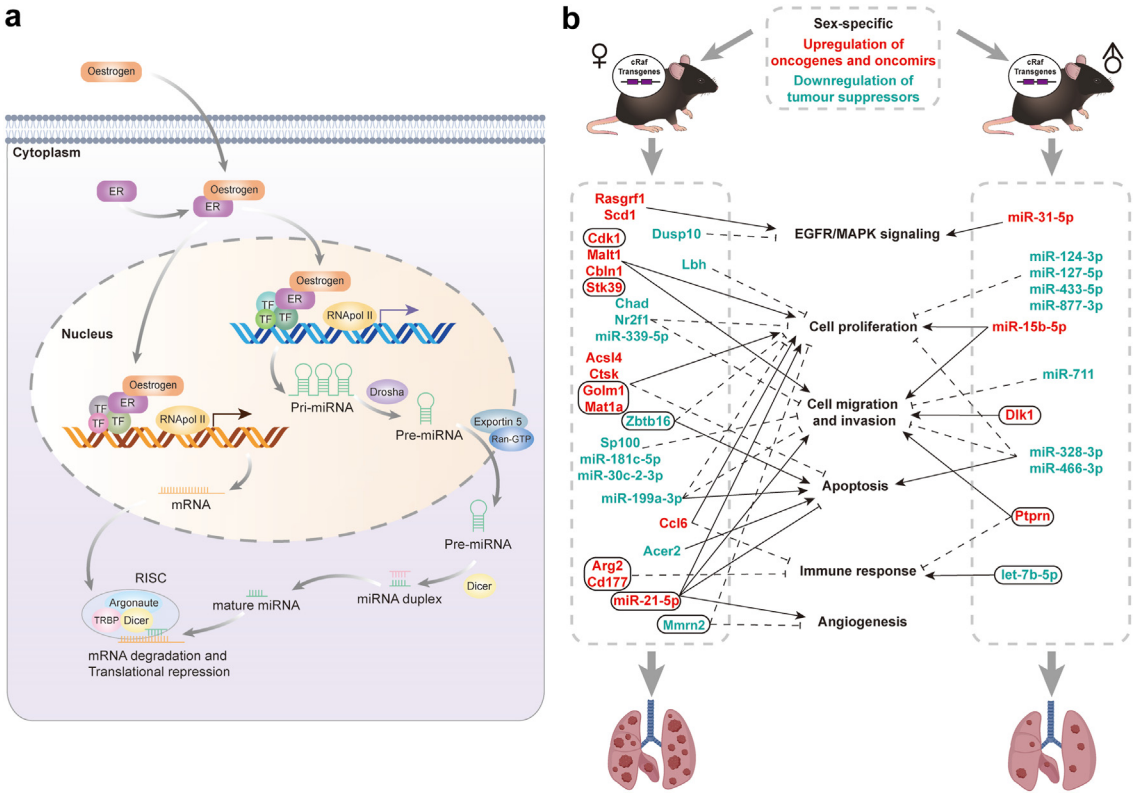

Fig. 12: Panel a: Schema to show the complex interplay between miRNA, oestrogen receptor and target genes. Panel b: Sex-specific regulation of oncogenes, oncomirs, and tumour suppressors in lung tumours of cRaf transgenic mice. Shown are sex-specific and therefore unique gene regulation in tumours of cRaf transgenic mice. Genes marked in red and turquoise refer to up- and downregulated genes, and the circled ones denote clinically validated genes in human LC. The function of the genes are highlighted by arrows (activation) or dashed lines (inhibition).

miR-181c-5p were repressed (2-3-fold) and their targets, i.e., Dusp5 and epiregulin were upregulated. Note, epiregulin stimulates EGFR signalling. Conversely, miR-21-5p was significantly upregulated and its target *MMP9* was repressed. MiR-21-5p is a target of the ERα receptor and functions as an oncomir. It is uniquely regulated in transgenic females and we likewise observed 2-fold higher expression of this miRNA in female NSCLC patients when compared to males (Supplementary Table S11). The function of the remaining 3 female-specific miRNAs are uncertain. Likewise of the 14 male specific and oestrogen receptor responsive miRNAs, 8 were repressed (range 2-6-fold), and these code for diverse functions as denoted for the tumour suppressors miR-574-3p and miR-711.

An important finding of our study is the function of the oestrogen receptor on immune cells in the tumour microenvironment, and Fig. 7f depicts the complex relationship between immunosurveillance and immunosuppressive cells in the tumour microenvironment. In total, there are 46 differentially expressed genes coding for immune response, and 39 are specifically regulated in female transgenic mice. Specifically, NK cells are of critical importance in immune surveillance and upon tumour cell recognition elicit an immune response. Unlike T cells, however, NK cells interact with tumour cells via receptors and the tumour cell recognition is independent of antigen presentation and processing.[129] Although activating or inhibiting NK receptors themselves were not regulated in cRaf transgenic mice, we observed significant regulation of 25 NK marker genes. Strikingly, 22 out of 25 NK marker genes are specifically regulated in females and the majority (17 genes) are down regulated (Fig. 4). Thus, in females NK immune responses are dampened, and likely contributed to the accelerated tumour growth (Fig. 2). A similar picture emerged with T cells. Here regulatory T cell marker genes are majorly down regulated in female transgenic mice and this supports an inflammatory microenvironment and tumour growth.[130] A further example relates to the mucosal-associated invariant T cells, whose marker genes are primarily regulated in females, and promote inflammation of the lung.[131] Furthermore, the complex interplay of dendritic, monocyte and macrophages is depicted in Fig. 4, and of the 23 regulated marker genes, 19 are specifically

regulated among transgenic females. Amphiregulin was selectively upregulated in females (4-fold) and this growth factor earmarks M1 polarized (=inflammatory) macrophages[132] to support an inflammatory microenvironment. Additionally, epiregulin is highly upregulated, i.e., 24- and 8-fold, respectively among male and female transgenic mice, and this growth factor stimulates proinflammatory cytokine production by antigen presenting cells.[133] Marker genes of dendritic cells were either up- or down regulated, and much to our surprise, tweety family member 3 (*Ttyh3*) is significantly repressed in transgenic females. This gene codes for a chloride anion channel, and is frequently upregulated in cancer. We observed *Ttyh3* upregulation in human NSCLC tissue samples (see below) while its down regulation in transgenic lung tumours might be linked to its regulatory function in neutrophils of the tumour microenvironment.[134,135]

