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Mechanistic insight into sex differences in lung cancer induced by cRaf

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Abstract

Women are at greater risk of developing lung cancer (LC); yet the underlying causes are unknown. To gain insight into sex difference in LC, we investigated tumor growth in a cRaf transgenic disease model. Overexpression of the cRaf kinase induced an extraordinary 8-fold increase in tumor growth among females, and nearly 70% of the 112 differentially expressed genes (DEGs) were female specific. We identified oncogenes, oncomirs, tumor suppressors, cell cycle regulators and MAPK/EGFR signaling molecules, which prompted sex-based differences in LC, and we report a regulatory gene-network, which protected males from accelerated tumor growth. Strikingly, 41% of DEGs are targets of hormone receptors, and the majority (85%) are estrogen receptor (ER) dependent. We confirmed the role of ER in a large cohort of LC patients and validated 40% of DEGs induced by cRaf in clinical tumor samples. Together, we report a molecular circuitry that prompted gender disparities in tumor growth, and propose the development of molecular targeted therapies by jointly blocking ER, CDK1 and arginase 2 activity in LC.

Keywords: Lung cancer, cRaf kinase, gender, MAPK/EGFR signaling, hormone receptors, transcription factor-miRNA gene regulatory network, clinical validation
Abbreviations:

LC: lung cancer
ICI: immune checkpoint inhibitor
HR: hazard ratio
TF: transcription factor
FFL: feed forward loop
DEG: differentially expressed gene
AAH: atypical adenomatous hyperplasia
DEM: differentially expressed miRNA
WT: wild type
TAM: tumor-associated macrophage
FDR: false discovery rate
FC: fold change
Areg: amphiregulin
Ereg: epiregulin
EGFR: epidermal growth factor receptor
Arg2: arginase 2
Rhbdl2: rhomboid veinlet-like 2
Cldn2: claudin 2
ER: estrogen receptor
Rasgrf1: Ras-specific guanine nucleotide-releasing factor 1
Rgl1: ral guanine nucleotide dissociation stimulator 1
Kdm2a: lysine demethylase 2A
Cdk1: cyclin 1 dependent kinase
Scd1: stearoyl-coenzyme A desaturase 1
Dlk1: delta-like 1 homolog
MMP9: matrix metallopeptidase 9
Cbln1: cerebellin 1 precursor
Malt1: mucosa-associated lymphoid tissue 1
Golm1: golgi membrane protein 1
Acsl4: acyl-CoA synthetase long-chain family member 4
Ctsk: cathepsin K
Ptprn: protein tyrosine phosphatase receptor type N
Acer2: alkaline ceramidase 2
Lbh: limb bud and heart
Zbtb16: zinc finger BTB domain containing 16
Chad: chondroadherin
Fga: fibrinogen alpha
Fgg: fibrinogen gamma
Gjb4: gap junction protein beta 4
Esr1: estrogen receptor 1
Esr2: estrogen receptor 2
Ar: androgen receptor
TFBS: transcription factor binding site
Maz: MYC-associated zinc finger protein
Nr2f1: nuclear receptor subfamily 2 group F member 1
Dusp5: dual specificity phosphatase 5
Mmnr2: multimerin 2
OS: overall survival
FETUB: fetuin B
GJB3: gap junction protein beta 3
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). There is conclusive evidence for tobacco product consumption to be the major risk factor for LC with 80% of cases being linked to cigarette smoking. Strikingly, the incidence rate of LC among women is increasing or even surpassing that of men to possibly suggest gender-related differences in the development of LC. 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. While the reasons remain uncertain, hormonal and genetic factors have been linked to gender disparities in LC, notably mutations in the p53 tumor suppressor and KRAS kinase, regulation of growth factor and DNA repair enzymes.

In regards to drug treatment responses there are also sex-related differences among LC patient. While immune checkpoint inhibitors (ICIs) and molecular targeted therapy become the mainstay of LC therapy, the results vary between smokers and non-smokers, and male and female patients. For example, the Keynote-024 study investigated the effectiveness of Pembrolizumab, i.e. a PD-1 antagonist as monotherapy in metastatic LC 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). 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 LC patients exceeded that of males, i.e. 21.4 versus 16 month (HR 0.66 vs.0.87).

Together, these and other observations are suggestive for sex-related differences in LC and demanded mechanistic investigations. Among the genetic events in LC, 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 LC patients, and oncomirs are of particular interest as they frequently target tumor 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.

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. Aberrant transcriptional regulations in cancers is the focus of oncogenomics, especially as deregulated TFs are one of the hallmarks of cancer. Adding to complexity are TF-miRNA-gene networks that function as feed-forward loops (FFLs) in the control of gene expression. However, miRNAs-gene regulatory networks and sex-related differences in tumor growth have not been investigated so far.

In the past, we reported the genomics of LC in a cRaf transgeneic 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\textsuperscript{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 signaling pathway\textsuperscript{16}. Although similar, the signaling outputs of RAF paralogs can differ\textsuperscript{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 reduced significantly tumor burden in a Kras\textsuperscript{G12V} oncogene-driven LC model\textsuperscript{18}. In addition, cRAF mutation have been identified in LC patients\textsuperscript{19} and the common KRAS\textsuperscript{G12C} mutation appears to be more frequent among women even at a lesser smoking history\textsuperscript{20}.

Our study aimed at investigating the sex-based differences in tumor growth in a cRaf transgenic LC disease model. We observed a highly significant disproportional 5 and 8-fold increase in tumor growth in the right and left lung, respectively of female transgenic mice. This prompted us to examine the complex interplay of miRNAs, TFs, hormone receptors and target genes in females. Next to histopathology, oncogenomic investigations and DNA-sequencing of tumor 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 LC patients. Importantly, we identified genes mechanistically linked to tumor growth and determined the prognostic value of highly regulated genes in Kaplan-Meier survival plots.

Overall, we report new insight into gender disparities in LC and highlight the role of hormone receptors, miRNAs and TF in the control of cell cycle regulators, tumor suppressors, oncogenes and oncomirs.

