

Aus dem Zentrum für Kinder- und Jugendmedizin der Universität zu Köln
Klinik und Poliklinik für Kinder- und Jugendmedizin
Direktor: Universitätsprofessor Dr. med. J. Dötsch

Assessment of the role of *FGFR1*^{N546K} mutations in neuroblastoma pathogenesis and treatment

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
Jana Boland
aus Essen

promoviert am 15. September 2026

Dekan: Universitätsprofessor Dr. med. G. R. Fink

1. Gutachter: Universitätsprofessor Dr. med. M. Fischer
2. Gutachter: Universitätsprofessor Dr. med. V. Neuschmelting

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Dissertationsschrift ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskriptes habe ich Unterstützungsleistungen von folgenden Personen erhalten:

Herr Prof. Dr. Matthias Fischer
Frau Dr. Lisa Werr
Frau Dr. Stefanie Höppner
Herr Dr. Christoph Bartenhagen
Frau Yvonne Kahlert
Frau Nadine Hemstedt
Frau Isabelle Pakulla
Herr Dr. Marcel Dammert
Herr Dr. Florian Malchers
Frau Priv.-Doz. Dr. Anne Schultheis
Prof. Dr. Holger Grüll

Weitere Personen waren an der Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe einer Promotionsberaterin/eines Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertationsschrift stehen.

Die Dissertationsschrift wurde von mir bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die in dieser Arbeit zugrundeliegenden Experimente sind nach entsprechender Anleitung von Herrn Prof. Dr. Matthias Fischer und Frau Dr. Lisa Werr größtenteils von mir selbst ausgeführt worden. Unterstützungen von anderen Personen bei einzelnen Arbeitsschritten sind im Folgenden genannt.

Die in dieser Arbeit durchgeführten Zellkultur-Experimente sind nach entsprechender Anleitung von Frau Dr. Lisa Werr von mir selbst ausgeführt worden. Ausnahmen stellen die Integration von *FGFR1* in den *pBABE-puro* Vektor und die Herstellung der modifizierten Zelllinien mithilfe einer Virus-Transduktion dar. Der Vektor wurde von Herrn Dr. Florian Malchers aus dem Zentrum für Biochemie zur Verfügung gestellt. Die Herstellung der Zelllinien erfolgte ohne meine Mitarbeit in der Abteilung für Translationale Genomik durch Herrn Dr. Marcel Dammert.

Die in dieser Arbeit durchgeführten Maus-Experimente sind von mir vor allem mit der Unterstützung von Frau Dr. Lisa Werr durchgeführt worden. Die Daten der Mauskohten mit dem *MYCN* und/oder *ALK* Transgen wurden mir als Kontrollen von der Arbeitsgruppe von Herrn Prof. Dr. Matthias Fischer zur Verfügung gestellt und ich war nur bei einem Teil der Mäuse dieser Kohorten selbst beteiligt. Die verwendeten transgenen *FGFR1* Mäuse wurden von der

Firma Taconic Biosciences GmbH hergestellt. Bei den Maus-Genotypisierungen unterstützten mich Frau Yvonne Kahlert sowie studentische Hilfskräfte. Beim Scoring und der Durchführung der MRT-Bildgebungen halfen neben Frau Dr. Lisa Werr auch Frau Dr. Stefanie Höppner, Studentin Frau Isabelle Pakulla sowie weitere studentische Hilfskräfte. Die MRT-Bildgebungen wurden im Institut für Radiologie der Uniklinik Köln nach entsprechender Anleitung von Herr Prof. Dr. Holger Grüll von mir und den genannten Personen durchgeführt und anschließend selbstständig von mir ausgewertet. Die Behandlung der Mäuse wurde von mir mit der Unterstützung von Frau Dr. Lisa Werr und Frau Isabelle Pakulla durchgeführt. Frau Dr. Lisa Werr hat ohne meine Hilfe die vier Mäuse der Behandlungskohorte mit der Dosis 30 mg/kg behandelt. Die Schnitte für weiterführende Analysen des Mausmaterials wurden von Frau Yvonne Kahlert und Frau Nadine Hemstedt angefertigt. Die immunhistochemischen Färbungen wurde ohne meine Mitarbeit vom Institut der Pathologie der Uniklinik Köln von Frau Alexandra Florin durchgeführt und gemeinsam mit Frau Priv.-Doz. Dr. Anne Schultheis ausgewertet.

Die statistische Analyse meiner Datensätze erfolgte mit der Unterstützung von Herrn Dr. Christoph Bartenhagen.

Erklärung zur guten wissenschaftlichen Praxis:

Ich erkläre hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten (Amtliche Mitteilung der Universität zu Köln AM 132/2020) der Universität zu Köln gelesen habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen.

Köln, den 06.02.2026

Unterschrift:

Acknowledgement

I would like to thank Prof. Dr. Matthias Fischer for giving me the possibility to work in his lab and for all his ideas within this project. This experience showed me how to work scientifically and how fascinating cancer research is. It will have a big impact on my future career path.

I would like to thank Dr. Lisa Werr for her motivation to push on this project. Her ambition improved the discovery of *FGFR1*^{N546K}.

I would also like to thank all members of the Fischer-lab for the scientific discussions and their support. Additionally, it was a great team of medical doctoral students who were all in the same situation and supported each other.

I would like to thank the Mildred Scheel School of Oncology for their financial support and for offering interesting presentations.

Finally, I would like to thank my parents, my sister and my boyfriend for their emotional support within the whole time.

This project would not have been the same without you!

I dedicate this thesis to my family, whose support and belief in me have made this possible.

Table of contents

ABBREVIATIONS	10
1. ZUSAMMENFASSUNG	13
2. INTRODUCTION	15
2.1. Neuroblastoma	15
2.1.1. Epidemiology and general information	15
2.1.2. Staging	16
2.1.3. Pathology	16
2.1.4. Molecular risk factors	16
2.1.5. Risk stratification and prognosis	17
2.1.6. Therapeutic strategies	17
2.2. Fibroblast Growth Factor Receptor	18
2.2.1. FGFR structur and signaling pathway	18
2.2.2. Dysregulation of FGFR signaling in cancer	18
2.2.3. Molecular <i>FGFR</i> alterations in distinct tumor entities	19
2.2.4. <i>FGFR1</i> ^{N546K} in neuroblastoma and other tumor entities	19
2.2.5. Selective FGFR inhibitors	20
2.2.6. Characteristics of the FGFR inhibitors erdafitinib and futibatinib	20
2.3. Aim of this study	211
3. MATERIAL AND METHODS	22
3.1. Material	22
3.1.1. Reagents	22
3.1.2. Primers	25
3.1.3. Antibodies	26
3.1.4. Devices	27
3.1.5. Software	28
3.1.6. Animal ethics vote	28
3.2. Methods	29
3.2.1. Site-directed mutagenesis of cloned <i>FGFR1</i>	29
3.2.2. Bacterial Ttransformation	30
3.2.3. Restriction digest	30
3.2.4. Polymerase chain reaction	30

3.2.5.	Agarose gel electrophoresis	31
3.2.6.	Preparation of dideoxy sequencing	32
3.2.7.	Cell lines	32
3.2.8.	Stable virus transduction	33
3.2.9.	DNA isolation	34
3.2.10.	Transformation assay	34
3.2.11.	Cytokine independence assay	34
3.2.12.	Generation of cell lysates	34
3.2.13.	Western blot analysis	35
3.2.14.	Treatment of cells for protein analysis	36
3.2.15.	Treatment of cells for viability analysis	36
3.2.16.	Cell viability assay	37
3.2.17.	Mouse tagging	37
3.2.18.	Lysis of mouse biopsies	37
3.2.19.	Mouse genotyping	37
3.2.20.	Magnetic resonance imaging	38
3.2.21.	Histopathology	39
3.2.22.	Immunofluorescence staining	39
3.2.23.	Treatment of mice with FGFR inhibitors	39
3.2.24.	Evaluation of anti-tumor effects	40
3.2.25.	Statistical analysis	40
4.	RESULTS	41
4.1.	Characterization of <i>FGFR1</i> ^{N546K} in <i>Ba/F3</i> cells	41
4.1.1.	Cloning of <i>FGFR1</i> ^{N546K} into a vector and transduction of <i>Ba/F3</i> cells	41
4.1.2.	Analysis of the oncogenic potential of <i>FGFR1</i> ^{N546K} in <i>Ba/F3</i> cells	42
4.1.3.	Treatment of <i>Ba/F3 FGFR1</i> ^{N546K} cells with <i>FGFR</i> inhibitors	44
4.2.	Characterization of <i>FGFR1</i> ^{N546K} in a transgenic mouse model	47
4.2.1.	Generation of genetically engineered mouse model	47
4.2.2.	Tumor development in <i>FGFR1</i> ^{N546K} mice	48
4.2.3.	Tumor development in <i>FGFR1</i> ^{N546K} ; <i>MYCN</i> mice	51
4.2.4.	Treatment of <i>FGFR1</i> ^{N546K} ; <i>MYCN</i> mice with futibatinib	53
5.	DISCUSSION	56
5.1.	<i>Ba/F3 FGFR1</i> ^{N546K} is an oncogenic driver in <i>Ba/F3</i> cells	56
5.2.	Treatment of <i>Ba/F3 FGFR1</i> ^{N546K} cells with <i>FGFR</i> inhibitors impairs cell viability and <i>FGFR</i> pathway down-regulation	57

5.3.	Targeted expression of <i>FGFR</i> ^{N546K} in mice	58
5.4.	Expression of <i>FGFR1</i> ^{N546K} in mice leads to spontaneously regressing neuroblastoma	58
5.5.	Co-expression of <i>FGFR1</i> ^{N546K} and <i>MYCN</i> in mice drives aggressive neuroblastoma progression	59
5.6.	Futibatinib inhibits tumor growth and prolongs survival in <i>FGFR1</i> ^{N546K} ; <i>MYCN</i> mice	59
5.7.	FGFR inhibitors may be a promising treatment option for FGFR-mutated pediatric malignancies despite recruitment challenges in clinical trials	60
6.	REFERENCES	62
7.	APPENDIX	70
7.1.	List of figures	70
7.2.	List of tables	70
7.3.	Appendix Figure 1: Scheme of <i>pBABE-puro</i> vector with <i>FGFR1</i> beta	71
7.4.	Appendix Table 1: Scoresheet	72
7.5.	Appendix Table 2: Early sacrifices of <i>R26-LSL-FGFR1-N546K</i> ^{fl/wt} ; <i>Th-IRES-Cre</i> ^{tg/wt} mouse cohort	74
7.6.	Appendix Table 3: <i>R26-LSL-FGFR1-N546K</i> ^{fl/wt} ; <i>Th-IRES-Cre</i> ^{tg/wt} mouse cohort	75
7.7.	Appendix Table 4: <i>R26-LSL-FGFR1-N546K</i> ^{fl/wt} ; <i>Th-MYCN</i> ^{tg/wt} ; <i>Th-IRES-Cre</i> ^{tg/wt} mouse cohort	77
7.8.	Appendix Table 5: <i>Th-ALK-F1174L</i> ^{tg/wt} ; <i>Th-MYCN</i> ^{tg/wt} ; <i>Th-IRES-Cre</i> ^{tg/wt} mouse cohort	80
7.9.	Appendix Table 6: <i>R26-LSL-FGFR1-N546K</i> ^{fl/wt} ; <i>Th-MYCN</i> ^{tg/wt} mouse cohort	81
7.10.	Appendix Table 7: <i>Th-MYCN</i> ^{tg/wt} mouse cohort	81
7.11.	Appendix Table 8: <i>Th-ALK-F1174L</i> ^{tg/wt} mouse cohort	82
7.12.	Appendix Table 9: <i>R26-LSL-FGFR1-N546K</i> ^{fl/wt} ; <i>Th-MYCN</i> ^{tg/wt} ; <i>Th-IRES-Cre</i> ^{tg/wt} mouse cohort after treatment with CMC-Na	84
7.13.	Appendix Table 10: <i>R26-LSL-FGFR1-N546K</i> ^{fl/wt} ; <i>Th-MYCN</i> ^{tg/wt} ; <i>Th-IRES-Cre</i> ^{tg/wt} mouse cohort after treatment with futibatinib	86

Abbreviations

AKT	protein kinase B
ALK	anaplastic lymphoma kinase
ALT	alternative lengthening of telomeres
ASCT	autologous stem cell transplantation
ATP	adenosine triphosphate
AttB	bacterial attachment site
BSA	bovine serum albumin
Bp	base pair
Cas	CRISPR-associated proteins
CMC-Na	sodium carboxymethylcellulose
CRISPR	clustered regularly interspaced short palindromic repeats
CTG	CellTiter-Glo®
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	ethylenedinitrilotetraacetic acid disodium salt dehydrate
ERK	extracellular signal-regulated kinase
FA	flip angle
FCS	fetal calf serum
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded tissue
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FI	floxed
FRS	FGF receptor substrate
HCl	hydrochloride
HE	hematoxylin and eosin
Het	heterozygous
IC ₅₀	half maximal inhibitory concentration
IDFR	image defined risk factors
IF	immunofluorescence
Ig	immunoglobulin
IHC	immunohistochemistry
IL-3	interleukin-3
¹²³ I-MIBG	¹²³ iodo-metaiodobenzylguanidine
INPC	International Neuroblastoma Pathological Classification

INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
LoxP	locus of x-over, P1
LTR	long terminal repeats
MAPK	mitogen-activated protein kinase
MKI	mitosis-karyorrhexis index
MRI	magnetic resonance imaging
MYCN	v-myc myelocytomatosis viral related oncogene
NaCl	sodium chloride
NaOH	sodium hydroxide
P	phosphorylated
P53	protein 53
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHOX2B	paired-like homeobox 2B
P/S	penicillin/streptomycin
ROSA16	Reverse Oriented Splice Acceptor, Clone 26
SD	standard deviation
SD	stable disease
STAT	signal transducer and activator of transcription protein
PI3K	phosphatidylinositol 3-kinase
PD	progressive disease
PR	partial response
PVDF	polyvinylidene fluoride
RAS	rat sarcoma virus
RPMI	Roswell Park Memorial Institute medium
TAE	tris-acetate-EDTA
TBS-T	tris-buffered saline with Tween
TE	echo time
Tg	transgene
Th	tyrosine hydroxylase
TK	tyrosine kinase domain
TKI	tyrosine kinase inhibitor
TM	transmembrane domain
TMM	telomere maintenance mechanisms
TR	repetition time

TSE	turbo-spin echo
RECIST	response evaluation criteria in solid tumors
WB	western blot
Wt	wild-type

1. Zusammenfassung

Das Neuroblastom ist durch divergente klinische Verläufe charakterisiert. Bei etwa der Hälfte der Patienten kommt es zu einer spontanen Tumorregression, während die andere Hälfte ein aggressives Tumorwachstum aufweist, das trotz multimodaler Therapie mit einer schlechten Prognose einhergeht. Die Ursache dieser Phänomene ist jedoch nicht endgültig geklärt. Um ein genaueres Verständnis für die Pathogenese zu erhalten, ist die Untersuchung klinischer und molekularer Marker Gegenstand der aktuellen Forschung. Ziel ist es, die Therapiemöglichkeiten, besonders für Hoch-Risiko-Patienten, zu verbessern.