In regards to monocytes, we observed regulation of 11 marker genes (6 up-, 5 downregulated) of which 8 were specifically regulated in female transgenic mice. A noticeable example relates to the significant upregulation of *Cxcl3* in males, and next to its role in inflammation and its production by TAMs,[136] this chemokine functions as a chemoattractant for neutrophils.[137] Indeed, the complex roles of neutrophils in cancer is the subject of a recent review,[135] and we identified 10 genes (5 up- and 5 downregulated), of which 9 were specifically regulated among cRaf females. We already emphasized the immunosuppressive tumour microenvironment in transgenic lung tumours and upregulation of *Cd177* results in ERK-mediated attenuation of chemokine signalling to modulate neutrophil migration.[138] Furthermore, TAMs secrete Ccl6 to support tumour cell migration[139] and this chemokine is also upregulated in pulmonary fibrosis and airway remodelling.[140] Additionally, we observed significant upregulation of the serine/threonine kinase 39 and the acute phase protein Lrg1. Note an earlier study demonstrated a Raf-1-dependent role of Stk39 in neutrophil adhesion[141] while upregulation of the leucine-rich α-2-glycoprotein serves as a biomarker in NSCLC patients[142] and is a critical effector in cell migration and invasion.[143] Lastly, upregulation of the inhibitor siglec receptor Siglecf (Siglec-5 alias CD170) is another example of the tumour suppressive environment in transgenic tumours. This receptor is expressed on tumour-infiltrating SiglecF^{high} neutrophils[144] and activated T cells, and its upregulation exemplifies the complex neutrophil-T cell interactions.[145]

As described above, amphiregulin and epiregulin are significantly induced in transgenic animals. Apart from their role in stimulating EGFR signalling (see below), amphiregulin enhances regulatory T cell-suppressive function via EGFR[146] and epiregulin regulates peptidoglycan-mediated proinflammatory cytokine production in antigen presenting cells.[133] Furthermore, both autocrine growth factor stimulate proangiogenic TAMs 93. Furthermore, complement factor I is 4-fold induced in cRaf females, and this factor inhibits C3b/C4b and therefore activity of the complement system.[147] In fact, various NSCLC cell lines secrete soluble inhibitors of the complement system and function as promoter of tumour progression.[148,149] Besides, marker genes of mast cells were specifically regulated in cRaf females and contribute to inflammation in the tumour microenvironment.[150] Finally, we identified arginase 2 (*Arg2*) as 3-fold upregulated in cRaf transgenic female mice and this mainly in myeloid cell expressed enzyme plays a key role in cancer immune response.[151] Overexpression of arginase inhibits proliferation of T cells and is associated with the downregulation of the CD3ζ chain, an essential component of the T cell receptor complex. It also causes T cell cycle arrest by reducing the phosphorylation of the retinoblastoma protein, which is a major component of the cyclin D/cyclin-dependent kinase complex.[152] Indeed, an independent immunohistochemistry evaluation of human NSCLC cases showed enhanced *Arg2* expression in the cytoplasm of NSCLC cells as well as cancer-associated fibroblasts.[91]

ICIs are truly game changers in the treatment of NSCLC, and targeting PD-1/PD-L1 and CTLA-4 improved significantly the survival of some cancer patients. However, clinical trials show that the overall efficacy was limited especially when considering OS, objective response rate, safety and time to treatment failure.[153] Given the variable expression of PD-1 in LC, the efficacy of ICIs remains controversial especially for tumours with only a small number of cells expressing the PD-1 target. Strikingly, in tumours of transgenic mice and in female LC patients, PD-L1 expression was unchanged while for male patients, its expression was significantly repressed but did not influence OS (Supplementary Fig. S5).

We view the regulation of *Arg2* as of great importance and found this enzyme to be specifically upregulated in cRaf females and in human lung adenocarcinoma regardless of sex. This enzyme is highly expressed in cancer-associated fibroblast and TAMs, and overexpression of arginase depleted arginine from the tumour microenvironment, which dampened T cell-mediated anti-tumour effects.[151,154] An earlier study reported inhibition of arginase 2 in dendritic cells to promote T cell proliferation,[155] and a recent study demonstrated significant regression of lung tumours in a mouse NSCLC model treated with an experimental arginase inhibitor.[156] Therefore, arginase may be a key regulator of the immune suppression in cancers and arginase inhibition combined with ICIs may evolve into a novel strategy to treat NSCLC.

There are important caveats to our study. First, our results are based on a transgenic disease model, and although the frequency of *CRAF* and *BRAF mutations* are similar in human NSCLC, the overall incidence is small. Notwithstanding, cRaf transgenicity caused an

