Functional analysis of *CD74-NRG1*
-
a new recurrent oncogenic gene fusion in lung adenocarcinoma

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Zusammenfassung

Das Lungen-Adenokarzinom ist mit einer Inzidenz von ca. 50% das häufigste Lungenkarzinom. In 25-50% der Fälle kann kein Onkogen als transfomierende Ursache in den Tumorproben detektiert werden, welche die Entstehung eines Tumors erklären kann (Cancer Genome Atlas Research Network, 2014; Pao & Hutchinson, 2012). Im letzten Jahrzehnt konnten neue Onkogene durch die Entdeckung von Fusionsgenen beschrieben werden (Rikova et al., 2007; Soda et al., 2007; Takeuchi et al., 2012). Das Ziel dieser Studie war die Identifizierung und Beschreibung neuer onkogener Fusionsgene mittels der Methode der Transkriptomsequenzierung. Die Grundlage hierfür bildeten 25 Patientenproben von Lungen-Adenokarzinomen, die negativ für *EGFR* und *KRAS* Mutationen waren, da diese bereits ca. 1/3 aller Mutationen beim Lungen-Adenokarzinom ausmachen (Pao & Hutchinson, 2012). Es zeigte sich, dass in 14/25 Tumorproben keine onkogenen Mutationen und in 10/25 Tumorproben nur bereits bekannte onkogene Veränderungen identifiziert werden konnten. Aber in einer Tumorprobe konnte eine bisher unbekannte Genfusion beschrieben werden - *CD74-NRG1*. Die Untersuchung einer erweiterten Onkogen-unbekannten Kohorte von 102 Lungen-Adenokarzinomen zeigte 4 weitere *CD74-NRG1*-positive Tumore. Alle wurden als invasiv muzinöses Adenokarzinom (Stadium I) diagnostiziert (4/15 invasiv muzinösen Adenokarzinonem innerhalb der Kohorte von Lungen-Adenokarzinomen).
CD74-NRG1 ist die erste identifizierte Genfusion, bei der ein Wachstumsfaktor mit einer potentiellen Ligandwirkung (*NRG1*) und einem anderen Gen (*CD74*) fusioniert. Alle bisherig beschriebenen onkogenen Genfusionen besitzen eine Kinase-Domäne, durch die die transformierende Wirkung ausgeübt wird. Der Mechanismus der *CD74-NRG1* Genfusion liegt in der membranständigen Expression des nicht in der gesunden Lunge exprimierten Liganden *NRG1 III β3,* welcher natürlicherweise vor allem für die Entwicklung von Schwannzellen und Kardiomyozyten benötigt wird (Hynes & Lane, 2005). Die exprimierte Domäne von NRG1 ist hierbei eine konservierte

EGF-like Domäne, die Interaktionen mit Proteinen der HER-Familie von Rezeptortyrosinkinasen eingehen kann. CD74-NRG1 aktiviert als Ligand den HER3 Rezeptor, welcher nach Bindung von NRG1 mit HER2 heterodimerisiert und dadurch trans-phosphoryliert wird. Dies führt in den Lungen-Adenokarzinomzelllinien H1568 und H322 sowohl zur Aktivierung des PI3-Kinase- als auch des MAP-Kinase Signalweges. Darüber hinaus kann die *CD74-NRG1* Genfusion in H1568 Zellen unter anderem das kontaktunabhängige Wachstum in Soft-Agar verstärken. Die Signaltransduktion von CD74-NRG1 über HER2 könnte therapeutisch relevant sein. Die Inhibierung von HER2 mit einem selektiven Inhibitor, Afatinib, kann die HER2-abhängige Phosphorylierung von HER3 und die daraus resultierende Aktivierung des PI3-Kinase Signalweges inhibieren. In der Zukunft muss die Onkogenität der *CD74-NRG1* Genfusion unter anderem noch in einem Mausmodell *in vivo* bestätigt werden. Durch diese Arbeit konnte mit der Beschreibung und Charakterisierung der Genfusion sowie des Designs des Mausvektors der Grundstein für weitere Analysen gelegt werden. Darüber hinaus konnte eine gezielte potentielle Therapie für Patienten mit der *CD74-NRG1* Genfusion aufgezeigt werden, welche bis jetzt unbehandelbar wären.

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Abbrevations

ABL - abelson murine leukemia oncogene homolog
AJCC - American Joint Committee on Cancer
AKT - synonym for proteinkinase B
ALK - anaplastic lymphoma kinase
AT - alveolar type
ATP - adenosine triphosphate
BASC - bronchoalveolar stem cell
BCR - breakpoint cluster region
BRAF - v-Raf murine sarcoma viral oncogene homolog B
CCG - Cologne Center for Genomics
CD74 - cluster of differenciation 74
CDKN2A - cyclin dependent kinase (gene for p14 and p16 gene)
cDNA - complementary DNA
CVD - cardiovascular diseases
CK - cytokeratin
c-src - cellular sarcoma protein
CRD - cysteine rich domain
CREBBP - cAMP responsive element binding protein
CTG - CellTiter-Glo®
DAPI - 4’,6-diamidino-2-phenylindole
DDR2 - discoidin domain-containing receptor 2
DMSO - dimethylsulfoxide
DNA - deoxyribonucleic acid
E.coli. - Escherichia Coli
ECL - enhanced chemiluminescence
ECM - extracellular matrix
EDTA - ethylenediaminetetraacetic acid
EGFR - epidermal growth factor receptor
EML4 - echinoderm microtubule-associated protein-like 4
ERBB - synonym for human epidermal growth factor receptor
ERK - extracellular-signal-regulated kinase
e.v. - empty vector
FCS - fetal calf serum
FGFR1 - fibroblast growth factor receptor 1
FISH - fluorescent in situ hybridization
FPKM - fragments per kilobase of exon per million fragments mapped
GI - growth inhibition
HA - hemagglutinin
HER - human epidermal growth factor receptor
HRP – horseradish peroxidase
IMA - invasive mucinous adenocarcinoma
IP - immunoprecipitation
KIF5B - kinesin-1 heavy chain
KRAS - kirsten rat sarcoma viral oncogene homolog
LKB1 - liver kinase B1

MAPK - mitogen-activated protein kinase
MHC - major histocompatibility complex
MLL - mixed lineage leukemia
NE - neuroendocrine
NFE2L2 - nuclear factor (erythroid-derived 2)-like 2
NRG - neuregulin
NSCLC - non-small cell lung cancer
NTRK1 - neurotrophic tyrosine kinase receptor
p53 - protein of TP53 gene
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate-buffered saline
PCR - polymerase chain reaction
PFA - paraformaldehyde
PFS - progression-free survival
PI3K - phosphoinositide-3-kinase
PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PNEC - pulmonary neuroendocrine cell
PVDF - polyvinylidene fluoride
PS - penicillin/streptomycin
RNA - ribonucleic acid
PTEN - phosphatase and tensin homolog
SCLC - small cell lung cancer
SDS - sodium dodecyl sulfate
RB1 - retinoblastoma protein
RET - rearranged during transfection
RSV - rous sarcoma virus
RTK - receptor tyrosine kinase
SCC - squamous cell carcinoma
TE - Tris-EDTA
TKI - tyrosine kinase inhibitor
TP53 - tumor protein p53
TP63 - tumor protein p63
TTF-1 - thyroid transcription factor 1
UICC - Union Internationale Contre le Cancer
US - United States of America
v-src - viral sarcoma protein
VEGF - vascular endothelial growth factors
VEGFR - vascular endothelial growth factor receptor
WB - western blot

The international system of units was used in this thesis.

1 - Introduction

1.1 Cancer

Cancer can be generally described as a disease caused by uncontrolled cell growth. In 2012 cancer led to around 8.2 million cases of death (IARC, 2012). However, cardiovascular diseases (CVD) are the deadliest diseases worldwide causing 17.3 million deaths in 2008 (cancer: 7.3 million (2008)) (WHO, 2011). Even now there are more cancer-related than CVD-related deaths in 10 European countries among men. Only in Denmark this holds also true for women (Nichols, Townsend, Scarborough, & Rayner, 2014). It is predicted that the total numbers of new cancer cases will increase to 21.4 million by 2030 (WHO, 2011).

1.2 Lung cancer

1.2.1 Epidemiology

Lung cancer is the most frequent and deadliest type of cancer accounting for 1.8 million new cases (12.9% of all new cancer cases) and leading to 1.59 million deaths (19.3%) in 2012 (IARC, 2012). Lung cancer is heavily associated with tobacco exposure with about 90% of lung cancer patients being active or former smokers (Hecht, 1999; 2002). In about 10% there are other factors causing lung cancer (Thun et al., 2008). The outcome for lung cancer patients is very poor - the five-year survival rate is only about 16% for all patients. Notably, the incidence rate for lung cancer in the United States of America (US) from 1975 - 2010 of men has dropped by 24% while it doubled within the same time for women. This might be explained by an increasing amount of female smokers, changes in smoking habits and changes in social behavior (Fig. 1) (National Cancer Institute, 2014).

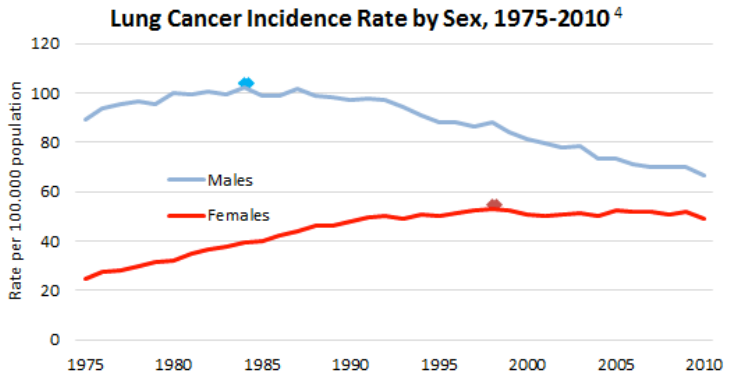

Figure 1: Incidence rate for lung cancer by sex from 1975-2010 in the US. http://www.lung.org/assets/images/chart-and-graphs/LC-Incidence-by-Sex.png

In general patients do not feel any side effects of early stage lung cancers, therefore about 70% of patients are diagnosed at late stage disease (stage III or IV). Unfortunately a diagnosis at late stage goes often in line with poor survival as low as 9.5 months median survival time for stage IV patients (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013). For the classification of lung cancer the *Union Internationale Contre le Cancer* (UICC) and the *American Joint Committee on Cancer* (AJCC) have published the 7th edition of the international standardized TNM staging system. The TNM staging system describes: (1) the size and growth stage of the tumor (T), (2) if the tumor already spread to the lymph nodes (N) and (3) if the tumor has already metastasized (M). These criteria are used to judge a patient's tumor stage and are updated regularly (Fig. 2) (Detterbeck, 2009).

T/M	Subgroup	N0	N1	N2	N3
T1	T1a	Ia	IIa	IIIa	IIIb
	T1b	Ia	IIa	IIIa	IIIb
T2	T2a	Ib	IIa	IIIa	IIIb
	T2b	IIa	IIb	IIIa	IIIb
T3	T3 $_{>7}$	IIb	IIIa	IIIa	IIIb
	T3 $_{Inv}$	IIb	IIIa	IIIa	IIIb
	T3 $_{Satell}$	IIb	IIIa	IIIa	IIIb
T4	T4 $_{Inv}$	IIIa	IIIa	IIIb	IIIb
	T4 $_{Ipsi\ Nod}$	IIIa	IIIa	IIIb	IIIb
M1	M1a $_{Contra\ Nod}$	IV	IV	IV	IV
	M1a $_{Pl\ Disem}$	IV	IV	IV	IV
	M1b	IV	IV	IV	IV

Figure 2: TNM staging system according to lung cancer stage (Detterbeck, 2009).

1.2.2 Classification

The classification of lung cancer is assessed according to histological stainings. It can be coarsely divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Petersen, 2011; Travis, Brambilla, & Riely, 2013). But due to the increasing amount of genomics data, there is a future trend to diagnose and classify lung cancers based on genomics data as every subtype has its own molecular profile. Therefore, molecular pathology can lead to a completely unbiased diagnosis and classification system in the near future and could already eliminate and further subdivide the group of large cell carcinomas (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013).

1.2.2.1 Small cell lung cancer

Small cell lung cancer accounts for around 15% of all diagnosed lung cancer cases worldwide (Wahbah, Boroumand, Castro, El-Zeky, & Eltorky, 2007) and is highly associated with smoking. More than 90% of SCLC patients being or having been heavy smokers (Rosell & Wannesson, 2012; van Meerbeeck, Fennell, & De Ruysscher, 2011). SCLC patients typically respond very well to initial standard chemotherapy with platinum and etoposide but show very soon a resistance phenotype and tumor relapse. The tumor is characterized by early metastasis and patients show a very poor 2-year survival of 5% for extensive stage or ~15% for limited stage disease (Pleasance et al., 2010). Up to now no gene could be identified as therapeutical target in SCLC. Only the tumor suppressor genes *tumor protein p53* (*TP53*) and *retinoblastoma protein* (*RB1*) are consistently inactivated in SCLC (Peifer et al., 2012; Rudin et al., 2012).

1.2.2.2 Non-small cell lung cancer

NSCLC can be divided into three main groups: adenocarcinoma, large cell carcinoma and squamous cell carcinoma. Other NSCLC subtypes are very rare.

Adenocarcinoma accounts for ~50% of all lung cancer cases and is the most frequent subtype of lung cancer. It is the leading cause for cancer-related mortality with more than one million deaths worldwide. The most common mutated driver oncogenes are *kirsten rat sarcoma viral oncogene homolog* (*KRAS*) and *epidermal growth factor receptor* (*EGFR*). These genes are mutated in about 30-40% of all lung adenocarcinomas. But still in 25-50% of lung adenocarcinomas the oncogenic driver is unknown (Fig. 3) (Cancer Genome Atlas Research Network, 2014; Pao & Hutchinson, 2012). In immunohistochemistry lung adenocarcinomas are typically positive for thyroid transcription factor 1 (TTF-1) and cytokeratin 7 (CK7) (Lau, Desrochers, & Luthringer, 2002; Su, Hsu, & Chai, 2006).

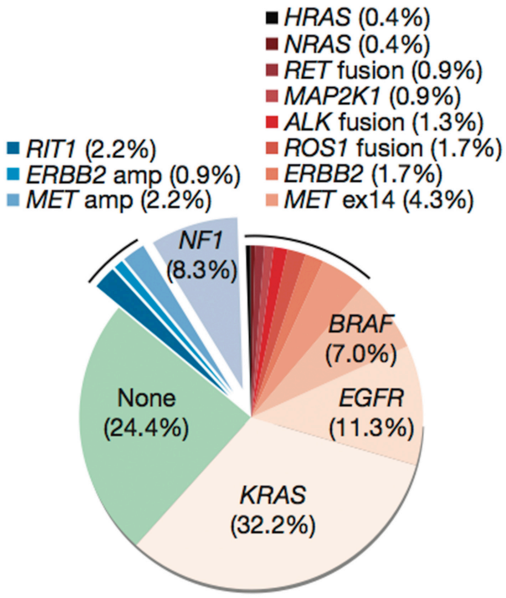

Figure 3: Distribution of oncogenic driver mutations in lung adenocarcinoma (Cancer Genome Atlas Research Network, 2014).

Squamous cell carcinoma (SCC) accounts for ~30% of NSCLC cases. Typically SCC is positive for tumor protein p63 (TP63) and cytokeratins 5/6 in immunohistochemistry. Common mutations for SCC are *TP53*, discoidin domain-containing receptor 2 (*DDR2*), nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) and fibroblast growth factor receptor 1 (*FGFR1*) (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013;

Hammerman et al., 2011; Perez-Moreno, Brambilla, Thomas, & Soria, 2012; Weiss et al., 2010).

Large cell carcinoma is a very heterogeneous group within lung cancer. It is poorly differentiated and accounts for about 10% of lung cancer cases. In immunohistochemistry it can only be characterized by negative TTF-1 stainings. Due to its poor differentiation and molecular similarities to other lung cancer subtypes there are thoughts that large cell carcinoma are only poorly differentiated variants of lung adenocarcinoma or squamous cell carcinoma. Therefore this subtype might be a more and more vanishing entity (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013).

1.2.3 Cell of origin

In the past a lot of research was aimed to decipher the cell of origin for every lung cancer subtype. But up to now the cellular origin of every lung cancer subtype is unknown (Fig. 4) (Sutherland et al., 2011). Recently, alveolar type (AT) 1/2 cells have been described as being capable of potentially initiating oncogenesis in mice (Desai, Brownfield, & Krasnow, 2014). From murine models it is known that the genetic alterations and the harboring cell type are very likely to be responsible for the tumor phenotype (Jackson et al., 2001; Meuwissen et al., 2003). Even transdifferentiation of one tumor type from one subtype to another have been described while tumors acquire additional genomic alterations (Han et al., 2014; Peifer et al., 2012). Notably cell type specific expression of *KRAS* G12D in AT2 and clara cells leads to lung adenocarcinoma while inactivation of *TP53* and *RB1* in clara, neuroendocrine and AT2 cells fosters the development of SCLC in mice (Fig. 4) (Sutherland et al., 2011; 2014).

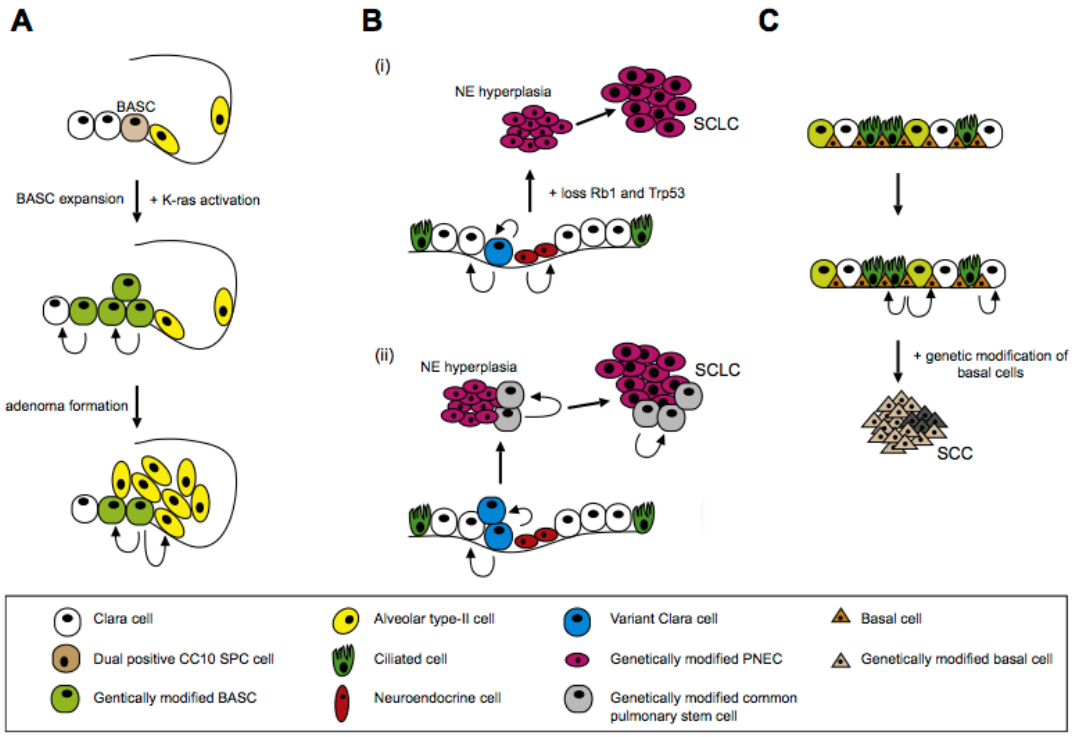

Figure 4: Putative model for the role of progenitor cells in the development of lung cancer. (A) The role of activated *kras* in bronchoalveolar stem cells (BASC) in NSCLS. (B) Two models for the development of neuroendocrine (NE) tumors in epithelial cells of bronchi. (i) NE tumor arise from pulmonary neuroendocrine cells (PNEC), (ii) another hypothesis would be that NE tumors arise from less-differentiated cells. (C) SCC is thought to evolve from basal cells of the trachea which harbors stem-cell-like properties (Sutherland & Berns, 2010).

1.2.4 Treatment

As distinct lung cancer types harbor distinct genomic alterations, proper molecular diagnosis for every subtype is indispensable. SCLC standard therapy consists of platinum- and etoposide-based chemotherapy and radiotherapy. Patients show a very high initial response to chemotherapy (70-90%) but relapse within months (Metro & Cappuzzo, 2009; Simon & Turrisi, 2007). The role of surgery remains controversial but there are indications that surgery does not prolong patient's life (Lad, 1994; Lim, Belcher, Yap, Nicholson, & Goldstraw, 2008).

Treatment guidelines for NSCLC are dependent on the tumor stage at diagnosis. In NSCLC patients that are staged up to IIIa a complete resection of the tumor is theoretically possible. Patients receive typically combined platinum-based chemotherapy and radiotherapy before and after surgery

(Vansteenkiste et al., 2013). Patients that harbor a NSCLC with stage IIIb or IV cannot be cured any more. For these patients histology and especially molecular pathology for the detection of potentially druggable oncogenic driver mutations is recommended (Reck et al., 2014). Personalized medicine was first proven to lead to a better survival in the IPASS and EURTAC trial showing that patients harboring mutated *EGFR* do benefit from targeted therapy of EGFR-inhibition with erlotinib or gefitinib (Fig. 5) (Maemondo et al., 2010; Rosell et al., 2012).