In einer kleinen Gruppe von Neuroblastomen konnte eine rekurrente Punktmutation im Gen für den Fibroblasten-Wachstumsfaktor-Rezeptor 1 (*FGFR1*) an Position 546 detektiert werden, die zu einem Austausch von Asparagin zu Lysin führte (p.N546K). Klinische Beobachtungen haben gezeigt, dass diese Patienten nur unzureichend auf eine Standard-Chemotherapie ansprach, woraus eine schlechte Prognose resultierte. Auch in anderen Tumoren, insbesondere in pädiatrischen Hirntumoren, konnte diese Mutation nachgewiesen werden.

Bei FGFR handelt es sich um einen Tyrosinkinase-Rezeptor, der über verschiedene Signalwege physiologische Prozesse, wie Zellproliferation und Angiogenese, steuert. Genetische Veränderungen in *FGFR1-4* können zur Krebsentstehung beitragen. Fusionen, Mutationen oder Amplifikationen konnten in verschiedenen Krebsentitäten, wie beispielsweise im Lungenkarzinom, Mammakarzinom und Urothelkarzinom, beobachtet werden. 2019 wurde der erste zielgerichtete FGFR Inhibitor namens Erdafitinib für erwachsene Patienten zur Behandlung vom *FGFR2*- oder *FGFR3*-mutierten fortgeschrittenen Urothelkarzinomen zugelassen; hierauf folgte die Zulassung der Inhibitoren Pemigatinib, Infigratinib und Futibatinib. Weitere FGFR Inhibitoren werden zurzeit in klinischen Studien getestet. Für Krebserkrankungen bei Kindern erfolgte bisher keine Zulassung von FGFR Inhibitoren.

In dieser Arbeit sollte mithilfe von *in vitro* und *in vivo* Modellen die Bedeutung der *FGFR1*^{N546K} Mutation in Neuroblastomen im Hinblick auf das onkogene Potenzial bewertet und der Effekt einer gegen *FGFR1*^{N546K} gerichteten Therapie getestet werden.

Zu diesem Zwecke wurden murine Ba/F3 Zellen mit ektopter *FGFR1*^{N546K} Expression hergestellt. Diese Veränderung führte zu einer Transformation der Zellen von einem Interleukin-3 abhängigen zu einem Interleukin-3 unabhängigen Wachstum und wies damit auf ein onkogenes Potenzial hin. In Übereinstimmung hiermit ließ sich eine erhöhte Phosphorylierung des mutierten FGFR1 und der nachgeschalteten Komponenten des Signalweges nachweisen. Eine Behandlung dieses Zellmodells mit Erdafitinib und Futibatinib zeigte eine Wachs-

tums-Inhibition im niedrig nanomolaren Konzentrationsbereich und eine Hemmung des Signalweges.

Die weitere Evaluation erfolgte in gentechnisch veränderten Mäusen, bei denen sich eine Expression von *FGFR1*^{N546K} im sympathischen Nervensystem induzieren ließ. Tötungen von *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} Mäusen im Alter von 7, 14 und 21 Tagen (je n = 3) wiesen auf das Vorhandensein von Neuroblastomen hin, wobei die Tumorgroße mit zunehmendem Alter mutmaßlich abnehmend war, was auf eine spontane Regression hindeutete. Im Verlauf entstand in 34 von 35 Mäusen kein Neuroblastom-assoziiierter letaler Phänotyp, wie die Untersuchung des Langzeit-Überlebens von *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} Mäusen (n = 35) erwartungsgemäß zeigte. Bei einer Ko-Expression des *FGFR1*^{N546K} und eines *MYCN* Transgens (n = 15) kam es hingegen mit einer Penetranz von 100% zur Neuroblastom-Entwicklung mit einem aggressiven Tumorstadium und Erreichen der Abbruchkriterien im Alter von 17-21 Tagen. Die Überlebensdauer von *Th-MYCN*^{tg/wt}; *Th-ALK-F1174*^{tg/wt} Mäusen war dagegen ungefähr zwei- bis dreimal so lang, was auf ein stärkeres onkogenes Potenzial von *FGFR1*^{N546K} im Vergleich zu *ALK*^{F1174L} hinwies. Eine Behandlung der *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt}; *Th-MYCN*^{tg/wt} Mäuse mit Futibatinib (30 mg/kg) zeigte eine stabile Erkrankung in 3/4 Mäusen und eine partielle Remission in einer Maus. Eine Reduktion der Dosis auf 5 mg/kg ergab eine stabile Erkrankung bei 10/12 Mäusen und jeweils eine partielle Remission und eine progressive Tumorerkrankung bei den anderen beiden Mäusen. Aufgrund der Nebenwirkungen, wie Durchfall und Gewichtsverlust, führte nur die geringere Dosis (n = 14) zu einem signifikant verlängertem Überleben mit 5-40 Tagen ab Behandlungsstart im Vergleich zur Kontrollkohorte (n = 13) mit 3-13 Tagen (p < 0.001).

Insgesamt lässt sich aus den Ergebnissen dieser Arbeit schließen, dass *FGFR1*^{N546K} ein starker onkogener Treiber in Neuroblastomen ist. FGFR Inhibitoren stellen eine vielversprechende Therapiemöglichkeit für diese Untergruppe von Hoch-Risiko-Neuroblastom-Patienten dar. Allerdings ist eine weitere Evaluation in Rahmen klinischer Studien notwendig.

2. Introduction

2.1. Neuroblastoma

2.1.1. Epidemiology and general information

Neuroblastoma is a malignant embryonal tumor of the developing sympathetic nervous system. It arises from the adrenal glands or the sympathetic trunk and is thus usually found in the neck, chest, abdomen or pelvis.¹ Neuroblastoma occurs mainly in infancy and early childhood, with a median age at diagnosis of 17 months.¹ It represents about 8% to 10% of all malignancies in children and is responsible for 12% to 15% of cancer-related mortality during childhood.²

In most cases neuroblastoma is a sporadic disease. Roughly 1% to 2% of neuroblastomas have a familial etiology, with germline mutations in the anaplastic lymphoma kinase gene (*ALK*) being the leading cause for hereditary disease.³ Congenital syndromes are associated with neuroblastoma in 5%.⁴

Due to no or few symptoms in early stages, neuroblastoma is diagnosed incidentally in many patients. Symptoms depend on localization and extent of the disease and may include abdominal or cervical swelling, abdominal pain, respiratory distress or neurological deficits. In addition, symptoms of metastatic disease are fever, weight loss, and bone pain.⁵ Rare, but specific symptoms are ecchymosis, monocular hematoma, Horner syndrome, spinal paraplegia, and opsoclonus-myoclonus-ataxia syndrome.^{6,7}

Neuroblastoma diagnosis comprises detection of tumor markers, such as increased levels of the urinary catecholamine metabolites vanillinic and homovanillinic acid or elevation of neuron specific enolase in serum. Furthermore, diagnostic imaging, such as ultrasound and magnetic resonance imaging (MRI), is required. For the assessment of tumor spreading, body scintigraphy using ¹²³Iodo-metaiodobenzylguanidine (¹²³I-MIBG) needs to be performed. Bone marrow infiltration is examined by puncture or biopsy. Final diagnosis requires the analysis of tumor histology and genetic alterations of the tumor, obtained by biopsy or resection.^{8–10}

Interestingly, the clinical course of the disease is highly variable. Roughly half of the patients have fatal disease progression, whereas spontaneous regression occurs frequently in other patients.¹¹

2.1.2. Staging

Two staging systems have been proposed: the International Neuroblastoma Staging System (INSS) and the International Neuroblastoma Risk Group Staging System (INRGSS). Both systems are important to determine the therapeutic strategy and prognosis.¹²

The INSS considers the anatomical tumor position, lymph node involvement, presence of metastases, and the extent of tumor resection. Stage 1 refers to a localized, completely resected tumor, and stage 2a to a localized, incompletely resected tumor. Stage 2b refers to additional involvement of lymph nodes on the ipsilateral side, and stage 3 to an incompletely resected tumor with extension to the contralateral side and/or involvement of lymph nodes on that side. Stage 4 is characterized by distant metastases, and stage 4S by metastases limited to skin, liver, and/or bone marrow in infants younger than 18 months.¹³

In 2009, a new staging system was established by the INRG, referred to as INRGSS, which allows pretreatment staging based on Image Defined Risk Factors (IDRF). Localized tumors in the absence and presence of IDRF are classified as stage L1 and L2, respectively, while metastasized disease and metastasized disease affecting the skin, liver, and/or bone marrow in children younger than 18 months are referred to as stage M and MS, respectively.^{12,14}

2.1.3. Pathology

The International Neuroblastoma Pathological Classification (INPC) takes the differentiation grade of the tumor cells and the extent of Schwannian stroma into account. Accordingly, tumors are classified as neuroblastoma, undifferentiated, poorly differentiated or differentiating, as ganglioneuroblastoma, intermixed or nodular, or as ganglioneuroma. In addition, patient age, and the Mitosis-Karyorrhexis Index (MKI) allows to distinguish between unfavorable and favorable histology, however, this classification is not being used in German risk stratification systems.¹⁵

2.1.4. Molecular risk factors

Genomic amplification of the proto-oncogene *MYCN* and loss of the short arm of chromosome 1, occurring in roughly 20%¹⁶ and 25%¹⁷, respectively, are risk factors for poor outcome in neuroblastoma that are currently used for risk estimation and therapy stratification in Germany.^{16,17}

In addition, the activating genomic alterations of the receptor tyrosine kinase *ALK* have been found in approximately 10% of neuroblastoma, offering an opportunity for targeted therapies.¹⁸ More recently, it has been reported that high-risk tumors are defined by the presence of telomere maintenance mechanisms, which may refine risk estimation in future trials.¹⁹

Despite much progress has been made over recent years, available molecular and clinical markers still do not fully explain the divergent phenotypes of neuroblastoma, which is a prerequisite for accurate risk assessment of patients and appropriate treatment tailoring.

2.1.5. Risk stratification and prognosis

Based on stage, histology, molecular markers, and age at diagnosis, neuroblastoma patients are allocated to three prognostic groups (low-, intermediate-, high-risk). Low-risk patients are defined by tumors without *MYCN* amplification in INSS stages 1, 2, 3 at an age younger than 2 years and 4S, and in INSS stages 2 and 3 without *chromosome 1p* aberration. Intermediate-risk includes patients with tumors bearing a *chromosome 1p* aberration but lacking *MYCN* amplification in INSS stages 2 and 3 at an age under 2 years, INSS stage 3 without *MYCN* amplification aged over 2 years, and INSS stage 4 without *MYCN* amplification and younger than 18 months at diagnosis. Patients are considered to be at high-risk, if they have metastatic INSS stage 4 diseases and are older than 18 months at diagnosis, or if their tumor cells bear genomic amplification of *MYCN*.¹¹

Non-high-risk patients have excellent overall survival rates over 90% despite limited or no therapy, and these tumors are often characterized by spontaneous regression.²⁰ By contrast, long-term survival in high-risk patients is still below 50% despite multimodal treatment.²¹

2.1.6. Therapeutic strategies

Therapeutic strategies for neuroblastoma patients depend on risk stratification and range from a watch-and-wait approach to intensive multimodal therapy.

Low-risk neuroblastoma patients may undergo either surgical tumor resection or a watch-and-wait approach for expected spontaneous regression. Chemotherapy is given only in case of tumor-associated symptoms.²⁰

In intermediate-risk patients, tumor resection is combined with chemotherapy and irradiation, if appropriate.¹¹

Patients with high-risk are intensively treated by multimodal strategies, including induction chemotherapy, surgery, radiotherapy, myeloablative chemotherapy and autologous stem cell transplantation (ASCT), and immunotherapy.^{5,22} The induction chemotherapy in German standard protocols consists of a total of six alternating N5 (cisplatin, vindesine, etoposide) and N6 (vincristine, dacarbazine, ifosfamide, doxorubicin) cycles. After several cycles, the primary tumor is resected, and the residual tumor volume is irradiated with a total dose of 36 Gray. Afterwards, myeloablative chemotherapy with busulfan and melphalan followed by ASCT is performed.⁵ As maintenance treatment, immunotherapy with the anti-GD2 antibody dinutuximab is given.²³

To improve response rates and outcome in high-risk patients, other treatment options, based on the individual tumor biology, are being developed. In a subgroup of neuroblastoma patients genomic alterations have been identified that may be accessible to targeted therapies. For example, ALK inhibitors, like crizotinib, lorlatinib, and ceritinib, have shown promising response rates in patients with relapsed or refractory *ALK*-mutated neuroblastoma^{24–26}, and are therefore being considered for first-line therapy in prospective trials for high-risk neuroblastoma patients.²⁷ Similarly, this project investigated whether *FGFR1* mutations may be a therapeutic target for specific *FGFR* inhibitors in neuroblastoma patients.

2.2. Fibroblast Growth Factor Receptor

2.2.1. *FGFR* structure and signaling pathway

FGFR signaling regulates physiologic cellular processes, such as tissue development and angiogenesis. *FGFR1* to 4 are transmembrane receptor tyrosine kinases, which consist of two (*FGFR1* beta) or three (*FGFR1* alpha) immunoglobulin-like domains, one transmembrane domain and two intracellular tyrosine kinase domains. *FGFR1* to 3 are structurally similar, while *FGFR4* differs from this structure. Twenty-two FGF ligands are known. The binding of these glycoproteins in a complex with heparin sulfate proteoglycans for stabilization of the interaction with the receptor lead to activation of RAS/MAPK (rat sarcoma virus, mitogen-activated protein kinase), PI3K/AKT (phosphatidylinositol-3 kinase, protein kinase B) and STAT (signal transducer and activator of transcription protein) pathways (Figure 1).^{28–30}

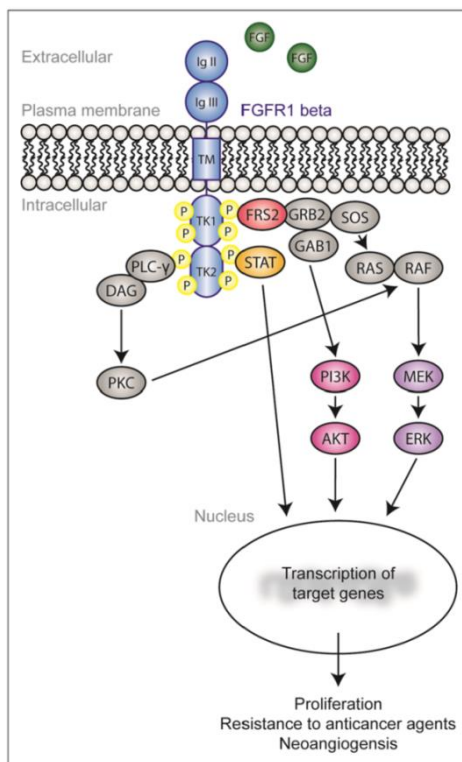


Figure 1: *FGFR* structure, interaction network, and dysregulation in cancer.