unprecedented induction of the EGFR ligand epiregulin and amphiregulin, and therefore we obtained evidence for an extensive cross-talk between MAPK and EGFR signalling. Thus, the disease model recapitulates a major signalling pathway in lung cancer. Second, we did not perform gene reporter and gene silencing assays in lung cancer cell lines to validate ER target genes. On the other hand the plethora of published results for ER regulated genes enabled an identification of ER target genes with high confidence. In fact, we only considered chromatin-IP proven gene targets and therefore rely on strong experimental evidence for the physical interaction of the oestrogen receptors with its targets. Third, by employing the surfactant C promoter, the transgene is targeted to alveolar epithelial cells and this results in high tumour multiplicity that is not commonly seen in clinical cases. On the other hand, the high tumour multiplicity revealed sex differences in tumour growth. Fourth, an analysis of genomic responses to cRaf hyperactivity enabled us to construct gene-miRNA and hormone receptor regulatory gene networks which still needs to be established in clinical studies. Notwithstanding, translational research provided clear evidence for commonalities between transgenic female mice and female NSCLC patients. Fifth, the proposed therapeutic intervention consisting of an anti-oestrogen, arginase 2 and CDK1 inhibition requires clinical studies. Sixth, a further limitation of our study is the built-in selection bias in HRs[157] and a possible overreliance on significance testing. Therefore, we report median difference and HR estimates with 95% CIs, and consider their plausibility based on clinical relevance and mechanistic knowledge in addition to strong experimental evidence. Seventh, we cannot exclude the possibility of "mixing effects" of the different factors contributing to sex differences in tumour growth, i.e., miRNAs, hormone receptors, other transcription factors and cRaf transgenicity on the regulation of tumour suppressors, oncogenes and oncomirs. Inevitably such confounding is difficult to establish, nonetheless might distort their true contribution to tumour growth.

In conclusion, our study highlights major differences in the genomic responses to cRaf hyperactivity with females being more sensitive to the tumour promoting effects of cRaf (Fig. 2). We gained insight into the complex interplay between cRaf, miRNAs, hormone receptors and TF and were able to construct sex specific regulatory gene networks. Thus, our study provided new insight into the role of cRaf in lung cancer, and we established clinical relevance by considering a large cohort of lung adenocarcinoma patients. The findings provide a rationale for the development of molecular targeted therapies by jointly blocking ER, inhibiting CDK1 and arginase 2 activity. Moreover, it is tempting to speculate that the combined use of immune checkpoint, arginase 2 and ER inhibitors will be more effective when compared to their single use.

Contributors

Conception of the Study and supervision of the experimental works (JB). Data analysis (SZ). Preparation of Figures and Tables (SZ). Comprehensive literature review (SZ, JB). Final manuscript preparation (JB). SZ and JB have accessed and verified the underlying data. All authors read and approved the final version of the manuscript.

Data sharing statement

The data to support the findings of this study are available from the authors upon reasonable request.

Declaration of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104763.

References

1 Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209–249.
2 Meza R, Meernik C, Jeon J, Cote ML. Lung cancer incidence trends by gender, race and histology in the United States, 1973-2010. *PLoS One*. 2015;10(3):e0121323.
3 North CM, Christiani DC. Women and lung cancer: what is new? *Semin Thorac Cardiovasc Surg*. 2013;25(2):87–94.
4 Ragavan M, Patel MI. The evolving landscape of sex-based differences in lung cancer: a distinct disease in women. *Eur Respir Rev*. 2022;31(163):210100. https://doi.org/10.1183/16000617.0100-2021. Print 2022 Mar 31.
5 MacRosty CR, Rivera MP. Lung cancer in women: a modern epidemic. *Clin Chest Med*. 2020;41(1):53–65.
6 Klein SL, Morgan R. The impact of sex and gender on immunotherapy outcomes. *Biol Sex Differ*. 2020;11(1):24.
7 Reck M, Rodríguez-Abreu D, Robinson AG, et al. Updated analysis of KEYNOTE-024: pembrolizumab versus platinum-based chemotherapy for advanced non-small-cell lung cancer with PD-L1 tumor proportion score of 50% or greater. *J Clin Oncol*. 2019;37(7):537–546.
8 West H, McCleod M, Hussein M, et al. Atezolizumab in combination with carboplatin plus nab-paclitaxel chemotherapy compared with chemotherapy alone as first-line treatment for metastatic non-squamous non-small-cell lung cancer (IMpower130): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2019;20(7):924–937.
9 Zhong S, Golpon H, Zardo P, Borlak J. miRNAs in lung cancer. A systematic review identifies predictive and prognostic miRNA candidates for precision medicine in lung cancer. *Transl Res*. 2021;230:164–196.
10 Magalhães M, Alvarez-Lorenzo C, Concheiro A, Figueiras A, Santos AC, Veiga F. RNAi-based therapeutics for lung cancer: biomarkers, microRNAs, and nanocarriers. *Expert Opin Drug Deliv*. 2018;15(10):965–982.
11 Lee TI, Young RA. Transcriptional regulation and its misregulation in disease. *Cell*. 2013;152(6):1237–1251.
12 Bushweller JH. Targeting transcription factors in cancer — from undruggable to reality. *Nat Rev Cancer*. 2019;19(11):611–624.
13 Arora S, Rana R, Chhabra A, Jaiswal A, Rani V. miRNA-transcription factor interactions: a combinatorial regulation of gene expression. *Mol Genet Genomics*. 2013;288(3–4):77–87.
14 Rohrbeck A, Borlak J. Cancer genomics identifies regulatory gene networks associated with the transition from dysplasia to advanced lung adenocarcinomas induced by c-Raf-1. *PLoS One*. 2009;4(10):e7315.
15 Rohrbeck A, Müller VS, Borlak J. Molecular characterization of lung dysplasia induced by c-Raf-1. *PLoS One*. 2009;4(5):e5637.