Results

Figure 1 depicts the workflow and supplementary Table S1 provides details of the experimental groups. We performed whole genome scans to identify DEGs and miRNAs (DEMs) and searched for gender-specific responses. Furthermore, we searched for gene targets of regulated miRNAs by querying the miRNet 2.0 repository, and only considered experimentally proven entries. We constructed TF-miRNA-gene networks by identifying TF binding sites in promotors of regulated genes. To define molecular circuitries in LC, we examined experimentally validated TF-miRNA and TF-gene interactions and investigated the role of hormone receptors in tumor-related gene regulations. Finally, we established clinical relevance by comparing the genomic data from cRAF transgenic mice to publically available human lung adenocarcinoma cases.
Lung tumor burden in cRaf transgenic mice

We previously reported the histopathology of cRaf transgenic mice and its progression from epithelial dysplasia to LC\textsuperscript{14,15}. Depicted in Figure 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 (Figure 2b) and multifocal tumor growth (Figure 2c) to adenocarcinomas that consume the entire lobe (Figure 2d). By employing the cRaf genetic disease model, we were able to follow the time-dependent sequence of events and we demonstrate high tumor multiplicity and tumor collisions (Figure 2d&2d). 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 tumor suppressors, i.e. Lmyc1, p53, Ts1c1 and Kras as evidenced by DNA sequencing (data not shown).

Depicted in Figure 2e and 2f are sections of lung tumors and infiltration of tumor-associated macrophages (TAMs). Their role in tumor associated immune responses will be discussed below.

To determine the effects of cRaf on lung tumor growth, we performed serial sectioning of lung tissue and counted the number of tumors sized $>200\mu m$ (Figure 2b&2c). 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 Figure 2g are the tumor counts for the left lung, and for transgenic females we observed a significant increase in tumor multiplicity when compared to males (p<0.0001). We also determined the number of tumors for the right lung of transgenic females and observed an increase in tumor multiplicity when compared to males (Figure 2h). We therefore demonstrate gender disparity in tumor growth.

Although the human TNM-staging of lung tumors cannot directly be applied to mice, i.e. in human pathology, the size, grade and staging of tumor is considered in relation to prognosis, a proximate of the staging of transgenic lung tumors would be pT2 and 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) $\leq 0.05$ and fold change (FC) $\geq |2|$. Depicted in Figure 3a and 3b 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 down-regulated (supplementary Table S3, Figure 3c). Likewise, we identified 57 DEMs of which 30 were up, 27 were downregulated (supplementary Table S3, Figure 3d).

We performed gene enrichment analysis and shown in Figure 3e&f were 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 (Figure 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 (Figure 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 and this defined 46 DEGs or 41% of total DEGs to code for immune responses
(supplementary Table S5). Depicted in Figure 4 are significantly regulated marker genes for different
immune cells and supplementary Figure S1 shows their general distribution among lymphoid and
myeloid cells.

Among highly regulated genes we noticed amphiregulin (Areg) and epiregulin (Ereg) as >4- and >24-
fold induced in transgenic animals. Apart from their role in stimulating epidermal growth factor receptor
(EGFR) signaling (see below), amphiregulin enhances regulatory T cell-suppressive function via
EGFR and epiregulin regulates peptidoglycan-mediated proinflammatory cytokine production in
antigen presenting cells. Furthermore, both autocrine growth factor stimulate proangiogenic TAMs.

Additionally, the complement factor I was 4-fold induced cRaf females, and this factor inhibits the C3
convertase of the complement system which promotes extravasation of macrophages and promotes
progression of cutaneous squamous cell carcinoma. In fact, various LC cell lines secrete soluble
inhibitors of the complement system and function as promoter of tumor progression.

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. Overexpression of
arginase inhibited 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
-dependent kinase complex. Indeed, an independent immunohistochemistry evaluation of human LC
cases showed enhanced Arg2 expression in the cytoplasm of LC cells as well as cancer-associated
fibroblasts.

Sex differences in genomic responses to cRaf overexpression

To identify genes and miRNAs regulated in cRaf transgenic mice, we compared the genomes of WT to
transgenic animals and explored gender-specific responses. Strikingly, of the 112 DEGs 77 are female
specific. Conversely, 6 DEGs were specifically regulated in male transgenic mice and 29 are
commonly regulated between both sexes (Figure 5a). Therefore, we observed a highly significant
gender disproportional regulation of genes. Similar, we identified 57 differentially expressed miRNAs
and will discuss their regulation below (Figure 5b). Initially, we performed a gene enrichment analysis
irrespective of sex and this defined EGFR signaling, epithelial cell proliferation and immune response
as significantly enriched terms (Figure 5c). Next we considered female-specific DEGs, and the GO
terms highlighted cellular response to growth factor stimulus, regulation of epithelial cell migration,
regulation of cell-cell adhesion, regulation of apoptotic signaling pathway and regulation of steroid
metabolic process (Figure 5d)
EGFR and MAPK signaling

cRaf influenced EGFR signaling 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 and previous research demonstrated EGF to stimulate Cldn2 expression and cyclin D1 nuclear retention in an EGFR dependent manner\textsuperscript{31,32}. The results infer a regulatory loop whereby cRaf activates EGFR signaling through induced expression of its ligands. Meanwhile, epiregulin serves as a marker of advanced disease in LC patients and confers invasive properties on EGFR-mutant cells\textsuperscript{33}.