FGFR1 beta comprises immunoglobulin-like (Ig), transmembrane (TM) and tyrosine kinase (TK) domains. Binding of FGF (green) to *FGFR* (blue) leads to receptor dimerization and trans-phosphorylation (P) of the tyrosine kinase domains. Activation of the RAS/MAPK (purple) and PI3K/AKT (pink) signaling pathways occurs via *FGFR* substrate 2 (red) and phospholipase C δ (grey). Other pathways, for example STAT-dependent signaling (orange), can also be activated. Several negative regulators influence the signaling pathway. Dysregulation of *FGFR* signaling is caused by various mechanisms, which may drive cancer development.²⁹

Figure based on Touat M, Ileana E, Postel-Vinay S, André F, Soria J-C. Targeting *FGFR* Signaling in Cancer. *Clinical Cancer Research* 2015.²⁹

2.2.2. Dysregulation of FGFR signaling in cancer

Dysregulation of FGFR signaling has been implicated in tumorigenesis of various cancer entities previously. Aberrant FGFR signaling in tumorigenesis can be caused by different mechanisms, including high availability of FGFs resulting in ligand-dependent FGFR signaling, or *FGFR* alterations generating a constitutively active kinase domain, which induces ligand-independent FGFR signaling. These alterations contribute to cancer development and progression through uncontrolled cell cycle progression with increased proliferation and survival, neoangiogenesis, and/or resistance to anticancer therapies.^{28–31}

2.2.3. Molecular *FGFR* alterations in distinct tumor entities

Aberrant FGFR signaling due to genomic alterations of *FGFR* genes, such as mutations, amplification or fusions, occurs in multiple solid tumors.²⁹

Urothelial bladder carcinoma is the most common tumor bearing *FGFR* alterations, predominantly mutations in *FGFR3* with a prevalence of up to 80%.³² *FGFR1* mutations have been most frequently described in pilocytic astrocytomas^{33–35}, *FGFR2* mutations in endometrial cancer³⁶ and *FGFR4* mutations in rhabdomyosarcoma³⁷.

Overexpression of *FGFR1* was mainly identified in breast cancer, lung cancer, and osteosarcoma³⁸, and *FGFR2* amplification has been explored in gastric and breast cancer³⁹. Studies in breast cancer have shown an association of *FGFR* amplification with early relapse, poor outcome and resistance to endocrine therapy.³⁰

Fusions between *FGFR1* to 3 and other genes, such as *TACC1*, *TACC3*, *BAIAP2L1*, *BICC1*, and *AHCYL1*, have been detected in glioblastoma⁴⁰, urothelial bladder carcinoma⁴¹, non-small cell lung cancer⁴², and cholangiocarcinoma⁴³.

2.2.4. *FGFR1*^{N546K} in neuroblastoma and other tumor entities

FGFR1 is recurrently mutated at codon 546 in cancer (*FGFR1*^{N546K}), which leads to an asparagine-to-lysine substitution within the tyrosine kinase domain 1. *FGFR1*^{N546K} mutations have been recently identified in diagnostic and relapsed neuroblastoma, albeit at low frequencies.^{22,44–52} The prevalence is 1% at diagnosis and 2% at relapse.^{45,53} The mutation tended to be associated with poor survival.^{45,53}

Additionally, *FGFR1*^{N546K} mutations have been found in some cases of other solid tumors. Tumors of the central nervous system, mainly low-grade gliomas in children, represent the most common entities with *FGFR1*^{N546K} mutations. Besides *FGFR1*^{N546K} mutations, *FGFR1*^{K656E} mutations and other *FGFR1* variants have been detected.⁵⁴ Overall, *FGFR1* point mutations in the tyrosine kinase domains have been described in the following histological tumor types of the central nervous system: dysembryoplastic neuroepithelial tumors^{55,56},

pilocytic astrocytomas^{33–35}, rosette-forming glioneuronal tumors^{57,58}, oligodendrogliomas^{55,59}, glioblastomas⁶⁰, and diffuse leptomeningeal tumors⁶¹. Sporadic cases with *FGFR1*^{N546K} mutation have also been observed in other tumor entities, for instance in breast cancer⁶² and Ewing sarcoma⁶³.

2.2.5. Selective FGFR inhibitors

Several novel agents inhibiting the kinase domain of FGFRs have entered clinical trials over the last few years, and four FGFR inhibitors, termed as erdafitinib, pemigatinib, infigratinib, and futibatinib, have been approved in adult patients by the US Food and Drug administration (FDA) to date.^{64–67} These inhibitors were developed for patients with tumors harboring FGFR alterations to selectively target FGFR and, thus, to reduce adverse effects elicited by non-selective tyrosine kinase inhibitors (TKI) and increase the inhibitory effect on FGFR. Clinical trials of FGFR inhibitors show that response rates differ between genetic alterations and therefore, it is necessary to determine clinical markers, which predict the response rate.⁶⁸

2.2.6. Characteristics of the FGFR inhibitors erdafitinib and futibatinib

In 2019, erdafitinib (JNJ-42756493) has been approved by the FDA, making it the first approved FGFR-targeted drug. This FGFR inhibitor has been described to effectively inhibit all four members of FGFR.⁶⁴ It was approved for treatment of patients with metastatic or unresectable urothelial carcinoma with *FGFR2* or *FGFR3* alterations. An overall response rate of 40%, a median progression-free survival of 5.5 months, and a median overall survival of 13.8 months were observed in patients with *FGFR*-altered tumors treated with erdafitinib. About half of the patients were affected by treatment-related grade 3 or higher grade adverse events, such as hyperphosphatemia, hyponatremia, stomatitis, asthenia, and ocular events.⁶⁴

Futibatinib (TAS-120) irreversibly inhibits FGFR1 to 4 and showed antitumor activity in heavily pretreated patients with *FGFR*-altered tumors, especially in intrahepatic cholangiocarcinoma with *FGFR2* fusion or rearrangement. In these patients, a response rate of 42%, a median progression-free survival of 9.0 months and a median overall survival of 21.7 months was observed. Adverse events included hyperphosphatemia, increased aspartate aminotransferase levels, stomatitis, and fatigue.⁶⁷

2.3. Aim of this study

We hypothesized that the amino acid substitution asparagine-to-lysine at position 546 leads to constitutive activation of FGFR1, and that mutated FGFR1 is thus an oncogenic driver in neuroblastoma. We also hypothesized that *FGFR1*^{N546K} may be an actionable target in these tumors for treatment with specific FGFR inhibitors.

Therefore, this thesis aimed to address the following questions on *FGFR1*^{N546K} mutant neuroblastoma:

- I. What is the oncogenic potential of the *FGFR1*^{N546K} mutation in neuroblastoma pathogenesis?
- II. Does the *FGFR1*^{N546K} mutation represent an actionable target in preclinical neuroblastoma models?

For this purpose, the molecular and phenotypic effects of *FGFR1*^{N546K} ectopically expressed in murine *Ba/F3* cells were analyzed. Additionally, a genetically engineered mouse model bearing an inducible *FGFR1*^{N546K} transgene in the presence and absence of ectopic *MYCN* expression was characterized. Finally, the anti-tumor effects using the approved FGFR inhibitors erdafitinib and futibatinib were evaluated *in vitro* and *in vivo*. The results of this study demonstrate that mutant *FGFR1*^{N546K} is a strong oncogenic driver in neuroblastoma, in particular in concert with *MYCN* overexpression, and a potential therapeutic target in this malignancy, providing a starting point for improved treatment in these patients.

3. Material and Methods

3.1. Material

3.1.1. Reagents

Name	Company	Order No.
Agar-Agar	Millipore	101614
Albumin Fraction V (BSA)	PanReac AppliChem	A1391,0100
AvrII, BamHI, HindIII, SbfI, SpeI	Biolabs	R0174, R0133, R0136, R0642, R0104
Carbenicillin	Carl Roth®	6344
Sodium carboxymethylcellulose (CMC-Na)	Selleckchem	S6703
Dimethylsulfoxide (DMSO)	Merck	101900
DPBS	Gibco™	14190144
CellTiter-Glo® (CTG)	Promega	G755A, G756A
Erdaftinib (JNJ-42756493)	Selleckchem	S8401
Ethanol	Carl Roth®	9065.2
Ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA)	Carl Roth®	8043.2
Fetal Bovine Serum (FBS)	Gibco™	10500064
Formaldehyde 4%	Sigma-Aldrich	1.00496
Futibatinib (TAS-120)	Selleckchem	S8848
GeneRuler DNA Ladder	Thermo Fisher Scientific	SM0311, SM0243
Halt™ Protease and Phosphatase Inhibitor Cocktail	Thermo Scientific™	78442
Heparin solution	Stemcell™ Technologies	07980
Human FGF-basic recombinant protein	Gibco™	PHG0024

Isofluran	Piramal Critical Care GmbH	30372.00.00
LB Broth (Lennox)	Sigma-Aldrich	L7275
Lipofectamine™ 2000 Transfection Reagent	Invitrogen™	11668019
Milk powder	Carl Roth®	T145
M-PER™ Mammalian Protein Extraction Reagent	Thermo Scientific™	78503
Nitrocellulose Membrane	Invitrogen™	LC2000
NuPAGE™ Antioxidans	Invitrogen™	NP0005
NuPAGE™, Gels, 4%-12%	Invitrogen™	NP0336BOX
NuPAGE® LDS Sample Buffer (4x)	Invitrogen™	NP0008
NuPAGE™ MOPS SDS Running Buffer (20x)	Novex®	NP0001
NuPAGE® Sample Reducing Agent (10x)	Invitrogen™	NP0004
NuPAGE® Transfer Buffer (20x)	Novex®	NP0006
One Shot™ TOP10 Electro-comp™ E.coli	Invitrogen™	C404050
Opti-MEM™ I Reduced Serum Medium	Gibco™	31985062
Paraformaldehyde	Sigma Aldrich	818715
pBABE-puro	Addgene	1764
pCL-Eco	Addgene	12371
Penicillin/Streptomycin (P/S)	Gibco™	15140122
Pierce® ECL Western Blotting Substrate	Thermo Scientific	32109
Plasmid Kits	Qiagen	12123, 12143, 12162
Platinum™ II Hot-Start PCR	Invitrogen™	14000012

Master Mix (2x)		
Polybrene [®]	Santa Cruz Biotechnology	SC-134220
Precision Plus Protein [™] Kaleidoscope [™] Prestained Protein Standards	Bio-Rad	1610375
Propanol	Carl Roth [®]	6752.3
Pure Link Genomic DNA Mini Kit	Invitrogen [™]	K182001
Puromycin	Sigma-Aldrich	P9620
Q5 High-Fidelity DNA Polymerase	Biolabs	M0491
QIAquick Gel Extraction Kit	Qiagen	28704
Q5 Site-Directed Mutagenesis Kit	Biolabs	E0554
Qubit [™] 1x dsDNA Assay-Kits	Invitrogen [™]	Q33230
Quick Start [™] Bradford 1x Dye Reagent	Bio-Rad	5000205
Recombinant Murine Interleukine-3 (IL-3)	PeptoTech	213-13
RedSafe [™] Nucleic Acid Staining Solution	iNtRON Biotechnology	21141
REDTaq [®] ReadyMix [™] PCR Reaction Solution	Sigma-Aldrich	R2523
ROTI [®] Free Stripping Buffer	Carl Roth [®]	3319
ROTI [®] Mix PCR 3	Carl Roth [®]	L785.2
RPMI 1640 Medium, Gluta-MAX [™] Supplement	Gibco [™]	61870010
Sodium chloride (NaCl)	Carl Roth [®]	3957
Sodium hydroxide (NaOH)	Carl Roth [®]	6771

Sponge Pad for Xcell II™ Blotting	Invitrogen™	EI9052
TransIT-LT1 Transfection Reagent	Mirusbio®	MIR 2304
TRIS-Acetate-EDTA Buffer	VWR® Life Science	K915
TRIS base	Tocris Bioscience™	3163
TRIS hydrochloride (HCl)	Carl Roth®	9090
Triton™ X-100	Merck	9036-19-5
Tween20®	PanReac AppliChem	A4974
Trypan blue	Boehringer	295833
Trypsin-EDTA, 0.05%	Gibco™	25300054
Antifade Mounting Medium	Vectashield®	VEC-H-1000
Antifade Mounting Medium with DAPI	Vectashield®	VEC-H-1200
Water, nuclease-free	Qiagen	129115

Table 1: Reagents used for experiments.

3.1.2. Primers

Name	Sequence	Length
pBABE_FGFR1_F1_SDM	ATATCATCAAGCTGCTGGGGG	21
pBABE_FGFR1_R1_SDM	TCTTATGCTTCCCGATCATC	20
pBABE_FGFR1_F4_Seq	GGTCTTAGGCAAACCCCTGG	20
pBABE_FGFR1_R4_Seq	GAGGCCAGATACTCCATGCC	20
F4_FGFR1_TG	CTTTATTAGCCAGAAGTCAGATGC	24
R4_FGFR1_TG	AATGGCGGACTCAAACGCCGCTG	23
F5_FGFR1_WT	AAGCACGTTTCCGACTTGAGTTG	23
R5_FGFR1_WT	GGGTGAGCATGTCTTTAATCTACC	24
MYCN_chr18F1	ACTAATTCTCCTCTCTCTGCCAGTATTTGC	30
MYCN_chr18R2	TGCCTTATCCAAAATATAAATGCCCAGCAG	30

MYCN_OUT1	TTGGCACACACAAATGTATATACACAATGG	30
TH5	CACCCTGACCCAAGCACT	18
TH3	CTTTCCTTCCTTTATTGAGAT	21
CreDU	GATACCTGGCCTGGTCTG	18

Table 2: Primers (5' to 3') used for PCR. All primers were purchased from Invitrogen™. The primers in the first four lines were used for *in vitro* studies and the others for mouse genotyping.

3.1.3. Antibodies

Protein	Company	Order No.	Dilution
Goat Anti-Mouse Immunoglobulins (Ig), HRP-linked	CiteAb	P0447	1:1,000 (WB)
Goat Anti-Rabbit IgG, HRP-linked	Cell Signaling	7074	1:1,000 (WB)
β-Actin	Abcam	ab49900	1:10,000 (WB)
FGFR1	Cell Signaling	9740	1:1,000 (WB) 1:200 (IF)
p-FGFR1 Tyr653/654	Thermo Fisher Scientific	44-1140	1:1,000 (WB)
FRS2	Abcam	Ab183492	1:1,000 (WB)
p-FRS2-α Tyr436	Cell Signaling	3861	1:1,000 (WB)
AKT	Cell Signaling	4685	1:1,000 (WB)
p-AKT Ser473	Cell Signaling	9271	1:1,000 (WB)
ERK1/2	Cell Signaling	4695	1:1,000 (WB)
p-ERK Thr202/Tyr204	Cell Signaling	4370	1:2,000 (WB)
STAT3	Cell Signaling	4909	1:2,000 (WB)
p-STAT3 Tyr705	Cell Signaling	9145	1:1,000 (WB)
Goat anti-Rabbit IgG, Alexa Fluor™ 555	Invitrogen	A27039	1:2,000 (IF)
Phox2b [Clone	Abcam	Ab183741	1:100 (IHC)

EPR14423]			
-----------	--	--	--

Table 3: Antibodies used for experiments including application-specific dilutions. P, phosphorylated; WB, Western Blot; IF, Immunofluorescence; IHC, Immunohistochemistry.