16 Lavoie H, Therrien M. Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol*. 2015;16(5):281–298.
17 Desideri E, Cavallo AL, Baccarini M. Alike but different: RAF paralogs and their signaling outputs. *Cell*. 2015;161(5):967–970.
18 Sanclemente M, Francoz S, Esteban-Burgos L, et al. c-RAF ablation induces regression of advanced kras/trp53 mutant lung adenocarcinomas by a mechanism independent of MAPK signaling. *Cancer Cell*. 2018;33(2):217–228.e4.
19 Noeparast A, Giron P, Noor A, et al. CRAF mutations in lung cancer can be oncogenic and predict sensitivity to combined type II RAF and MEK inhibition. *Oncogene*. 2019;38(31):5933–5941.
20 Dogan S, Shen R, Ang DC, et al. Molecular epidemiology of *EGFR* and *KRAS* mutations in 3,026 lung adenocarcinomas: higher susceptibility of women to smoking-related *KRAS*-mutant cancers. *Clin Cancer Res*. 2012;18(22):6169.
21 Kerkhoff E, Fedorov LM, Siefken R, Walter AO, Papadopoulos T, Rapp UR. Lung-targeted expression of the c-Raf-1 kinase in transgenic mice exposes a novel oncogenic character of the wild-type protein. *Cell Growth Differ*. 2000;11(4):185–190.
22 Del Vescovo V, Meier T, Inga A, Denti MA, Borlak J. A cross-platform comparison of affymetrix and Agilent microarrays reveals discordant miRNA expression in lung tumors of c-Raf transgenic mice. *PLoS One*. 2013;8(11):e78870.
23 R Core Team. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing; 2020. https://www.R-project.org/.2020.
24 Zhou Y, Zhou B, Pache L, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun*. 2019;10(1):1523.
25 Wickham H. Data analysis. In: *ggplot2*. Anonymous Springer; 2016:189–201.
26 Chang L, Zhou G, Soufan O, Xia J. miRNet 2.0: network-based visual analytics for miRNA functional analysis and systems biology. *Nucleic Acids Res*. 2020;48(W1):W224–W251.
27 Otasek D, Morris JH, Bouças J, Pico AR, Demchak B. Cytoscape automation: empowering workflow-based network analysis. *Genome Biol*. 2019;20(1):185.
28 Goldman MJ, Craft B, Hastie M, et al. Visualizing and interpreting cancer genomics data via the Xena platform. *Nat Biotechnol*. 2020;38(6):675–678.
29 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
30 Ikari A, Watanabe R, Sato T, et al. Nuclear distribution of claudin-2 increases cell proliferation in human lung adenocarcinoma cells. *Biochim Biophys Acta*. 2014;1843(9):2079–2088.
31 Ikari A, Sato T, Watanabe R, Yamazaki Y, Sugatani J. Increase in claudin-2 expression by an EGFR/MEK/ERK/c-Fos pathway in lung adenocarcinoma A549 cells. *Biochim Biophys Acta*. 2012;1823(6):1110–1118.
32 Zhang J, Iwanaga K, Choi KC, et al. Intratumoral epiregulin is a marker of advanced disease in non-small cell lung cancer patients and confers invasive properties on EGFR-mutant cells. *Cancer Prev Res (Phila)*. 2008;1(3):201–207.
33 White MA, Vale T, Camonis JH, Schaefer E, Wigler MH. A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. *J Biol Chem*. 1996;271(28):16439–16442.
34 Wagner KW, Alam H, Dhar SS, et al. KDM2A promotes lung tumorigenesis by epigenetically enhancing ERK1/2 signaling. *J Clin Invest*. 2013;123(12):5231–5246.
35 Li Z, Zhu W, Xiong L, Yu X, Chen X, Lin Q. Role of high expression levels of STK39 in the growth, migration and invasion of non-small cell type lung cancer cells. *Oncotarget*. 2016;7(38):61366–61377.
36 Sen U, Coleman C, Sen T. Stearoyl coenzyme A desaturase-1: multitasker in cancer, metabolism, and ferroptosis. *Trends Cancer*. 2023;9(6):480–489.
37 Zhang J, Song F, Zhao X, et al. EGFR modulates monounsaturated fatty acid synthesis through phosphorylation of SCD1 in lung cancer. *Mol Cancer*. 2017;16(1):127.
38 Szklarczyk D, Gable AL, Nastou KC, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res*. 2021;49(D1):D605–D612.
39 Li L, Tan J, Zhang Y, et al. DLK1 promotes lung cancer cell invasion through upregulation of MMP9 expression depending on Notch signaling. *PLoS One*. 2014;9(3):e91509.
40 Takagi H, Zhao S, Muto S, et al. Delta-like 1 homolog (DLK1) as a possible therapeutic target and its application to radioimmunotherapy using 125I-labelled anti-DLK1 antibody in lung cancer models (HOT1801 and FIGHT004). *Lung Cancer*. 2021;153:134–142.