Although EGFR itself was not regulated at the transcript level, we identified key molecules of the MAPK signaling 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 signaling 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 Figure 5e the signaling pathways in lung tumors of cRaf transgenic mice and highlight the cross-talk between the EGFR and MAPK signaling pathway and the estrogen 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 signaling pathway among females. Additionally, the ral gunanine nucleotide dissociation stimulator 1 (Rgl1), which uncouples Ras from activation of Raf1\textsuperscript{34}, was down regulated. Likewise, the lysine demethylase 2A (Kdm2a) was repressed, and this histone demethylase activates ERK1/2 signaling through epigenetic repression of the DUSP3\textsuperscript{35}. Together, the findings imply upstream effector-loop regulations for the sustained Raf1 activation. Indeed, failure of Kdm2a to repress DUSP3 allows for continuous MAPK signaling. Strikingly, 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 and blocked p53 dependent apoptosis in LC\textsuperscript{36}. Another example relates to the 4 fold induced stearoyl-coenzyme A desaturase 1 (Scd1). This enzyme activates the EGFR/PI3K/AKT signaling pathway and promotes tumor growth in LC\textsuperscript{37}. Further examples are highlighted in Figure 5e.

The expression of MAPK signaling molecules is also influenced by the ER and the importance of steroid hormone receptor in the sex-dependent genomic responses in cRaf transgenic mice will be discussed below. Supplementary Figure S2 informs on the protein-protein interaction networks of MAPK signaling molecules and we obtained evidence for their physical interaction based on information retrieved from the String database\textsuperscript{38}.

Specifically in tumors of male transgenic mice, we observed 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 LC. Nonetheless, its
expression did not differ between male and female LC patients (supplementary Table S11). DLK1 promotes LC cell invasion through upregulation of matrix metallopeptidase 9 (MMP9) in an NOTCH dependent manner\textsuperscript{39}. However, in transgenic females \textit{Mmp9} was repressed. Currently DLK1 is explored as a therapeutic target for radioimmunotherapy in LC\textsuperscript{40}.

**Oncogenes and tumor suppressors**

As described above, we identified 112 DEGs in tumors of cRaf transgenic mice (Figure 5a) of which 47 code for oncogenes and tumor suppressors and 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 down regulated) and tumor suppressors (8 down, 3-up regulated) while for males only 2 oncogenes were upregulated. Furthermore, of the 29 common DEGs (Figure 5a) 13 and 1 code for oncogenes and tumor suppressors (Figure 5f, supplementary Table S6).

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 LC\textsuperscript{41}. Moreover, the mucosa-associated lymphoid tissue 1 (\textit{Malt1}) was nearly 3 fold induced in cRaf females and promoted the progression of EGFR-induced LC by activating NF-kappa B\textsuperscript{42}. Other examples included the 2-3 fold upregulation of golgi membrane protein 1 (\textit{Golm1}), acyl-CoA synthetase long-chain family member 4 (\textit{Acsl4}) and cathepsin k (\textit{Ctsk}), which stimulate cell proliferation and metastasis\textsuperscript{43-45}. Furthermore, we identified upregulation of \textit{Cd177} and this surface protein is expressed in tumor infiltrating Treg’s and suppresses immune response\textsuperscript{46}. Additionally, in female transgenic mice we observed 2-fold upregulated methionine adenosyltransferase I. The enzyme confers chemoresistance in LC and bladder cancer\textsuperscript{47,48}. Further upregulated oncogenes are \textit{Cdk1}, \textit{Rasgrf1}, \textit{Cldn2}, \textit{Areg}, \textit{Ereg} and \textit{Rhbdl2} and we already described their functions.

The only 2 oncogenes regulated in males were \textit{Dlk1} (15-fold upregulated) which promotes cell invasion\textsuperscript{59} as well as protein tyrosine phosphatase receptor type N (\textit{Ptprn}) which promotes transformation\textsuperscript{49} (3-fold upregulated).

Among the 11 tumor suppressors specifically regulated in females, 8 were repressed and 3 were upregulated. For instance, the tumor suppressor alkaline ceramidase 2 (\textit{Acer2}) was highly repressed in cRaf females to about 12% of non-transgenic controls and this enzyme induces apoptosis and autophagy in a p53 dependent manner\textsuperscript{50}. Likewise, the TFs limb bud and heart (\textit{Lbh}) and zinc finger BTB domain containing 16 (\textit{Zbtb16}) were 3- and 5-fold repressed in female cRaf transgenic mice and function as a negative regulator of ER signaling and MAPK signaling\textsuperscript{51} and programmed cell death\textsuperscript{52}. Their repression is suggestive for sustained ER and survival signaling in cRaf induced tumorigenesis. Likewise, in cRaf females the tumor suppressor chondroadherin (\textit{Chad}) was >2-fold repressed and through ECM receptor interactions supported migration of tumor cells\textsuperscript{53}.

Of the commonly regulated DEGs (Figure 5a) 13 code for oncogenes and all were upregulated (range 2-24 fold). These code for EGFR/MAPK signaling, cell proliferation, metastasis and inhibition of cell death. We already discussed the importance of \textit{Areg}, \textit{Ereg}, \textit{Cldn2}, and \textit{Rhbdl2} and now wish to highlight the regulation of fibrinogen alpha (\textit{Fga}) and gamma (\textit{Fgg}) which were 4- and 6-fold upregulated in males and females. Note, \textit{FGG} is significantly elevated in LC tissue and is a
determinant of the metastatic potential of circulating tumor cells\textsuperscript{54}. Likewise, 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 LC\textsuperscript{55}. Further examples included induced expression of St100a14 and this calcium binding protein stimulates cell migration and invasion\textsuperscript{56} while induced expression of sialyltransferase St8sia6, i.e. a siglec molecule elicits immune response, macrophage polarization and augments arginase 2 expression\textsuperscript{57}. We also observed upregulation of tandem C2 domain, and this oncogene inhibits p53 signaling in lung cancer\textsuperscript{58}. Conversely, induced Tnfsf9 expression promotes immunosuppressive activity of regulatory T-cells in LC\textsuperscript{59}. Further information can be found in supplementary Table S6 and we show highly regulated oncogenes and tumor suppressors in Figure 5g.

**MiRNA-gene networks in lung tumors of cRaf transgenic mice**

We identified 57 differentially expressed miRNAs in tumors of cRaf transgenic mice of which 27 and 30 were up- and down-regulated (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 gender specific regulations of DEGs. In fact, there are only 4 DEMs regulated in common (Figure 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 females. Likewise, miR-16-5p and miR-335-5p were 2 and 3-fold upregulations among both sexes.