3.1.4. Devices

Name	Model	Company
Automated Liquid Handler	Biomek 4000	Beckman Coulter
Bioanalyzer Instrument	2100 Bioanalyzer	Agilent
Cell counter	Cedex XS	Roche
Centrifuge for cell culture	Cryofuge 6000	Heraeus Sepatech
Fluorescence Microscope	DM5500 B	Leica Biosystems
Heating block	AccuBlock™ Digital Dry Bath	Labnet
Incubator	Galaxy 170 S	New Brunswick
Imager for Electrophoresis and Western Blot	CHemiDoc™ MP Imaging System	BIO-RAD
Microbiological cabinet	Heraeus HLB 2448	Heraeus
Microcentrifuge	Microcentrifuge 5418 R	Eppendorf
Micropulser Electroporation	MicroPulser Electroporator	BIO-RAD
Microscope for cell culture	Axiovert 25 Inverted Microscope	Carl Zeiss
Module Western Blot	Mini-Gel-Tank	Invitrogen™
MRI	3.0T MRI system	Philips
MRI coil	40-mm diameter	Philips Research Europe
Microplate Reader	SpectraMax i3x	Molecular Devices
PCR-Cycler	UNO II Thermoblock	Biometra®
Pipet Controller	BrandTech™ accu-jet™ pro	Fisher Scientific
Pipets 0.5-10 µl, 10-100 µl, 100-1000 µl	Research® plus single channel pipets	Eppendorf
Power Supply Western Blot	PowerEase™ Touch 120 W	Invitrogen™

Shaker	Sunflower Mini-Shaker	Grant Bio
Shaker cabinet	MaxQ™ 6000	Thermo Scientific™
Spectralphotometer	V-1200	VWR®
Stainer	Leica BOND-MAX stainer	Leica Biosystems
Vortexer	Reax 2000	Heidolph
Waterbath	Precision Series	Carl Roth®

Table 4: Devices used for experiments.

3.1.5. Software

- Adobe Illustrator, version 2024 28.3.0
- Biomek Software, version 4.1
- Cedex XS Software, version 1.0.3
- Cytovision®, version 7.7
- FIJI, version 1.52p
- GraphPad Prism, version 10.1.2
- Geneious Prime, version 2022.0.2
- Image Lab™ Software, version 5.2
- Microsoft Excel, 14.0.7261.5000
- Microsoft PowerPoint, version 14.0.7268.500
- NEBaseChanger®, version 1.3.3
- RadiAnt DICOM Viewer, version 2021.2
- R, version 4.0.4
- SoftMax® Pro 6, version 6.6

3.1.6. Animal ethics vote

Animal experiments were conducted in accordance with FELASA recommendations. The mice were housed in a specific-pathogen-free facility. Breedings and experiments were approved by the local animal care committee and the relevant authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, AZ: 81-02.04.2019.A009; 84-02.04.2020.A146). The condition of the mice was evaluated according to a scoresheet (see Section 7.4.).

3.2. Methods

3.2.1. Site-directed mutagenesis of cloned *FGFR1*

The retroviral *pBABE-puro* backbone vector⁶⁷ was generated and the coding sequence of *FGFR1 beta* (Transcript ID ENST00000397091.9) (see Section 7.3.) was cloned into the vector by Dr. Florian Malchers (University Hospital of Cologne). A C to G nucleotide exchange at position c.1638 of the *FGFR1* coding sequence, leading to p.N546K, was introduced into the cloned *FGFR1* sequence using the Q5 Site-Directed Mutagenesis (SDM) Kit (Biolabs) according to the manufacturer's instructions (Figure 2).⁵³ Mutagenic primers (*pBABE_FGFR1_F1_SDM* and *pBABE_FGFR1_R1_SDM*, see Table 2) were designed and an annealing temperature of 62°C was calculated by the tool NEBaseChanger®.⁶⁹ For inserting the specific nucleotide substitution, polymerase chain reaction (PCR) using the appropriate primers was conducted and an enzyme mix was added to link the deoxyribonucleic acid (DNA) ends and remove the template. The mutated vector was integrated in 5-alpha competent *Escherichia coli* (E.coli) cells by heat shock to amplify the DNA.⁵³

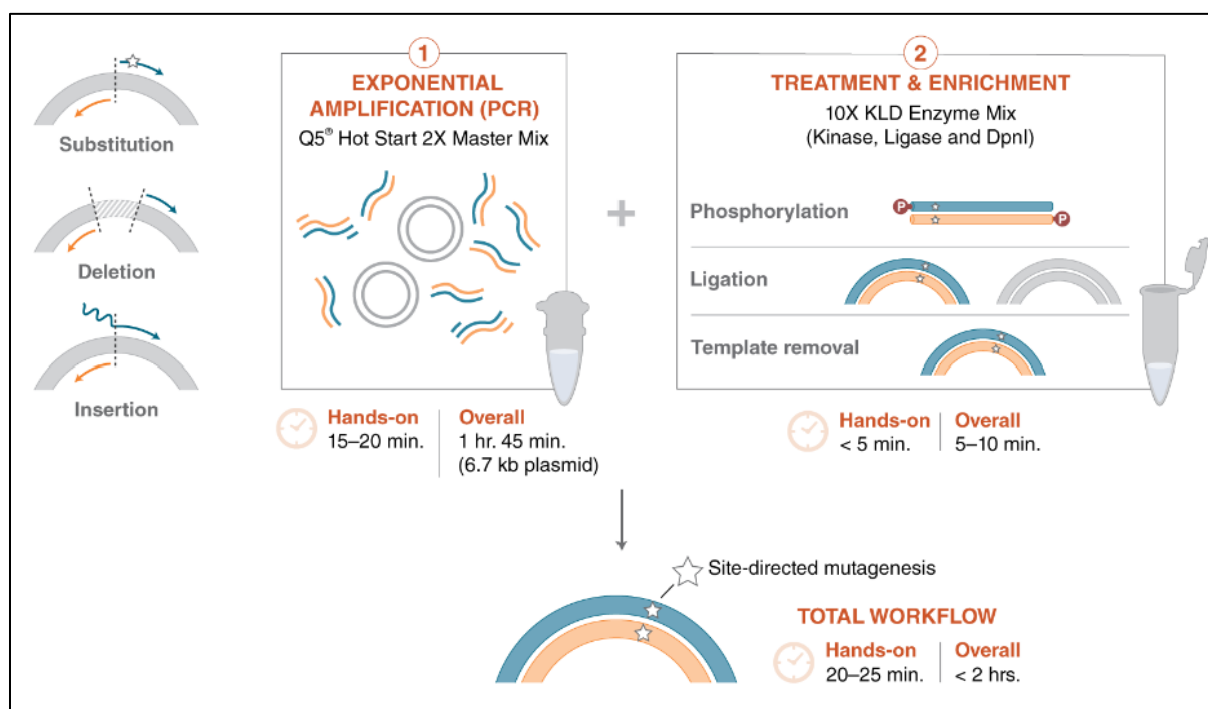


Figure 2: Workflow of site-directed mutagenesis.

The process of inserting the specific substitution by site-directed mutagenesis consists of two steps. First, the template DNA was amplified by PCR using mutagenic primers. Second, the PCR product was treated with an enzyme mix consisting of kinase, ligase and DpnI. This mix phosphorylates and ligases the mutated DNA and removes the template. These steps result in a plasmid containing the desired point mutation.^{70,71}

Figure based on New England Biolabs®. Site Directed Mutagenesis. <https://www.neb.com/en/applications/cloning-and-synthetic-biology/site-directed-mutagenesis> (accessed January 25th, 2025).⁷⁰

3.2.2. Bacterial transformation

For plasmid amplification, bacterial transformation by electroporation was performed. 1 µl plasmid (100 ng/µl) was added to Electrocomp E.coli on ice. For electroporation the shock was implemented in Gene Pulser/MicroPulser Electroporation Cuvettes 0.1 cm by Micropulser. After adding LB medium (LB Broth solved in distilled water) the bacteria were shook at 37 °C, which is the specific growth temperature for the bacteria, at 220 rpm for 1 hour. Volumes ranging from 5 to 100 µl were spread on LB plates with carbenicillin (1:1,000), which is more stable than ampicillin, as selection antibiotic. The plates were incubated overnight at 37 °C. Single colonies were picked with pipette tips and grew in 3 ml LB medium with antibiotic (1:1,000) for 14 hours. To increase the amount of plasmid, the bacteria were subsequently cultured again in another 15 ml LB medium for additional 6 hours.⁵³ Plasmid isolation was performed according to the protocols of the QIAGEN Plasmid Mini, Midi, and Maxi kits (Qiagen). For long-time storage, liquid overnight culture and glycerol were mixed 1:1 in cryovials and stored at -80 °C.

3.2.3. Restriction digest

To verify the modified *pBABE-puro* plasmid by fragment length analysis, the following enzymes were used in various combinations: AvrII, HindIII, SpeI, BamHI, and SbfI (see Section 7.3.). The reaction was performed using the appropriate reaction buffer referring to the instructions for the distinct enzymes.

The following reagents were combined:

- 1 µl DNA (500 ng – 1 µg)
- 0.5 µl of each restriction enzyme
- 1.5 µl 10x reaction buffer
- X µl distilled water (to bring volume to 15 µl)

The mix was incubated at 37 °C for 1 hour and visualized on a 1% agarose gel.

3.2.4. Polymerase chain reaction

PCR was conducted for the process of site-directed mutagenesis (see Section 3.2.1), the preparation of the appropriate sequence for dideoxy sequencing (see Section 3.2.6) and genotyping of mice (see Section 3.2.19) to amplify DNA. The PCR mix in most experiments contained the PlatinumTM II Hot-Start PCR Master Mix 2x (consisting of Platinum II Taq Hot-Start DNA-Polymerase, Platinum II PCR-Buffer, and dNTPs), nuclease-free water, and DNA (see scheme below). For genotyping of the *Th-IRES-Cre* transgene in mice, Q5 Polymerase was used (see scheme below).

The PCR mix (1x) was generated according to the following scheme using Platinum II Taq Hot-Start DNA-Polymerase:

- 12.5 µl Platinum™ II Hot-Start PCR Master Mix 2x
- 1 µl each Forward Primer
- 1 µl each Reverse Primer
- 1 µl or 2 µl (in case of mouse tissue) DNA
- X µl nuclease-free water (to bring volume to 25 µl)

The PCR mix (1x) was generated according to the following scheme using Q5 Polymerase (only for *Th-IRES-Cre* mouse genotyping PCR):

- 5 µl Q5 Buffer
- 5 µl Enhancer
- 0.25 µl Q5 Polymerase
- 0.5 µl dNTPs
- 1 µl each Forward Primer
- 1 µl each Reverse Primer
- 2 µl DNA of mouse tissue
- X µl nuclease-free water (to bring volume to 25 µl)

PCR was performed in a thermo-cycler using different heating steps, called denaturation, annealing, and extension, in multiple cycles. The annealing temperatures were calculated by NEBaseChanger. The exact temperatures and number of cycles depended on the characteristics of the primers and PCR products and are given in the appropriate sections. For cycling conditions of dideoxy sequencing see Section 3.2.6, and for mouse genotyping Section 3.2.19.

3.2.5. Agarose gel electrophoresis

All PCR products were evaluated by agarose gel electrophoresis. 10 µl of the samples were mixed with Loading Dye and run on a 1.5% agarose gel consisting of 150 ml Tris-Acetate-EDTA (TAE) Buffer 1x, 2.3 g agarose, and 7.5 µl RedSafe™ Nucleic Acid Staining Solution. A GeneRuler 100 bp DNA Ladder was used as a reference. The gel was analyzed in a BIO-RAD ChemiDoc™ MP Imaging System.

3.2.6. Preparation of dideoxy sequencing

First, the appropriate *FGFR1* sequence comprising the mutation was amplified by PCR. Second, PCR products with premixed primers were prepared and dideoxy sequencing was performed by Microsynth Seqlab GmbH.

The PCR mix was generated according to the scheme shown in Section 3.2.4. Sequencing primers (pBABE_FGFR1_F4_Seq and pBABE_FGFR1_R4_Seq) were designed by Genious (see Table 2). PCR was performed under the following cycling conditions:

Step 1: Initial Denaturation	94 °C	2:00 min.
Step 2: Denaturation	94 °C	0:30 min.
Step 3: Annealing	60 °C	0:30 min.
Step 4: Elongation	68 °C	0:40 min.
Step 5: Final Elongation	72 °C	5:00 min.

Steps 2 to 4 were conducted in 35 cycles.

The expected PCR product was 414 bp long. In case of unspecific products, the appropriate DNA was extracted from the gel according to the QIAquick Gel Extraction Kit (Qiagen).

Mixes containing about 7.5 ng/μl of the DNA template and the forward or reverse sequencing primers, respectively, were sent to Microsynth Seqlab GmbH for further processing. Sequencing data were aligned to the unmutated *FGFR1* sequence to detect the mutation.

3.2.7. Cell lines

Ba/F3, which is a murine IL-3 dependent pro-B cell line, was cultured in Roswell Park Memorial Institute medium (RPMI) + GlutaMAX, 20% FCS, 1% P/S, and 10 ng/ml recombinant murine IL-3.⁷² *HEK293T* cells grew in DMEN + GlutaMAX, 10% FCS, and 1% P/S. All cell lines were incubated at 37 °C and 5 % CO₂ and were negatively tested for mycoplasma.

Cells were split at 80% to 90% confluency. For passaging, adherent cells, like *HEK293T*, were detached from the ground by Trypsin ETDA. Trypsin reaction was inactivated by adding culture medium and the cells were split appropriately. To split cells in suspension, such as *Ba/F3*, the appropriate amount of medium was removed and fresh medium was added.

For long-time storage, cells were frozen in cryovials at -80 °C. In the following, volumes that allow freezing of four cryovials are given. Cells in suspension or detached cells were centrifuged at 500 g for 5 minutes and were resuspended in 1.5 ml FCS and 0.5 ml of the appropriate medium. After an incubation time of 5 to 10 minutes at room temperature, a freezing

solution, consisting of 0.6 ml FCS, 1 ml medium, and 0.4 ml DMSO, was added. 1 ml of the mix was quickly filled into each cryovial. The cryovials were initially stored in freezing boxes to slowly cool down to -80 °C overnight.

For thawing cells, e.g., to control the success of the freezing procedure or to use the cells in cell culture, the cells were transferred to a falcon tube and resuspended in the appropriate medium first by drops and then faster. After 10 minutes the DMSO was released and the cells were centrifuged and transferred to a cell culture flask. Media were changed at the following day and cell viability was evaluated.