41 Qu P, Roberts J, Li Y, et al. Stat3 downstream genes serve as biomarkers in human lung carcinomas and chronic obstructive pulmonary disease. *Lung Cancer*. 2009;63(3):341–347.
42 Pan D, Jiang C, Ma Z, Blonska M, You MJ, Lin X. MALT1 is required for EGFR-induced NF-κB activation and contributes to EGFR-driven lung cancer progression. *Oncogene*. 2016;35(7):919–928.
43 Aruna, Li LM. Overexpression of golgi membrane protein 1 promotes non-small-cell carcinoma aggressiveness by regulating the matrix metallopeptidase 13. *Am J Cancer Res*. 2018;8(3):551–565.
44 Castillo AF, Orlando UD, Maloberti PM, et al. New inhibitor targeting Acyl-CoA synthetase 4 reduces breast and prostate tumor growth, therapeutic resistance and steroidogenesis. *Cell Mol Life Sci*. 2021;78(6):2893–2910.
45 Kim MC, Borcherding N, Ahmed KK, et al. CD177 modulates the function and homeostasis of tumor-infiltrating regulatory T cells. *Nat Commun*. 2021;12(1):5764.
46 Martin KA, Hum NR, Sebastian A, et al. Methionine adenosyltransferase 1a (MAT1A) enhances cell survival during chemotherapy treatment and is associated with drug resistance in bladder cancer PDX mice. *Int J Mol Sci*. 2019;20(20):4983. https://doi.org/10.3390/ijms20204983.
47 Jiang F, Shen Q, Zhang F, et al. ADH1C facilitates cisplatin resistance of lung adenocarcinoma cells. *DNA Cell Biol*. 2022;41(6):631–640.
48 Song X, Jiao X, Yan H, et al. Overexpression of PTPRN promotes metastasis of lung adenocarcinoma and suppresses NK cell cytotoxicity. *Front Cell Dev Biol*. 2021;9:622018.
49 Wang Y, Zhang C, Jin Y, et al. Alkaline ceramidase 2 is a novel direct target of p53 and induces autophagy and apoptosis through ROS generation. *Sci Rep*. 2017;7:44573.
50 Ai J, Wang Y, Tan K, et al. A human homolog of mouse Lbh gene, hLBH, expresses in heart and activates SRE and AP-1 mediated MAPK signaling pathway. *Mol Biol Rep*. 2008;35(2):179–187.
51 Wang X, Wang L, Guo S, et al. Hypermethylation reduces expression of tumor-suppressor PLZF and regulates proliferation and apoptosis in non-small-cell lung cancers. *FASEB J*. 2013;27(10):4194–4203.
52 Deng X, Wei W, Huang N, et al. Tumor repressor gene chondroadherin oppose migration and proliferation in hepatocellular carcinoma and predicts a good survival. *Oncotarget*. 2017;8(36):60270–60279.
53 Palumbo JS, Kombrinck KW, Drew AF, et al. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. *Blood*. 2000;96(10):3302–3309.
54 Lin YP, Wu JI, Tseng CW, Chen HJ, Wang LH. Gjb4 serves as a novel biomarker for lung cancer and promotes metastasis and chemoresistance via Src activation. *Oncogene*. 2019;38(6):822–837.
55 Ding F, Wang D, Li X, et al. Overexpression of S100A14 contributes to malignant progression and predicts poor prognosis of lung adenocarcinoma. *Thorac Cancer*. 2018;9(7):827–835.
56 Friedman DJ, Crotts SB, Shapiro MJ, et al. ST8Sia6 promotes tumor growth in mice by inhibiting immune responses. *Cancer Immunol Res*. 2021;9(8):952–966.
57 Hao XL, Han F, Zhang N, et al. TC2N, a novel oncogene, accelerates tumor progression by suppressing p53 signaling pathway in lung cancer. *Cell Death Differ*. 2019;26(7):1235–1250.
58 Cho J, Son J, Ha S, Lee I. Systems biology analysis identifies TNFRSF9 as a functional marker of tumor-infiltrating regulatory T-cell enabling clinical outcome prediction in lung cancer. *Comput Struct Biotechnol J*. 2021;19:860–868.
59 Zhong Z, Xia Y, Wang P, Liu B, Chen Y. Low expression of microRNA-30c promotes invasion by inducing epithelial mesenchymal transition in non-small cell lung cancer. *Mol Med Rep*. 2014;10(5):2575–2579.
60 Meng W, Li Y, Chai B, Liu X, Ma Z. miR-199a: a tumor suppressor with noncoding RNA network and therapeutic candidate in lung cancer. *Int J Mol Sci*. 2022;23(15):8518. https://doi.org/10.3390/ijms23158518.
61 Tong F, Ying Y, Pan H, Zhao W, Li H, Zhan X. MicroRNA-466 (miR-466) functions as a tumor suppressor and prognostic factor in colorectal cancer (CRC). *Bosn J Basic Med Sci*. 2018;18(3):252–259.