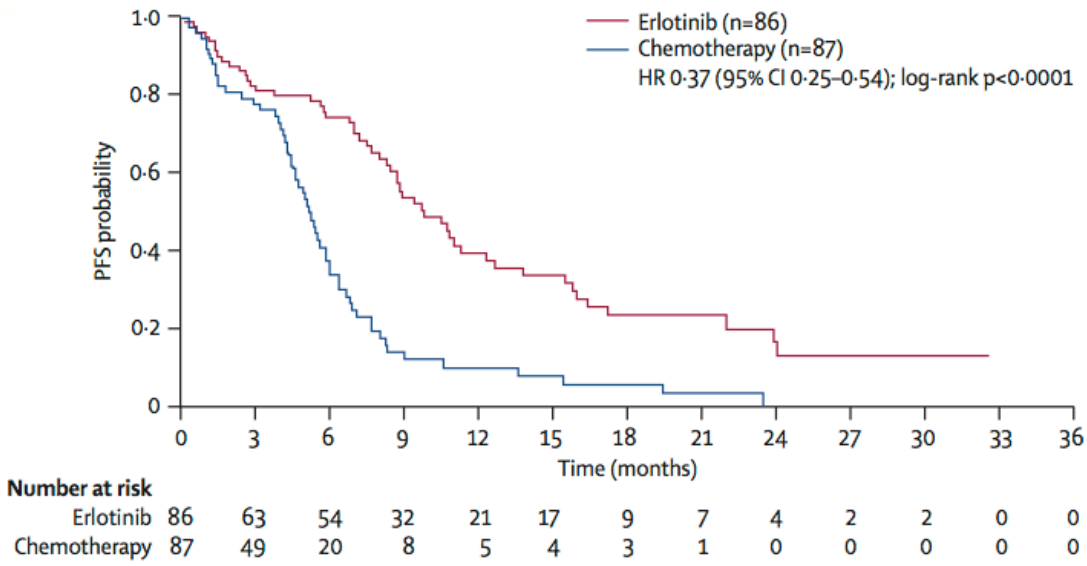

Figure 5: Gain of progression-free survival (PFS) by targeted therapy compared to chemotherapy. Kaplan-Meier plot of PFS of *EGFR*-mutated NSCLC patients treated either with chemotherapy or an EGFR-inhibitor (erlotinib) (Rosell et al., 2012).

In Germany according to patient's health conditions and cancer center, different treatment options are applied. As molecular diagnostics takes about 2-4 weeks patients are treated in the meantime with platinum-based chemotherapy. With the application of molecular diagnostics the patient could gain additional and beneficial treatment options such as EGFR tyrosine kinase inhibitors (TKI) in the case of *EGFR*-mutated NSCLC as these cancers are dependent on EGFR signaling (Fig. 6) (Reck et al., 2014; Rosell et al., 2012). The broader the molecular diagnostics panel, the bigger is the chance of having additional treatment options for specific driver lesions. Many clinical

trials are now conducted to analyze if targeted therapy of druggable oncogenic mutations leads to oncogenic dependency in these patients, another example beside EGFR TKIs would be crizotinib which targets anaplastic lymphoma kinase (ALK) and leads to a better PFS in these patients as well (Shaw et al., 2013).

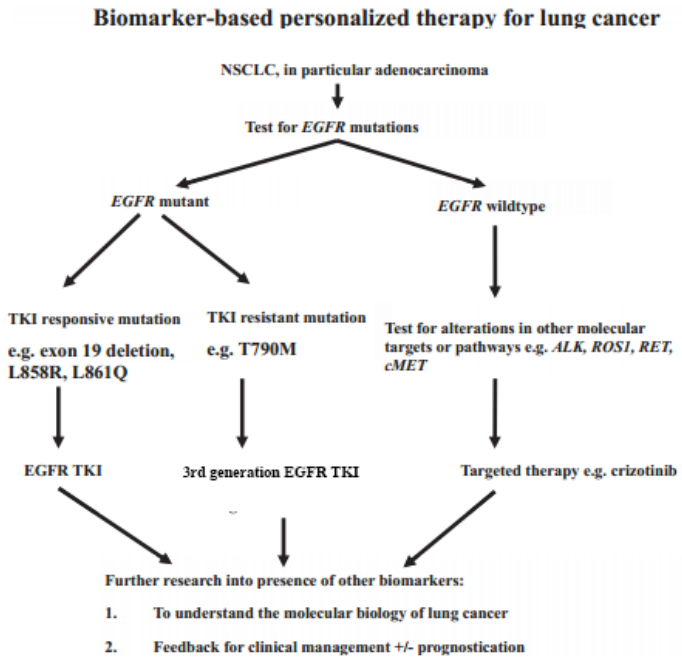

Figure 6: Relevance of molecular pathology for treatment of NSCLC (adapted from (W. A. Cooper, Lam, O'Toole, & Minna, 2013)).

1.3 Tumor development

Cancer is a disease of the genome and develops in a multi-step process. It is influenced by many different factors, e.g. cells get exposed to carcinogens from the environment. Those factors can finally lead to an altered expression of one or a set of genes, alter the genomic sequence or facilitate cells of developing cancer that are already predisposed by inherited somatic mutations (Fig. 7) ((Irigaray et al., 2007) and reviewed in (Anand et al., 2008; Arteaga et al., 2014)).

A

Cancer	Associated Gene
Leukemias and lymphomas	ATM
All cancers	BLM
Breast, ovarian, pancreatic and prostate cancers	BRCA1, BRCA2
Breast, thyroid and endometrial cancers	PTEN
Colorectal cancer	APC
Melanoma	CDKN2A
Retinal cancer	RB1
Leukemia	FACC, FACA
Colorectal cancer	MLH1, MSH2, MSH6, PMS6
Pancreatic cancer	PRSS1, SPINK1
Leukemias, breast, brain and soft tissue cancers	TP53
Pancreatic cancers, pituitary adenomas, benign skin and fat tumors	MEN1
Thyroid cancer, pheochromacytoma	RET, NTRK1
Pancreatic, liver, lung, breast, ovarian, uterine and testicular cancers	STK11/LKB1
Tumors of the spinal cord, cerebellum, retina, adrenals and kidneys	VHL
Kidney cancer	WT1
Skin cancer	XPD, XPB, XPA

B

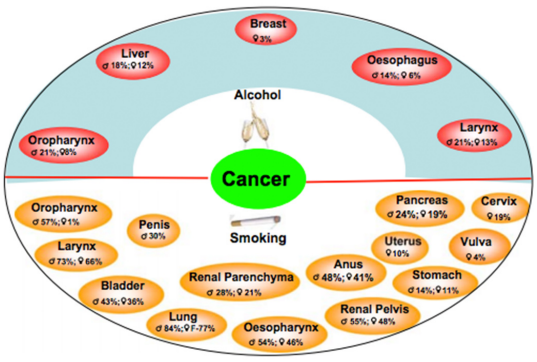

Figure 7: Genetical predisposition and enviromental factors develop to different cancer types. (A) Inherited mutations that predispose to different cancer types. (B) Association of alcohol usage and smoking linked to different types of cancer (adapted from (Anand et al., 2008; Arteaga et al., 2014)).

In many cases proto-oncogenes or tumor suppressor genes are altered by genomic aberrations or epigenetic modifications which cause or predispose to cancer development (Chin & Gray, 2008). Other possibilities include genomic rearrangements thereby creating fusion genes, alternative splicing or deletions of regulatory regions of proteins that influence tumor development as well (Cancer Genome Atlas Research Network, 2014; Ekstrand, Sugawa, James, & Collins, 1992; Soda et al., 2007).

1.3.1 Oncogenes and tumor suppressor genes

The oncogene hypothesis of cancer was first established by Hübner and Todaro in 1969 (Huebner & Todaro, 1969). Oncogenes can be defined as genes that encode for proteins that are capable to drive the activation of a potential oncogenic pathway upon deregulation. These pathways have in general important roles in signal transduction for cell growth, cell divisions and differentiation. Traditionally the first viral oncogene described was viral sarcoma protein (*v-src)* from rous sarcoma virus (RSV) by J. Michael Bishop's lab (Bernstein, MacCormick, & Martin, 1976; Swanstrom, Parker, Varmus, & Bishop, 1983). RSV could transform chicken embryos and led to transformation of mouse fibroblasts. Already in 1910 Peyton Rous could show

that a chicken tumor was transplantable from one chicken to another and that he could transmit the cancer without any cells (Rous, 1910; 1911). Only a small change in nucleotide sequences could lead to tumor formation when human wild-type *c-src* was turned into viral derived *v-src* (G. M. Cooper, 2000; Swanstrom et al., 1983).

There are different ways that lead to deregulation of proto-oncogenes. The most common ways for deregulation are either by gain-of-function mutations or amplifications therefore leading to constitutive and increased pathway activation. Structural aberrations leading to genomic rearrangements or translocations and segmental deletions of regulatory elements are observed as well to activate oncogenes constitutively (Chin & Gray, 2008). In lung cancer the most frequently mutated oncogenes are *KRAS*, *EGFR*, *PIK3CA*, *BRAF* and *ERBB2* (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013) (reviewed in (W. A. Cooper et al., 2013)).

In contrast tumor-suppressors are negative regulators of cell growth and do act in a recessive fashion compared to the dominant acting oncogenes. *RB1* gene was the first tumor suppressor that has been shown to be involved in cell-cycle control and progression. A biallelic loss-of-function mutation of *RB1* leads to retinoblastoma which is a rapidly developing cancer of the eye in childhood. In general both allels of a tumor suppressor gene have to be inactivated to fully predispose a cell or an organism to cancer (Knudson, 1971). The recessive fashion of tumor suppressor genes and the fact that both allels have to be hit to get full predisposition to cancer is reflected in their function. Most tumor suppressor genes are involved in cell cycle control, DNA repair or DNA damage sensing, cell differentiation, migration or protein degradation. Therefore it is very hard to treat patients with aberrations in tumor suppressor genes therapeutically as their function needs to be reconstituted instead of inhibited. This can up to now only work if the gene is epigenetically silenced and reactivation is achieved e.g. via histone deacetylase or DNA methyltransferase inhibitors which lead to global re-expression of genes including tumor suppressor genes (Bolden, Peart, & Johnstone, 2006; Cameron, Bachman, Myöhänen, Herman, & Baylin, 1999). An inactivating mutation of *TP53* in lung cancer already lowers the probability

of survival. Survival is even more decreased with additional oncogenic mutations of oncogenes and typically if already one allel is lost, the non-mutated allel is lost by loss of heterozygosity leading to the complete loss of both allels (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013). Typical tumor suppressor genes showing genomic aberrations in lung cancer are *TP53*, *PTEN*, *RB1*, *LKB1* and *p16/CDKN2A* and do mainly act in cell cycle control pathways (W. A. Cooper et al., 2013).

1.3.2 Hallmarks of cancer

In the famous reviews “Hallmarks of Cancer” (2000) and “Hallmarks of Cancer: the next generation” (2011) Douglas Hanahan and Robert Weinberg describe essential alterations which alter and transform healthy cells and lead to tumor development by influencing cell metabolism and cell physiology. Drugs that interfere with these emerging hallmarks are used in clinical trials to treat human cancers (Fig. 8). Nevertheless the central hallmarks of cancer are:

- resisting cell death
- sustaining proliferative signaling
- evading growth suppressors
- activating invasion and metastasis
- enabling replicative immortality
- inducing angiogenesis

In the updated version from 2011 two emerging hallmarks – deregulating cellular energetics and avoiding immune destruction – and two enabling characteristics – genome instability and mutation and tumor-promoting inflammation – were added (Hanahan & Weinberg, 2011). But to understand the general biology of cancer this introduction focuses on the core hallmarks of cancer from 2000.

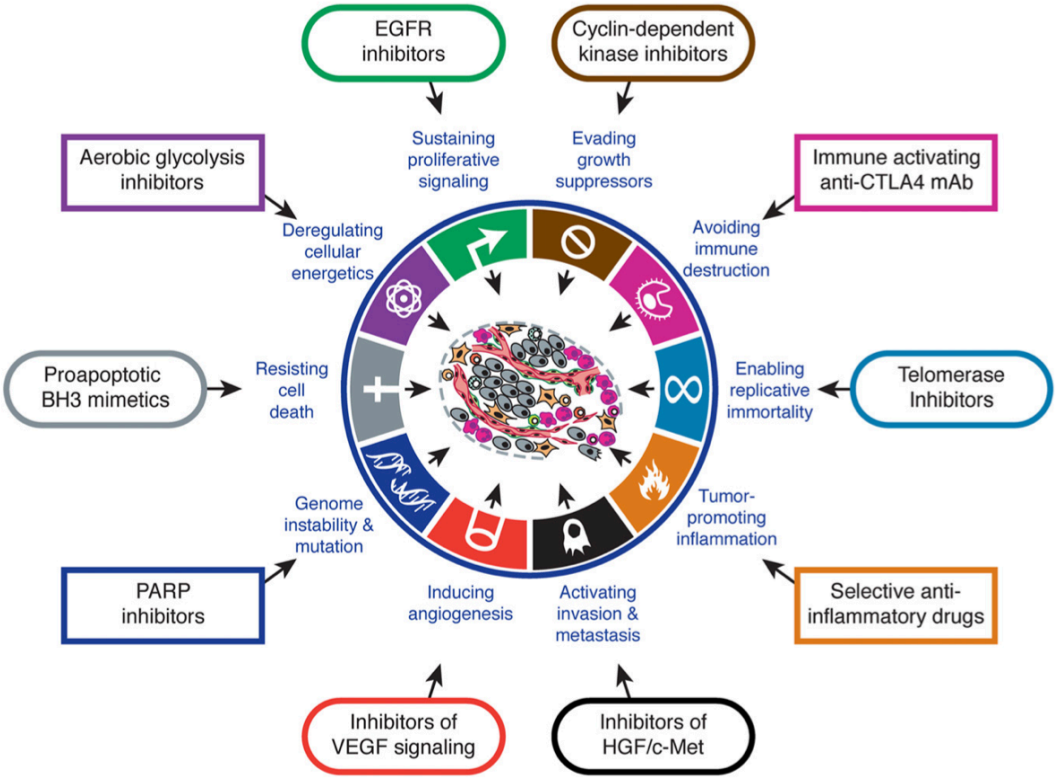

Figure 8: Hallmarks of cancer and examples for druggable targets.

1.3.2.1 Resisting cell death

Cell death or apoptosis is a strongly controlled mechanism of the human body that is balanced via pro- and anti-apoptotic proteins. It is an important mechanism to eliminate damaged cells, cells that are misplaced or no longer needed or for cells that are recognized as “non-self”. This can be controlled either by immune cells or compounds via activation of death receptors or via intrinsic signaling e.g. upon DNA damage (Wang et al., 2013; N. S. Wilson, Dixit, & Ashkenazi, 2009). The most important enzymes signaling towards apoptosis are the caspase enzymes (Ashkenazi, 2008). *TP53* was called “guardian of the genome” as p53 is one of the most important proteins sensing DNA damage or stress (D. P. Lane, 1992). It can sense DNA damage and accumulates upon DNA damage in the nucleus and arrests the cells in G_1 phase in order to get the DNA damage repaired. If this is not possible, p53 initiates the caspase pathway by upregulating pro-apoptotic proteins which lead to apoptosis to prevent the transfer of damaged or mutated DNA to the

next generation (Ashkenazi, 2008; Kruse & Gu, 2009). In cancer cells apoptosis is actively inhibited by inactivating pro-apoptotic signaling pathways and survival is supported by the activation of anti-apoptotic pathways. In cancer *TP53* is the most frequently mutated gene. In general it gets inactivated and therefore cannot trigger apoptosis any more. By this mechanism, cancer cells can evade apoptosis even under circumstances like cellular stress and accumulation of mutations (Levine & Oren, 2009). Especially in SCLC (up to 100%) and NSCLC (more than 50%) *TP53* is inactivated in many patients (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013; Peifer et al., 2012).

1.3.2.2 Sustaining proliferative signaling

Sustaining proliferative signaling is one of the most fundamental mechanisms of cancer cells. Healthy cell proliferation is tightly controlled and does need different stimuli for proliferation. The most prominent pathways are the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways which are essential and key pathways in many cancers. They are generally used for driving cell growth and proliferation, survival, angiogenesis and inhibit apoptosis. Therefore constitutive activation of these pathways is important for many cancers and can mediate resistance to therapy. Key molecules of those pathways are currently used as targets in running clinical trials (Baselga, 2011; Burris, 2013; P. Liu, Cheng, Roberts, & Zhao, 2009). The PI3K and MAPK pathways are activated by receptor tyrosine kinases (RTKs) which are anchored in the cellular membrane. Each RTK has different specificities to different ligands (growth factors). These growth factors are either produced in a para- or autocrine manner. By controlling the activated state of RTKs by a specific ligand the downstream pathway can be carefully controlled. Tumor cells can therefore overexpress the ligand, overexpress the RTK or acquire activating mutations within the RTK itself or increase the affinity of the ligand to the RTK, leading to constitutive activation of the pathway (Greulich et al., 2005; Huang, Xu, & White, 2009; Zadeh, Bhat, & Aldape, 2013). Another way of leading to a

sustained proliferative signaling within this network is via inactivation of *PTEN*, a tumor suppressor gene involved in dephosphorylation and therefore inactivation of the PI3K pathway. As all of the mentioned mechanisms are used or hijacked by cancer cells, many pathways and key proteins are pharmacologically used to tackle the oncogenic signaling of the tumor. Mutations in the PI3K and MAPK pathway are often found in lung adenocarcinomas and can be therapeutically targeted (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013; Ding et al., 2008; Rosell et al., 2012).

1.3.2.3 Evading growth suppressors

The cell cycle is well controlled in normal cells to prevent the transfer of mutations to daughter cells or to prevent improper tissue formation. This is achieved via a resting (G_0- or G_1-phase) phase in cell cycle e.g. by the retinoblastoma protein 1 (RB1) before cells enter the S-phase. This tumor suppressor protein is important in the transmission of anti-growth signals classically by binding E2F – a strong transcriptional activator. *RB1* is mutated, deleted or inactivated especially in up to 100% of SCLC but often inactivated in other cancers as well (Burkhart & Sage, 2008; Classon & Harlow, 2002; Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013; Peifer et al., 2012). If *RB1* is mutated and inactivated the cells cannot control their cell cycle any more and cannot arrest in G_0. Only about 4% of lung adenocarcinomas are affected by inactivation of *RB1*. But this pathway is affected in about 15% of lung adenocarcinomas by *CDKN2A* mutations which acts upstream of P53 and RB1 and leads to the inactivation of a negative feedback loop for RB1 (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013; Ding et al., 2008).

1.3.2.4 Activating invasion and metastasis

Tumor cells acquire invasive and metastasizing properties during their development. Normal cells depend on cell-cell interactions and cell-matrix interaction with the extracellular matrix (ECM). These interactions are mainly

provided by cadherins and intergrins that interact with the ECM and neighboring cells providing pro-survival and pro-proliferative signals. Normal cells induce apoptosis or anoikis if they loose contact with other cells or the ECM (Frisch & Francis, 1994; Lelièvre et al., 2012). Malignant cancer cells have often lost E-cadherin, one of the main proteins for cell-cell interactions, and do not depend on survival signals from neighboring cells or the ECM anymore. These tumor cells gained the ability to migrate via the blood or lymph vessel system to more distant organs and some of them are even able to establish a metastasis at a distant site (Chiang & Massagué, 2008; Klein, 2008). As 90% of all cancer patients die from metastasis it is surprising that this process is not fully understood. Therefore ongoing intensive research focuses to slow down or even prevent metastatic spread in the future (Arteaga et al., 2014).

1.3.2.5 Enabling replicative immortality

The replicative capacity of most human body cells is limited due to the length of the telomeres which protect the caps of the chromosomes. During cell division they get shortened with each division. If their length falls below a critic threshold for maintaining genomic integrity, cells can induce senescence or apoptosis. The number of cell divisions up to this state is named Hayflick limit (Hayflick, 1965; Shay & Wright, 2000). Cancer cells as well as stem cells can bypass this limit by expressing telomerase - an enzyme that can lengthen the telomeres again. Therefore these cells can divide for an unlimited time. Telomerase inhibitors are under development in clinical trials for different types of cancer - especially as reexpression of telomerase is very common in NSCLC and SCLC and many other cancers (C.-H. Chen & Chen, 2011; Harley, 2008; Shay & Bacchetti, 1997).

1.3.2.6 Inducing angiogenesis

After tumor establishment a tumor can grow up to 1-2mm in diameter but then stops or becomes necrotic as oxygen or nutrition supply cannot reach the tumor center any more via diffusion (N. Nishida, Yano, Nishida, Kamura, &

Kojiro, 2006). To circumvent the growth limits, tumor cells use a process called tumor-associated neovascularization. This process is induced by cytokines, angiopoietins and vascular endothelial growth factors (VEGFs) which increase permeability and induce sprouting of new blood vessels. Secretion of VEGFs and binding to their corresponding receptor (VEGF receptors 1-4 (VEGFR)) can secure oxygen and nutrition supply of cancer cells in a multistep process (Hanahan & Folkman, 1996; Saaristo, Karpanen, & Alitalo, 2000; Senger et al., 1983). Inducing angiogenesis is therefore important for tumors to grow and survive. This is reflected in a wide range of tumors showing elevated levels of VEGF or VEGFR (Goel & Mercurio, 2013). Treatment efforts towards VEGF signaling inhibition decreases the amount of blood vessels but at the same time other drugs cannot reach the tumor as efficient as if the tumor was fully vascularized. In addition inhibition of VEGF or VEGF receptors can result in increased invasion and metastasis by several escape mechanisms (Ebos et al., 2009; Goel & Mercurio, 2013; Paez-Ribes et al., 2009). One possibility to escape the inhibition is for example the upregulation of placental growth factor that can activate VEGFR1, too (Fischer et al., 2007).