With females, we identified 17 regulated miRNAs (Figure 5b) of which 10 and 3, respectively code for tumor suppressors (6 up-, 4 down-regulated) and oncomirs (1 up-, 2 down-regulated). For instance, the tumor suppressor miR-30c-2-3p was reduced to 15% of WT controls, and this tumor suppressor is commonly repressed in various cancers and inhibits EMT in LC\textsuperscript{60}. A further example relates to the 2-fold repressed tumor suppressor miR-199a-3p which targets ARG2\textsuperscript{61}. As described above, ARG2 is highly expressed in tumor associated macrophages and its induced expression is unique to cRaf females (supplementary Table S3).

Conversely, with males 36 DEMs are regulated (Figure 5b) of which 15 and 5, respectively code for tumor suppressors (7 up-, 8 down-regulated) and oncomirs (2 up-, 3 down-regulated). 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 tumor suppressors inhibit cell proliferation\textsuperscript{62,66}, migration and invasion\textsuperscript{67,68}, as well as immune response\textsuperscript{69}.

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 signaling pathway, regulation of epithelial cell proliferation, positive regulation of cell-cell adhesion, as well as negative regulation of apoptotic signaling pathway (Figure 6a).
Within the network (Figure 6a), 12 miRNAs code for tumor suppressors of which 5 were up- and 6 down-regulated. 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 LC tumorigenesis by regulating the tumor immune microenvironment\textsuperscript{61,69}, induce apoptosis, inhibit cell proliferation\textsuperscript{70}, EM transitions\textsuperscript{67,71}, and inhibit cell invasion\textsuperscript{62,72}. On the other hand, miR-130a-3p, miR-16-5p, miR-146b-5p, miR-335-5p and miR-34a-5p were upregulated (range 2-3-fold) and these miRNAs block tumor growth by regulating cell cycle\textsuperscript{73,74} and apoptosis\textsuperscript{75}. 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 LC\textsuperscript{76}.

The gender disparities in the regulation of miRNAs is of critical importance and provided a molecular rationale for sex difference in tumor growth. In Figure 6b, we highlight miRNAs upregulated in male transgenic mice which target oncogenes and tumor 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 \textit{Scd1}, \textit{Stk39}, \textit{Acsl4} and \textit{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 tumor suppressors \textit{Zbtb16}, \textit{Sp100}, \textit{Dusp10}, \textit{Mmrn2}, \textit{Lbh}, \textit{Acer2} and \textit{Nr2f1}. Thus, repression of these miRNAs protected males from dysfunction of these tumor suppressors as seen in females (Figure 6b).

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

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

The role of the estrogen and androgen receptor in gender-specific genomic responses

To understand gender-specific regulations of DEGs and DEMs, we searched for targets regulated by the estrogen (\textit{Esr1} and \textit{Esr2}) and androgen receptor (\textit{Ar}). We queried the GSEA, Transmir v2.0 and hTFTarget databases and considered chromatin-IP proven binding sites in promoters of tumor specific DEGs and DEMs. As shown in Figure 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 S7). We identified 26 and 7 DEGs, respectively as targets of the estrogen and androgen receptors, while an additional 13 DEGs contained binding sites for both hormone receptors.
(supplementary Table S7, Figure 7a). However, the Esr1&2 and the Ar receptors itself were not
regulated at the transcript level.

Subsequently, we searched for hormone receptor binding sites in promoters of DEMs and this
revealed 1 and 23 miRNAs which are targets of the androgen and estrogen receptors, while 3 were
regulated by both hormone receptors (Figure 7b). 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\textsuperscript{77,78}. Of the 23 Esr1&2 targets 8 and 14 miRNAs, respectively
were uniquely regulated in females and males. The fact that 14 Esr1&2 responsive miRNAs were
uniquely regulated in males was a surprise finding. Nonetheless, estrogens do play an important role
in male physiology as well\textsuperscript{79}. In regards to female-specific and Esr1&2 responsive miRNAs, we
identified 4 which code for tumor 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 miR-181c-5p were repressed (2-3-fold) and their targets, i.e. Dusp5 and epiregulin were
upregulated. Note, epiregulin stimulates EGFR signaling. Conversely, miR-21-5p was significantly
upregulated and its target MMP9 was repressed. MiR-21-5p is a target of the estrogen 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 LC 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 estrogen receptor responsive miRNAs, 8 were repressed (range 2-6-fold), and these code
for diverse functions as denoted for the tumor suppressors miR-574-3p and miR-711.

Depicted in Figure 7c is the complex interplay between miRNAs and genes targeted by sex hormone
receptors and the network consisted of 46 DEGs and 27 DEMs. Note 39 out of 46 DEGs are targets of
the estrogen receptors and 32 are specifically regulated in females. In fact, only 6 DEGs are regulated
in common in male and female transgenic mice. Moreover, sulfotransferase 2B is the only gene
regulated in males and this gene is also a target of the ER (Figure 7d). Even more astonishingly is the
fact that of the 20 DEGs targeted by the androgen receptor 18 were specifically regulated in females
while the remaining 2 are common to both genders (Figure 7e). Therefore, the hormone receptor
dependent regulation of genes provided a molecular rationale for the gender disproportional tumor
growth seen in the present study.

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

Shown in Figure 7a 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\textsuperscript{80}. 
Although cMyc itself was not regulated, we found several Maz target genes as highly regulated in cRaf transgenic mice (Figure 8a). Specifically, Maz is a repressor of Myc activity\(^{81}\) and was significantly repressed (3-fold) in cRaf females (supplementary Table S3). A recent review summarized the functions of Maz in various cancers though its role in LC remains elusive\(^ {82}\). Specifically repression of Maz in cRaf females may be one of the reasons for its increased tumor burden (Figure 2g&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 tumor suppressor\(^ {83}\). In cRaf females, \(Nr2f1\) was >2-fold repressed. Importantly, through binding to its TFBS, \(Nr2f1\) functions as a transcriptional repressor of the estrogen receptor\(^ {84,85}\). Although \(Esr1\) itself was not regulated in cRaf transgenic mice, the female-specific increase in tumor burden (Figure 2g&h) tends to suggest deliberate repression of the \(Nr2f1\) tumor suppressor function. Indeed, NR2F1 activation causes growth arrest in various cancer cell lines and suppresses metastasis in vivo\(^ {83}\).