62 Li J, Chen M, Yu B. miR-433 suppresses tumor progression via Smad2 in non-small cell lung cancer. *Pathol Res Pract*. 2019;215(10):152591.
63 Chen J, Wang M, Guo M, Xie Y, Cong Y. miR-127 regulates cell proliferation and senescence by targeting BCL6. *PLoS One*. 2013;8(11):e80266.
64 Lu J, Lin J, Zhou Y, Ye K, Fang C. MiR-328-3p inhibits lung adenocarcinoma-genesis by downregulation PYCR1. *Biochem Biophys Res Commun*. 2021;550:99–106.
65 Li S, Zhu Y, Liang Z, et al. Up-regulation of p16 by miR-877-3p inhibits proliferation of bladder cancer. *Oncotarget*. 2016;7(32):51773–51783.
66 Xiao W, Li D, Tang Y, et al. Inhibition of epithelial-mesenchymal transition in gastric cancer cells by miR-711-mediated down-regulation of CD44 expression. *Oncol Rep*. 2018;40(5):2844–2853.
67 Ma W, Ma C, Zhou N, Li X, Zhang Y. Up- regulation of miR-328-3p sensitizes non-small cell lung cancer to radiotherapy. *Sci Rep*. 2016;6:31651.
68 Zhang Q, Pan J, Xiong D, et al. Pulmonary aerosol delivery of let-7b microRNA confers a striking inhibitory effect on lung carcinogenesis through targeting the tumor immune microenvironment. *Adv Sci (Weinh)*. 2021;8(17):e2100629.
69 Wang L, Kang F-B, Sun N, et al. The tumor suppressor miR-124 inhibits cell proliferation and invasion by targeting B7-H3 in osteosarcoma. *Tumor Biol*. 2016;37(11):14939–14947.
70 Li X, Wu P, Tang Y, et al. Down-regulation of MiR-181c-5p promotes epithelial-to-mesenchymal transition in laryngeal squamous cell carcinoma via targeting SERPINE1. *Front Oncol*. 2020;10:544476.
71 Li Y, Zhang X, Yang Z, Li Y, Han B, Chen LA. mir-339-5p inhibits metastasis of non-small cell lung cancer by regulating the epithelial-to-mesenchymal transition. *Oncol Lett*. 2018;15(2):2508–2514.
72 Song G, Xiao M, Wan X, et al. MiR-130a-3p suppresses colorectal cancer growth by targeting Wnt family member 1 (WNT1). *Bioengineered*. 2021;12(1):8407–8418.
73 Li Y, Zhang H, Dong Y, et al. MiR-146b-5p functions as a suppressor miRNA and prognosis predictor in non-small cell lung cancer. *J Cancer*. 2017;8(9):1704–1716.
74 Chen T, Xiao Q, Wang X, et al. miR-16 regulates proliferation and invasion of lung cancer cells via the ERK/MAPK signaling pathway by targeted inhibition of MAPK kinase 1 (MEK1). *J Int Med Res*. 2019;47(10):5194–5204.
75 Bica-Pop C, Cojocneanu-Petric R, Magdo L, Raduly L, Gulei D, Berindan-Neagoe I. Overview upon miR-21 in lung cancer: focus on NSCLC. *Cell Mol Life Sci*. 2018;75(19):3539–3551.
76 Rothenberger NJ, Somasundaram A, Stabile LP. The role of the estrogen pathway in the tumor microenvironment. *Int J Mol Sci*. 2018;19(2):611. https://doi.org/10.3390/ijms19020611.
77 Bossone SA, Asselin C, Patel AJ, Marcu KB. MAZ, a zinc finger protein, binds to c-MYC and C2 gene sequences regulating transcriptional initiation and termination. *Proc Natl Acad Sci U S A*. 1992;89(16):7452–7456.
78 Izzo MW, Strachan GD, Stubbs MC, Hall DJ. Transcriptional repression from the c-myc P2 promoter by the zinc finger protein ZF87/MAZ. *J Biol Chem*. 1999;274(27):19498–19506.
79 Zheng C, Wu H, Jin S, Li D, Tan S, Zhu X. Roles of Myc-associated zinc finger protein in malignant tumors. *Asia Pac J Clin Oncol*. 2022;18(6):506–514.
80 Khalil BD, Sanchez R, Rahman T, et al. An NR2F1-specific agonist suppresses metastasis by inducing cancer cell dormancy. *J Exp Med*. 2022;219(1):e20210836. https://doi.org/10.1084/jem.20210836. Epub 2021 Nov 23.
81 Klinge CM, Silver BF, Driscoll MD, Sathya G, Bambara RA, Hilf R. Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. *J Biol Chem*. 1997;272(50):31465–31474.
82 Jiang JG, Bell A, Liu Y, Zarnegar R. Transcriptional regulation of the hepatocyte growth factor gene by the nuclear receptors chicken ovalbumin upstream promoter transcription factor and estrogen receptor. *J Biol Chem*. 1997;272(7):3928–3934.
83 Shuck SC, Short EA, Turchi JJ. Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. *Cell Res*. 2008;18(1):64–72.
84 Pantic B, Trevisan E, Citta A, et al. Myotonic dystrophy protein kinase (DMPK) prevents ROS-induced cell death by assembling a hexokinase II-Src complex on the mitochondrial surface. *Cell Death Dis*. 2013;4(10):e858.