1.3.3 Fusion-genes

The first chromosomal translocation was discovered in 1960 by Peter Nowell and David Hungerford in a patient with chronic myeloid leukemia (Nowell & Hungerford, 1960). The *BCR-ABL* gene fusion results from a chromosomal translocation of chromosome 9 and 22. This fusion protein leads to cellular transformation and can cause leukemias (Sawyers, 1993). Due to hyperactive kinase activity of the ABL nuclear kinase the cell undergoes uncontrolled cell growth. Another decade after demonstrating that *BCR-ABL* is tumorigenic in mice, treatment with imatinib and other tyrosine kinase inhibitors were shown to be effective in tumor treatment mediated by the inhibition of ABL kinase activity (Deininger, Buchdunger, & Druker, 2005; Druker, 2003). More than 50,000 cases of chromosomal rearrangement creating gene fusions have been found across all main tumor types up to now (Mitelman, Johansson, &

Mertens, 2007). The most interesting fusion genes are the ones generated *in-frame* and therefore leading to an actual fusion protein. The best characterized fusion proteins are those that hijack the promoter of the 5' part of gene A and fuse it to the kinase part of gene B resulting in a deregulated hyperactive tyrosine kinase such as in *BCR-ABL*. This method destroys regulatory elements of kinases or kinases get expressed in a tissue in which they are generally not expressed or only during development. Common gene fusions found in lung cancer are *EML4-ALK, KIF5B-RET* and *CD74-ROS1* (Fig. 9). In summary all described fusion genes lead to hyperactive kinases and to cellular transformation that can be targeted and thereby inhibited with specific molecules (Kohno et al., 2012; Rikova et al., 2007; Soda et al., 2007; Takeuchi et al., 2012). Other chromosomal translocations lead to the loss of function of tumor suppressor genes such as *CREBBP*, *CHEK2* or *MLL* either by an *out-of-frame* fusion or by destroying functional domains of the protein (Jin, Mertens, Kullendorff, & Panagopoulos, 2006; Pleasance et al., 2010; Xia, Anderson, Diaz, & Zeleznik-Le, 2003).

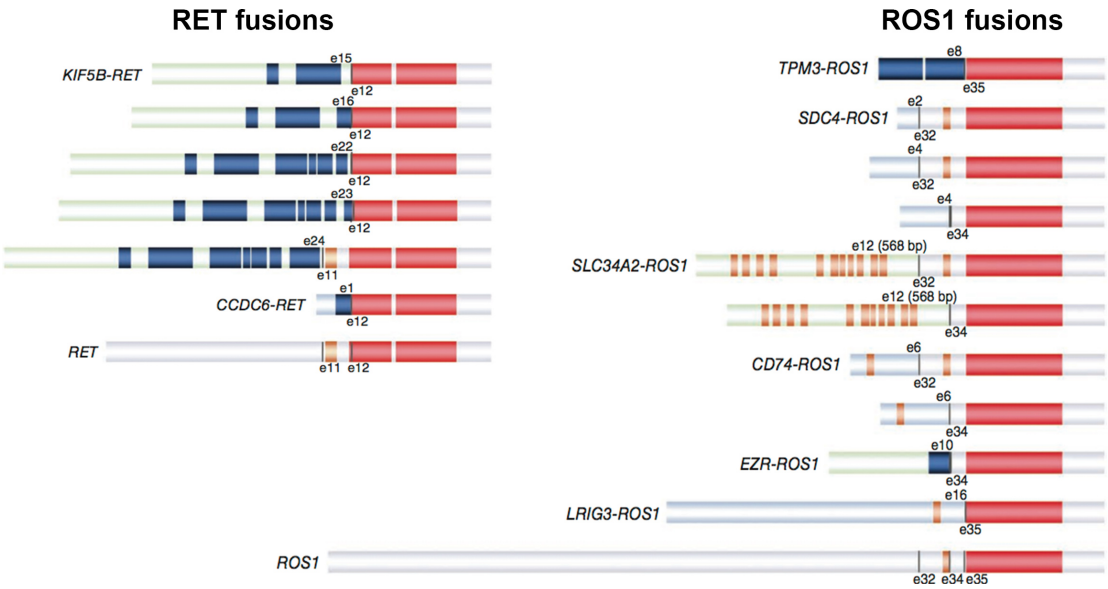

Figure 9: Different *RET* and *ROS1* fusion genes found in NSCLC. The kinase domain (red) is conserved in every fusion protein (modified from (Takeuchi et al., 2012)).

1.4. Neuregulin-HER2:HER3 signaling pathway

The family of neuregulins (NRGs) is a very broad family of growth factors with more than 30 isoforms. They mediate cell-cell interactions in neuronal cells, cardiocytes, breast and epithelial cells. NRGs are divided in 6 major families. While types I, II, IV-VI are soluble ligands, NRG1 type III stays membrane-tethered (Falls, 2003; L. Mei & Xiong, 2008).

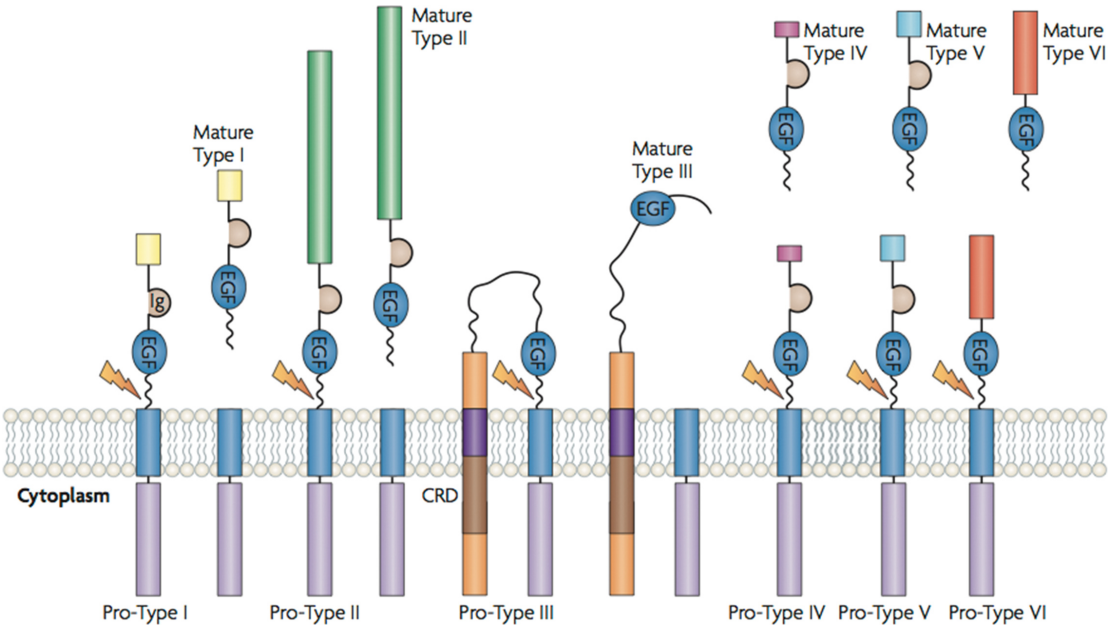

Figure 10: Different isoforms and processing of NRG1. Only NRG1 type III contains a cysteine rich domain (CRD) in its amino terminus determining the type of NRG1 and stays membrane-tethered compared to the other isoforms (L. Mei & Xiong, 2008).

NRG1 type III is a crucial survival factor for Schwann cell precursors in embryonic nerves but is expressed in axons as well and regulates myelin sheath thickness (Falls, 2003; Jessen & Mirsky, 2005; L. Mei & Xiong, 2008; Wolpowitz et al., 2000). These functions are triggered via NRGs binding affinity to humane epidermal growth factor receptor 3 (HER) and HER4. Both are RTKs that are localized on the plasma membrane for signaling via the PI3K and MAPK pathway (Fig. 11) (Hynes & Lane, 2005; Sithanandam et al., 2003; Yarden & Sliwkowski, 2001).

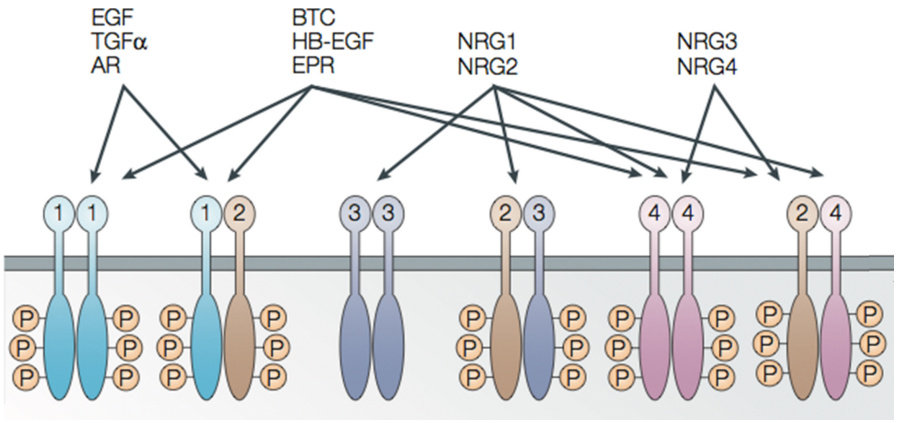

Figure 11: Interaction network of HER receptors 1-4. Different HER receptors can be activated by different ligands. Binding affinity of ligands to different homo- and heterodimers influences the signaling (modified from (Hynes & Lane, 2005)).

HER3 was discovered in 1989 and is the biggest HER receptor (Kraus, Issing, Miki, Popescu, & Aaronson, 1989; Roskoski, 2014). It has the most PI3K (p85 subunit binding sites of PI3K) phospho-tyrosine binding sites but has an impaired kinase domain activity that cannot or ~1,000 times less efficient phosphorylate itself or other kinases. This is due to the nonconservatively substitution of important amino acid residues within the kinase domain in comparison to the other HER family members (Hynes & Lane, 2005; Roskoski, 2014; Shi, Telesco, Liu, Radhakrishnan, & Lemmon, 2010). The best characterized dimerization partner of HER3 is HER2 which preferentially forms heterodimers upon NRG1 binding to HER3 and trans-phosphorylates HER3 (Baselga & Swain, 2009; Li, Mei, Liu, & Zhou, 2007; Plowman et al., 1990; Yarden & Sliwkowski, 2001). Many tumors show elevated HER3 expression levels or upregulate expression of HER3 as a resistance mechanism upon treatment. Thus they harbor alternative possibilities to trigger the PI3K and MAPK signaling pathway (Engelman et al., 2007; Sergina et al., 2007). Beside membranous localization, HER3 can relocalize to the nucleus in a cancer-specific manner. This has been discovered in NSCLC and correlated with increased vascular and lymphatic invasion and an overall poor survival (Baselga & Swain, 2009; Begnami et al., 2011; Maurer et al., 1998). By this interaction network NRG1 can trigger HER3 receptor activation including its pro-oncogenic PI3K and MAPK signaling pathways and can even

establish a resistance mechanism when targeted therapies inhibit other pro-oncogenic pathways (T. R. Wilson et al., 2012).

2 - Aim of this study

Lung cancer is the world's leading type of cancer conducting to death (IARC, 2012; Siegel, Ma, Zou, & Jemal, 2014). Lung adenocarcinoma accounts for about 50% of all lung cancer cases and in about 50% of all lung adenocarcinoma cases the oncogenic driver mutation is still unknown (Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013; Pao & Hutchinson, 2012). The most common driver mutations for lung adenocarcinoma are *EGFR* and *KRAS* mutations, which account for about 1/3 of all lung adenocarcinomas (Pao & Hutchinson, 2012). Since the identification of the *EML4-ALK* gene fusion in 2007, additional gene fusions have been found involving *NTRK1*, *RET* and *ROS1* kinases (Davies et al., 2012; Kohno et al., 2012; Soda et al., 2007; Takeuchi et al., 2012; Vaishnavi et al., 2013). These gene fusions lead to oncogene dependency which can be therapeutically used for specific treatment and therefore help to prolong patient's survival (Bergethon et al., 2012; Camidge, Pao, & Sequist, 2014; Shaw et al., 2013). Therefore there were two objectives for this study after the discovery of the gene fusion in the index patient sample:

1. Functional characterization of *CD74-NRG1* gene fusion.

As *CD74-NRG1* gene fusion is a completely unknown gene fusion there was first the need to investigate basic properties of the gene fusion such as localization, interactions partners and functional relevant domains. The next step was to investigate biochemical effect in cells harboring the *CD74-NRG1* gene fusion as well as showing a functional change within these cells such as pathway activation or beneficial growth properties such as colony formation capabilities.

2. Therapeutical relevance of CD74-NRG1 gene fusion

Due to the initial study design, the finding of a novel oncogenic driver mutation might harbor therapeutic potential. Therefore on the basis of the functional characterization results, *CD74-NRG1* transduced cells were screened with different inhibitors. They were as well analyzed for inhibition of direct and indirect interaction partners and effects on downstream pathways. Thus,

potential treatment strategies were determined as well as possible resistance mechanisms due to *CD74-NRG1* itself. The final results should help to translate the findings into clinical studies and to help targeting *CD74-NRG1*-positive tumors in the future.

3 - Material and Methods

3.1 Antibodies

protein	company	order no.	dilution
β-actin-HRP	Santa Cruz	sc-47778	1:5000
AKT	Cell signaling	#9272	1:1000
p-AKT	Cell signaling	#9271	1:1000
CD74	Abcam	ab22604	1:1000 (WB) 1:80 (IP)
CD74-PE	Biolegend	#357603	1:100
ERK1/2	Cell signaling	#9102	1:1000
p-ERK1/2	Cell signaling	#9106	1:2000
HA-tag	Abcam	ab130275	1:1000 (WB) 1:100 (flow cytom.)
HER2	Cell signaling	#2242	1:1000
p-HER2	Cell signaling	#2243	1:1000
HER3	Cell signaling	#4754	1:1000
HER3 XP	Cell signaling	#12708	1:1000 (WB) 1:50 (IP)
p-HER3	Cell signaling	#4791	1:1000
MEK1/2	Cell signaling	#9122	1:1000
p-MEK1/2	Cell signaling	#9121	1:1000
NRG1 β1		AF-396-NA	1:200 (WB) 1:20 (flow cytom.)
S6	Cell signaling	#9202	1:1000
p-S6	Cell signaling	#9205	1:1000
gt-anti-rb-HRP	Millipore	#12-348	1:3000
gt-anti-ms-HRP	Millipore	#12-349	1:3000
dk-anti-gt-HRP	Millipore	AP-180P	1:3000
gt-anti-rb-488	Life Technologies	A-11008	1:800
dk-anti-gt-488	Life Technologies	A-11055	1:800
gt-anti-ms-488	Life Technologies	A-10680	1:800

Table 1: List of antibodies including application-specific concentrations.

3.2 Compounds

Compounds were either purchased at Selleck chemicals, Sigma-Aldrich or were a kind gift of Daniel Rauh, PhD and the Lead Discovery Center.

3.3 DNA and RNA handling

General DNA and RNA handling procedures were done according to standard manufacturer's instructions. Extraction of plasmid DNA of bacterial transformation was done with Machery Nagel NucleoSpin mini-kit (mini), Machery Nagel NucleBond Xtra Midi EF kit (midi) or Machery Nagel NucleoSpin Gel and PCR clean-up kit. For elution of DNA TE-buffer (5mM Tris/HCl, pH 8.5) was used in an assay dependent volume. RNA extraction was performed with RNeasy mini kit (Qiagen).

3.4 cDNA synthesis

After RNA isolation 1µl of RNA was used for first-strand cDNA synthesis with the SuperScript III reverse transcriptase kit (LifeTechnologies). All reactions were mixed on ice.

1µl	oligo$(dT)_{18}$ (100µM) (Thermo Fisher Scientific)
1µl	RNA template
1µl	dNTP mix (10mM) (Thermo Fisher Scientific)
up to 13µl	H_2O

The mix was heated in a thermocycler to 65°C for 5 minutes and cooled down on ice for 1 minute to let the oligo(dT) primers anneal to the poly-A overhangs of 3' mRNA. Following the standard procedures of the protocol the mix was placed again in a thermocycler for 50 minutes at 50°C following inactivation for 15 minutes at 70°C. Subsequently 1µl RNase H (Thermo Fisher Scientific) was added to the reaction for 20 minutes at 37°C to remove RNA that might interfere in downstream applications by the formation of RNA:DNA hybrids. Inactivation was taken out for 10 minutes at 65°C.

3.5 Sanger sequencing

All Sanger sequencing reactions were performed at the Cologne Center for Genomics (CCG) by dideoxy-sequencing with BigDye Terminator v3.1 (Applied Biosystems). For Sanger sequencing of specific regions from mRNA a mRNA-specific pre-amplification step with Maxima Hot Start Taq DNA

polymerase or Pfu DNA polymerase (Thermo Fisher Scientific) needed to be performed following general sequencing procedures.

3.6 Molecular cloning

3.6.1 PCR and restriction enzyme based cloning

Molecular cloning is being used in molecular biology to amplify DNA. It can be used for many purposes e.g. investigation of protein functions, gene-/protein editing, for therapeutics (recombinant Insulin, (Crea, Kraszewski, Hirose, & Itakura, 1978)) and many more. For conventional cloning restriction enzymes that can cut sequence-specific DNA motifs are used because only sequence compatible ends can ligate and assemble in a ligation reaction. All restriction enzymes were purchased from Fermentas or New England Biolabs. Transformation of Escherichia Coli (E.coli.) bacteria (DH5α, XL-Gold or NEB 5-alpha) were performed via standard protocols by heat shock. The *CD74-ROS* and *SLC-ROS* (long) genes were subcloned from the pcDNA3.1(+) vector to the standard retroviral pBabe-puro backbone via EcoRI and XhoI digest.

FIG-ROS (kind gift of Kwock-Kin Wong, MD, PhD), *HER2*, *HER3* and *CD74-NRG1* (cDNA) were PCR amplified with adapter primers containing restriction sites using proofreading PCR polymerases (Pfu-Ultra, Agilent; Q5 or Phusion, New England Biolabs) and cloned into pBabe-puro, pBabe-hygro and/or pBabe-neo retroviral vector. The truncated version of *CD74-NRG1* lacking the EGF-like domain of *NRG1* was PCR amplified with primers containing suitable restriction sites and an additional stop codon.

Hemagglutinin tag (HA) was added to full-length *CD74-NRG1* using gene-specific primers with a HA tag containing overhang.

3.6.2 Gibson assembly

Gibson assembly is a technique developed by Daniel Gibson at the J. Craig Venter Institute in 2009. The assembly is completely independent of restriction enzymes and is based on an isothermal reaction with three different

enzymes (5' T5 exonuclease, Q5 DNA polymerase, Taq DNA ligase) in one reaction (Fig. 12).

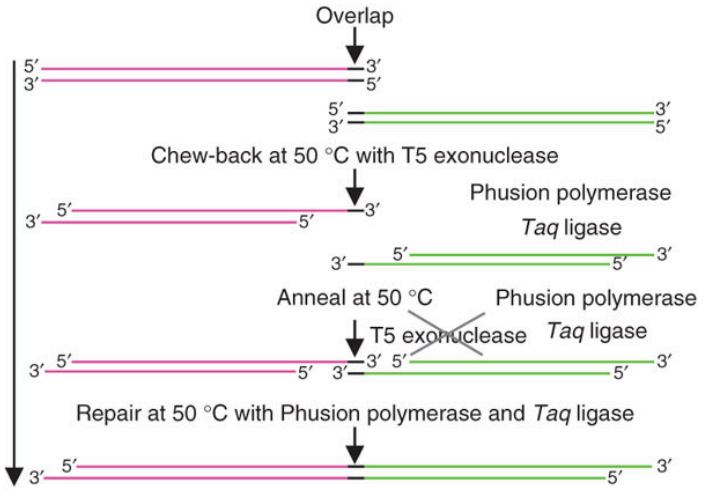

Figure 12: Mechanism of isothermal Gibson-Assembly reaction. Schematic cartoon of the three steps co-occurring in the isothermal Gibson assembly reaction (Gibson et al., 2009).

As the reaction takes place at 50°C, which is a trade-off of the optimal working temperatures of the three enzymes, the most critical step is that the melting temperature of the complementary overhangs created by the 5' T5 exonuclease (first step) do match an annealing temperature of at least 50°C. The second and third step cannot be divided into independent steps. When the overlapping single-stranded DNA overhangs anneal to each other the DNA polymerase fills up the gaps between the chew backs and the DNA ligase seals the nicks in between (Fig. 12). Gibson assembly is therefore the method of choice being independent of any restriction enzyme and joining several DNA parts in one reaction to a completely new vector and/or gene fusion. The *mCherry* fluorescent protein and *CD74-NRG1*/*CD74-NRG1_del* have been joined via Gibson assembly to the pBabe-mCherry-CD74-NRG1(_del) vector and used for immunofluorescent studies. The same approach has been used for generating the inducible mouse-targeting vector SERCA-Frt-STOP-FRT_LoxP-CD74-NRG1_LoxP (SERCA vector was a kind gift of Thomas Wunderlich, PhD (Belgardt et al., 2008)).