3.2.8. Stable virus transduction

Stable virus transduction was kindly performed by Dr. Marcel Dammert (during PhD thesis, University Hospital of Cologne). The *pBABE-puro* retroviral vectors containing *FGFR1*^{WT} (wild-type) or mutated *FGFR1*^{N546K} were stably integrated in *Ba/F3* cells. *HEK293T* cells were used for retrovirus production. When *HEK293T* cells were grown at 70% confluency, transfection of the *pBABE-puro* retroviral vectors and a *pCI-Eco* retroviral packaging vector was performed.⁵³

The transfection mixture consisted of the following components:⁵³

- 4.6 µg *pBABE* with gene of interest
- 4.6 µg *pCI-Eco*
- 486 µl Opti-MEM
- 14 µl TransIT-LT1

After an incubation time of 20 minutes, the mix was added dropwise to the cells. The supernatant containing the viruses was collected after 48 hours and centrifuged 2-times at 40 g for 4 minutes to remove residual cells. 3 ml supernatant was sterilely filtered through a 0.45 µm nitrocellulose filter. The retroviruses were directly used for infection of cells or stored at -80 °C.⁵³

Ba/F3 cells were seeded at 50% confluency and stably transduced with the previously generated retroviruses. The viral supernatant (2/3 of final volume) and fresh media (1/3 of final volume) supplemented with 0.7 µl/ml polybrene to increase transduction efficacy were added. The media were replaced after 48 hours by fresh media containing 3 µg/ml puromycin to negatively select non-transduced cells. The antibiotic was withdrawn after 10 passages and the expression of the gene of interest was confirmed by western blotting and dideoxy sequencing.⁵³

3.2.9. DNA isolation

DNA was isolated from cell culture to confirm the success of stable virus transduction of *FGFR1*^{N546K} and *FGFR1*^{WT} into the cells by dideoxy sequencing. The cells were centrifuged at 500 g for 5 minutes and the cell DNA pellet was washed with PBS. Another centrifugation step followed and afterwards the supernatant was removed. Then, DNA isolation was performed according to the Pure Link Genomic DNA Mini Kit instructions (Invitrogen™). To determine DNA concentrations the Qubit™ Assay (Invitrogen™) was used.

3.2.10. Transformation assay

Ba/F3 cells usually need IL-3 to grow, but ectopic oncogene expression may allow IL-3 independent growth.⁷² For *Ba/F3* transformation, withdrawal of IL-3 in *Ba/F3 FGFR1*^{N546K} cells was conducted independently in different cell culture bottles over weeks. Media were changed at least once a week. When sufficient numbers of viable cells were detected, distribution on further cell culture bottles was conducted to expand the transformed cells. These cells were frozen for long-time storage, and three *FGFR1*^{N546K} mutant cell lines, referred to as replicates, were used for experiments to assess the phenotypic and molecular effects of *FGFR1*^{N546K}.⁵³

3.2.11. Cytokine independence assay

Parental *Ba/F3* cells and *Ba/F3* cells with *FGFR1*^{WT} or mutated *FGFR1*^{N546K} were seeded at 1 x 10⁵ cells/ml. Cells were cultured with 10 ng/ml and 0 ng/ml IL-3. Cell numbers and viability were determined by Cedex XS Cell Analyzer after 144 hours. Data from triplicate experiments were documented with Excel and plotted with GraphPad Prism.⁵³

3.2.12. Generation of cell lysates

Cells were centrifuged and cell pellets were washed with cold PBS. Cells were lysed in M-PER Mammalian Protein Extraction Reagent supplemented with 40 µl/ml Protease and Phosphatase Inhibitor Cocktail and 10 µl/ml EDTA. The mix was incubated on ice for 30 minutes and centrifuged at 4 °C with high speed for 30 minutes. The supernatant was transferred into another tube. For determination of protein concentrations, 5 µl of the lysates were added to 1 ml Bradford and a photometer was used for measurement.⁵³

The following reagents were mixed to produce lysates:

- Volume lysate [µl] = 20 to 30 µg protein / protein concentration [µg/µl]
- Volume water [µl] = 14 µl – volume lysate
- 1 µl Reducing Agent

- 5 µl Loading Buffer

The mix was heated at 70 °C for 10 minutes. The prepared mixtures were either directly used for western blotting or stored at -20 °C.

3.2.13. Western blot analysis

Twenty to 30 µg proteins and the Precision Plus Protein Kaleidoscope as a reference for the protein size were loaded on a 4%-12% polyacrylamide gel. To conduct protein separation, MOPS SDS Running Buffer (1x) supplemented with 500 µl Antioxidant was added to the chambers. Electrophoresis was performed at 170 Volt until the migration front reached the bottom of the gel.⁵³

Proteins were transferred to a 0.45 µm polyvinylidene fluoride (PVDF) membrane by constructing a sandwich, as follows (from the bottom to the top): cathode, two sponges, one whatman paper, gel, membrane, one whatman paper, two sponges, anode. The materials were soaked in Transfer Buffer (1x) and cold water was given into the chambers. Blotting was performed for 2 hours at 30 Volt.⁵³

Then membranes were blocked in either 5% non-fat milk or bovine serum albumin (BSA) solved in Tris-buffered saline (9 g Tris Base + 68.5 g Tris HCl + 87.8 g NaCl, 1:10 dilution with water) with 0.1% Tween 20 (TBS-T) for 1 hour. All antibodies binding to phosphorylated epitopes were solved in BSA to obtain better signals, whereas the others were solved in milk. Membranes were incubated overnight at 4°C with primary antibodies against: FGFR1, phospho-FGFR1 Tyr653/654, FRS2, phospho-FRS2-α Tyr436, STAT3, phospho-STAT3 Tyr705, AKT, phospho-AKT Ser473, p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2) Thr202/Tyr204, and β-Actin (see Table 3). The next day, two washing steps with TBS-T were performed to remove unbound unspecific antibodies, and membranes were incubated with the following secondary antibodies for 1 hour at 4 °C: Polyclonal Goat anti-Mouse Immunoglobulins/HRP or anti-Rabbit IgG, HRP-linked Antibody (see Table 3). After two more washing steps, Detection Reagent (1:1 dilution of Detection Reagent 1 and 2) was dripped on the membranes. Protein-antibody complexes were visualized using the chemiluminescence detection system.⁵³

Membranes were stripped to detect multiple proteins on the same membrane. Stripping Buffer was added and the membranes were shaken at room temperature for 45 minutes. After washing and blocking, the membranes were incubated with the appropriate primary antibody overnight. The further procedure was performed as outlined above.⁵³

3.2.14. Treatment of cells for protein analysis

Cells were seeded at 4.4×10^6 cells/ml in serum starved media, and after 24 hours, cells were treated with 10 nM, 50 nM, or 100 nM erdafitinib (JNJ-42756493; Selleckchem), or DMSO as control for 1 hour. Cells were then stimulated with 1 ng/ml recombinant FGF2 ligand and 10 μ g/ml Heparin sodium as a cofactor for 20 minutes prior to preparing lysates.⁷³ Generation of cell lysates and western blot analyses were performed according to the protocol outlined above (see Sections 3.1.12. and 3.1.13.).⁵³

3.2.15. Treatment of cells for viability analysis

Treatment was performed using the robot system Biomek 4000 according to a semiautomatic pipetting protocol established in the working group. 100 μ l media with 10,000 suspended *Ba/F3* cells were pipetted into each well. At the same day, the cells were treated with various concentrations of FGFR inhibitors, ranging between 0.0001 μ M and 100 μ M, and DMSO as control (see Figure 3). Erdafitinib and futibatinib were diluted and then added in triplicates.⁵³

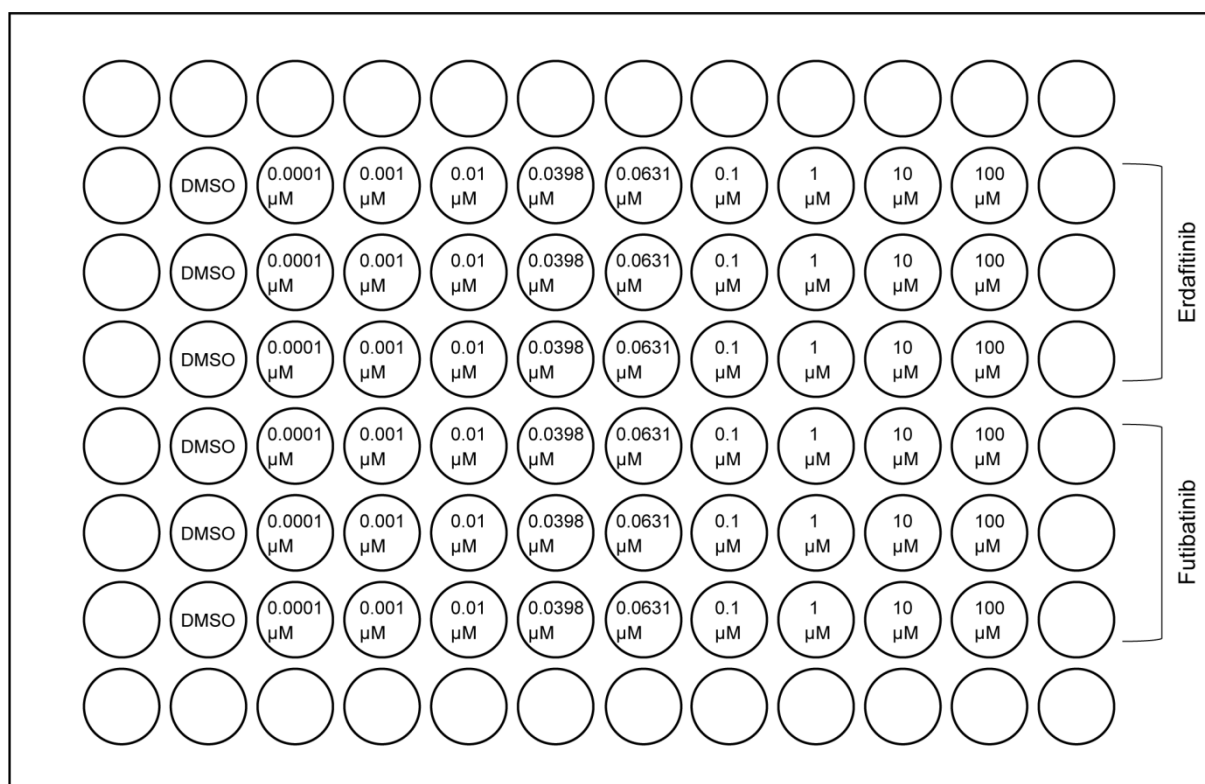


Figure 3: Scheme of FGFR inhibitor concentrations used for *Ba/F3* cell treatment in 96-well plates.

Ba/F3 cells were plated into inner wells and outer wells were left blank. DMSO as control was added to the second row of wells. Cells of the upper part of the plate were treated with erdafitinib, while those of the lower part were treated with futibatinib. The following concentrations were added in triplicates each: 0.0001 μ M, 0.001 μ M, 0.01 μ M, 0.0398 μ M, 0.0631 μ M, 0.1 μ M, 1 μ M, 10 μ M, and 100 μ M.

3.2.16. Cell viability assay

After 72 hours, a cell viability assay was performed. CellTiter-Glo® (CTG) was added to the wells under light-protected conditions. Luminescent signals were measured using a SpectraMax i3x instrument after 15 minutes. The luminescent signal is proportional to the amount of adenosine triphosphate (ATP), which allows quantifying the viable cells. Half maximal inhibitory concentrations (IC₅₀), defined as concentrations of the compound, which inhibit 50% of the cell growth, were calculated by GraphPad Prism.⁵³

3.2.17. Mouse tagging

Mice were tagged by ear hole markings according to an internal scheme at the age of 21 days. For treatment experiments, tail tip biopsies were taken from mice at the age of 10-12 days and the tails were marked with permanent marker. The tissue was used for genotyping.

3.2.18. Lysis of mouse biopsies

Mouse tissue of the ear or tail was lysed to extract the DNA. 50 µl of Lysis Buffer (2.5 ml 10 M NaOH + 1 ml 0.5 M EDTA (pH 8.0) + nuclease-free water to bring volume to 1l) was added and heated at 95 °C for 1 hour. The mix was neutralized with 50 µl 40 mM Tris HCl (pH 7) and the supernatant was used for genotyping PCRs.

3.2.19. Mouse genotyping

Genotyping PCRs were performed according to standard protocols mentioned in section 3.2.4. The following primers were used for mouse genotyping: F4_FGFR1_TG, R4_FGFR1_TG, F5_FGFR1_WT and R5_FGFR1_WT for the *FGFR1* transgene, MYCN_chr18F1, MYCN_chr18R2 and MYCN_OUT1 for the *MYCN* transgene, and TH5, TH3 and CreDU for the presence of *Th-IRES-Cre* (see Table 2). For *FGFR1* and *MYCN* PCR Platinum™ II Hot-Start PCR Master Mix 2x was used, and for *Th-IRES-Cre* PCR the mix was generated using Q5 polymerase according to Section 3.2.4.

FGFR1 PCR was performed under the following cycling conditions:

Step 1: Initial Denaturation	94 °C	3:00 min.
Step 2: Denaturation	94 °C	0:30 min.
Step 3: Annealing	60 °C	0:35 min.
Step 4: Elongation	72 °C	0:35 min.
Step 5: Final Elongation	72 °C	5:00 min.

Steps 2 to 4 were repeated in 35 cycles.

MYCN PCR was performed under the following cycling conditions:

Step 1: Initial Denaturation	95 °C	3:00 min.
Step 2: Denaturation	95 °C	0:30 min.
Step 3: Annealing	60 °C	0:30 min.
Step 4: Elongation	72 °C	0:40 min.
Step 5: Final Elongation	72 °C	5:00 min.

Steps 2 to 4 were repeated in 40 cycles.

Th-IRES-Cre PCR was performed under the following cycling conditions:

Step 1: Initial Denaturation	98 °C	3:00 min.
Step 2: Denaturation	98 °C	0:45 min.
Step 3: Annealing	56 °C	0:45 min.
Step 4: Elongation	72 °C	0:60 min.
Step 5: Final Elongation	72 °C	10:00 min.

Steps 2 to 4 were repeated in 35 cycles.

The length of amplicons was determined by gel electrophoresis (see Section 3.2.5), and was 607 bp for *FGFR1* wildtype, 184 bp for the *FGFR1* transgene, 500 bp for *MYCN* wildtype, 295 bp for the *MYCN* transgene, and 430 bp and 290 bp in the presence and absence of *Th-IRES-Cre*, respectively.