85 Itoh K, Ebata T, Hirata H, et al. DMPK is a new candidate mediator of tumor suppressor p53-dependent cell death. *Molecules*. 2019;24(17):3175. https://doi.org/10.3390/molecules24173175.
86 Chen M, Guan B, Xu H, Yu F, Zhang T, Wu B. The molecular mechanism regulating diurnal rhythm of flavin-containing monooxygenase 5 in mouse liver. *Drug Metab Dispos*. 2019;47(11):1333–1342.
87 La Sala L, Mrakic-Sposta S, Micheloni S, Prattichizzo F, Ceriello A. Glucose-sensing microRNA-21 disrupts ROS homeostasis and impairs antioxidant responses in cellular glucose variability. *Cardiovasc Diabetol*. 2018;17(1):105–112.
88 Loeb GB, Khan AA, Canner D, et al. Transcriptome-wide miR-155 binding map reveals widespread noncanonical microRNA targeting. *Mol Cell*. 2012;48(5):760–770.
89 Kidger AM, Rushworth LK, Stellzig J, et al. Dual-specificity phosphatase 5 controls the localized inhibition, propagation, and transforming potential of ERK signaling. *Proc Natl Acad Sci U S A*. 2017;114(3):E317–E326.
90 Lorenzon E, Colladel R, Andreuzzi E, et al. MULTIMERIN2 impairs tumor angiogenesis and growth by interfering with VEGF-A/VEGFR2 pathway. *Oncogene*. 2012;31(26):3136–3147.
91 Giatromanolaki A, Harris AL, Koukourakis MI. The prognostic and therapeutic implications of distinct patterns of argininosuccinate synthase 1 (ASS1) and arginase-2 (ARG2) expression by cancer cells and tumor stroma in non-small-cell lung cancer. *Cancer Metab*. 2021;9(1):28–37.
92 Ma R, Black A, Qian B. Macrophage diversity in cancer revisited in the era of single-cell omics. *Trends Immunol*. 2022;43(7):546–563.
93 Matsubara E, Komohara Y, Esumi S, et al. SPP1 derived from macrophages is associated with a worse clinical course and chemoresistance in lung adenocarcinoma. *Cancers (Basel)*. 2022;14(18):4374. https://doi.org/10.3390/cancers14184374.
94 Pan Y, Yu Y, Wang X, Zhang T. Tumor-associated macrophages in tumor immunity. *Front Immunol*. 2020;11:583084.
95 Degirmenci U, Wang M, Hu J. Targeting aberrant RAS/RAF/MEK/ERK signaling for cancer therapy. *Cells*. 2020;9(1):198. https://doi.org/10.3390/cells9010198.
96 Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417(6892):949–954.
97 Holderfield M, Deuker MM, McCormick F, McMahon M. Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. *Nat Rev Cancer*. 2014;14(7):455–467.
98 Marchetti A, Felicioni L, Malatesta S, et al. Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations. *J Clin Oncol*. 2011;29(26):3574–3579.
99 Paik PK, Arcila ME, Fara M, et al. Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. *J Clin Oncol*. 2011;29(15):2046–2051.
100 Cardarella S, Ogino A, Nishino M, et al. Clinical, pathologic, and biologic features associated with BRAF mutations in non-small cell lung cancer. *Clin Cancer Res*. 2013;19(16):4532–4540.
101 O'Leary CG, Andelkovic V, Ladwa R, et al. Targeting BRAF mutations in non-small cell lung cancer. *Transl Lung Cancer Res*. 2019;8(6):1119–1124.
102 Jameson KL, Mazur PK, Zehnder AM, et al. IQGAP1 scaffold-kinase interaction blockade selectively targets RAS-MAP kinase-driven tumors. *Nat Med*. 2013;19(5):626–630.
103 Yan N, Guo S, Zhang H, Zhang Z, Shen S, Li X. BRAF-mutated non-small cell lung cancer: current treatment status and future perspective. *Front Oncol*. 2022;12:863043.
104 Matthews HK, Bertoli C, de Bruin RAM. Cell cycle control in cancer. *Nat Rev Mol Cell Biol*. 2022;23(1):74–88.
105 Enserink JM, Kolodner RD. An overview of Cdk1-controlled targets and processes. *Cell Div*. 2010;5:11.
106 Ubersax JA, Woodbury EL, Quang PN, et al. Targets of the cyclin-dependent kinase Cdk1. *Nature*. 2003;425(6960):859–864.
107 Huang Z, Shen G, Gao J. CDK1 promotes the stemness of lung cancer cells through interacting with Sox2. *Clin Transl Oncol*. 2021;23(9):1743–1751.
108 Kang J, Sergio CM, Sutherland RL, Musgrove EA. Targeting cyclin-dependent kinase 1 (CDK1) but not CDK4/6 or CDK2 is selectively lethal to MYC-dependent human breast cancer cells. *BMC Cancer*. 2014;14:32.
109 Zhang P, Kawakami H, Liu W, et al. Targeting CDK1 and MEK/ERK overcomes apoptotic resistance in BRAF-mutant human colorectal cancer. *Mol Cancer Res*. 2018;16(3):378–389.