3.7 Primer

3.7.1 Cloning Primer (5' to 3'):

Name	length	sequence
FIG-ROS1F_BamHI	23	GCATGGATCCATGTCGGCGGGCG
FIG-ROS1R_SalI	35	GCATGTCGACTTAATCAGACCCATCTCCATATCCA
CD74-NRG1F_BamHI	24	ctatGGATCCATGCACAGGAGGAG
CD74-NRG1R_SalI	28	gatcGTCGACTTATACAGCAATAGGGTC
NRG1_truncated+SalI_R	37	GCATGTCGACCTAGAAAGTTTTCTCCTTCTCCGCACA
CD74-NRG1+HA-TAG_SalI	73	GATCGTCGACCTACTAGGAGGCGTAGTCAGGCACGTCGTATGGGTAGGCCATTTCAGGCAGAGACAGAAAGGG
ErbB3_F_SalI	27	GCATGTCGACATGAGGGCGAACGACGC
ErbB3_R_SalI	31	GCATGTCGACCGTTCTCTGGGCATTAGCCTT
HER3-pB-Eco-Sal_F	41	AGTGTGGTGGTACGTAGGAATTCATGAGGGCGAACGACGCT
EGFP_R+pB_Sal	43	CACACATTCCACAGGGTCGACTAGCTACTAGCTAGTCGAGATC
HER3-EGFP_R	27	CTCACCATCGTTCTCTGGGCATTAGCC
HER3-EGFP_F	26	AGAGAACGATGGTGAGCAAGGGCGAG
pB-Eco-Sal_mCherry	41	AGTGTGGTGGTACGTAGGAATTCATGGTGAGCAAGGGCGAG
mCherry-CD74-NRG1_R	28	CTGTGCATCTTGTACAGCTCGTCCATGC
CD74-NRG1 (mCherry)_F	27	CTGTACAAGATGCACAGGAGGAGAAGC
pB-Eco-Sal_mCherry-CD74-NRG1_R	42	CACACATTCCACAGGGTCGACTATTCAGGCAGAGACAGAAAG
pB-Eco-Sal_mCherry-CD74-NRG1_DEL_R	42	CACACATTCCACAGGGTCGACTAGAAAGTTTTCTCCTTCTCC
CAG_NEW_SERCA_F	67	AAACTCTTCGCGGTCTTTCCAGTGGTTAATTAAAGTTATAATCGCTGAGGTAATATTTAAAATCATT
CAG_Reverse_new	45	CCACGAAGGAGTGAGGGCTGGATATGGAAAGACCGCGAAGAGTTT
KANA_Stop_new_Kill_PacI_F	78	TATCCAGCCCTCACTCCTTCGTGGGAAGTTCCTATTTCTCTAGAAAGTATAGGAACTTCGGATCCGAACAAACGACCCA
KANA-NEW_25nt_added_R	93	GGTCGACCACTGTGCTGGCGAATTCGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCAAGCTTACTTACCATGTCAGATCCAG

CD74-NRG1_25nt_added_LoxP	97	GGCGCGCCGAATTCGCCAGCACAGTGGTCGAC CATAACTTCGTATAGCATACATTATACGAAGTTAT CGCCGCCACCATGCACAGGAGGAGAAGCAG
CD74-NRG1_NEW_SERCA_R	95	CGGCCGCTCTAGAACTAGTGGATCCCCCGGGAT AACTTCGTATAATGTATGCTATACGAAGTTATTTT ATTCTATTCAGGCAGAGACAGAAAGGG

3.7.2 Sequencing Primer (5' to 3'):

Name	length	sequence
pBabe_F	20	CTTTATCCAGCCCTCAC
pBabe_R	20	CAAATCAACCACAAGCTGGA
Fig-ROS_1_R	20	CTCGCCCCATATACTTCAGC
Fig-ROS_2_F	20	CAAATCAACCACAAGCTGGA
Fig-ROS_2_R	20	ATCCACTTCAGGAGCCACAT
Fig-ROS_3_F	20	TGACTTGAAACGACCAATGC
Fig-ROS_3_R	20	CATCAGATGTGCCTCCTTCA
Fig-ROS_4_F	19	CCAAGGAAGGGGTGACAGT
Fig-ROS_4_R	20	GCTGGATAAGGCTGATGACC
Fig-ROS_5_F	22	TGGTTGACCTTGTAGACCTGTG
Fig-ROS_5_R	20	GGCATAGTTCAGGCCTTCAG
Fig-ROS_6_F	24	CAGGACCAACTTCAGTTATTCAGA
Fig-ROS_6_R	20	CTTGCCAGAAGGGCAGTAAG
CD74-ROS_1F	20	CCCCTTGAACCTCCTCTTTC
CD74-ROS_1R	19	ATCCGCATCTTGCTCACAG
CD74-ROS_2F	19	AAGCCTGTGAGCAAGATGC
CD74-ROS_2R	20	CAGCTCTTTGTCCTCGTTGA
CD74-ROS_3F	20	GCGGCTGAAGAATCAGAAGT
CD74-ROS_3R	20	CACAGGTCCACCAGATCCAC
CD74-ROS_4F	20	TGTATCTGGAACGCATGCAC
CD74-ROS_4R	20	CGAAGCTCTCATTGATCACG
CD74-ROS_5F	20	GGCGTGATCAATGAGAGCTT
CD74-ROS_5R	20	ACTTTCCACACCTGGTTGCT
SLC-ROS_1F	20	CCAGTGTGGTGGTACGTAGG
SLC-ROS_1R	20	TCTGGTTCTGCAGGTTGTTG
SLC-ROS_2F	20	GCTGCCGGATCACCTACTAC
SLC-ROS_2R	20	CCTTCTTCAGGGTTTTCACG
SLC-ROS_3F	18	ACCGCCGTGGATATTCTG
SLC-ROS_3R	20	GATCTCCCAGATCAGGATGC
SLC-ROS_4F	20	CTGATGGACGGCATCTTCAC
SLC-ROS_4R	19	GGGCCCTCAGACTTTTCCT
SLC-ROS_5F	20	GCCCTGATGGAAACAAAGAA
SLC-ROS_5R	20	CCACACCTGGTTGCTGACTA
CD74-NRG1_seq1_F	25	GCAGAATGCCACCAAGTATGGCAAC
CD74-NRG1_seq1_R	25	GTTGCCATACTTGGTGGCATTCTGC

CD74-NRG1_seq2_F	24	CATCATGTGTGTGGTGGCCTACTG
CD74-NRG1_seq3_F	24	GAAATGTCTCCACCCGTGTCCAGC
ErbB3_1R	20	ACAGCTTCTGCCATTGTCCT
ErbB3_2F	20	CCCTCAGGACACAGACTGCT
ErbB3_2R	20	TGTTGCTCGAGTCCACAGTC
ErbB3_3F	20	CCACATGCACAACTTCAGTG
ErbB3_4F	20	CGGGCTCTGATACTTGTGCT
ErbB3_5F	20	TCCCTGAGGGTGAATCAATC
ErbB3_6F	20	TGGTGTGACAGTTTGGGAGT
ErbB3_7F	20	GGAGTCTTGCCAGGAGTCTG
ErbB3_3R	20	TCGACAGGACAAGCACTGAC
ErbB3_4R	20	CGTTCCAAGTATCGCCTCAT
ErbB3_5R	20	AAATCTGCCACCTGAACCTG
ErbB3_6R	20	GTTGTCCTCCTCTGCTTCCA
ErbB3_7R	20	CCCAGGACAGAACTGAGACC
ErbB3_5'UTR_F	19	ATTTGCAACCTCCGCTGCC
ErbB3_3'UTR_R	20	GGAAAAGGGGCTGGGACCTG
ErbB2_5'UTR_F	19	AATGGCCAGGACAAACGCA
ErbB2_3'UTR_R	20	AGCCATCTGGGAACTCAAGC
ErbB2_1R	19	GCGTCAGGCTCTGACAATC
ErbB2_2F	20	CCCAGCTCTTTGAGGACAAC
ErbB2_2R	19	GTCCCCATCAAAGCTCTCC
ErbB2_3F	20	GCCCTGGTCACCTACAACAC
ErbB2_3R	20	TGCCTGGCATTCACATACTC
ErbB2_4F	19	CTACTCGCTGACCCTGCAA
ErbB2_4R	20	AAGCACCTTCACCTTCCTCA
ErbB2_5F	20	CGGTGTGAAACCTGACCTCT
ErbB2_5R	19	ATCCACTTGATGGGCACCT
ErbB2_6F	20	AAGCATACGTGATGGCTGGT
ErbB2_6R	20	GCCACTCCTGGTAGATGAGC
ErbB2_7F	19	CCCCATCTGCACCATTGAT
ErbB2_7R	20	GGGTCCTGGTCCCAGTAATA
ErbB2_8F	20	TTTGATGGTGACCTGGGAAT
EGFR _1116_R	20	TGATGGAGGTGCAGTTTTTG
EGFR _120_R	20	TGCCTTGGCAAACTTTCTTT
EGFR _1266_F	20	GACCTCCATGCCTTTGAGAA
EGFR _1609_R	20	CTGACATTCCGGCAAGAGAC
EGFR _1760_F	20	CCAGTGTGCCCACTACATTG
EGFR _2135_R	20	CCTCAAGAGAGCTTGGTTGG
EGFR _2265_F	20	GAAGCAACATCTCCGAAAGC
EGFR _2635_R	22	TGCATGGTATTCTTTCTCTTCC
EGFR _2755_F	20	CCAAGCCATATGACGGAATC
EGFR _3133_R	20	AGAGAGCTCAGGAGGGGAGT
EGFR _3277_F	20	TCCTCCCAGTGCCTGAATAC
EGFR _3608_R	20	TTCTGCATTTTCAGCTGTGG
EGFR _608_R	20	ATTGGGACAGCTTGGATCAC

EGFR _752_F	19	GAGAGCGACTGCCTGGTCT
EGFR_267_F	20	CAGGAGGTGGCTGGTTATGT
EGFR_81_F	18	GAGTCGGGCTCTGGAGGA
KRAS-cDNA-A-F	19	GGAGAGAGGCCTGCTGAAA
KRAS-cDNA-A-R	21	TGGCAAATACACAAAGAAAGC
KRAS-cDNA-B-F	20	TACAGTGCAATGAGGGACCA
KRAS-cDNA-B-R	23	TGCTGATGTTTCAATAAAAGGAA
KRAS-cDNA-C-F	23	GGAAATAAATGTGATTTGCCTTC
KRAS-cDNA-C-R	22	CTAACAGTCTGCATGGAGCAGG
SERCA-Stop_1F	20	CCTCGCTAGCATCTGTAGGG
SERCA-Stop_1R	20	GCGTTGGCTACCCGTGATAT
SERCA-Stop_2F	20	CATGGGTCACGACGAGATCC
SERCA-Stop_2R	20	GCCCTGAATGAACTGCAGGA
SERCA-Stop_3F	20	GTGCCCAGTCATAGCCGAAT
SERCA-Stop_3R	20	GCCATATTTGGTGTCCCCGA
SERCA-Stop_4F	20	GAAACCACCGTTGCCGTAAC
SERCA-Stop_4R	24	GCCTCATCATCACTAGATGGCATT
SERCA-Stop_5F	21	AGCAGTGGTGGAATGCCTTTA
SERCA-Stop_5R(IRES)	19	GGCGGAATTGGGGTACCCT
SERCA-Stop_6F	20	CTCCCCCTGAACCTGAAACA

Table 2: List of primer sequences used for the conducted experiments.

3.8 Cell lines

Ba/F3 cells were a kind gift from Nikolas von Bubnoff. NIH-3T3 cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). HEK293T, H322, H1395, H1563, H1568, HCC827, PC9 and SKBR-3 have been purchased from the American Type Culture Collection (ATCC). H3122 cells were a kind gift from Christine Lovly, MD, PhD.

NIH-3T3 cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco) and 1% penicillin/streptomycin (PS) (Gibco) (≙ 50units/ml penicillin; ≙ 50µg/ml streptomycin). Ba/F3 cells, HEK293T, H322, H1395, H1563, H1568, HCC827, PC9 and SKBR3 cells were cultured in RPMI (Gibco) supplemented with 10% FCS and 1% PS. All cells were grown in a humidified incubator at 37°C and 5% CO_2. For passaging adherent cells were split at 80 – 90% confluence. Cells were washed with Phosphate Buffered Saline (PBS) (Gibco) and incubated in 0.25% Trypsin/EDTA (Gibco).

After detachment of cells from the flask, Trypsin reaction was inactivated with at least twice the amount of culture medium and split appropriately.
For long-term storage of cells, a suitable amount of cells was washed, trypsinized and centrifuged at 300g for 5 minutes. The cells were resuspended in freezing media (DMEM or RPMI, 10% FCS, 1% PS and 10% DMSO) and frozen in cryo vials. The initial freezing step was done in polyfoam freezing boxes to let the cells slowly cool down to -80°C, before taking the cells to a liquid nitrogen tank (-196°C) for long-term storage within the next days.

3.9 Virus production

HEK293T cells were plated to 70% of confluence in according growth media (HEK293T cells can adapt their metabolism with different media) supplemented with 10% FCS and 1% PS at 37°C, 5% CO_2 over night. The next day transfection reaction is set up for retroviral packaging vector (pCL-eco for mouse cells; pCl-ampho for human cells, Imgenex) and the pBabe retroviral vector containing the gene of interest (Tab.3).

	6cm / T25	T75	T175
TransIT-LT1 (µl)	14	42	84
Opti-MEM (µl)	486	1358	2716
DNA (µg)	4.6	14	28
Media (ml)	3.5	10	20

Table 3: Amounts of TransIT, Opti-MEM, DNA and growth media used for transfection.

For transfection reaction TransIT-LT1 (Mirus) was added dropwise to Opti-MEM+Glutamax media (Gibco) and incubated for 5 minutes. The plasmid DNA containing pBabe retroviral vector and packaging vector are added to the Opti-MEM:TransIT-LT1 mixture and incubated for 25 minutes at room temperature in order to let the TransIT-LT1:DNA complexes being formed. The mixture is added dropwise to the HEK293T cells into complete growth media and virus-containing supernatant is collected after 48 – 60 hours. The

supernatant is centrifuged at 300g for 5 minutes and sterile filtered through a 0.45µm nitrocellulose filter to make sure no cellular debris is left in the supernatant. The virus containing supernatant is kept for up to 3 months at 4°C.

3.9.1 Stable transduction of cells

For stable expression of the desired gene cells were seeded to an appropriate flask to 50% confluence. Viral supernatant (80% of final volume) and 20% fresh growth media were mixed and 8µg/ml polybrene (Santa Cruz) was added to the cells. The viral supernatant was kept on the cells for 24 – 48 hours to allow proper infection of cells. After 48 hours of the initial transduction the media was exchanged with complete growth media containing an appropriate amount of antibiotics (3µg/ml puromycin, 800µg/ml hygromcyin, 800µg/ml geneticin; LifeTechnologies). Antibiotics were exchanged every 4 days until control cells (non-transduced cells) were negatively selected. Proper expression of gene of interest was checked via immunoblot and backups of cells were frozen for long-term storage in liquid nitrogen.

3.10 Preparation of cell lysates

Cells were treated prior lysing the cells accordingly. For starving cells, cells were washed with PBS and grown in media with 0% FCS and 0% PS for at least 24 hours. Cells were washed with cold PBS to remove residual media. Cells were lysed in lysis buffer (Cell Signaling: 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1µg/ml leupeptin; Roche: 1x Compete mini; Roth: 1mM PMSF; Calbiochem: 200µl Phophatase inhibitor cocktail set III; for 10ml of 1x lysis buffer) and incubated on ice for 10 minutes. Lysates were centrifuged for 10 minutes, 14,000rpm at 4°C. Supernatant was collected to a fresh tube, kept on ice and used for determining protein concentrations using BCA Protein Assay (Thermo Fisher Scientific). Cell lysates were either directly used for SDS polyacrylamide gel electrophoresis

or stored at -80°C. Prior loading the lysates on a SDS polyacrylamide gel, samples were mixed with 5x Laemmli buffer (250mM Tris (pH 6.8), 50% glycerol, 5% β-mercaptoethanol, 10% SDS and 0.05% bromphenol blue) and boiled at 95°C for 5 minutes to denature the proteins in the cell lysates and apply a general negative charge to each protein according to their mass (Shapiro, Viñuela, & Maizel, 1967).

3.11 SDS-polyacrylamide gel electrophoresis (PAGE)

30 – 50µg boiled cell lysates were loaded on Novex® 4-12% Tris-Glycin polyacrylamide gels. As a protein size standard PageRuler™ Plus Prestained Protein Ladder (Fermentas) was used. The proteins were separated at 120V in SDS running buffer (192mM Glycin, 25mM Tris-HCl, 0.1% SDS) until the migration front of bromphenol blue has reached the end of the gel.

3.12 Western Blot / Immunoblot

For blotting the proteins on a polyvinylidene fluoride (PVDF) membrane the PVDF membrane had to be incubated in methanol for 1 minute and equilibrated in transfer buffer (25mM Tris-HCl, 192mM Glycin, 20% methanol). The polyacrylamide gel was placed in a sandwich between PVDF membrane, whatman paper and sponges to guarantee a proper electric field in between anode and cathode to let the protein migrate and bind on the PVDF membrane. The transfer was run for 90 minutes at 25V. After the transfer the membrane was blocked in 5% skimmed milk/TBST (50mM Tris-HCl, 150mM NaCl, pH 7.4, 0.05% Tween-20) for 1 hour at room temperature. Primary antibodies were diluted according to table 1 in TBST and incubated over night at 4°C on a rotator. The next day membranes were washed extensively with TBST prior incubation with isotype-specific secondary HRP-conjugated antibody for 1 hour at room temperature. After extensive washing to remove unbound unspecific antibody with TBST at room temperature, membranes were incubated with enhanced chemiluminescence reagent (ECL) (GE

Healthcare) and western blots were captured and developed on ECL Hyperfilm (GE Healthcare).

3.13 Co-Immunoprecipitation

A co-immunoprecipitation is used to study the interaction of different proteins with each other as part of a complex. In a final volume of 150µl 400µg of protein lysate was preincubated with 25µl of prewashed agarose beads (Santa Cruz) for 1h at 4°C for preclearing the lysates from unspecific binding proteins to the beads. Beads and lysates were separated via centrifugation at 1000g, 4°C for 5 minutes. For enrichment of CD74-NRG1 or HER3 protein, lysates were incubated with a CD74 or HER3 antibody at 4°C over night and immunoprecipitated with Protein A-agarose (mouse) or Protein G-PLUS-agarose beads (rabbit). Beads were washed extensively with PBS. After the final washing step, beads were resuspended in 30µl of Laemmli buffer and boiled for 5 minutes at 95°C. Beads were pelleted and supernatant was directly loaded on a NOVEX® 4-12% polyacrylamide gel following standard western blotting procedure. Full lysates, lysate incubated only with agarose beads or only with antibody were used as controls.

3.14 Supernatant concentration

Supernatant of NIH-3T3 cells transduced with *empty vector* control or *CD74-NRG1* vector was placed on ice and centrifuged at 300g, 4°C for 5 minutes to pre-clear the supernatant from cellular debris. The supernatant was subjected to a Vivaspin® 6 centrifugal concentrator (Sartorius) with a pore size of 3kDa in a swinging bucket cooling centrifuge at 3000g, 4°C for 2 hours. Supernatant was stored at -80°C. For immunoblotting 50µl was mixed with 5x Laemmli buffer and loaded on a Novex® 16% polyacrylamide gel (LifeTechnologies) following standard procedures for western blotting.

3.15 Soft agar assay

2% and 1.2% Agarose Type IX-A ultra low (Sigma-Aldrich) was dissolved in ddH_2O and heated in the microwave until the agarose was dissolved. Agarose solution was sterile aliquoted and solidified at 4°C over night. Prior use 2% and 1.2% Agarose was boiled for 30 minutes in a thermomixer at 95°C and cooled down to 60°C. 2x RPMI supplemented with 20% FCS and 2% PS was prewarmed to 37°C. For preparing the bottom agar equal volumes of 2% Agarose solution and 2x RPMI were mixed and 500µl was used per well in a 24-well plate. The plate was stored at 4°C for 30 minutes to allow the 1% Agarose:RPMI media to solidify. The plate was allowed to warm to room temperature for 15 minutes. H1568 cells were split using standard protocol and cell numbers were assessed in a Z2 particle counter (Beckman Coulter). 1.2% Agarose solution was slowly cooled down to 44°C in a thermomixer and mixed with equal volumes of 2x RPMI. 8000 cells were mixed with 500µl of 0.6% Agarose:RPMI media and plated on the solidified bottom agar. The plate was placed at 4°C for 10 minutes to let the top agar solidify and incubated at 37°C, 5% CO_2 over night. The next day, top agar was carefully covered with RPMI media and incubated for 14 days. Brightfield pictures were taken with a Zeiss Axiovert 40 CFL microscope at 100x magnification. Colony size was assessed with ImageJ.

3.16 Proliferation assay

H322 and H1568 cells were seeded in 6 wells (initial start 40,000 cells). At day 1, 2 and 5 duplicates of cells were trypsinized and counted with a Z2 particle counter (Beckman Coulter). Data were analyzed with Excel and graphs were plotted with R.

3.17 Viability assay

Cells were plated at low density in a white-bottom 96-well plate (Corning®) in 90µl media/well over night. Compounds were prepared by serial dilution. DMSO was added to control wells in the highest dilution used in the assay.

The cells were treated for 96 hours with the compounds following determination of ATP content as surrogate for viability by CellTiter-Glo® assay (CTG) (Promega). CTG was incubated for at least 20 minutes on the cells up to 4 hours without light. Luminiscence was assessed on an Infinite 200 Pro microplate reader (Tecan). Data were analyzed in Excel and plotted with R.