**MiRNA-TF-gene regulatory network**

To better understand the sex dependent gene regulations in tumors 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 Figure 8b the network which consisted of 7 miRNAs and 15 target genes all of which were significantly regulated in cRaf animals (supplementary Table S3). Strikingly, all miRNA-gene targets are female-specific (Figure 8b), and we highlighted their functions. Of the 7 miRNAs, 5 code for tumor 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 Figure 8b, the oncomir miR-21-5p is a target of the \(Ar\) and \(Esr1\) receptor, and this miRNA regulates 4 genes in cRaf females, all of which contained estrogen 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\(^ {86}\). Similar, the serine/threonine kinase \(Dmpk\) was 3 fold repressed and this kinase protects cells from oxidative stress\(^ {87}\); and is a target of p53 signaling\(^ {88}\). Another example relates to the 2.5 fold repressed flavin-containing monooxygenase \(Fmo5\), and this xenobiotic defense enzyme is regulated by the circadian rhythm\(^ {89}\). Moreover, upregulation of miR-21-5p enhanced ROS levels by inhibiting the metabolism of superoxide to hydrogen peroxide\(^ {90}\). 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 estrogen 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 signaling. Furthermore, miR-181c-5p controls translation of Dusp5 and in tumors of cRaf females, this tumor 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. Therefore, DUSP5 takes on a dual role in the control of ERK signaling. Adding to complexity is the fact that the histone lysine-specific demethylase Kdm2a regulates epigenetically Erk1/2 signaling, and in cRaf females, Kdm2a was repressed. Together, we obtained evidence for a complex interplay involving Dusp5, miR-181c-5p, the estrogen receptor and miR-130a-3p, which we found 2 fold upregulated in cRaf females and the latter miRNA targets Kdm2a.

In the network, we also show DEGs coding for angiogenesis (Figure 8b). For instance, multimerin 2 (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 VEGFR.

Mmrn2 repression therefore supports angiogenesis in cRaf females.

Clinical validation - Hormone receptor expression in human LC tumor 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 Figure 2g&h the tumor growth is strongly influenced by gender and the tumor multiplicity increased significantly in females.

Depicted in Figure 9a&b are H&E stained sections of lung tumor 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 tumors exhibited an acinar growth pattern with invasive glands and poorly formed glandular spaces (bI&bII), as well as invasive nests of tumor cells that produced glandular lumina without solid components (bIII). The immunohistochemistry evidenced marked expression of the estrogen and androgen receptor. Notwithstanding the expression of the androgen receptor is less in case II (Figure 9dII, see also Figure captions for more details). Therefore, we and others provided evidence for sex hormone receptors to be significantly regulated in lung adenocarcinoma.

Clinical genomics of LC tumor 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 tumor versus 58 adjacent, histologically proven non-tumorous samples of LC patients. This revealed 5,395 DEGs (3373 up-, 2022 down-regulated). In the same way we compared tumor associated miRNAs of LC patients (513 tumor, 45 controls) and identified 414 DEMs as significantly
regulated (251 up-, 163 down-regulated) (supplementary Figure S3, supplementary Table S9). The Venn diagram in Figure 10a shows the commonly regulated DEGs and DEMs between human and mouse lung tumor samples. We identified 27 up-, 9 down-regulated DEGs, as well as 4 up- and 3 down-regulated 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 LC patients.

We found high expression of ARG2, CLDN2 and molybdenum cofactor synthesis 1 to be associated with 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 reduced survival (Figure 10b). Likewise, high expression of the tumor 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 (Figure 10c).

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 tumor arginase expression and activity across different tumors. In fact, we observed opposite regulation of arginase 1 and 2 in human LC 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 tumor associated macrophages, we investigated expression of TAM marker genes in LC patients based on TAM genes reported by Ma et al (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 LC patients and this macrophage derived cytokine confers drug resistance in human LC. Similar, matrix metalloproteinase 12 was highly induced (13 and 14-fold in female and male LC patients) and promotes angiogenesis. The regulation of arginase 1 and 2 and TAM marker genes was sex-independent. However, unlike LC 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 LC is less clear and controversial. We found high expression of arginase 2 to be associated with better survival (Figure 10b).

To probe for gender-specific regulations, we compared female LC tumor samples to adjacent non-tumorous tissues and this defined 5070 DEGs (3097 up- and 1973 down-regulated) and 392 DEMs (211 up- and 181 down-regulated) (supplementary Figure S3). Similar, we compared male LC tumors versus adjacent non-tumorous tissues and obtained 5237 DEGs (3207 up- and 2030 down-regulated) and 315 DEMs (241 up- and 74 down-regulated) (supplementary Figure S3). Subsequently, we constructed Venn diagrams to determine gender-specific regulations among female and male LC samples. This defined 376 and 486 genes which were specifically upregulated in female and male LC patients (supplementary Figure S4a, supplementary Table S11). We show the Metascape enriched GO terms in supplementary Figure S4b. With female LC patients, innate immune response, peptide-cross linking and cell-cell recognition were enriched terms, while for males, cell surface receptor signaling, cell morphogenesis involved in differentiation, Notch signaling and epidermis
morphogenesis were specific annotations. Similar, for the 273 and 330 downregulated genes the
Metascape annotations are given in supplementary Figure S4d. With females, enriched GO terms
were cell junction assembly, negative regulation to canonical Wnt signaling pathway, cellular response
to hormone stimulus, cell-cell adhesion via plasma membrane adhesion molecules. Conversely, for
males the 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 down-regulated) and 93 (86 up-, 7 down-regulated) miRNAs regulated in female and
male LC patients (supplementary Figure S4e).