3.2.20. Magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed on a clinical 3.0 Tesla MRI system using a small rodent solenoid coil with an inner diameter of 40 mm. Mice were anesthetized by a 1.5% to 2.5% isoflurane inhalation and body temperature was kept constant during examination by an implemented heating system. The abdomen and pelvis of the mice were scanned to detect neuroblastoma. High resolution axial and coronal T2-weighted MR images were generated using a multishot turbo-spin echo (TSE) sequence (repetition time (TR) = 5729 ms (coronal) and 7639 ms (axial), echo time (TE) = 60 ms, flip angle (FA) = 90°, acquired resolution = 0.19 x 0.26 mm² in-plane, 1 mm slice thickness, turbo factor = 10). Images were exported in DICOM format and the maximal axial diameters of each tumor part were measured using the RadiAnt software.⁵³

3.2.21. Histopathology

Mouse tissue was fixed in 4% formaldehyde and after 48 hours, the samples were embedded into paraffin and subsequently sliced into sections. Three μm sections from formalin-fixed paraffin-embedded tissue (FFPE) were deparaffinized and hematoxylin and eosin (HE) and immunohistochemistry staining was performed according to standard protocols of the routine diagnostics pipeline (Institute for Pathology, University Hospital Cologne). HE-stained sections were evaluated in a blinded fashion by a board-certified pathologist according to the INPC tumor classification (see Section 2.1.3). Immunohistochemistry using a primary antibody against PHOX2B, which is a known marker of neural crest cells, was performed using the Leica BOND-MAX stainer (Leica Biosystems).⁵³

3.2.22. Immunofluorescence staining

Mouse samples were cut into 6 μm thick sections and transferred to object slides, which were stored at $-20\text{ }^{\circ}\text{C}$. For immunofluorescence staining, sections were thawed for 30 minutes and the tissues were circled. After the first washing step in 1x PBS for 5 minutes, the samples were fixed with 4% formaldehyde. Another washing step followed and the samples were permeabilized with 0.3% Triton X-100 in PBS for 10 minutes. The sections were then rinsed in 1x PBS for 5 minutes and blocked with 1% BSA/Glycine/0.1% Tween for 45 minutes. The sections were incubated at $4\text{ }^{\circ}\text{C}$ overnight with the primary antibody anti-FGFR1 (Cell Signaling #9740, 1:200) in 1% BSA/TBS/0.1% sodium-acid. The samples were rinsed three times in 1x PBS for 5 minutes and incubated with the appropriate fluorescence-conjugated secondary antibody for 2 hours at room temperature under light-protected conditions (goat anti-rabbit IgG, Superclonal Recombinant, Alexa Fluor 555, Invitrogen, 1:2,000 in PBS). Another three washing steps with PBS followed and then an increasing alcohol series was conducted. DAPI (1:2 in Antifade Mounting Medium) was dropped on the dried samples. Immunofluorescence staining was visualized using an Epi-fluorescence microscope (Leica DM5500) and images were taken at 40x magnification using Cytovision.⁵³

3.2.23. Treatment of mice with FGFR inhibitors

Mice were genotyped at the age of 10-12 days and *R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt};Th-MYCN^{tg/wt}* mice were selected for treatment studies. MRI was performed weekly, beginning at the day of treatment start, to evaluate the tumor diameters. Mice bearing neuroblastoma were randomized to treatment groups. For intervention trials, animals were treated with 5 mg/kg and 30 mg/kg futibatinib solved in 0.5% sodium carboxymethylcellulose (CMC-Na) with 10% DMSO, and with 0.5% CMC-Na containing 10% DMSO as control. The solutions were administered by gavage daily with a volume of 10 ml/kg. Drug holiday was conducted when mice lost more than 10% weight.⁵³

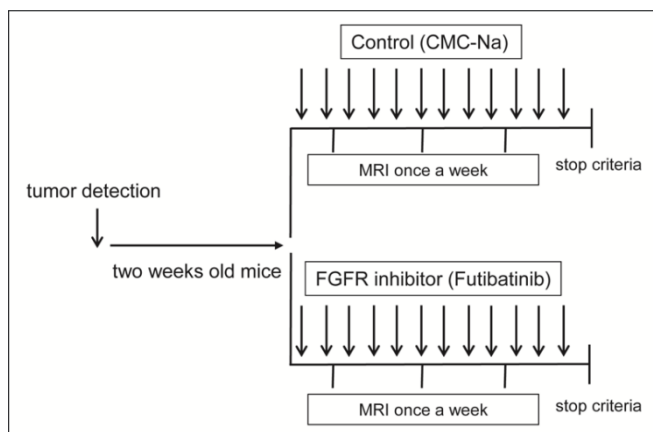


Figure 4: Scheme of mouse treatment with futibatinib or control solution.

Treatment of R26-LSL-FGFR1-N546K^{fl/wt}; Th-IRES-Cre^{tg/wt}; Th-MYCN^{tg/wt} mice bearing neuroblastoma was started at the age of 2 weeks with the FGFR inhibitor futibatinib or CMC-Na as control. The effect was evaluated using weekly MRI until stop criteria were reached.

3.2.24. Evaluation of anti-tumor effects

Overall survival and tumor size were determined to evaluate the anti-tumor effect of futibatinib. Mice were sacrificed when stop criteria were reached, and the time from treatment start to scarification was used for survival analysis. Response evaluation criteria in solid tumors (RECIST), distinguishing between complete response (CR), partial response (PR), progressive disease (PD) and stable disease (SD), were used to assess response to treatment. Therefore, maximal diameters of all tumor lesions in treated mice were measured on the MRI images. Tumor lesions of the left and right adrenal glands, the space between the adrenal glands, and the pelvis were taken into account for measurement. The relative change of tumor sizes were calculated by considering the sum of all target lesions. Complete response referred to disappearance of all tumor lesions, and partial response to reduction of at least 30% of the sum of diameters compared to the baseline sum of diameters. Progressive disease was defined as increase of at least 20% of the sum of diameters, taking the smallest sum of diameters as reference. Stable disease was diagnosed if neither partial response nor progressive disease occurred. The best overall response was the best response that was observed from treatment start to progression.⁷⁴

3.2.25. Statistical analysis

Statistical analysis was performed by R or GraphPad Prism. Boxplots indicate the median, 1st/3rd quartiles, and minimum/maximum values. P-values were calculated by one-sided Wilcoxon rank-sum tests. Disease-specific survival for observation mouse cohorts was calculated from birth to time of death due to neuroblastoma, with a maximum observation period of 365 days, and the overall survival from birth to time of death due to any reason and not restricted to neuroblastoma. For the treatment cohorts, it was calculated from treatment start until death. Survival is shown by Kaplan-Meier curves, and p-values were calculated by log-rank tests. P-values were classified as follows: $p > 0.05$, not significant (n.s.); $p < 0.05$, significant (*); $p < 0.01$, significant (**); $p < 0.001$, significant (***) ; $p < 0.0001$, significant (****).

4. Results

4.1. Characterization of *FGFR1*^{N546K} in *Ba/F3* cells

4.1.1. Cloning of *FGFR1*^{N546K} into a vector and transduction of *Ba/F3* cells

The human transcript variant *FGFR1 beta* was cloned into the retroviral expression vector *pBABE-puro* from position 5,215 bp to 7,416 bp between the cloning sites attB1 and attB2 (bacterial attachment sites 1 and 2) (see Section 7.3.). In comparison to *FGFR1 alpha*, exon 3 is deleted in the splice variant *FGFR1 beta*, resulting in the absence of the immunoglobulin-domain I and a higher affinity to FGF1/2 as a consequence.⁷⁵ Site-directed mutagenesis using site-specific primers was conducted to introduce a base change from G to C, which leads to an asparagine-to-lysine substitution at codon 546 in *FGFR1*.

Integration of *FGFR1 beta* in the *pBABE-puro* vector was confirmed by its fragment length analysis after restriction digest (Figure 5A, see Section 7.3.). The nucleotide substitution in the *FGFR1*^{N546K} sequence was validated after transduction of the construct into *Ba/F3* cells by dideoxy sequencing (Figure 5B).

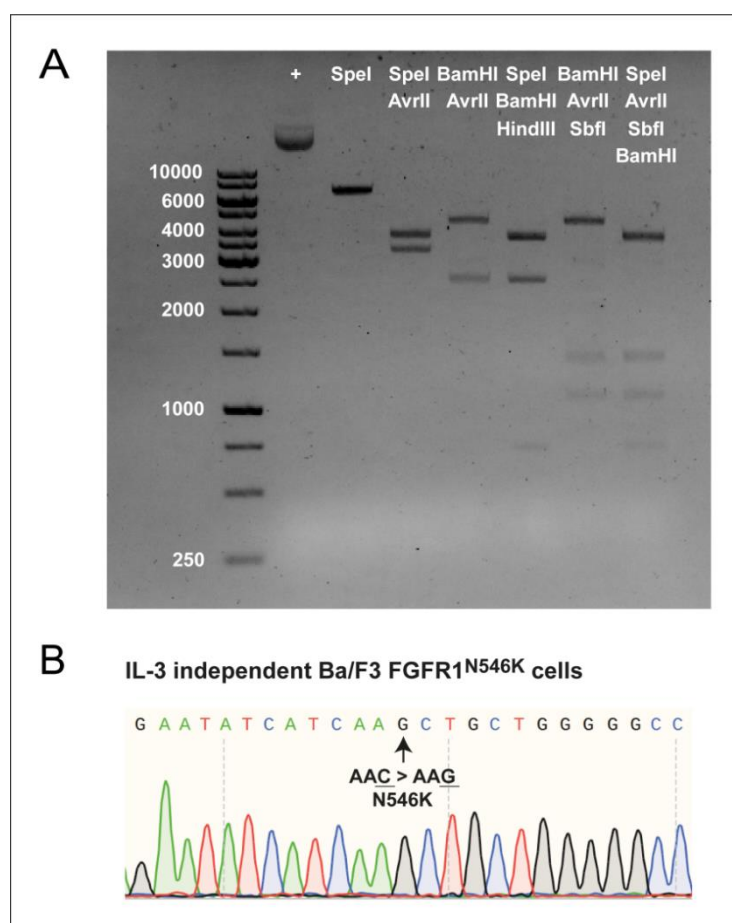


Figure 5: *pBABE-puro* vector with integrated *FGFR1*^{N546K}.

A: Gel electrophoresis after restriction digest with AvrII (371 bp), HindIII (387 bp), SpeI (4,415 bp), BamHI (5,162 bp), and SbfI (6,668 bp) of the *pBABE-puro* vector with *FGFR1* (7,427 bp in total). As expected, the fragments have the following lengths (from left to right): > 10,000 bp (vector without restriction enzyme); 7,427 bp; 4,098 and 3,329 bp; 4,791 and 2,636 bp; 747, 2,652 and 4,028 bp; 1,130, 1,506 and 4,791 bp; 747, 1,130, 1,506 and 4,044 bp.

B: Representative validation of the nucleotide substitution C to G at position p.546 of *FGFR1* in IL-3 independent *Ba/F3* cells after stable virus transduction of the modified *pBABE-puro* vector.

4.1.2. Analysis of the oncogenic potential of *FGFR1*^{N546K} in *Ba/F3* cells

Ba/F3 cells stably expressing *pBABE-puro* vectors containing *FGFR1*^{WT} or *FGFR1*^{N546K} were generated to assess the transforming potential of the mutation. *Ba/F3*, which is an IL-3 dependent pro-B cell line, is an established system to evaluate the oncogenic potential of mutated tyrosine kinase receptors. It is based on the observation that these cells rely on IL-3 to grow while dying after withdrawal of IL-3. Oncogene expression transforms the cells to oncogene addicted growth without IL-3 supplementation.⁷²

A cytokine independence assay was conducted to evaluate the transforming potential of *FGFR1*^{N546K} expression. Parental *Ba/F3* cells and *Ba/F3* cells expressing *FGFR1*^{WT} or *FGFR1*^{N546K} were cultured in media containing 10 ng/ml IL-3 or without IL-3. The concentration of viable cells was measured after 144 hours. As expected, the viable cell concentration did not differ between parental *Ba/F3*, *Ba/F3 FGFR1*^{WT}, and *Ba/F3 FGFR1*^{N546K} in the presence of 10 ng/ml IL-3, because cell growth did depend on IL-3 in all three cases. After withdrawal of IL-3, however, the viable cell concentration of *Ba/F3 FGFR1*^{N546K} cells was significantly higher than that of *Ba/F3* parental cells. By contrast, this cell concentration of parental and *Ba/F3 FGFR1*^{WT} cells did not differ. These observations indicate that *Ba/F3* cells expressing *FGFR1*^{N546K} developed oncogene-addicted growth, which allowed cells to survive in the absence of IL-3 (Figure 6A).⁵³

To use this cell model for further experiments, IL-3 was withdrawn in three independent cell culture bottles of *Ba/F3 FGFR1*^{N546K} cells. After about one month viable *FGFR1*^{N546K} expressing cells adapted to IL-3 independent growth and started to proliferate. All following experiments were conducted with stably proliferating cells. In contrast, *Ba/F3* parental and *Ba/F3 FGFR1*^{WT} cells completely died without IL-3.

Western blot analysis was performed to compare FGFR1 pathway activation between IL-3 dependent and IL-3 independent cells. *Ba/F3* parental, *Ba/F3 FGFR1*^{WT}, and *Ba/F3 FGFR1*^{N546K} cells grown in media containing IL-3 and three independent *Ba/F3 FGFR1*^{N546K} cell replicates without IL-3 were analyzed. Parental *Ba/F3* cells did not endogenously express *FGFR1* (Figure 6B).⁷⁶ Western blot analysis confirmed that FGFR1 was expressed in all transduced cell lines. Strong phosphorylation of FGFR1, its adaptor protein FRS2-α, and other down-stream components in IL-3 independent cells, particularly in replicate 1 and 2, in comparison to the IL-3 dependent controls demonstrated activation of the FGFR1 signaling pathway. This result indicates ligand independent FGFR1 pathway activation through a constitutively active tyrosine kinase induced by mutated *FGFR1*^{N546K} in IL-3 independent cells. Interestingly, activation of the down-stream components varied between the distinct IL-3 independent *FGFR1*^{N546K} cell lines. In approach 1, ERK and AKT were mainly phosphorylated, whereas it was STAT3 in approach 2. In approach 3, neither FGFR1 nor the downstream

components were significantly upregulated, indicating that these cells may not depend on the FGFR1 signaling pathway. Thus, another mechanism may allow IL-3 independent growth in these cells, which has to be considered for interpretation of further experiments (Figure 6B).⁵³

Since these results suggested that cell growth of *Ba/F3 FGFR1^{N546K}* replicates 1 and 2 in the absence of IL-3 depend on activation of the FGFR1 signaling pathway through *FGFR1^{N546K}*, we proceeded with these models to evaluate the inhibitory effect of FGFR inhibitors.