3.18 Flow cytometry

For flow cytometry 200,000 cells were resuspended in 100µl PBS supplemented with 2% FCS (FACS buffer) in 96-well plates with a U-shaped well bottom. For extracellular staining cells were incubated with primary antibody for 30 minutes in 30µl FACS buffer covered from light.
For intracellular staining cells were fixed in 100µl 4% paraformaldehyde/PBS solution (PFA) for 30 minutes protected from light and washed extensively. For perforating the cell membrane, cells were resuspended in Saponin-buffer (0.5% Saponin, 5% FCS in PBS) and incubated in 30µl Saponin buffer with primary antibody. Cells were washed 3 times with 150µl FACS buffer and incubated with secondary antibody for 30 minutes in 30µl FACS buffer/Saponin buffer covered from light. Cells were washed extensively and resuspended in 100µl FACS buffer. Flow cytometry was performed on a BD LSR II at the University Hospital Bonn by Dr. Juliane Daßler. Results were analyzed with FlowJo (Treestar).

3.19 Immunofluorescence

Cells were plated on glass cover slips at 60% confluence over night. The next day cells were washed with PBS and fixed with 4% PFA/PBS solution for 20 minutes protected from light. Fixation was stopped via extensive washing with PBS. Cells were embedded in ProLong® Gold antifade mounting media with DAPI (LifeTechnologies). Pictures were taken on a Leica TCS SP8 gSTED confocal microscope at the CECAD imaging facility at the Universitiy Hospital of Cologne.

4 - Results

4.1 Identification of *CD74-NRG1* gene fusion

The objective of this study was the identification of new oncogenic driver gene fusions in lung adenocarcinoma. Therefore tumor specimens from 26 lung adenocarcinoma patients of never-smokers that were negative for *KRAS* and *EGFR* mutations were used for transcriptome sequencing. After applying a novel detection algorithm that was tweaked to detect fusion genes from RNAseq data in 14/25 cases no oncogenic driver mutation could be found. In one patient sample an amplification of the *EGFR* locus was found and in 9/25 cases known fusion genes were detected. In one sample from a 64-year-old female patient with stage I invasive mucinous adenocarcinoma (IMA) subtype the novel *CD74-NRG1* gene fusion was discovered for the first time (Tab. 4).

SAMPLE	AGE	SEX	STAGE UICC	SMOKING STATUS	DRIVER
S00214	63	female	Ia	never	EGFR amp
S00686	68	male	Ia	never	EML4-ALK
S00688	46	female	Ia	never	EML4-ALK
S00754	39	female	IIIa	never	EML4-ALK
S00664	56	female	IIIb	never	CD74-ROS1
S00687	60	female	Ia	never	CD74-ROS1
S00545	68	female	IIIb	never	EZR-ROS1
S00751	65	female	IIa	never	KIF5B-RET
S01465	75	male	Ia	never	KIF5B-RET
S01276	66	female	IIIb	never	CCDC6-RET
S01052	64	female	Ib	never	**CD74-NRG1**
S00557	72	female	IV	never	Unknown
S00585	74	male	IV	never	Unknown
S00611	50	female	IV	never	Unknown
S00684	70	female	Ia	never	Unknown
S00726	79	female	IIa	never	Unknown
S00737	72	male	IV	never	Unknown
S00738	59	male	Ib	never	Unknown
S00747	63	female	IIIa	never	Unknown
S00752	48	male	IIIb	never	Unknown
S00755	71	female	IIIa	never	Unknown
S01156	74	female	IIIa	never	Unknown
S01194	73	male	Ib	never	Unknown
S01272	80	female	Ib	never	Unknown
S01337	66	female	IIIa	never	Unknown

Table 4: Clinical information on patients used in the screening study. Patient information, clinical stage and detected driver mutation of samples used for the transcriptome analysis.

In an additional screening cohort at the Aichi cancer center in Nagoya in 4/94

lung adenocarcinoma patients that were never-smoker and negative for *EGFR*, *KRAS*, *ALK*, *HER2* and *ROS1* mutations the *CD74-NRG1* gene fusion could be identified. The breakpoint analysis was performed at the Cologne Center for Genomics by Sanger sequencing from patient cDNA and validated to harbor the identical breakpoint as in the first index-case. Within this cohort all *CD74-NRG1* patients (4/15) were diagnosed with IMA. IMA is very prone to *KRAS* mutations which is reflected in the cohort. 6/15 (40%) of the cases harbored *KRAS* mutations, 4/15 (27%) possessed the *CD74-NRG1* gene fusion and in 5/15 (33%) the driver is still unknown (Fernandez-Cuesta et al., 2014; Maeda et al., 2012). Therefore *CD74-NRG1* gene fusion might be a new diagnostic marker and oncogenic event specific for IMA – especially as this finding could be validated in two completely independent cohorts (Gow, Wu, Chang, & Shih, 2014; Nakaoku et al., 2014).

4.2 Characterization of CD74-NRG1's general cellular properties

4.2.1 *CD74* promoter leads to expression of *CD74-NRG1* gene fusion

NRGs are generally not expressed in lung cells. Therefore, expression of the *CD74-NRG1* gene fusion needs to be driven by the *CD74* portion in patients harboring the fusion. Analysis of expression levels - fragments per kilobase of exon per million fragments mapped (FPKM) - of each individual gene (*CD74* and *NRG1*) was performed on patient samples from RNAseq reads. Expression levels of *CD74* were distributed in a wide range starting from 49 FPKM up to 941 FPKM (median 289 FPKM) (Fig. 13 A, upper part). In the index-case expression of *CD74* was 195 FPKM. Compared to this no *NRG1* isoform was expressed in any of the lung cancer specimens analyzed beside *NRG1 III-β3* in the index-case harboring the *CD74-NRG1* gene fusion with 74 FPKM (Fig. 13 A, lower part). The exact breakpoint of the CD74-NRG1 gene fusion is mapped to an intronic region of *CD74* (chromosome 5: 149,783,493) and *NRG1* (chromosome 8: 32,548,502). Notably reads of *NRG1 III-β3* were only detected after the breakpoint (Fig. 13 B). This is in line with the break-

apart fluorescence in situ hybridization (FISH) analysis showing that only one allel is affected and the other allel stays wild-type (Fig. 13 C) (FISH was performed at the Aichi cancer enter in Nagoya and analyzed by Yasushi Yatabe, MD, PhD) (Fernandez-Cuesta et al., 2014).

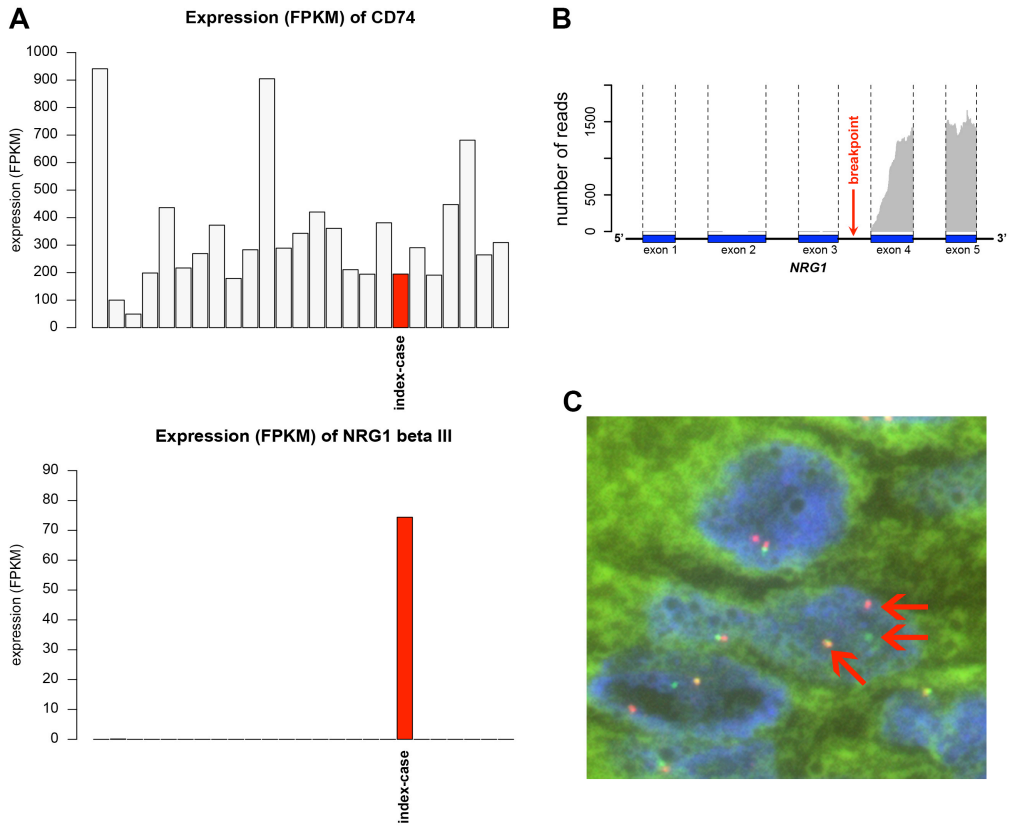

Figure 13: Expression analysis of *CD74-NRG1* gene fusion. (A) The *CD74* (upper part) promoter is used for the expression of the *NRG1 III-β3* (lower part) part of the *CD74-NRG1* gene fusion. (B) *NRG1 III-β3* reads could only be detected after the breakpoint. (C) *NRG1* break-apart FISH picture of the index-case patient showing that only one allel (red arrows) of *NRG1 III-β3* is affected by the chromosomal rearrangement (Fernandez-Cuesta et al., 2014).

4.2.2 Cellular localization of the *CD74-NRG1* gene fusion

To investigate the localization of the newly discovered *CD74-NRG1* gene fusion, NIH-3T3 mouse fibroblast were transduced with the pBabe retroviral vector system containing either *empty vector* (e.v.) control, *CD74-NRG1 or* carboxy-terminal hemagglutinin-tagged (HA) *CD74-NRG1* (CD74-NRG1-HA) (Fig. 14 A).

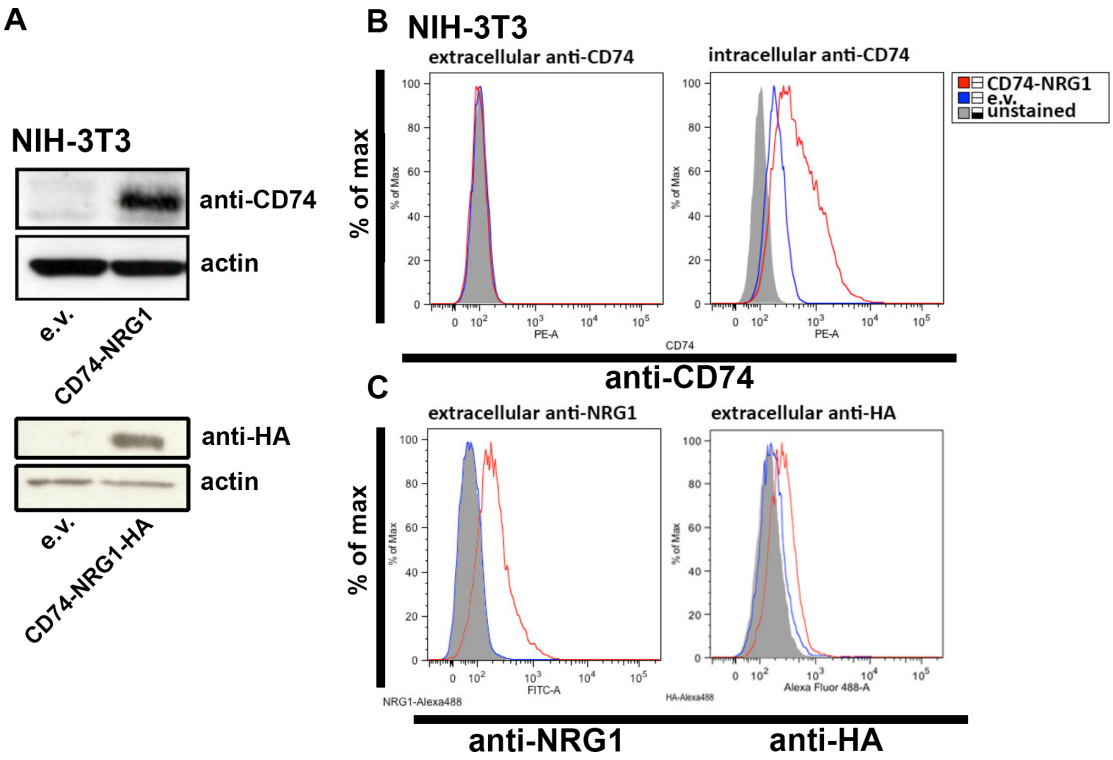

Figure 14: Analysis of CD74-NRG1 localization on the cellular membrane by flow cytometry. (A) Immunoblot analysis of NIH-3T3 cells for protein expression of CD74-NRG1 (upper part) and CD74-NRG1-HA (lower part). (B, C) Flow cytometric analysis of NIH-3T3 cells transduced with *empty vector* (e.v.), *CD74-NRG1* or *CD74-NRG1-HA* for extra- and intracellular binding of labeled antibodies (CD74, NRG1 and HA) (Fernandez-Cuesta et al., 2014).

Flow cytometric analysis for CD74 showed no shift in fluorescence intensities for extracellular stainings (without perforating the cell membrane) but showed a clear binding of the antibody for intracellular CD74 staining in *CD74-NRG1* transduced NIH-3T3 cells compared to *e.v.* control cells (Fig. 14 B). Extracellular stainings for NRG1 showed a shift in fluorescence intensities for *CD74-NRG1* transduced cells. As this shift was not as strong as with the CD74 antibody this finding was confirmed by staining against HA. CD74-NRG1-HA transduced cells showed an intensity shift with the HA antibody compared to *e.v.* control cells (Fig. 14 C). This analysis suggests that the CD74-NRG1 fusion gene can be localized within the cell membrane with the carboxy-terminal NRG1 part being expressed on the cell surface and the amino-terminal part of the fusion protein being localized inside of the cell which resembles the wild-type situation of NRG1 type III proteins (compare

Fig. 10). (The flow cytometry data were assessed together with Dr. Juliane Daßler, University Hospital Bonn.)

Localization studies were conducted via confocal microscopy imaging in NIH-3T3 cells transduced with *e.v.*, or *CD74-NRG1* and *CD74-NRG1_del* (truncated version of CD74-NRG1 lacking EGF-like domain) that were tagged at the amino-terminus of CD74-NRG1 with *mCherry* fluorescent protein (Xie, Qiao, Wu, & Tang, 2011). Localization of CD74-NRG1 and CD74-NRG1_del were assessed throughout the cell beside the nucleus (Fig. 15).

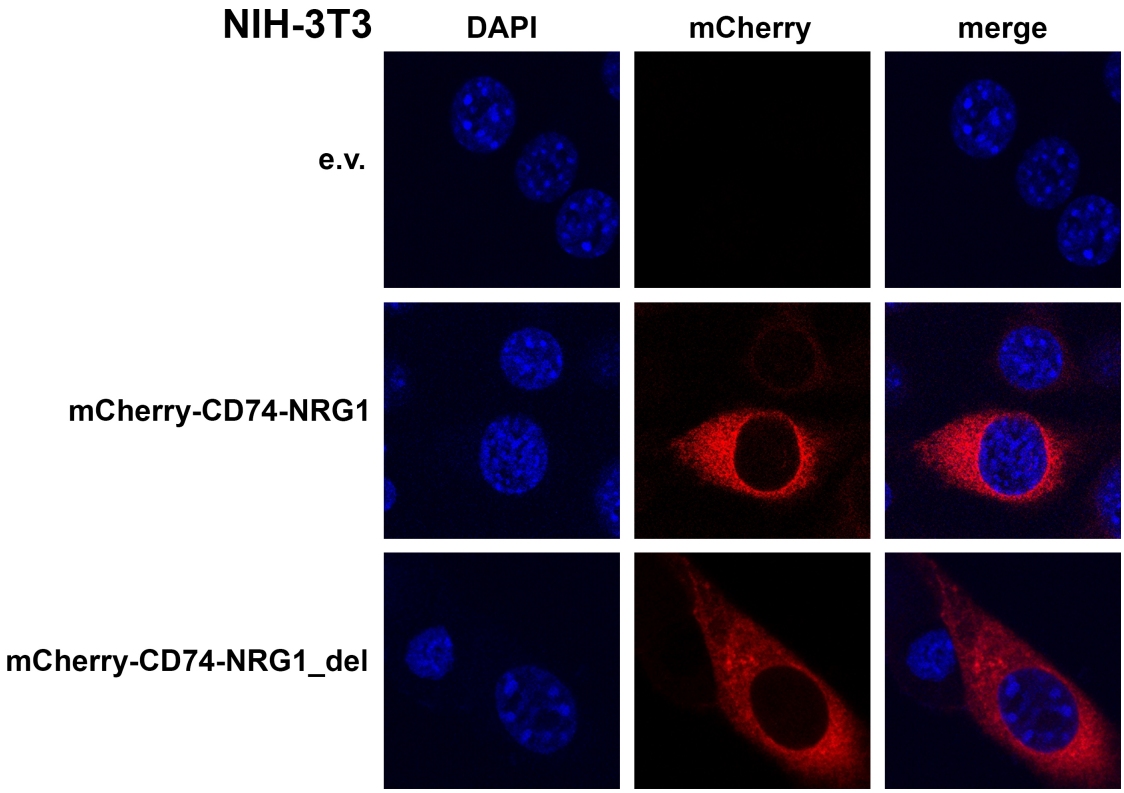

Figure 15: Confocal microscopy images of CD74-NRG1 and CD74-NRG1_del. NIH-3T3 cells were transduced with retrovirus containing *e.v.*, a *mCherry*-tagged version of *CD74-NRG1* or *CD74-NRG1_del*. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

In summary localization of the CD74-NRG1 gene fusion is not only restricted to the cell membrane but is as well localized in the cytoplasm of cells. Only the nucleus is clearly spared out for expression of the CD74-NRG1 gene fusion. This would be in line with immunofluorescence pictures published for CD74 and/or NRG1 expression showing cytoplasmic and membranous

stainings but no staining of the nucleus (compare ab22603 (CD74) and ab53104 (NRG1), Abcam and (Xie et al., 2011)).

4.2.3 CD74-NRG1's function is achieved via the NRG1-part of the gene fusion

When comparing different *CD74*-associated gene fusions in lung cancer it is striking that common gene fusions with *CD74* as fusion partner do harbor all the breakpoint within the same region. For example the breakpoint of *CD74-ROS* leads to a fusion on protein level at the exact amino acid position as in *CD74-NRG1* (Fernandez-Cuesta et al., 2014; Takeuchi et al., 2012). In *CD74-NTRK1* the fusion leads to a breakpoint prolonged *CD74* part of about 20 amino acids compared to the breakpoints of *CD74-NRG1* and *CD74-ROS* (Fig. 16 A) (Vaishnavi et al., 2013).

When transducing Ba/F3 murine pro-B-cells with retrovirus containing *CD74-ROS*, *FIG-ROS*, *SLC-ROS* or *EGFR L858R*, they become independent of IL-3 signaling. At the same time they developed an oncogene dependency on ROS1 or EGFR signaling. Targeting of ROS1 by crizotinib or EGFR by erlotinib inhibits ROS1-kinase or EGFR thereby inducing apoptosis in these cell lines but not vice versa (Fig. 16 B). These results indicate that the functionally active part and oncogenic switch is triggered by the ROS1-kinase or EGFR in these cells. The 5' fusion partners *CD74*, *FIG* and *SLC* do only lead to expression in the patients and may influence the localization but do not have an oncogenic function in terms of intrinsic pathway activation (Fig. 16 B) (Charest et al., 2003; Paez et al., 2004; Rikova et al., 2007). In analogy to the described gene fusions and the exact breakpoint position on protein level *NRG1* should trigger the oncogenic function in *CD74-NRG1*. Compared to the discovered gene fusions, *CD74-NRG1* represents the first gene fusion involving a ligand (NRG1) instead of a kinase (Fernandez-Cuesta et al., 2014).

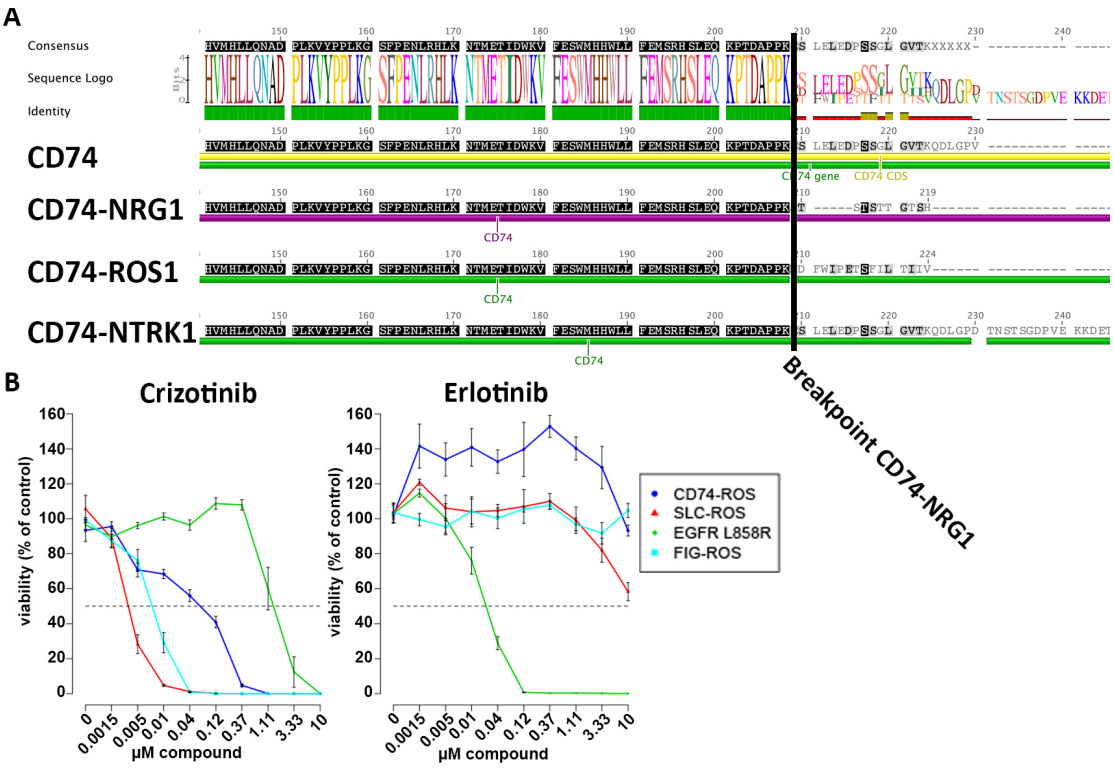

Figure 16: Comparison of different gene fusions to CD74-NRG1. (A) Amino acid sequence alignment of CD74-NRG1, CD74-ROS1 and CD74-NTRK1 on CD74. (B) Viability assay with CellTiter-Glo® on Ba/F3 cells transduced with corresponding gene fusions and screening with crizotinib or erlotinib. Error bars are calculated by standard deviations of the mean.