Furthermore, we compared DEGs from LC tumors of cRaf transgenic mice with human LC samples.
This revealed 31 commonly regulated DEGs (23 up-, 8 down-regulated) between female LC patients
and female mice, while for males, 14 DEGs (14 upregulated) were commonly regulated (Figure 11a).
Furthermore, 3 (3 upregulated) DEMs were common between female LC patients and female mice,
and 2 (1 up-, 1 down-regulated) were common between male LC patients and male mice (Figure 11b).
The results imply that 40% of DEGs regulated in LC tumors of cRaf transgenic mice are likewise
regulated in human LC. With DEMs, 14% and 5%, respectively of female and male cRaf mice were
commonly regulated with human LC 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 LC patients. Higher expression of
CDK1, FETUB, GJB3 and miR-21-5p was associated with poor OS among female LC patients (Figure
11c), while higher expression of EREG was associated with worse outcome in male patients (Figure
11d). Collectively, we confirmed clinical relevance of cRaf tumor associated genes with implication for
OS.

Discussion

Our study aimed at an identification of sex-specific differences in lung tumor growth, and we
demonstrate overexpression of the kinase domain of cRaf to cause complex genomic responses in
lung epithelial cells. We report the regulation of 112 DEGs of which 10% coded for MAPK signaling
molecules (Figure 5d).

In general, the MAPK signaling pathway stimulates cell proliferation and blocks apoptosis, and has
been the focus of targeted therapies for LC. Apart from an exaggerated MAPK signaling, the
genomic data were highly suggestive for an impaired p53 activity. Associated herewith, we noted
repression of tumor suppressors and upregulation of oncogenes and oncomirs. In fact 42% of the 112
DEGs code for oncogenes and tumor suppressors and of these, 20 and 11, respectively were uniquely
regulated in females. Their regulation provided a molecular rationale for an increased lung tumor
burden among female transgenic mice. On the contrary, there are only 2 oncogenes specifically
regulated in cRaf males (supplementary Table S6). Furthermore, we identified miRNAs specifically
regulated in males which protected animals from the upregulation of oncogenes and repression of tumor suppressor as seen in females. Therefore, with females, a larger number of tumor suppressors were repressed while oncomirs and oncogenes were significantly upregulated.

To better comprehend the sex differences in tumor growth, we constructed gene networks and assessed the role of hormone receptors on tumor specific gene regulations. This revealed a complex interplay between the estrogen receptor, miRNAs and genes in the control of tumor growth. Figure 12 provides an overview of the genes strongly associated with sex disparities in tumor growth. Finally, we identified sex dependent gene regulations in human LC and demonstrate relevance of the findings with impact on OS. The genes validated in human LC are highlight in Figure 12.

Specifically, the incidence of CRAF mutations in human lung cancers is similar to that of BRAF19,97,98, and mutations cause uncontrolled serine/threonine kinase activity, and therefore exaggerated MAPK signaling. Interestingly, some studies suggested the BRAF V600E mutation to be more prevalent in female LC patients99-101 and one study reported even an incidence of 8.6% vs 0.9% between female and male LC cases (HR=11.29; P <0.001)99. However, the OS for advanced-stage patients with BRAF mutations did not differ when compared to other driver mutations102. 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 signaling. Importantly, inhibition of IQGAP1 disrupted RAF signaling and tumor growth103.

Although the cRaf LC disease model differs in its mode of action, i.e. overexpression of the non-mutated kinase domain as compared to the driver mutation, both conditions result in hyperactivity of the kinase and therefore recapitulate important aspects of the molecular pathology of RAF mutated lung carcinogenesis. The use of BRAF inhibitors in molecular stratified LC patients has been recently summarized104. 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 signaling outputs was summarized in the seminal review of Desideri and colleagues17.

Genomic responses to cRaf hyperactivity

We identified 77 DEGs uniquely regulated in cRaf females, of which 20 and 11, respectively code for oncogenes (13 up-, 7 down-regulated) and tumor suppressors (3 up-, 8 down-regulated). These function in cell signaling, proliferation, apoptosis, metastasis, angiogenesis and immune response.

For instance, we observed upregulation of the oncogenes Rasgrf1, Scd1 and repression of the tumor suppressor Dusp10. Their aberrant expression is linked to an activated MAPK signaling. Furthermore, MAPK signaling 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 tumor suppressors inhibit cell proliferation. Correspondingly, their repression promoted cell proliferation.
Among cyclin dependent kinases, we identified Cdk1 as significantly upregulated in tumors of female cRaf transgenic mice and confirmed the result in human LC. 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 mitosis-phase\textsuperscript{105}. So far there are approximately 75 known protein targets of CDK1\textsuperscript{106,107} and we identified 8 as significantly regulated in human LC (range 2-15-fold upregulated for both sexes, supplementary Table S11).

We and others reported poor outcome for high CDK1 expression in LC patients, and upregulation of CDK1 promoted cancer stemness\textsuperscript{108}. Strikingly, and irrespective of gender, CDK1 is significantly induced in human LC. However, only in females high CDK1 expression is associated with worse outcome (Figure 11c). Similar results were obtained for epiregulin. Although regulated in both sexes, only with males its induced expression is associated with worse OS. A similar relationship was seen with has-miR-21-5p. It is upregulated in all LC patients, but in females, it is associated with poor outcome (Figure 11c). In recent years, the development of CDK1 inhibitors was the focus of research to treat breast, colon and pancreatic cancer\textsuperscript{109-111}, yet, the clinical efficacy of CDK1 inhibitors awaits confirmation.