110 Wijnen R, Pecoraro C, Carbone D, et al. Cyclin dependent kinase-1 (CDK-1) inhibition as a novel therapeutic strategy against pancreatic ductal adenocarcinoma (PDAC). *Cancers (Basel)*. 2021;13(17):4389. https://doi.org/10.3390/cancers13174389.
111 Lau SK, Chu PG, Weiss LM. Immunohistochemical expression of estrogen receptor in pulmonary adenocarcinoma. *Appl Immunohistochem Mol Morphol*. 2006;14(1):83–87.
112 Lindberg MK, Movérare S, Skrtic S, et al. Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. *Mol Endocrinol*. 2003;17(2):203–208.
113 Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology*. 1997;138(11):4613–4621.
114 Chen P, Li B, Ou-Yang L. Role of estrogen receptors in health and disease. *Front Endocrinol (Lausanne)*. 2022;13:839005.
115 Maitra R, Malik P, Mukherjee TK. Targeting estrogens and various estrogen-related receptors against non-small cell lung cancers: a perspective. *Cancers (Basel)*. 2021;14(1):80. https://doi.org/10.3390/cancers14010080.
116 Mukherjee TK, Malik P, Hoidal JR. The emerging role of estrogen related receptorα in complications of non-small cell lung cancers. *Oncol Lett*. 2021;21(4):258.
117 Hershberger PA, Vasquez AC, Kanterewicz B, Land S, Siegfried JM, Nichols M. Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands. *Cancer Res*. 2005;65(4):1598–1605.
118 Smida T, Bruno TC, Stabile LP. Influence of estrogen on the NSCLC microenvironment: a comprehensive picture and clinical implications. *Front Oncol*. 2020;10:137.
119 Rodriguez-Lara V, Avila-Costa MR. An overview of lung cancer in women and the impact of estrogen in lung carcinogenesis and lung cancer treatment. *Front Med (Lausanne)*. 2021;8:600121.
120 Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F. Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential. *Steroids*. 2014;90:13–29.
121 He M, Yu W, Chang C, et al. Estrogen receptor α promotes lung cancer cell invasion via increase of and cross-talk with infiltrated macrophages through the CCL2/CCR2/MMP9 and CXCL12/CXCR4 signaling pathways. *Mol Oncol*. 2020;14(8):1779–1799.
122 Kawai H, Ishii A, Washiya K, et al. Estrogen receptor alpha and beta are prognostic factors in non-small cell lung cancer. *Clin Cancer Res*. 2005;11(14):5084–5089.
123 Fasco MJ, Hurteau GJ, Spivack SD. Gender-dependent expression of alpha and beta estrogen receptors in human nontumor and tumor lung tissue. *Mol Cell Endocrinol*. 2002;188(1–2):125–140.
124 Dougherty SM, Mazhawidza W, Bohn AR, et al. Gender difference in the activity but not expression of estrogen receptors alpha and beta in human lung adenocarcinoma cells. *Endocr Relat Cancer*. 2006;13(1):113–134.
125 Daugaard I, Sanders KJ, Idica A, et al. miR-151a induces partial EMT by regulating E-cadherin in NSCLC cells. *Oncogenesis*. 2017;6(7):e366.
126 Zhu C, Wang S, Zheng M, et al. miR-31-5p modulates cell progression in lung adenocarcinoma through TNS1/p53 axis. *Strahlenther Onkol*. 2022;198(3):304–314.
127 Yu F, Liang M, Huang Y, Wu W, Zheng B, Chen C. Hypoxic tumor-derived exosomal miR-31-5p promotes lung adenocarcinoma metastasis by negatively regulating SATB2-reversed EMT and activating MEK/ERK signaling. *J Exp Clin Cancer Res*. 2021;40(1):179–187.
128 Cooke PS, Nanjappa MK, Ko C, Prins GS, Hess RA. Estrogens in male physiology. *Physiol Rev*. 2017;97(3):995–1043.
129 Liu S, Galat V, Galat Y, Lee YKA, Wainwright D, Wu J. NK cell-based cancer immunotherapy: from basic biology to clinical development. *J Hematol Oncol*. 2021;14(1):7.
130 Greten FR, Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. *Immunity*. 2019;51(1):27–41.
131 Wen X, Zhang X, Nian S, et al. Title of article: mucosal-associated invariant T cells in lung diseases. *Int Immunopharmacol*. 2021;94:107485.
132 Meng C, Liu G, Mu H, Zhou M, Zhang S, Xu Y. Amphiregulin may be a new biomarker of classically activated macrophages. *Biochem Biophys Res Commun*. 2015;466(3):393–399.
133 Sugiyama S, Nakabayashi K, Baba I, Sasazuki T, Shirasawa S. Role of epiregulin in peptidoglycan-induced proinflammatory cytokine production by antigen presenting cells. *Biochem Biophys Res Commun*. 2005;337(1):271–274.
134 Yen M, Yeh I, Liu K, et al. Next-generation sequencing predicts interaction network between miRNA and target genes in lipoteichoic acid-stimulated human neutrophils. *Int J Mol Med*. 2019;44(4):1436–1446.
135 Hedrick CC, Malanchi I. Neutrophils in cancer: heterogeneous and multifaceted. *Nat Rev Immunol*. 2022;22(3):173–187.
136 Butler KL, Clancy-Thompson E, Mullins DW. CXCR3(+) monocytes/macrophages are required for establishment of pulmonary metastases. *Sci Rep*. 2017;7:45593.
137 Metzemaekers M, Gouwy M, Proost P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. *Cell Mol Immunol*. 2020;17(5):433–450.
138 Bai M, Grieshaber-Bouyer R, Wang J, et al. CD177 modulates human neutrophil migration through activation-mediated integrin and chemoreceptor regulation. *Blood*. 2017;130(19):2092–2100.
139 Masetti M, Carriero R, Portale F, et al. Lipid-loaded tumor-associated macrophages sustain tumor growth and invasiveness in prostate cancer. *J Exp Med*. 2022;219(2):e20210564. https://doi.org/10.1084/jem.20210564. Epub 2021 Dec 17.
140 Ma B, Zhu Z, Homer RJ, Gerard C, Strieter R, Elias JA. The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol*. 2004;172(3):1872–1881.
141 Pillinger MH, Feoktistov AS, Capodici C, et al. Mitogen-activated protein kinase in neutrophils and enucleate neutrophil cytoplasts: evidence for regulation of cell-cell adhesion. *J Biol Chem*. 1996;271(20):12049–12056.
142 Li Y, Zhang Y, Qiu F, Qiu Z. Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. *Electrophoresis*. 2011;32(15):1976–1983.
143 Lin M, Liu J, Zhang F, et al. The role of leucine-rich alpha-2-glycoprotein-1 in proliferation, migration, and invasion of tumors. *J Cancer Res Clin Oncol*. 2022;148(2):283–291.
144 Engblom C, Pfirschke C, Zilionis R, et al. Osteoblasts remotely supply lung tumors with cancer-promoting SiglecF(high) neutrophils. *Science*. 2017;358(6367):eaal5081. https://doi.org/10.1126/science.aal5081.
145 Minns D, Smith KJ, Hardisty G, Rossi AG, Gwyer Findlay E. The outcome of neutrophil-T cell contact differs depending on activation status of both cell types. *Front Immunol*. 2021;12:633486.
146 Zaiss DM, van Loosdregt J, Gorlani A, et al. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. *Immunity*. 2013;38(2):275–284.
147 Nilsson SC, Sim RB, Lea SM, Fremeaux-Bacchi V, Blom AM. Complement factor I in health and disease. *Mol Immunol*. 2011;48(14):1611–1620.
148 Okroj M, Hsu YF, Ajona D, Pio R, Blom AM. Non-small cell lung cancer cells produce a functional set of complement factor I and its soluble cofactors. *Mol Immunol*. 2008;45(1):169–179.
149 Revel M, Daugan MV, Sautés-Fridman C, Fridman WH, Roumenina LT. Complement system: promoter or suppressor of cancer progression? *Antibodies (Basel)*. 2020;9(4):57. https://doi.org/10.3390/antib9040057.
150 Varricchi G, Galdiero MR, Loffredo S, et al. Are mast cells MASTers in cancer? *Front Immunol*. 2017;8:424.
151 Munder M. Arginase: an emerging key player in the mammalian immune system. *Br J Pharmacol*. 2009;158(3):638–651.
152 Grzywa TM, Sosnowska A, Matryba P, et al. Myeloid cell-derived arginase in cancer immune response. *Front Immunol*. 2020;11:938.
153 Pasello G, Pavan A, Attili I, et al. Real world data in the era of immune checkpoint inhibitors (ICIs): increasing evidence and future applications in lung cancer. *Cancer Treat Rev*. 2020;87:102031.
154 Zea AH, Rodriguez PC, Culotta KS, et al. L-Arginine modulates CD3zeta expression and T cell function in activated human T lymphocytes. *Cell Immunol*. 2004;232(1–2):21–31.
155 Dunand-Sauthier I, Irla M, Carnesecchi S, et al. Repression of arginase-2 expression in dendritic cells by microRNA-155 is critical for promoting T cell proliferation. *J Immunol*. 2014;193(4):1690–1700.
156 Miret JJ, Kirschmeier P, Koyama S, et al. Suppression of myeloid cell arginase activity leads to therapeutic response in a NSCLC mouse model by activating anti-tumor immunity. *J Immunother Cancer*. 2019;7(1):32.
157 Hernán MA. The hazards of hazard ratios. *Epidemiology*. 2010;21(1):13–15.