4.2.4 CD74-NRG1 interacts via its EGF domain with the HER3 receptor

The only functional domain within CD74-NRG1 fusion protein is the EGF-like domain that is preserved from NRG1 III-β3. To further characterize the functional effect of the EGF-like domain, a truncated version of CD74-NRG1 named CD74-NRG1_del lacking the EGF-like domain was generated (Fig. 17).

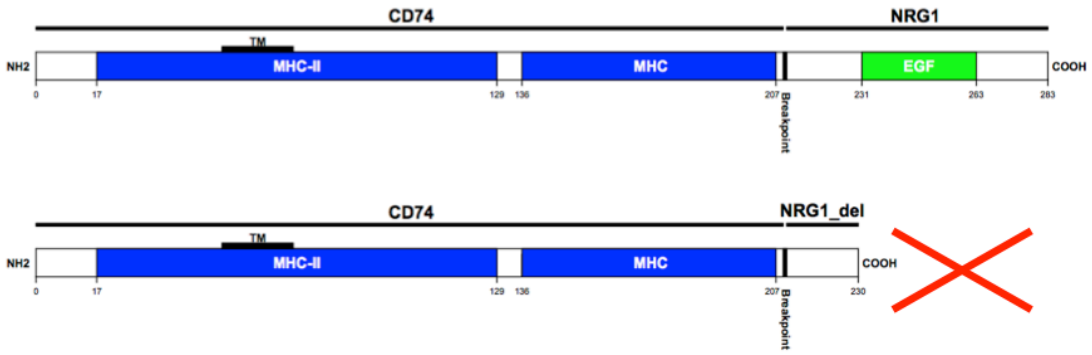

Figure 17: Schematic figure of CD74-NRG1 full-length and truncated variant CD74-NRG1_del. Structure of CD74-NRG1 (upper part) fusion protein and truncated version CD74-NRG1_del (lower part) lacking the EGF-like domain compared to full-length CD74-NRG1. (TM – transmembrane domain)

Due to earlier publications, NRG1 is described as a ligand of HER3 and HER4 receptors (Hynes & Lane, 2005; Sithanandam et al., 2003; Yarden & Sliwkowski, 2001). As only HER3 and not HER4 was expressed in the patient cohort (average of 31.4 FPKM (HER3) and 0.2 FPKM (HER4)) the interaction of NRG1:HER3 was further investigated. For this purpose co-immunoprecipitations of CD74-NRG1 and the HER3 receptor were conducted to elucidate if the CD74-NRG1 fusion protein is capable of physically binding to the HER3 receptor. Therefore NIH-3T3 cells expressing *HER2* and *HER3* together with either *empty vector* (*ev*), *CD74-NRG1* or *CD74NRG1_del* were generated. Additionally NIH-3T3 cells expressing *HER2* and *HER3* were generated as well as NIH-3T3 cells expressing *empty vector* (ev), *CD74-NRG1* or *CD74-NRG1_del* to further investigate the role of an auto- or paracrine binding mode.

The immunoblot analysis shows a clear expression and detection of the individual overexpressed proteins in the NIH-3T3 cells in the full lysate lanes (Fig. 18, lanes 1-3). Interaction of CD74-NRG1 fusion protein with the HER3 receptor could only be detected in the HER3 co-immunoprecipitation lysates from cells which were transduced additionally with full-length *CD74-NRG1* gene fusion including EGF-like domain. In cells transduced with *CD74-NRG1_del* or *empty vector* in addition to *HER2* and *HER3* no CD74-NRG1 protein could be detected in the HER3 co-immunoprecipitation (Fig. 18 upper part, lanes 4-6).

To check for a specific paracrine interaction (from one cell to another) of CD74-NRG1 fusion protein with HER3, 1:1 mixed cultures of NIH-3T3 cells transduced with *HER2* and *HER3* and NIH-3T3 cells transduced with *empty vector*, *CD74-NRG1* or *CD74-NRG1_del* were generated. Physical interaction could only be confirmed in mixed cultures of NIH-3T3 cells transduced with CD74-NRG1 mixed with NIH-3T3 cells transduced with HER2 and HER3 indicating a paracrine interaction of the NRG1 portion from the CD74-NRG1 fusion protein with the HER3 receptor via the EGF-like domain (Fig.18, lower part, lanes 4-6). Therefore CD74-NRG1 fusion shows interaction with surrounding cells and binding to the HER3 receptor thus very likely stimulating HER3 receptor signaling. In addition this experiments show that full-length CD74-NRG1 fusion protein binds to HER3 and does not need to be further processed (e.g. shed) for interaction.

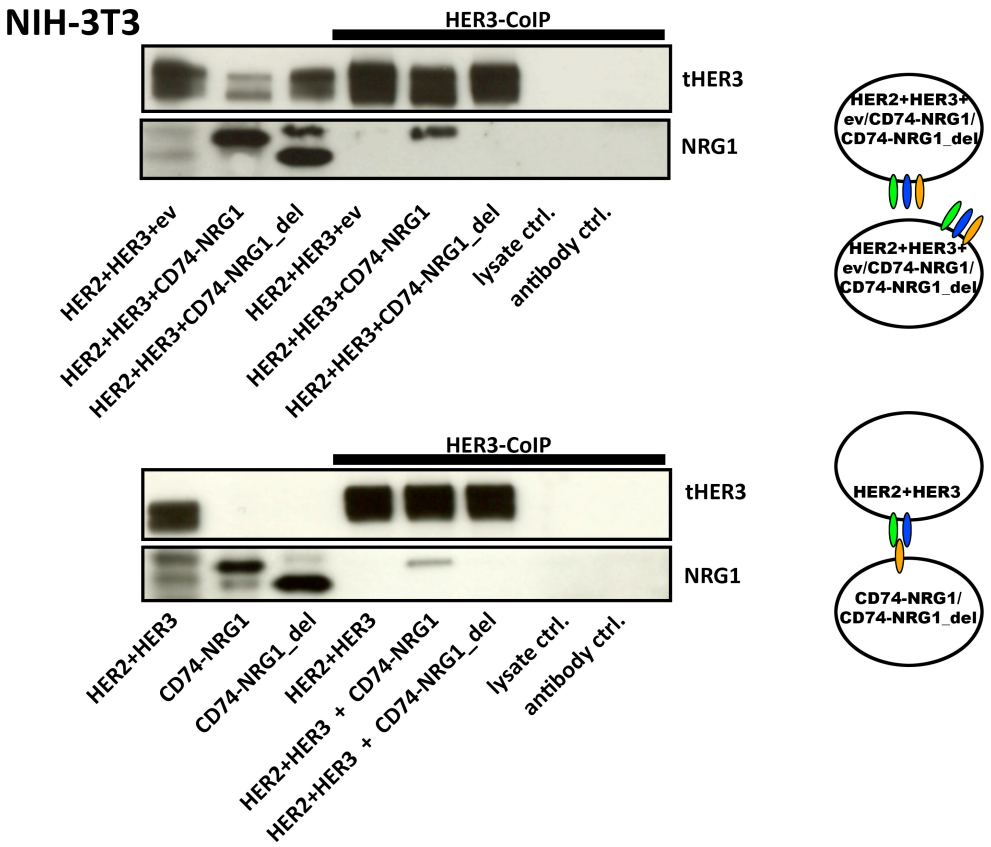

Figure 18: Interaction analysis of CD74-NRG1 with HER3 by HER3 co-immunoprecipitation. Immunoblot analysis including HER3 co-immunoprecipitation (CoIP) of NIH-3T3 cells transduced with *HER2* and *HER3* and/or *empty vector*, *CD74-NRG1* or *CD74-NRG1_del*. All genes are expressed in every NIH-3T3 cell

(upper part) or individual cells expressing *empty vector*, *CD74-NRG1* or *CD74-NRG1_del* were generated and mixed 1:1 with NIH-3T3 cells expressing HER2 and HER3 receptor (lower part) to investigate paracrine interaction of neighboring cells. One representative experiment is shown.

In the reciprocal experiment the identical cell lysates were used for the co-immunoprecipitation of CD74-NRG1 with a CD74 antibody and subsequent analysis of HER3 detection. The results confirm the immunoblot analysis of the HER3 co-immunoprecipitation. Only in cells or co-cultured cells which express full-length CD74-NRG1 fusion protein including the EGF-like domain, HER3 receptor is detected in co-immunuprecipitated samples indicating a physical interaction of the EGF-like domain of the NRG1 portion with the HER3 receptor as well. No HER3 receptor could be detected in cells transduced with *empty vector* or *CD74-NRG1_del* (Fig. 19, upper part). These results show a robust interaction of the EGF-like domain of CD74-NRG1 fusion with the HER3 receptor and confirm that cells expressing the *CD74-NRG1* gene fusion are capable of interacting as well as wild type *NRG1 III-β3* with the HER3 receptor in a paracrine mode of action. Notably, no additional processing of full-length CD74-NRG1 fusion protein was necessary to interact with the HER3 receptor confirming the results of HER3 co-immunoprecipitations. No shed NRG1 portion could be detected as described for other isoforms of NRG1 or claimed by Takashi Kohno's group for CD74-NRG1 fusion protein (Fig. 19, lower part) (Nakaoku et al., 2014).

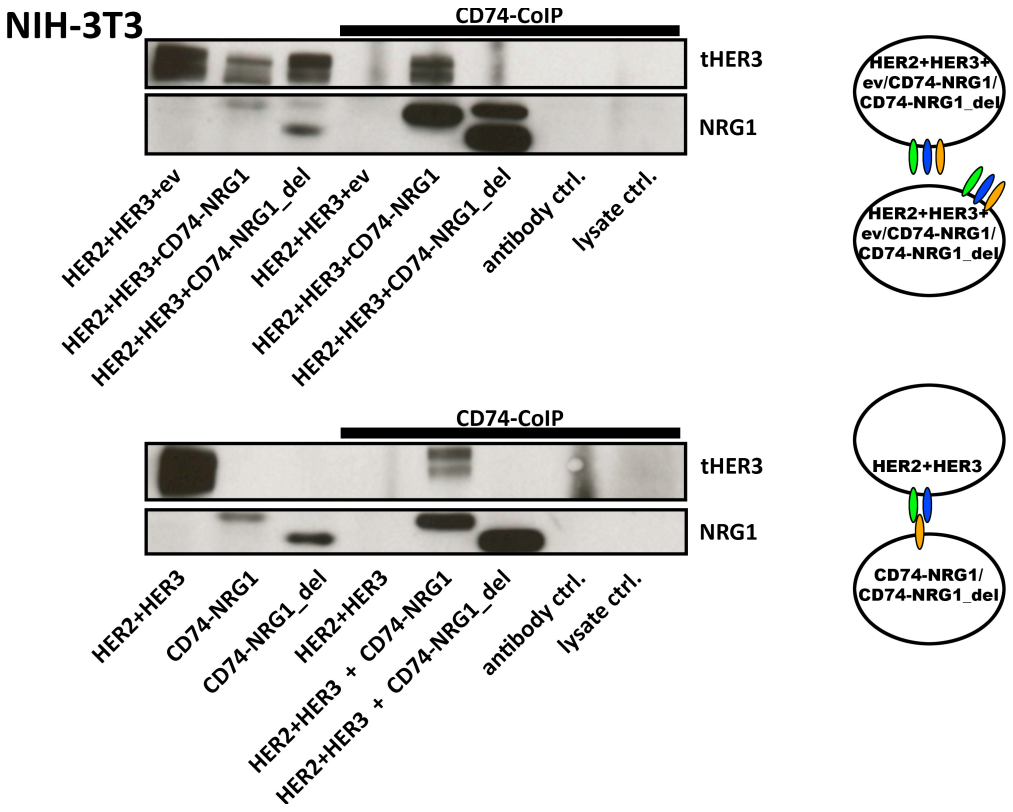

Figure 19: Interaction analysis of CD74-NRG1 with HER3 by CD74 co-immunoprecipitation. Immunoblot analysis including CD74 co-immunoprecipitation of different NIH-3T3 cells transduced with either *HER2*, *HER3* and *empty vector*, *CD74-NRG1* or *CD74-NRG1_del* (upper part) or to check for paracrine interaction in 1:1 mixed cell cultures (lower part).

4.2.5 CD74-NRG1 does not become shed in NIH-3T3 cells

Due to reports that NRG1 III-β3 harbors an own shedding site and is not exclusively membrane-tethered compared to the other NRG1 isoforms, CD74-NRG1 was investigated to its ability of becoming shed (Nakaoku et al., 2014). Shedding could already not been detected in co-immunoprecipitations of lysates from NIH-3T3 cells. Only full-length CD74-NRG1 could be detected in immunoblots of full lysates as well as of co-immunoprecipitated lysates (Fig. 18, 19). The NRG1 portion of the CD74-NRG1 fusion protein is predicted to show a molecular weight of 8kDa (http://bioinformatics.org/sms/prot_mw.html). Therefore supernatant of NIH-3T3 cells transduced with *empty vector* or *CD74-NRG1* was collected and applied on sample concentrating columns to concentrate the supernatant for proteins. Immunoblot analysis of the concentrated supernatants of NIH-3T3

transduced cells with *empty vector* or *CD74-NRG1* revealed no detectable protein with an NRG1 antibody below 10kDa. H1568 cells transduced with *empty vector* (e.v.) or *CD74-NRG1* were used as controls to show specific antibody binding (Fig. 20). Similar to the results of the co-immunoprecipitation no NRG1 protein could be detected with a polyclonal NRG1 antibody that is specifically directed against the EGF-like domain of NRG1. Therefore CD74-NRG1 seems at least not to become shed in NIH-3T3 cells indicating either i) a cell-type specific effect for processing membrane-bound NRG1 or ii) only a very minor portion of CD74-NRG1, fusion protein being processed that is undetectable in immunoblots. Beside this evidence from NIH-3T3 cells no further processed CD74 portion of CD74-NRG1 fusion protein could be detected in any immunoblot neither for murine nor in human lung adenocarcinoma cell lines (Fig. 14, 18, 19, 20, 21).

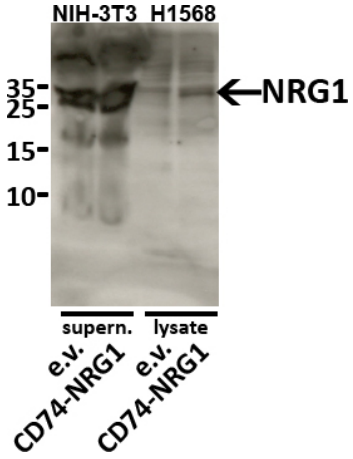

Figure 20: Immunoblot of concentrated supernatants of NIH-3T3 cells. Immunoblot against NRG1 protein of concentrated supernatant (supern.) from NIH-3T3 cells transduced with *empty vector* (e.v.) or *CD74-NRG1*. Lysates of H1568 lung adenocarcinoma cell line transduced with *empty vector* (e.v.) or *CD74-NRG1* served as antibody control. One representative experiment is shown.

4.3 Functional relevance of *CD74-NRG1* gene fusion

4.3.1 CD74-NRG1 can lead to increased HER3 phosphorylation and can activate major downstream pathways

As shown in Fig. 18 and 19 CD74-NRG1 can physically interact with the HER3 receptor, but its ability for HER3 receptor activation needs to be

proven. For this purpose H322 and H1568 lung adenocarcinoma cell lines that were wild-type for *KRAS*, *EGFR*, *HER2* and *HER3* were transduced with *empty vector* (*e.v.*), *CD74-NRG1* or *CD74-NRG1_del*. HER3 receptor activation was assessed via phospho-HER3 (pHER3) antibody and typical downstream targets of PI3K (phospho-AKT (pAKT) and phospho-S6 (pS6)) and MAPK (phospho-ERK (pERK)) pathway (Baselga & Swain, 2009; Yarden & Sliwkowski, 2001). Robust HER3 receptor activation could be observed in *CD74-NRG1* transduced H1568 and H322 cells compared to *empty vector* control and *CD74-NRG1_del* transduced cells. Subsequent PI3K and MAPK pathway activation could be observed in H1568 and H322 cells only harboring the full-length CD74-NRG1 fusion protein compared to control cells. In line with reports that NRG1 induces trans-phosphorylation of HER3 by HER2, no differences could be observed for activation of the HER2 receptor (Li et al., 2007). Therefore the oncogenic effect of the CD74-NRG1 fusion may be triggered by its ability to activate oncogenic HER3 receptor signaling and therefore augment survival and cell growth properties of cells (Fig. 21 A). In addition no HER4 receptor expression was detected by targeted sequencing from cDNA (data not shown) limiting the observed effects to CD74-NRG1:HER3:HER2 interaction.

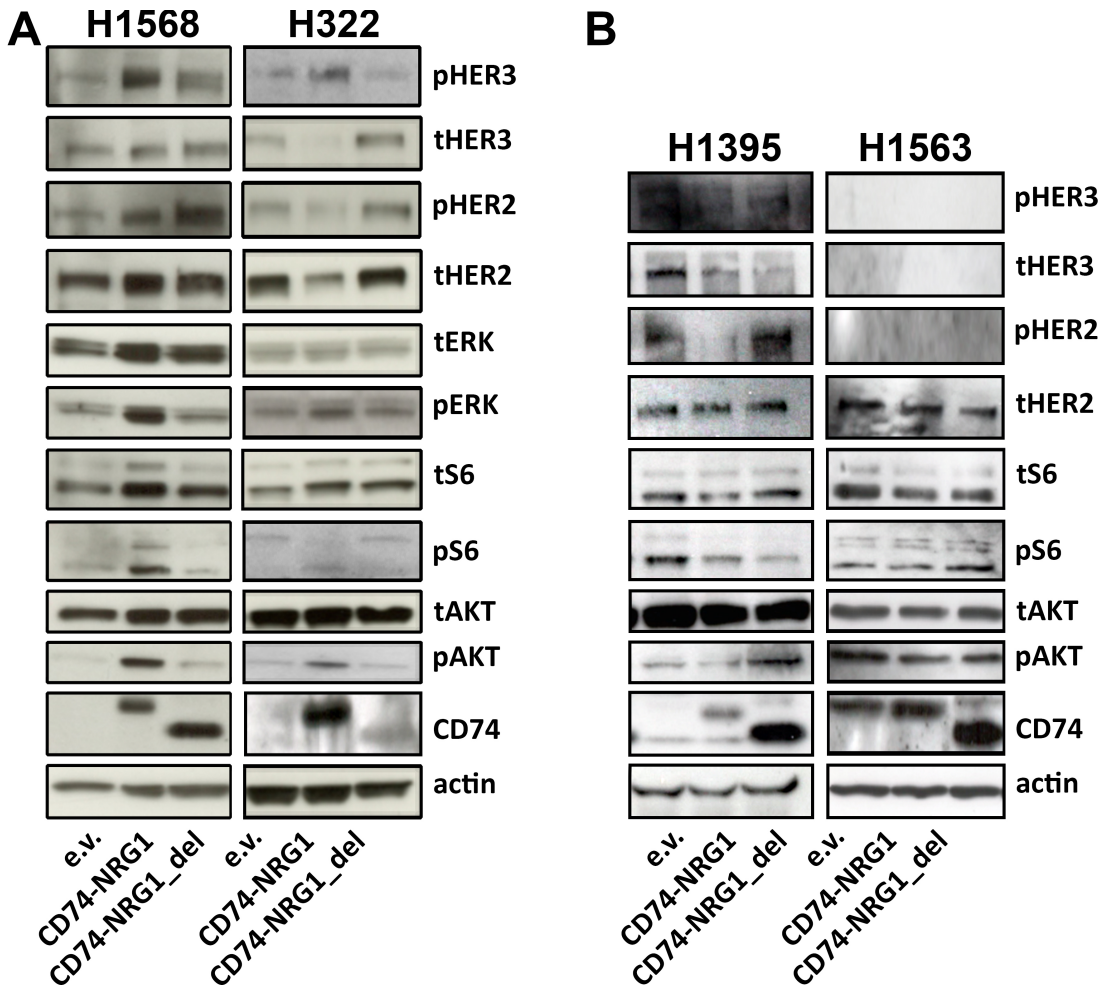

Figure 21: Immunoblot analysis of lung adenocarcinoma cell lines for effects on downstream activation of HER3. (A) H1568 and H322 cells show increased downstream pathway (PI3K and MAPK) activation by the CD74-NRG1 fusion protein compared to control cells. (B) H1395 and H1563 cells do not show a CD74-NRG1 fusion dependent activation of HER3 receptor and PI3K pathway. Equal loading was assessed via actin. (t=total; p=phospho) One representative experiment is shown.