Moreover, we identified Arg2, Cd177 and Ccl6 as specifically upregulated in cRaf females, while the tumor suppressor P2ry13 was repressed. These code for immune response and tumor microenvironment in LC, and immune escape is a critical mechanism of tumor growth. ICIs are truly game changers in the treatment of LC, 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 (<20%)\textsuperscript{112}. Given the variable expression of PD-1 in LC, the efficacy of ICIs remains controversial especially for tumors with only a small number of tumor cells expressing the PD-1 target. Strikingly, in tumors 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 Figure 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 LC regardless of sex. This enzyme is highly expressed in cancer-associated fibroblast and TAMs, and overexpression of arginase depleted arginine from the tumor microenvironment, which dampened T cell-mediated anti-tumor effects\textsuperscript{28,113}. An earlier study reported inhibition of arginase-2 in dendritic cells to promote T cell proliferation\textsuperscript{114}, and a recent study demonstrated significant regression of lung tumors in a mouse LC model treated with an experimental arginase inhibitor\textsuperscript{115}. 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 LC.

We identified 39 DEGs as targets of the estrogen receptors of which 32 are specifically regulated in females while 6 are common for both genders. (Figure 7d). 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 (Figure 7e). Together, we provide strong evidence for the hormone receptor dependent
regulation of genes in lung tumors of cRaf transgenic mice.

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

Furthermore, we show 12.6% of DEGs are targets of the ER in female LC patients (supplementary Table S11) even though the ESR itself was not transcriptionally regulated. Currently, an antiestrogen therapy in LC is being evaluated. Intriguingly, and as a result of its oxidative metabolism, estrogen itself may cause DNA damage and therefore become mutagenic.

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 tumor suppressors regulate cell proliferation, apoptosis and EMT. Furthermore, the oncomir miR-21-5p was specifically upregulated in cRaf females, and miR-21-5p is a target of the Esr1. Altogether, there are 57 DEMs (Figure 5b) of which 26 and 4 are regulated by the estrogen and the androgen receptor. While there are more DEMs regulated in males most are targets of the estrogen receptor.

In conclusion, our study highlights major differences in the genomic responses to cRaf hyperactivity with females being more sensitive to the tumor promoting effects of cRaf (Figure 2). We gained insight into the complex interplay between cRaf, miRNAs, hormone receptors and TF and were able to construct gender 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 LC 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.

Methods

SPC cRaf transgenic mice

We performed the 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). 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 tumor growth. 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 S1 gives an overview of the experimental groups. We used the Mann-Whitney U test to compare the number of tumors sized >200µm.

Patients characteristics and tissue specimens

FFPE tissue blocks were obtained 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). Further details are given in supplementary Table S2.

Histopathology and immunohistochemistry

The left and right lung of 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.

Whole genome miRNA profiling

We previously reported a cross-platform comparison of the Affymetrix and Agilent microarrays based miRNA expression analysis in lung tumors of cRaf transgenic mice. Affymetrix microarray platform: We isolated from each lung 200 ng of total RNA and labelled nucleic acids with the FlashTag Biotin HSR labeling 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 100ng of total RNA and performed 3’ end labeling with the Cy3-pCp dye, purified the samples with Micro Bio-Spin columns, and hybridized the samples onto arrays with the miRNA Microarray System labeling 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 analyzed 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. Briefly, we prepared independent pools of four mice/pool, thus totaling 16 animals per study group. We 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. 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 Labeling 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 hours 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 analyze hybridization intensity data. Finally, we applied default parameters of the Affymetrix software package for analysis.

Data processing and statistical analysis
Differentially expressed genes (DEGs)

We applied the unpaired t-test to compare the average signal values between cRaf transgenic and WT animals (supplementary Table S1).

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, respectively. We computed the principal component analysis 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. 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.

Only genes and miRNAs with a false discovery rate (FDR) < 0.05 and fold change (FC) ≥ |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 down-regulated DEGs. We analyzed the data with Metascape (https://metascape.org/) 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 in R.

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). Specifically, we compared DEMs identified in lung tumors 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 that were visualized with the software Cytoscape 3.9.1.
To identify transcription factor binding sites (TFBSs), we performed promoter analysis of DEGs. First, DEGs were converted 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’). Enriched TFBSs in the Yes set data were considered 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 actually binding of TF proteins to recognition sites in promoters of regulated genes.

We queried the Xena database to obtain miRNA and mRNA sequencing data of TCGA-LUAD dataset as well as other clinical information. For the DEG analysis, a total of 510 tumor samples and 58 adjacent non-cancerous samples were considered. For the DEM analysis, 513 tumor samples and 45 adjacent non-cancerous samples were considered. To determine gender-specific regulation, we compared female tumor samples to female adjacent non-cancerous controls, and male tumor samples to male adjacent non-cancerous controls. DEGs and DEMs were identified using Deseq2 package in R. Genes and miRNAs with |FC| > 2, FDR < 0.05 were considered significantly regulated. We summarize the grouping information and the results in supplementary Figure S3.

For survival analysis, we included 491 LC patients with OS information. For gender-dependent genes/miRNAs, we considered 265 females and 226 males LC 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.

We used R or geneXplain software to perform statistical analysis. If not otherwise specified, all tests were two-tailed, and a P value or FDR < 0.05 was considered to be statistically significant.
The data to support the findings of this study are available from the authors upon reasonable request.

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Author contributions

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)

Competing interests

The authors declare no competing interests.
Figure 1. Workflow showing the experimental works, the genomic data analysis and data retrieval from public repositories.
Figure 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 tumor growth in the left lung of cRaf transgenic mice.

Panel d: Lung adenocarcinoma of cRaf transgenic mice. Lung section of a collision tumor with pleural fibrosis and desmoplastic reaction. Tumor cells infiltrate the pleura.

Panel e-f: Lung sections of cRaf transgenic mice highlighting the tumor microenvironment and tumor infiltrating macrophages.

Panel g and H: Multiplicity of >200 µm tumors in the left and right lung of male and female transgenic mice.

**** p<0.0001, two tailed Mann-Whitney U test.
Figure 3. Identification of genes and miRNAs in lung tumors cRaf transgenic mice.