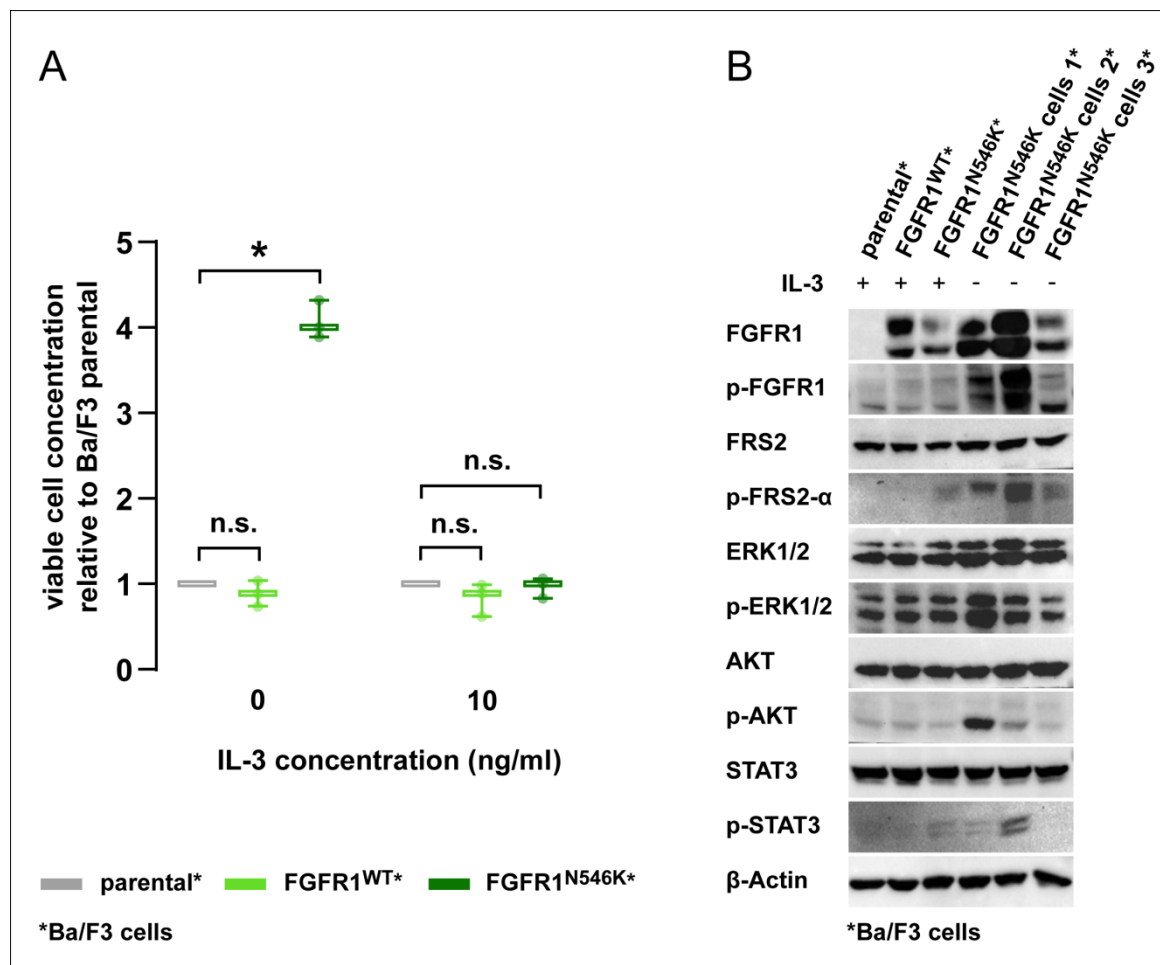


Figure 6: Transforming potential and FGFR1 signaling pathway in *Ba/F3 FGFR1^{N546K}* cells.

A: Boxplots of the viable cell concentration of *Ba/F3* cells expressing *FGFR1^{WT}* (light green) or *FGFR1^{N546K}* (dark green) relative to parental *Ba/F3* cells (grey) in the presence of IL-3 (10 ng/ml) and after withdrawal of IL-3 after 144 hours. Replicates of three independent experiments are shown. Parental vs. *FGFR1^{N546K}* *Ba/F3* cells in the absence of IL-3, $p = 0.038$ by Wilcoxon rank-sum test.

B: Western blot analysis of FGFR1 signaling pathway proteins of IL-3 dependent *Ba/F3* cells (parental, *FGFR1^{WT}*, *FGFR1^{N546K}*) and IL-3 independent *FGFR1^{N546K}* *Ba/F3* cells 1-3. Antibodies against total and phosphorylated FGFR1, FRS2-α, AKT, ERK, and STAT3 were used; β-Actin served as a loading control.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

4.1.3. Treatment of *Ba/F3 FGFR1^{N546K}* cells with FGFR inhibitors

To assess whether *FGFR1^{N546K}* mutated cells are sensitive or resistant to FGFR inhibitors, IL-3 independent *Ba/F3* cells expressing *FGFR1^{N546K}* were treated with erdafitinib or futibatinib using concentrations between 0.1 and 100,000 nM. These two FGFR inhibitors were chosen because erdafitinib was the first approved FGFR inhibitor in adult patients, and because futibatinib, which is also approved in adult patients, has shown strong inhibitory results in murine *NIH3T3* cells expressing *FGFR1^{N546K}* by others.⁷⁷

Ba/F3 FGFR1^{N546K} replicates 1-3 in the absence of IL-3 and IL-3 dependent *Ba/F3* parental cells as control were treated with erdafitinib and futibatinib for 72 hours, followed by analysis of cell viability by luminescent cell viability assay. The experiment demonstrated that both FGFR inhibitors significantly inhibited viability of IL-3 independent *Ba/F3 FGFR1^{N546K}* replicates 1 and 2 in comparison to parental *Ba/F3* cells (Figure 7A-D). In addition, futibatinib inhibited cell viability at lower concentrations than erdafitinib, indicating a higher on-target efficacy of the drug. In contrast to replicates 1 and 2, cell viability of replicate 3 did not differ from parental cells under exposure to both erdafitinib and futibatinib (Figure 7A-D). While the exact resistance mechanism of these cells remains unexplained, the result was in line with the absence of FGFR1 pathway activation in native cells, as shown by western blot analysis (Figure 6B).⁵³

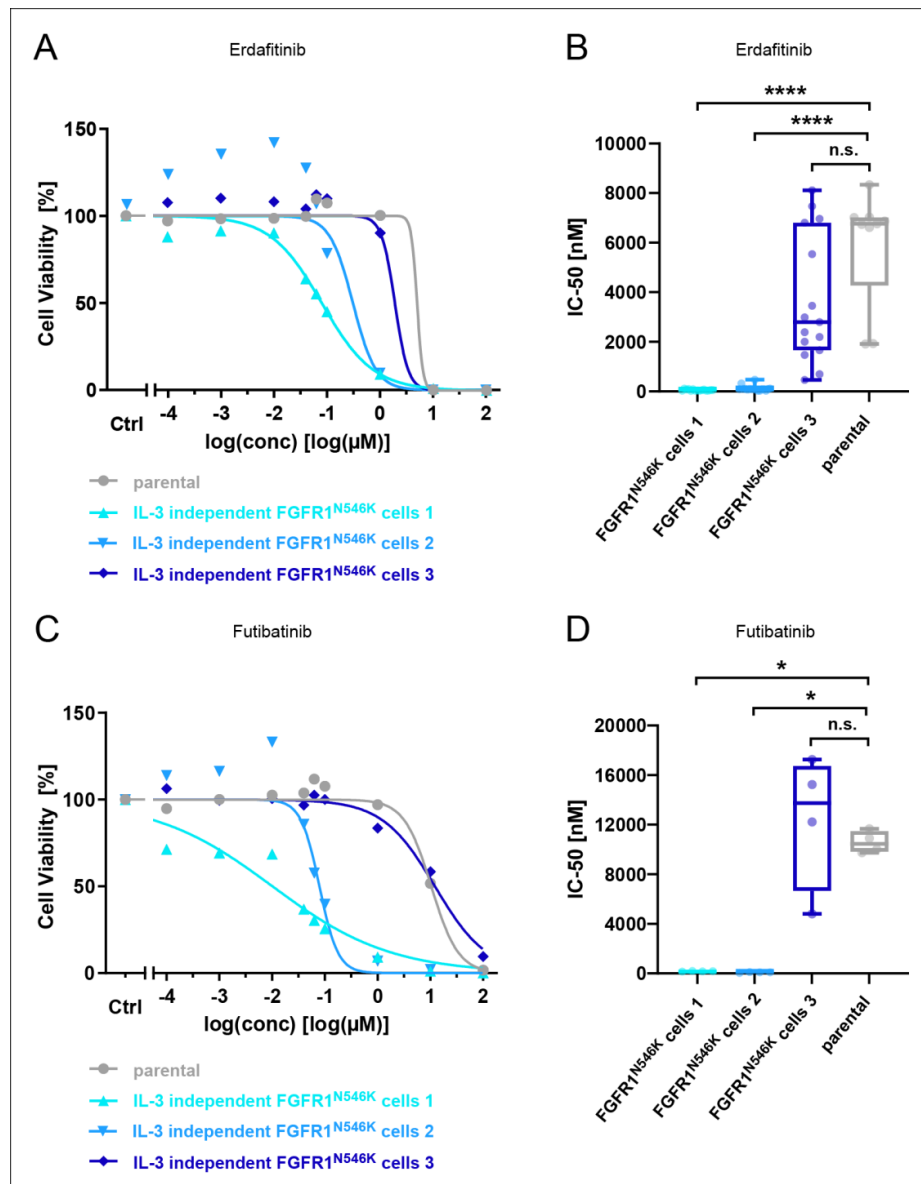


Figure 7: Cell viability after treatment of *Ba/F3 FGFR1^{N546K}* cells with erdafitinib or futibatinib.

A/C: Cell viability (y-axis) after treatment of IL-3 independent *Ba/F3 FGFR1^{N546K}* cells 1-3 (blue) and parental *Ba/F3* cells (grey) as control with erdafitinib (**A**) and futibatinib (**C**) for 72 hours. A range of concentrations (0.0001 μM, 0.001 μM, 0.01 μM, 0.0398 μM, 0.0631 μM, 0.1 μM, 1 μM, 10 μM, 100 μM; x-axis) of the FGFR inhibitors were used and plotted on a logarithmic scale. Growth inhibition curves and means of four independent experiments consisting of triplicates each are shown.

B/D: Boxplots of IC₅₀ values (y-axis) after treatment of IL-3 independent *Ba/F3 FGFR1^{N546K}* cells 1-3 (blue) and parental *Ba/F3* cells (grey) as control with erdafitinib (**B**) and futibatinib (**D**) for 72 hours. Results of nine (parental) and fifteen (replicates 1, 2, 3) independent experiments for erdafitinib (**B**) and four for futibatinib (**D**) consisting of triplicates each are shown. Replicate 1 vs. parental, $p < 0.001$; replicate 2 vs. parental, $p < 0.001$ (**B**); replicate 1 vs. parental, $p = 0.014$; replicate 2 vs. parental, $p = 0.014$ (**D**); all comparisons by Wilcoxon rank-sum test.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

To examine whether exposure of IL-3 independent *Ba/F3 FGFR1^{N546K}* cells to FGFR inhibitors results in repression of FGFR1 signaling, western blot analysis was performed after treatment with 10 nM, 30 nM, and 100 nM erdafitinib and DMSO as control. Consistent with the findings of the cell viability assay, this experiment revealed dose-dependent inhibition of the FGFR1 signaling pathway: In the sensitive replicates 1 and 2, the signal of phosphorylated FGFR1, its adaptor protein FRS2- α , and the downstream components ERK and STAT3 was significantly reduced upon FGFR inhibition. The impact on AKT was not consistent between replicate 1 and 2. Replicate 3 again showed lack of FGFR1 and downstream protein phosphorylation, except for weak FRS- α phosphorylation and dose-dependent inhibition after treatment (Figure 8). This observation is in line with the findings of the cell viability assay, and is likely due to low expression of the FGFR1 transgene. Taken together, erdafitinib induced inhibition of the FGFR signaling pathway in *FGFR1^{N546K}* addicted cells, which indicate that *FGFR1* bearing this specific point mutation may be a promising therapeutic target.⁵³

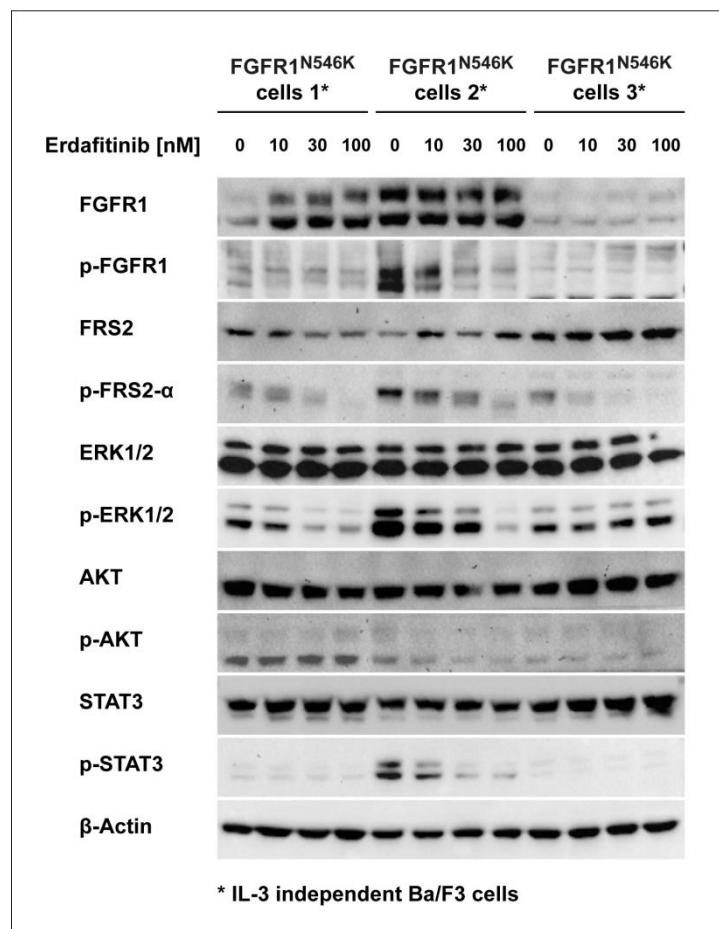


Figure 8: FGFR1 signaling pathway after treatment of *Ba/F3 FGFR1^{N546K}* cells with erdafitinib.

Western blot analysis of FGFR1 signaling pathway proteins in IL-3 independent *Ba/F3* replicates 1-3 after treatment with 0 nM, 10 nM, 30 nM, and 100 nM erdafitinib. Antibodies against total and phosphorylated FGFR1, FRS2- α , AKT, ERK, and STAT3 were used; β -Actin served as a loading control.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

4.2. Characterization of *FGFR1*^{N546K} in a transgenic mouse model

4.2.1. Generation of genetically engineered mouse models

For *in vivo* experiments, mice bearing *FGFR1*^{N546K} and *Th-IRES-Cre* transgenes alone or in combination with a *Th-MYCN* transgene were used to assess the oncogenic potential and potential therapeutic value of *FGFR1*^{N546K}.⁷⁸

FGFR1^{N546K} genetically engineered mice (*R26-LSL-FGFR1-N546K*^{fl/wt}) were generated by Taconic Biosciences GmbH. The coding sequence of the *FGFR1*^{N546K} transgene was integrated into the *ROSA26* (Reverse Oriented Splice Acceptor, Clone 26; R26) locus by CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated proteins).⁵³ The *ROSA26* locus is a preferred position for the integration and ubiquitous expression of transgenes.⁷⁹ The introduction of a stop cassette flanked by loxP (locus of x-over, P1) sites prevented transcription of the transgene. For generation of C57BL/6N *R26-LSL-FGFR1-N546K*^{fl/wt} mice, the targeting vector was introduced into embryonic stem cells. Successfully transfected cells were taken to generate chimeras, which were backcrossed, and mice with successful germline transmission were used for further mating.⁷⁹

Afterwards, cross-breeding of *R26-LSL-FGFR1-N546K*^{fl/wt} mice with Cre-transgenic *Th-IRES-Cre*^{tg/wt} mice was performed (Figure 9A).⁵³ Cre recombinase mediated deletion of the stop cassette flanked by *loxP* recombination sites under the control of the tyrosine hydroxylase (Th) promotor.^{79,80} This mechanism enabled conditional induction of mutated *FGFR1* in the sympathetic nervous system during a defined period of mouse development. A fraction of these mice were mated with mice bearing a *Th-MYCN* transgene.⁷⁸

All mice were genotyped to detect whether the animals expressed *FGFR1*^{N546K}, *Th-IRES-Cre*, and/or *MYCN* in a heterozygous manner (Figure 9B).⁵³ Mice having the correct genotype were used for the experiments.