H1395 and H1563 lung adenocarcinoma cell lines were confirmed to be wild-type for *KRAS*, *EGFR*, *HER2* and *HER3* by Sanger sequencing (data not shown). H1395 shows only very low HER2 expression levels while H1563 does not express the HER3 receptor at all (confirmed by target sequencing on cDNA and immunoblot analysis). Immunoblot analysis revealed no increased PI3K pathway activation in *CD74-NRG1* transduced cells compared to control cells (Fig. 21 B). These results might show the dependency of CD74-NRG1 signaling on HER3 receptor expression itself or on a minimal threshold level of HER2 receptor expression and/or phosphorylation in order to trigger HER3 receptor activation. No HER4 expression could be detected by target

sequencing of cDNA in H1395 and H1563 cells excluding any interaction of CD74-NRG1:HER4 (data not shown).

4.3.2 CD74-NRG1 does not transform Ba/F3 cells

Ba/F3 cells were transduced with *HER2* and *HER3* under different selectable markers and kept under IL-3. When adding virus containing *empty vector*, *CD74-NRG1* or *CD74-NRG1_del* to the cells and waiting for selection of stable cells, cells die without addition of IL-3. This experiment points out that the fusion might only add little additional effect that leads to oncogenic transformation. When transducing Ba/F3 cells with CD74-ROS1 or FIG-ROS they instantly become independent from IL-3 signaling and can be used e.g. for screening purposes (Fig. 16). Therefore Ba/F3 cells are not a suitable system for analyzing oncogenic effects of *CD74-NRG1* gene fusion.

4.3.3 *In vitro* proliferation assay reveals no increased proliferation rate of *CD74-NRG1* transduced cell lines

After proving the physical interaction and activation of HER3 receptor downstream signaling the question arose for a functional consequence and translational relevance of the *CD74-NRG1* gene fusion. As PI3K and MAPK pathway can induce cell cycle progression and proliferation H1568 and H322 cells transduced with empty vector, CD74-NRG1 or CD74-NRG1_del were used in an *in vitro* cell proliferation assay (P. Liu et al., 2009; Zhang & Liu, 2002). No differences in growth properties could be detected between fusion transduced cells or control cells for H1568 and H322 although H322 have been reported to show increased proliferation when supplemented with recombinant NRG1 (Yen et al., 2000).

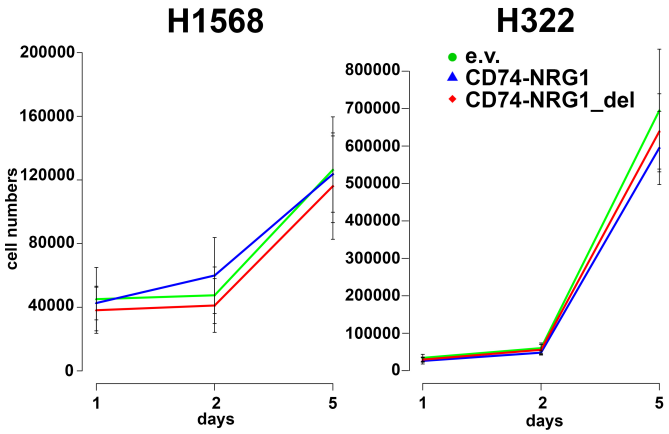

Figure 22: *In vitro* proliferation assay of H1568 and H322 cells. H1568 and H322 cells transduced with *e.v.*, *CD74-NRG1* or *CD74-NRG1_del*. Cells were counted on days 1, 2 and 5 in duplicates (n=4).

4.3.4 CD74-NRG1 increases anchorage independent growth in H1568 cells

PI3K and MAPK pathway can increase survival of cells and thereby helping cells to survive under more demanding conditions. H1568 cells harboring the *CD74-NRG1* gene fusion and H1568 cells transduced with corresponding control plasmids (*empty vector* (*e.v.*) and *CD74-NRG1_del*) were used in a soft-agar assay to analyze their colony formation capabilities. Indeed *CD74-NRG1* fusion transduced H1568 cells showed increased colony formation capabilities. Colony size was about 5 times higher in *CD74-NRG1* transduced H1568 cells compared to *e.v.* and *CD74-NRG1_del* transduced cells (Fig. 23). Although H1568 cells could already form colonies without any additional oncogene, triggering PI3K and MAPK pathways via CD74-NRG1:HER3 could further increase colony size indicating an increased oncogenic potential of cells signaling via CD74-NRG1 fusion. Notably H322 could not from any colonies at all and could therefore not used in this experimental setting.

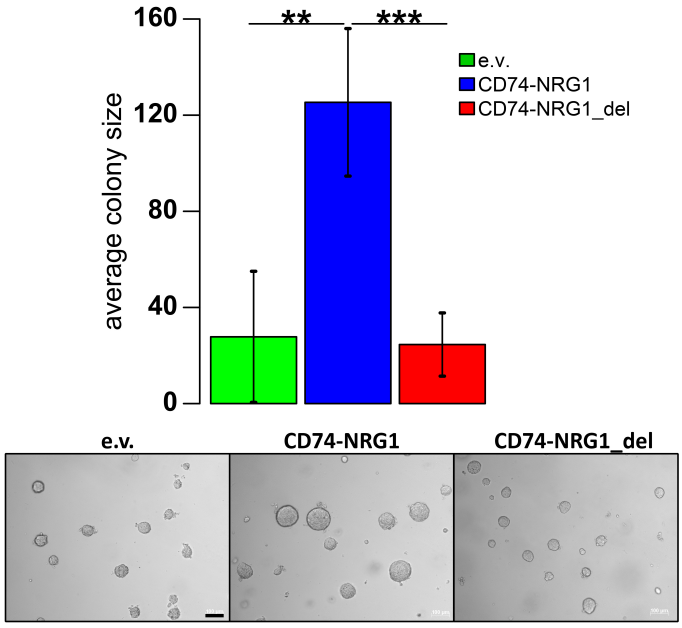

Figure 23: Soft-agar assay of H1568 cells. (A) H1568 cells transduced with *empty vector* (*e.v.*), *CD74-NRG1* or *CD74-NRG1_del* to measure anchorage independent growth capabilities. Average colony size normalized to e.v. control cells is shown of n=4 experiments. Error bars represent standard deviations. Significance p-values were determined by two-tailed t-test. ** ≤ 0.01, *** ≤ 0.001. (B) Representative pictures of soft-agar assay from H1568 cells were taken with phase-contrast microscope (100x magnification). The black scalebar represents 100µm.

4.4 Translational relevance of *CD74-NRG1* gene fusion

4.4.1 CD74-NRG1 fusion protein leads to a HER2 dependent HER3 trans-phosphorylation

Targeting NRG1 in patients harboring a CD74-NRG1-positive tumor can only be efficient in those cases when HER receptors become activated by NRG1 and not by other mechanism. Only a few studies have been conducted with anti-NRG1 antibodies. It seems that invasive capacity and proliferation of breast cancer cell lines SKBR-3 and MDA-MB-231 can be inhibited with antibodies directed against NRG1 (Montero et al., 2008). Other therapeutic strategies may aim towards inhibiting the interaction of HER3 or HER2 preventing trans-phosphorylation of HER3 by HER2 upon NRG1 binding the HER3 receptor (Li et al., 2007).

Therefore afatinib, a strong type II non-ATP-competitive inhibitor that selectively inhibits the EGFR and HER2 receptor, is used to inhibit trans-phosphorylation of HER3 (Blanc, Geney, & Menet, 2013; Eskens et al., 2008; Y. Liu & Gray, 2006; Solca et al., 2012). H1568 cells harboring the *CD74-NRG1* gene fusion show increased HER3 phosphorylation and increased activation of downstream signaling pathways compared to control cells (Fig. 21, 24) (Fernandez-Cuesta et al., 2014). Inhibition of EGFR and HER2 with 100nM afatinib (equivalent to GI_{40} value in H1568 cells, Fig. 25) leads to a complete shutdown of HER3 phosphorylation as well as inactivation of PI3K pathway (pAKT) after 12h and 48h (Fig. 24). Therefore HER2 inhibitors seem to be a promising treatment option for patients that harbor a *CD74-NRG1*-positive tumor and whose signaling is driven by the interaction of NRG1 with the HER3 receptor.

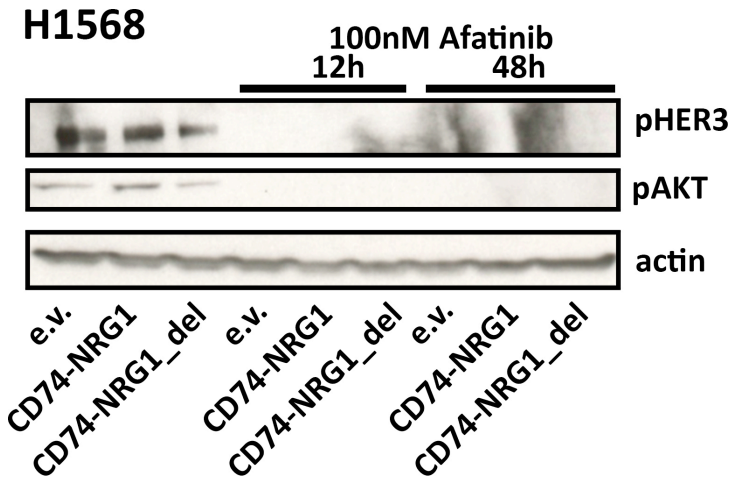

Figure 24: Immunoblot analysis of H1568 cell line treated with afatinib. H1568 cells transduced with *empty vector* (*e.v.*), *CD74-NRG1* or *CD74-NRG1_del*. Cells were treated for 12h or 48h with 100nM afatinib. Inhibition was assessed via phosphorylation of HER3 receptor (pHER3) and phosphorylated AKT (pAKT). Actin was used as loading control. One representative experiment is shown.

4.4.2 Survival of H1568 and H322 cell lines harboring *CD74-NRG1* gene fusion do not show increased viability upon EGFR and HER2 receptor inhibition.

As HER2 inhibition leads to inhibition of PI3K pathway in H1568 cells (Fig. 24), survival upon HER2 inhibition was investigated in the following experiment. H1568 and H322 cells transduced with *empty vector* (*e.v.*),

CD74-NRG1 or *CD74-NRG1_del* were used in a compound screen with afatinib and lapatinib. Compared to afatinib, lapatinib is a type I inhibitor binding to the active conformation of EGFR and the HER2 receptor (Baselga & Swain, 2009; Solca et al., 2012). No viability differences could be observed in any of the H1568 or H322 cell lines (Fig. 25). As the oncogenic driver gene of H1568 and H322 is unknown these results indicate that no oncogenic dependency of these cells has been established towards the *CD74-NRG1* gene fusion. Due to lack of a specific druggable target in H1568 and H322 cells the next experiment included the selection of lung adenocarcinoma cell lines that were precisely described to depend on one specific oncogenic driver that signals via PI3K pathway. As there are some well characterized cell lines available harboring activating mutations in EGFR and therefore depend on PI3K signaling, these cell lines were transduced with the *CD74-NRG1* gene fusion to signal in parallel via PI3K pathway. By inhibition of EGFR while harboring the gene fusion at the same time, the next goal was to induce a resistance phenotype in these cell lines. This should be achieved via their alternative possibility of triggering PI3K pathway activation and thereby leading to a pathway switch from EGFR to HER2:HER3 signaling.

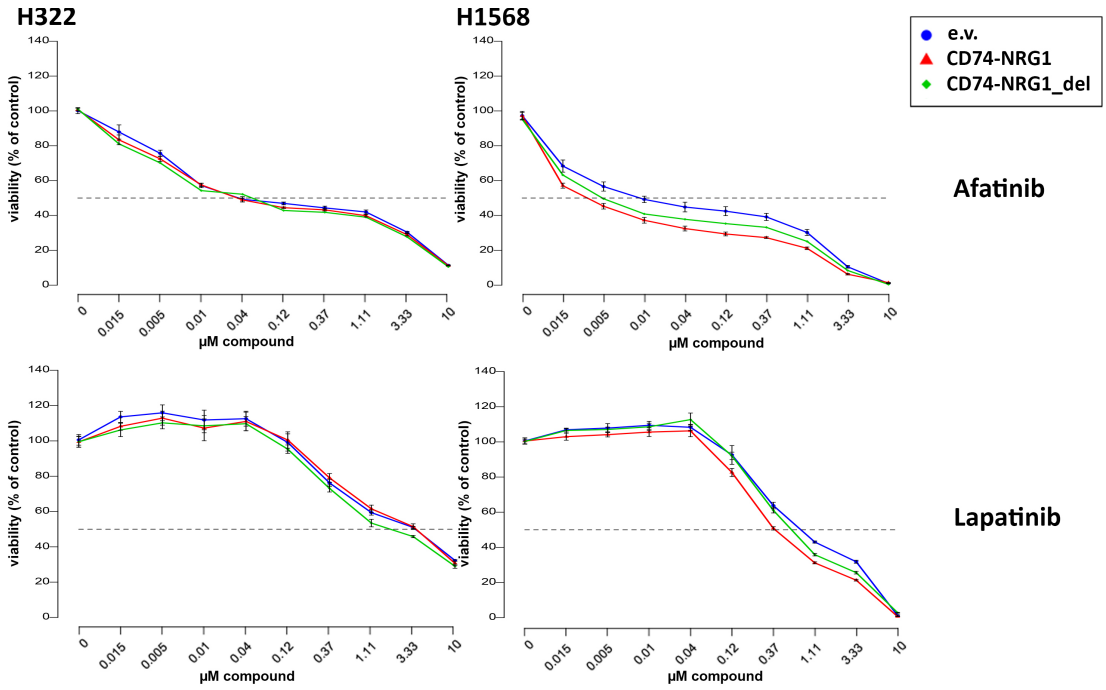

Figure 25: Compound screen with afatinib and lapatinib on H1568 and H322 cells. H1568 and H322 lung adenocarcinoma cell lines transduced with *empty vector* (*e.v.*), *CD74-NRG1* or *CD74-NRG1_del* were screened for viability against afatinib and lapatinib by CellTiter-Glo® assay.

4.4.3 CD74-NRG1 gene fusion does not lead to resistance in EGFR-dependent cell lines

HCC827 and PC-9 lung adenocarcinoma cell lines do both harbor exon 19 deletions in EGFR which lead to strong EGFR signaling dependency (Cavazzoni et al., 2012). Both cell lines are highly sensitive to treatment with EGFR inhibitors in already low nM range such as erlotinib and gefitinib (Hua Cheng et al., 2011; Sharma, Bell, Settleman, & Haber, 2007). However, when treating *CD74-NRG1* gene fusion transduced HCC827 and PC-9 cells with erlotinib or afatinib no changes in viability could be observed. This indicates that no pathway switch and resistance phenotype could have evolved. Only the strong EGFR dependency could be confirmed as treatment already with low nM doses of the cells with erlotinib and afatinib leads to strong reduction of cell viability (Fig. 26). As pathway switch requires HER2 and HER3 expression, both cell lines are confirmed to express both receptors (Ono et al., 2004; Yonesaka et al., 2011). One publication by Jeff Settleman's lab described no induction of resistance of HCC827 and PC-9 cell lines upon

NRG1 treatment. In contrast, the HER2 receptor amplified breast cancer cell line SKBR-3 and *EML4-ALK* expressing lung adenocarcinoma cell line H3122 are instead described as showing a resistance phenotype upon HER2 receptor or EML4-ALK inhibition while adding recombinant NRG1 (T. R. Wilson et al., 2012).

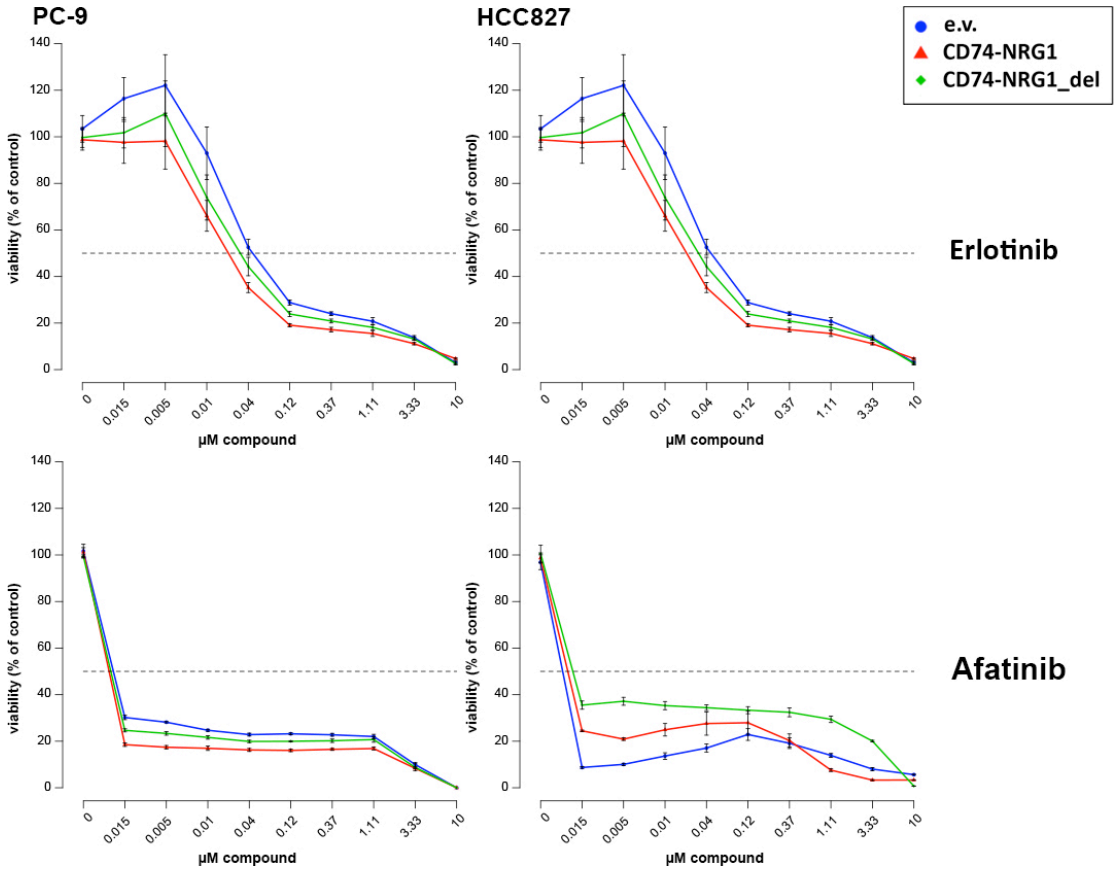

Figure 26: Compound screen of fusion and control virus transduced PC-9 and HCC827 cell lines. PC-9 and HCC827 cell lines were treated with erlotinib (EGFR inhibitor) and afatinib (EGFR and HER2 inhibitor). Cell viability was assessed by CellTiter-Glo® assay.

4.4.4 NRG1 as a resistance mechanism in SKBR-3 and H3122 cell lines

NRG1 is getting more and more into the focus as oncogene and/or resistance mechanism in cancer (Falls, 2003; Hynes & Lane, 2005; T. R. Wilson et al., 2012; Yarden & Pines, 2012). As a resistance mechanism NRG1 drives PI3K and MAPK pathway activation as an in addition or an alternative to the oncogenic driver present in the cancer cell. This has been shown by Jeff Settleman's lab by screening experiments of well-characterized cell lines with

and without various growth factors. NRG1 could be shown to induce resistance in SKBR-3 (*HER2* amplified) breast cancer cell lines when inhibited with lapatinib. Another cell line being more resistant upon addition of NRG1 was H3122 (*EML4-ALK* positive) lung cancer cell line treated with TAE-684 (ALK inhibitor) (T. R. Wilson et al., 2012). These results could be confirmed in own experiments showing a prolonged viability and higher resistance levels when treated with lapatinib (SKBR-3) or TAE-684 (H3122) while adding recombinant NRG1b (Fig. 27 A). As the EGF-like domain of recombinant NRG1 is identical to EGF-like domain of CD74-NRG1 fusion protein the same effects were expected for full-length CD74-NRG1 gene fusion transduced SKBR-3 and H3122 cells (Fig. 27 B). However H3122 and SKBR-3 cells were not able of being transducable with *CD74-NRG1* or *CD74-NRG1_del*. Only stable cell lines expressing *empty vector* could be generated. To solve this issue cells were transiently transfected with *empty vector* (*e.v.*), *CD74-NRG1* or *CD74-NRG1_del* containing expression plasmids. But only a very minor shift of viability could be observed in SKBR-3 cells with the *CD74-NRG1* gene fusion (Fig. 27 C). Therefore judging on *CD74-NRG1*'s capability of inducing resistance in these cells needs to be further analyzed.