Panel: a-b Heatmaps of the DEGs (A) and DEMs (B). The Euclidian distance algorithm segregates DEGs and DEMs by gender.

Panel c-d: Venn diagrams of up- and down-regulated DEGs (C) and DEMs (D) in lung tumors 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.
Figure 4 Regulation of immune cell marker genes in lung tumors 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.
Figure 5 Sex-dependent genomic responses in lung tumors of cRaf transgenic animals.

Panel a-b. Venn diagrams showing gender 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 tumors of cRaf transgenic mice. Together 18 DEGs are regulated in the cross-talk between the MAPK/EGFR signalling cascade and the estrogen 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.

Panel f: The flow diagram shows the number of oncogenes, oncomirs and tumor suppressors regulated in a sex-dependent manner in lung tumors of cRaf transgenic mice.

Panel g: Histograms of highly regulated oncogenes and tumor suppressors. Shown are genes which were >3-fold regulated. All oncogenes are upregulated. However, in females the tumor suppressors are down regulated.
Figure 6. MiRNA-gene regulations.

Panel a: MiRNA-gene regulatory network in lung tumors 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 tumor suppressor genes in tumors of cRaf transgenic mice. In females, 5 oncogenes are upregulated and 7 tumor suppressors are down regulated. Conversely, in males 2 oncogenes are upregulated but, none of the tumor suppressors are regulated. Apart from the sex dependent regulation of oncogenes and tumor suppressors, we identified 13 oncogenes and 1 tumor suppressor regulated in common between both sexes. DEGs and DEMs are marked in red and turquoise.

ns = not significant, * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
Figure 7. The role of sex hormone receptors in tumor specific gene regulations.

Panel a-b: Venn diagrams showing the number of DEGs (A) and DEMs (B) containing Ar and Esr1&2 binding sites.

Panel c: The importance of the androgen and estrogen receptor in the control of gene expression. The network consisted of 46 DEGs and 27 DEMs. The hormone receptors bind to the promoters of miRNAs and target genes and the majority of the target genes are regulated by the estrogen receptor.

Panel d-e: Venn diagrams showing the number of DEGs targeted by Ar and Esr1&2 in lung tumors of female and male transgenic mice.

Ar: androgen receptor; Esr1&2: estrogen receptor 1&2.
Figure 8. TF-miRNA-gene regulatory network in lung tumors of cRaf transgenic mice.

Panel a. TF-gene regulatory networks. The transcription factor MAZ and Nr2f1 are repressed in lung tumors 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.
Figure 9: Histopathology and hormone receptor expression in human LC 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 tumors exhibited an acinar growth pattern (BI&BII), as well as invasive nests of tumor cells that produced glandular lumina without solid components (BIII).

Panel c. Immunohistochemistry staining of the estrogen receptor in LC patients. The tumor cells display marked expression of the estrogen receptor.

Panel d. Immunohistochemistry staining of the androgen receptor in LC patients. Unlike case I and III, the expression of the androgen receptor in tumor cells of female case II is very slight to none.
Figure 10: Genomics of human lung adenocarcinoma

Panel a: Clinical validation of DEGs and DEMs which we identified in lung tumors of cRaf transgenic mice. Depicted are Venn diagrams of commonly regulated DEGs and DEMs between LC patients and cRaf transgenic mice.

Panel b-c: Kaplan-Meier survival curves for 11 DEGs and 3 DEMs commonly regulated between LC patients and cRaf transgenic mice.
Figure 11. Sex specific gene regulations in human lung adenocarcinoma.

Panel a: Clinical validation of sex-specific DEGs identified in lung tumors of cRaf transgenic mice. Venn diagrams of commonly regulated DEGs between LC patients and cRaf transgenic mice.

Panel b: Clinical validation of sex-specific DEMs identified in lung tumors of cRaf transgenic mice. Venn diagrams of commonly regulated DEMs between LC patients and cRaf transgenic mice.

Panel c: Kaplan-Meier survival plots for sex-specific gene regulations. Shown are survival plots for DEGs and DEMs commonly regulated among cRaf transgenic mice and human LC.
Figure 12. Sex-specific regulation of oncogenes, oncomirs and tumor suppressors in lung tumors of cRaf transgenic mice. Shown are sex-specific and therefore unique gene regulation in tumors of cRaf transgenic mice. Genes marked in red and turquoise refer to up- and down-regulated 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).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS3.DifferentiallyexpressedgenesandmiRNAsinlungtumorsofRaftransgenicmice.xlsx
- SupplementaryTableS8EnrichedtranscriptionfactorbindingsitesinpromotersofDEGs.xlsx
- SupplementaryTableS7.ListofDEGsandDEMswithpromoterbindingsitesfortheandrogenandestrogenreceptor.xlsx
- SupplementaryTableS12.ListofCDK1targetgenesidentifiedinfemaleandmaleclinicalsamplesoftheTCGAdatabase.xlsx
- SupplementaryTableS12.pdf
- SupplementaryTableS5.Listofimmunecellmarkergenes.WeretrievedtheinformationfromtheCellMarkerdatabaseandpublishedliterature.xlsx
- SupplementaryTableS4.EnrichedGOtermsforDEGsbasedonannotationsbytheMetascapeandgeneXplainsoftware.xlsx
- SupplementaryTableS10.TumorassociatedmacrophagegenesregulatedinclinicalsamplesoftheTCGAdatabase.xlsx
- SupplementaryTableS6.ListofoncogenesoncomirsandtumorsuppressorsregulatedinlungtumorsofRaftransgenicmice.xlsx
- SupplementaryTableS13.EstrogenreceptortargetgenesidentifiedinfemaleandmaleclinicalsamplesoftheTCGAdatabase.xlsx
- SupplementaryTableS11.SexdisparitiesinDEGsandDEMssidentifiedinclinicalsamplesoftheTCGAdatabase.xlsx
- SupplementaryTableS9.ListofDEGsandDEMssidentifiedinclinicalsamplesfromtheTCGAdatabase.xlsx
- Supplementaryfigures.docx