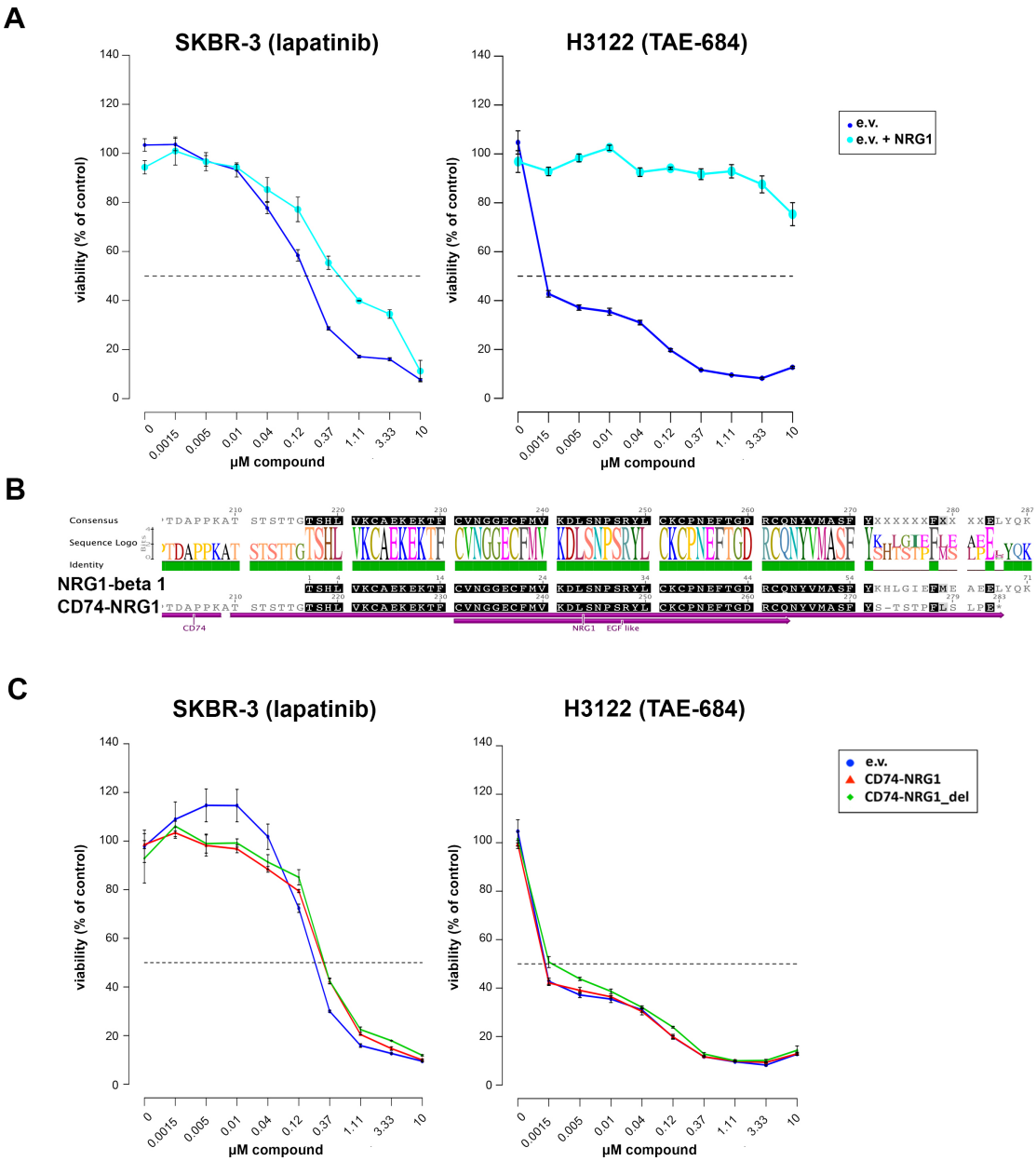

Figure 27: SKBR-3 and H3122 cells induce resistance upon addition of recombinant NRG1. (A) Viability screen showing NRG1 inducing resistance in SKBR-3 and H3122 cell lines treated with lapatinib or TAE-684. (B) Alignment of recombinant NRG1-beta 1 and CD74-NRG1 on amino acid level showing 100% sequence identity of EGF-like domain. (C) SKBR-3 and H3122 cell transiently transfected with *empty vector*, *CD74-NRG1* or *CD74-NRG1_del* inducing no resistance phenotype.

4.5 Generation of an inducible CD74-NRG1 mouse

To proof *CD74-NRG1* gene fusion being oncogenic *in vivo* an inducible mouse-targeting vector was designed. The mouse-targeting vector is designed via short- and long arm of homology (SAH, LAH) to integrate via

homologous recombination into the ROSA26 locus to achieve generalized expression (Friedrich & Soriano, 1991; Soriano, 1999). The expression is driven by the CAG promoter and is inhibited via a kanamycin-stop cassette (KANA/STOP) harboring a SV40 poly-A terminator (Miyazaki et al., 1989; Niwa, Yamamura, & Miyazaki, 1991). The cassette is flanked by Frt sites (flp recombinase recognition sites) to start expression upon recombination with flp recombinase (Zhu & Sadowski, 1995). *CD74-NRG1* gene fusion is flanked by LoxP recombination sites being able in the case of tumor formation and dependency to shutdown expression of *CD74-NRG1* via the cre recombinase which might lead to tumor breakdown to ultimately proof oncogenicity of CD74-NRG1 fusion *in vivo* (Fig. 28) (Sauer & Henderson, 1988). As the generation of genetically-manipulated mice takes already about one year following breading and inducing *CD74-NRG1* expression in lung via inhalation of flp-recombinase containing viral particles, these experiments are not part of the thesis and will be conducted in the near future. To investigate CD74-NRG1's oncogenic potential a dual-recombinase strategy is ideal, as start and stop of CD74-NRG1 expression can be tightly controlled and give important insights into tumor establishment and dependency. Right now the embryonic stem cells containing the CD74-NRG1 gene fusion are being generated at Wunderlich-lab.

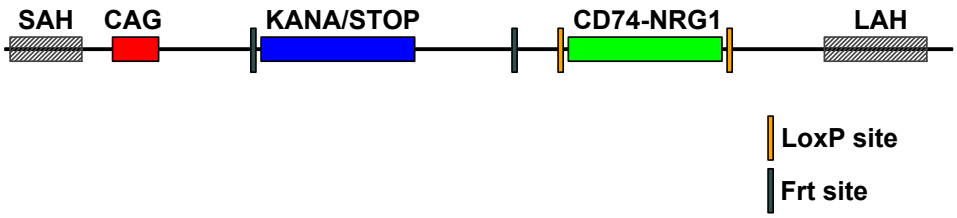

Figure 28: Dual recombinase mouse-targeting vector for *CD74-NRG1*-mouse. Schematic design of an inducible mouse-targeting vector to introduce *CD74-NRG1* gene fusion into the murine ROSA26 locus.

5 - Discussion

In this study the *CD74-NRG1* gene fusion was described and characterized for the first time (Fernandez-Cuesta et al., 2014). Other working groups discovered the *CD74-NRG1* gene fusion independently showing recurrence and confirming the initial discovery (Gow et al., 2014; Nakaoku et al., 2014). It seems as if *CD74-NRG1* gene fusion might be a specific event for IMA as all patients that were *CD74-NRG1*-positive were diagnosed with an IMA. All patients were from Asian population but up to now only small cohorts were screened for *CD74-NRG1* gene fusion. Frequencies from all 3 studies range from 7 – 27% within IMA patients and about 1.7% of all lung adenocarcinoma patients, may be leading to other frequencies in Caucasian population. Notably 10/11 diagnosed *CD74-NRG1*-positive cases were female and never-smokers, only one case was a male who stopped smoking 8 years before diagnosis (Fernandez-Cuesta et al., 2014; Gow et al., 2014; Nakaoku et al., 2014). As IMA is prone for >30% *KRAS* mutations, *CD74-NRG1* may be another important driver gene in IMA with up to 27% of cases affected depending on the screening cohort (Maeda et al., 2012).

Identically to all known *CD74*-positive gene fusions the *CD74* promoter drives the expression of the functional active 3'-part (*ROS*, *NTRK1* or *NRG1*) of the fusion (Fig. 13, 16) (Charest et al., 2003; Fernandez-Cuesta et al., 2014; Vaishnavi et al., 2013). However how the CD74 promoter is regulated or if regulation is changed in fusion-positive patients remains unclear. CD74's main function is the regulation of trafficking of major histocompatibility complex (MHC) II complexes to the cell surface in antigen presenting cells (Beswick & Reyes, 2009). Beside assisting in antigen-presentation MAPK pathway activation in cells has been observed via migratory inhibitory factor binding to CD74 and when primed with interferon-γ (Leng et al., 2003). Therefore its function in non-immune competent cells as all sequenced lung adenocarcinomas of this study remains elusive.

CD74 is characterized as a type II transmembrane protein with an intracellular amino-terminus and an extracellular carboxy-terminus (Leng et al., 2003).

Beside localizing to the plasma membrane there is a constant portion of CD74 localized in the cytoplasm (Xie et al., 2011). This is in line with the flow cytometry analyses and confocal microscopy pictures that proof a membrane bound and extracellular portion of CD74-NRG1 while another portion is localized in the cytoplasm (Fig. 14, 15). The *CD74-NRG1* gene fusion is the first oncogenic fusion protein that involves a ligand (NRG1) as functionally active part in contrast to a kinase domain. Therefore interaction studies of the well characterized NRG1:HER3 complex confirmed only an interaction in full-length *CD74-NRG1* transduced cells proofing that the EGF-like domain is the critical domain for binding HER3 (Fig. 17, 18, 19) (Baselga & Swain, 2009; Falls, 2003; Hynes & Lane, 2005; Yarden & Sliwkowski, 2001). NRG1 type I, II and IV – VI are described as being shed from the membrane and becoming a soluble and functional active ligand while NRG1 type III is described as membrane-tethered (Falls, 2003; L. Mei & Xiong, 2008; Montero et al., 2008; Talmage, 2008). Especially after one study claimed the *CD74-NRG1* gene fusion (which harbors a NRG1 type III 3'-part) being shed from the membrane, deeper analysis was necessary to further characterize the processing of CD74-NRG1 (Nakaoku et al., 2014). Three points have to be discussed when looking at processing of CD74-NRG1 fusion. i) In not a single immunoblot CD74-NRG1 was detected with a CD74 antibody with a smaller size indicating any shedding of CD74-NRG1 (Fig. 21). However this would not exclude efficient proteasomal degradation. ii) In the co-immunoprecipitations for proving the physical interaction of the CD74-NRG1 fusion protein with HER3 only full-length CD74-NRG1 was detected to interact with the HER3 receptor. This does not exclude the possibility of processing but already proofs that physical interaction with HER3 can occur by full-length and non-shed CD74-NRG1 fusion protein – even in a paracrine fashion from one cell to the other (Fig. 18/19). iii) When concentrating the supernatant of stable transduced NIH-3T3 cells, no shed portion of NRG1 could be detected (Fig. 20). Therefore it is very unlikely that CD74-NRG1 becomes shed – especially not to a high degree. Another point to keep in mind is that the co-immunoprecipitations and supernatant concentrations have been conducted

in a murine fibroblast cell line that may be does not express the protease that would be necessary for shedding.

H1568 and H322 lung adenocarcinoma cell lines transduced with *CD74-NRG1* showed as expected enhanced PI3K and MAPK pathway activation compared to control cells (Fig. 21, left). However H1395 and H1563 cell lines did not show any increase in PI3K pathway activation upon transduction with *CD74-NRG1* (Fig. 21, right). As NRG1 signaling is dependent on the HER receptor family it is critical that HER3 (receptor for NRG1 but kinase dead) and HER2 (trans-phosphorylating HER3) or HER4 (receptor for NRG1 and not kinase dead) are expressed (Hynes & Lane, 2005; Yarden & Sliwkowski, 2001). H1563 cells do not express any HER3 receptor neither on cDNA level nor on protein level (no HER4 expression on cDNA level). Therefore CD74-NRG1 is not able to trigger HER receptors and cannot activate downstream signaling especially as HER2 receptors are not phosphorylated at all. In H1395 cells HER2 and HER3 are expressed but not HER4 (validated on cDNA level). However in immunoblots HER2 and HER3 expression seems to be very low and other requirements are necessary for PI3K pathway activation by CD74-NRG1 (Fig. 21, right). As all lung adenocarcinoma cell lines are unknown for their driver lesion but were confirmed to be wild-type for *KRAS*, *EGFR*, *HER2* and *HER3* it is impossible to judge which additional factors are required for CD74-NRG1 to activate its oncogenic signaling. As *HER2* and *HER3* are expressed in H1395 but no additional effect of the CD74-NRG1 fusion could be observed a minimal threshold of both receptors is very likely to be important for activation of the downstream signaling pathways. The same reason may be true for the non transforming effect of *CD74-NRG1* gene fusion in Ba/F3 cells transduced with *HER2* and *HER3* as Ba/F3 cells just died upon IL-3 withdrawal (data not shown). NRG1 stimulation was linked to accelerating cell growth in H322 cells (Yen et al., 2000). However when investigating functional effects of the *CD74-NRG1* gene fusion in H1568 and H322 cells no increased proliferation could be detected in *CD74-NRG1* transduced cells compared to control cells (Fig. 22). As PI3K and MAPK pathways were activated by CD74-NRG1 fusion protein may be

even higher levels of NRG1 are necessary to increase proliferation. When investigating anchorage independent growth in H1568 cells transduced with *CD74-NRG1* or control plasmids (H322 cells were not able to grow in soft-agar), colony size was strongly increased in *CD74-NRG1* transduced cells compared to *empty vector* and *CD74-NRG1_del* transduced H1568 cells (Fig. 23). H1568 were already transformed and able to form colonies in control cells, but the CD74-NRG1 fusion protein increased colony size even more indicating a strong additional oncogenic effect of CD74-NRG1 fusion in H1568 cells (Fernandez-Cuesta et al., 2014).

Due to missing treatment options for patients with *CD74-NRG1* gene fusion, the effect of HER2 inhibition was investigated in H1568 cells as HER2 trans-phosphorylates HER3 upon binding of CD74-NRG1. Low nM concentrations of afatinib could inhibit HER3 and AKT phosphorylation (Fig. 24). The indirect inhibition of the HER3:NRG1 interaction network via HER2 inhibition answers several questions. i) The activating effects of *CD74-NRG1* gene fusion can be effectively inhibited via HER2 inhibition *in vitro*. ii) HER3 phosphorylation is clearly dependent on trans-phosphorylation via HER2 due to the strong selectivity of afatinib on the HER2 receptor.

In compound screenings with selective HER2 inihibitor (afatinib and lapatinib) on H1568 and H322 cells no induction of resistance by CD74-NRG1 could be observed (Fig. 25). However as the oncogenic driver is unknown in these cell lines and as these cells were already transformed, no change in pathway dependency could be detected as these cells were already well growing prior to transduction with the *CD74-NRG1* gene fusion. Therefore PC-9 and HCC827 cells were chosen for follow up experiments as they are EGFR mutated and depending on EGFR signaling. However when transducing these cells with *CD74-NRG1* gene fusion or control plasmids and treating with erlotinib (Hua Cheng et al., 2011; Sharma et al., 2007) or afatinib no increased viability levels could be detected (Fig. 26). However these results confirm published results that NRG1 cannot induce resistance to erlotinib in both cell lines (T. R. Wilson et al., 2012). As the breast cancer cell line SKBR-3 and the lung adenocarcinoma cell line H3122 were reported to induce resistance to lapatinib or TAE-684 both cell lines were transduced with *CD74-*

NRG1 gene fusion or control plasmids. Both cell lines showed resistance to inhibition with lapatinib (SKBR-3) or TAE-684 (H3122) while adding recombinant NRG1 protein, but SKBR-3 cells to a much lower extend than reported (Fig. 27 A) (T. R. Wilson et al., 2012). H3122 cells treated with recombinant NRG1 showed even at 10µM of TAE-684 a high viability around 75% compared to untreated cells. However it was up to the end of this study not possible to establish cells stably expressing the *CD74-NRG1* and *CD74-NRG1_del* gene fusion which are needed for proper experiments. Neither SKBR-3 cells stably expressing CD74-NRG1 or CD74-NRG1_del showed higher viability levels under lapatinib treatment, nor H3122 transiently transfected with empty vector, CD74-NRG1 or CD74-NRG1_del plasmids showed a resistance effect when treated with TAE-684. It remains questionable how SKBR-3 cells are able to induce resistance under NRG1 addition and lapatinib treatment, as HER2 cannot trans-phosphorylate HER3. In some breast cancer cell lines HER4 is not able to induce its oncogenic effect without HER2 when treated with NRG1 (Mill et al., 2012). Therefore it remains even more elusive how resistance is induced in SKBR-3 cells treated with NRG1. H3122 cells treated with TAE-684, which is a highly selective ROS and ALK inhibitor, can induce resistance when bypassing the ALK signaling with NRG1 and activate PI3K and MAPK pathways via HER2 and HER3. These differences in pathway inhibition may be reflected in the viability screen when adding recombinant NRG1. H3122 remain nearly completely resistant under NRG1 addition while SKBR-3 cells only showed a small shift in viability. Further experiments needs to be done and stable H3122 cells need to be generated in order to make a clear statement if *CD74-NRG1* gene fusion can induce resistance as seen with recombinant NRG1 (Fig. 27 C).

All discussed results were accomplished *in vitro*, but to ultimately judge on CD74-NRG1's oncogenic capabilities it is necessary to proof the oncogenic effect *in vivo*. This poof will have important effects on patient treatment and to include *CD74-NRG1* gene fusion in molecular diagnostic panels. The CD74-NRG1 mouse (fusion mouse) will be generated in cologne in collaboration with Thomas Wunderlich, PhD (CECAD, Cologne) and Ingo Voigt (Head of Transgenic Core Facility, MPI of Ageing, Cologne). Therefore the *in vivo* proof

of CD74-NRG1's oncogenic potential will take some time but will give important and valuable results that help to further decipher CD74-NRG1's mechanism in tumor establishment. At the same time the fusion mouse is a very important animal model for testing potential treatment strategies. These results will help to treat patients in the future that harbor a tumor that is *CD74-NRG1*-positive and will help to cure or enhance survival of these patients. To discover patients harboring *CD74-NRG1* gene fusion the EUCROSS clinical trial has been established at the University Hospital of Cologne. Patients are screened for ROS-translocations. Negative patients will undergo a second screening round for evaluation of phosphorylation levels of HER3 (as predictor for activation) by immunohistochemistry leading to the identification of potential *CD74-NRG1*-positive patients as *CD74-NRG1* diagnosis has not been established in molecular diagnostic panels yet. Therefore the results of this study will help to identify and treat *CD74-NRG1*-postive patients in the future.

6 - Summary

Lung adenocarcinoma accounts for about 50% of all lung cancer cases. In 25-50% of lung adenocarcinoma the oncogenic driver is still unknown. Especially an increasing amount of gene fusions as oncogenic driver lesion have been found within the last decade (Cancer Genome Atlas Research Network, 2014; Pao & Hutchinson, 2012). Therefore the goal of this study was to identify new oncogenic fusion genes. For this purpose the tumors of 25 patients with lung adenocarcinoma known to be negative for *EGFR* and *KRAS* mutations were used for transcriptome sequencing. In 14/25 patient samples no oncogenic alteration and in 10/25 patient samples already known oncogenic alterations could be detected. But in 1/25 patient sample a new gene fusion - *CD74-NRG1* - was discovered. *CD74-NRG1* is the first identified gene fusion which involves a growth factor as ligand that is fused to a fusion partner instead of a kinase domain. In an extended screening cohort of 102 lung adenocarcinoma patients negative for common driver lesions 4 additional *CD74-NRG1*-positive patients could be identified. All were diagnosed as stage I invasive mucinous adenocarcinoma (4/15 invasive mucinous adenocarcinoma). Mechanistically *CD74-NRG1* is a membrane-tethered fusion protein. The 5' *CD74* portion leads to the expression of *NRG1 III β3* in the tumor patients. The 3' *NRG1* portion harbors an EGF-like domain which is able to bind and activate the HER3 receptor. Thereby HER3 heterodimerizes with the HER2 receptor and becomes trans-phosphorylated by the HER2 receptor (Li et al., 2007). Expression of *CD74-NRG1* in lung cancer cell lines H1568 and H322 leads to the activation of PI3K and MAPK pathways and can increase anchorage independent growth in H1568 cells.

Due to the involvement of HER2 in the activation mode of *CD74-NRG1* gene fusion signaling the inhibition of HER2 with the selective inhibitor afatinib is a potential treatment option in *CD74-NRG1*-positive lung adenocarcionomas. The effect of afatinib could already be shown in H1568 cells. In the future, *CD74-NRG1*'s oncogenic capabilities need to be proven with a dual recombinase strategy *in vivo* to proof oncogenic driver capabilities and tumor

dependency. In conclusion this study describes the *CD74-NRG1* gene fusion for the first time and may help to improve treatment options for IMA patients that lack up to now any effective therapy.

7 - References

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8 - Publications

Fernandez-Cuesta, L.*, **Plenker, D.***, Osada, H., Sun, R., Menon, R., Leenders, F., et al. (2014). CD74-NRG1 Fusions in Lung Adenocarcinoma. *Cancer Discovery*, *4*(4), 415–422. doi:10.1158/2159-8290.CD-13-0633

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Malchers, F., Dietlein, F., Schottle, J., Lu, X., Nogova, L., Albus, K., …**Plenker, D.**, … et al. (2014). Cell-autonomous and non-cell-autonomous mechanisms of transformation by amplified FGFR1 in lung cancer. *Cancer Discovery*, *4*(2), 246–257. doi:10.1158/2159-8290.CD-13-0323

Peifer, M., ndez-Cuesta, L. F. A., Sos, M. L., George, J., Seidel, D., Kasper, L. H., **Plenker, D.**,… et al. (2012). Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nature Genetics*, 1–9. doi:10.1038/ng.2396

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11 - Eidesstattliche Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Roman Thomas und Prof. Dr. Thomas Langer betreut worden.

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Unterschrift