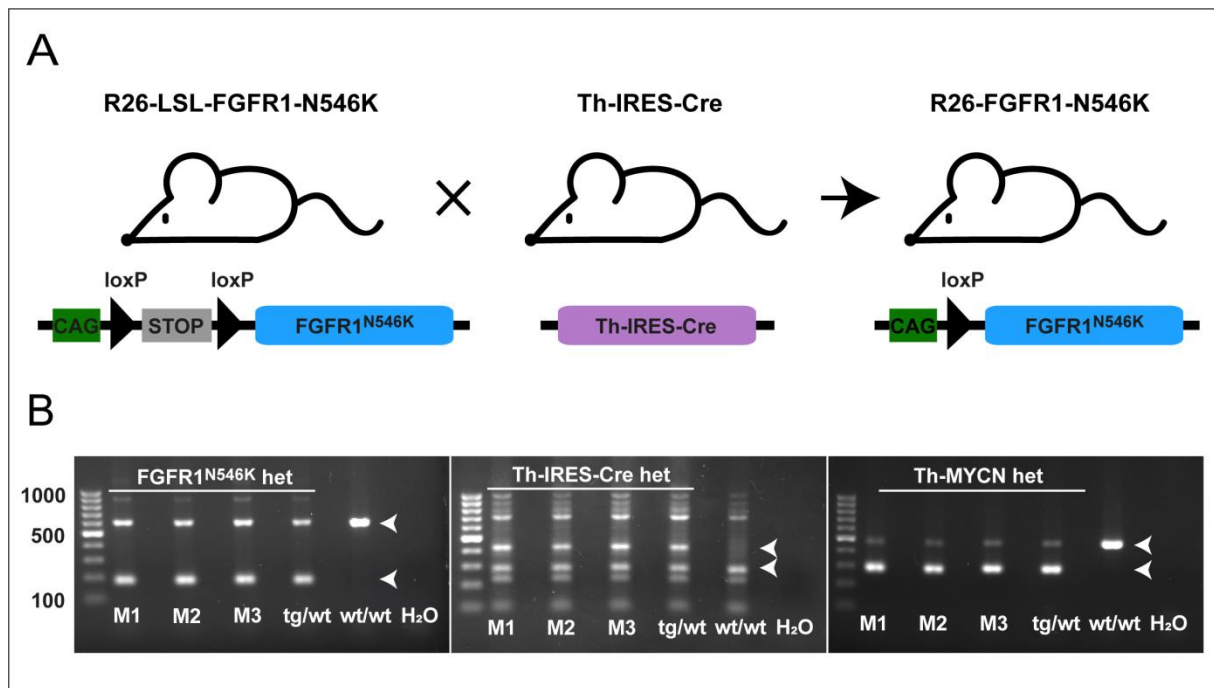


Figure 9: Mating and genotyping of transgenic mice expressing *FGFR1*^{N546K} and/or *MYCN*.

A: *R26-LSL-FGFR1-N546K*^{fl/wt} mice harbored the coding sequence of human *FGFR1*^{N546K} (blue) in the *ROSA26* locus. A stop signal (grey) flanked by *loxP* sites (black) prevented the expression of the transgene. Crossing of these mice with *Th-IRES-Cre*^{tg/wt} mice led to deletion of the stop sequence by Cre recombinase under the control of the Th-promotor. This step resulted in activation of the *FGFR1*^{N546K} transgene in cells of the sympathetic nervous system under the CAG promoter (green).⁷⁹

B: Genotyping PCRs of *FGFR1*^{N546K}, *Th-IRES-Cre*, and *Th-MYCN*. Three mice (M1, M2, M3) heterozygous for each transgene were analyzed; one mouse known to be heterozygous for each transgene, one mouse known to be wild-type, and no template (H₂O) were used as controls. The appropriate lengths for the amplicons are indicated by arrows. Tg, transgene. Het, heterozygous. Wt, wild-type.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

4.2.2. Tumor development in *FGFR1*^{N546K} mice

To evaluate potential tumor development within the first weeks of life, *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} mice were sacrificed at the ages of 7 days (n = 3), 14 days (n = 3) and 21 days (n = 3; see Section 7.5.). Small tumors occurred in the adrenal glands of these mice with 100% penetrance. All tumors were histologically confirmed to be neuroblastomas using hematoxylin and eosin (HE) staining (9/9) and paired-like homeobox 2b (PHOX2B) immunohistochemistry (2/2) (Figure 10A). Protein expression of FGFR1 was validated by immunofluorescence (Figure 10B). Interestingly, comparison of tumor sizes from mice sacrificed at the ages of 7 and 21 days revealed larger tumors in the former, suggesting spontaneous tumor regression within the first weeks of life (Figure 10C).⁵³

Long-term survival was observed in another subgroup of *R26-LSL-FGFR1-N546K^{fl/wt};Th-IRE5-Cre^{tg/wt}* mice (n = 35, see Section 7.6.). Despite tumor detection within the first three weeks of life, tumors were not macroscopically detectable by MRI analysis, starting at the age of 21 days, in the majority of cases (33/35). This is explainable by the small and presumably decreasing tumor size over the time. Two mice (2/35) had macroscopically detectable tumors seen by MRI. Partial tumor regression occurred in one of these five mice (Figure 10D), whereas slow but constant tumor progression occurred in another one, leading to death at the age of 361 days. Histology of the suprarenal glands of part of the mice was analyzed after scarification and tumors *in situ* were still microscopically detectable (15/16). The median survival was 114 days, restricted due to papilloma and not neuroblastoma. These observations suggest that *FGFR1^{N546K}* alone may be capable of driving neuroblastoma development but may not be sufficient for driving a lethal phenotype.⁵³

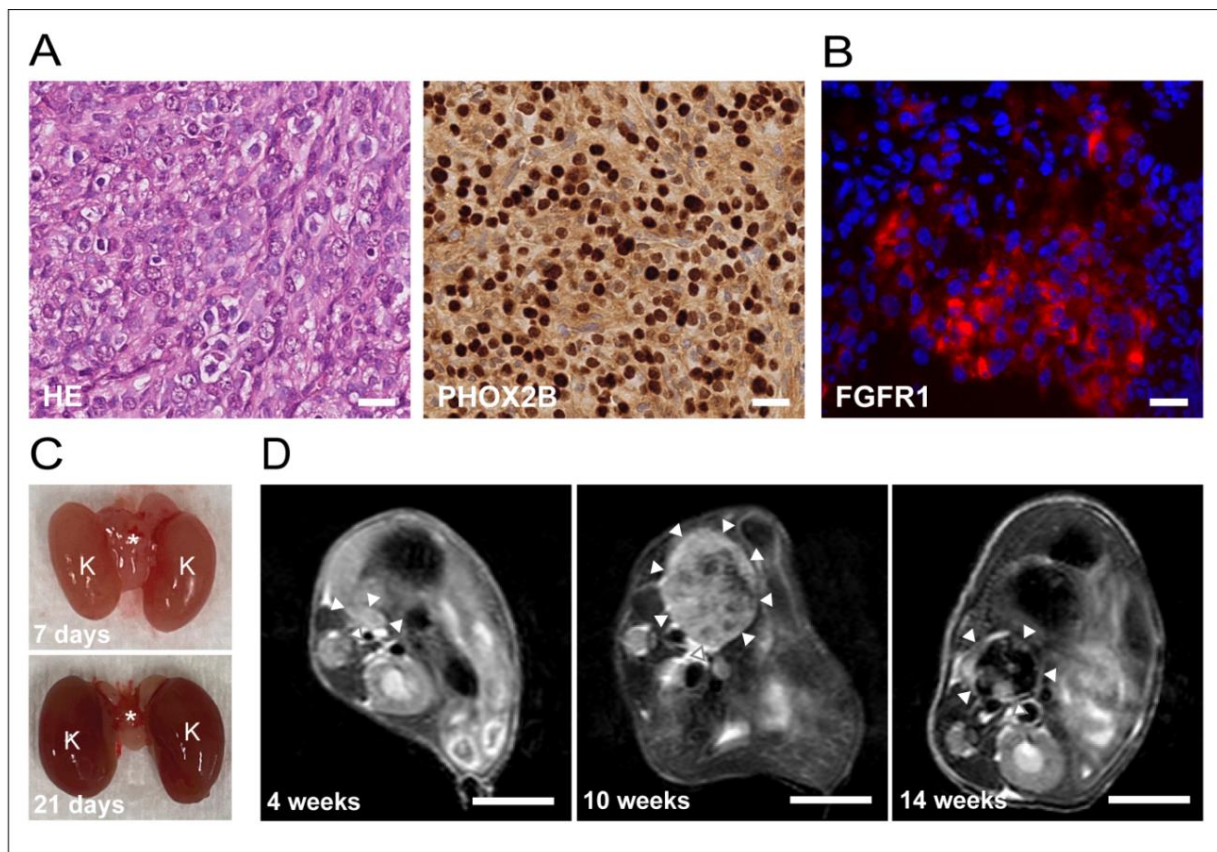


Figure 10: Neuroblastoma development in *FGFR1^{N546K}* mice.

A: HE (left) and PHOX2B (right) staining of a tumor of a 7-days old *FGFR1-N546K^{tg/wt}* mouse. Tumor was classified as differentiating neuroblastoma positive for PHOX2B (brown). Scale bars, 20 μ m.

B: Immunofluorescence staining of FGFR1 (red) of a tumor of a *FGFR1-N546K^{tg/wt}* mouse at the age of 7 days; cell nuclei were counterstained with DAPI (blue). Scale bar, 20 μ m.

C: Macroscopic preparations of tumors (*) and kidneys (K) from *FGFR1-N546K^{tg/wt}* mice at the ages of 7 days (top) and 21 days (bottom).

D: Progression followed by spontaneous regression of a tumor (arrows) in a *FGFR1-N546K^{tg/wt}* mouse at the age of 4, 10, and 14 weeks, as determined by MRI. Scale bars, 0.5 cm.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

By contrast, survival of *R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt}* mice (n = 35, see Section 7.6.) was limited by the development of papilloma (occurring in 25/35 mice, Figure 11A), defined as a benign tumor of the squamous epithelium of the skin or the mucosa, and spindle cell sarcoma (9/35, Figure 11C) in a fraction of cases.⁵³ Mice with papilloma and sarcoma were sacrificed at the ages of 121 and 253 days on average, respectively, indicating that development of these tumors is a late event. Papillomas were predominantly observed at the tail or in the mucosa of the anus, mouth or eyes. FGFR1 expression in the epithelium was validated by immunofluorescence (Figure 11B). Published gene expression data of mice revealed that expression of tyrosine hydroxylase not only occurs in the sympathetic nervous system but also the skin, particularly epithelium, as well as skeletal musculature and connective tissue, which may explain the development of papillomas and sarcomas in these mice.⁸¹

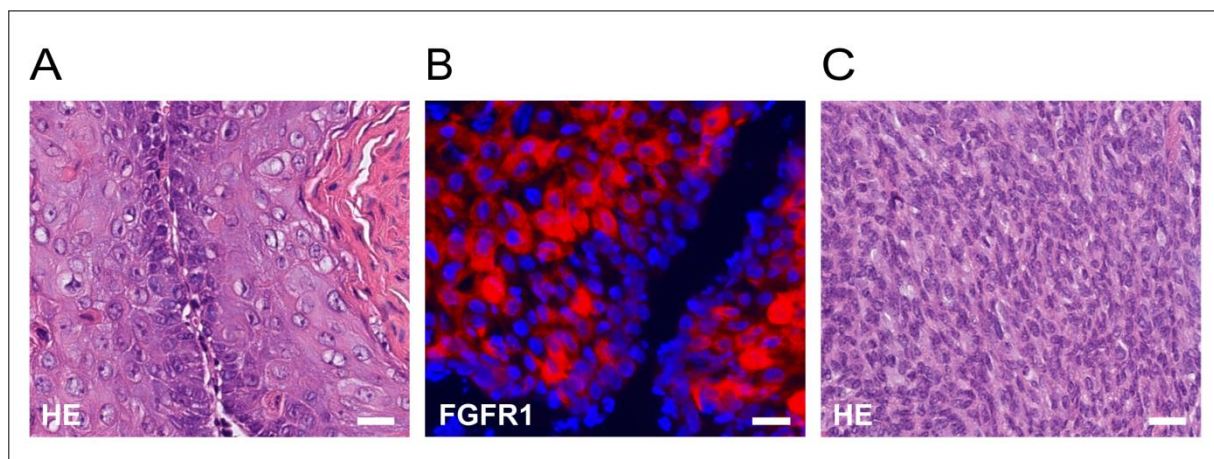


Figure 11: Papilloma of the tail in *FGFR1^{N546K}* mice.

A: HE staining of a papilloma in a *FGFR1-N546K^{tg/wt}* mouse. Thickened, differentiated squamous epithelium (purple) and stroma (pink, right margin) can be seen. Scale bar, 20 μ m.

B: Immunofluorescence staining of FGFR1 (red) of a papilloma of a *FGFR1-N546K^{tg/wt}* mouse; cell nuclei were counterstained with DAPI (blue). Scale bar, 20 μ m.

C: HE staining of a sarcoma consisting of spindle-shaped cells in a *FGFR1-N546K^{tg/wt}* mouse. Scale bar, 20 μ m.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

4.2.3. Tumor development in *FGFR1*^{N546K};*MYCN* mice

R26-LSL-FGFR1-N546K^{fl/wt};*Th-IRES-Cre*^{tg/wt} mice were mated with *Th-MYCN* mice to determine the oncogenic effect of the *FGFR1*^{N546K} transgene in combination with *MYCN* overexpression, which corresponds to the genotype of combined *FGFR1*^{N546K} mutation and *MYCN* amplification in neuroblastoma patients.⁴⁵ *R26-LSL-FGFR1-N546K*^{fl/wt};*Th-MYCN*^{tg/wt};*Th-IRES-Cre*^{tg/wt} (n = 15, see Section 7.7.) mice developed multifocal tumors in the abdomen and the pelvis, presumably deriving from the suprarenal glands and the sympathetic ganglia, within the first days of life with 100% penetrance (Figure 12A). Tumors progressed rapidly, which led to death at the age of 17-21 days due to symptoms that met the stop criteria, such as weight loss and increased abdominal circumference (Figure 12B). HE (11/11) and PHOX2B (7/7) staining confirmed that tumors represented neuroblastoma (Figure 12C). Protein expression of FGFR1 in the tumor cells was validated by immunofluorescence staining (Figure 12D). Thus, the *FGFR1*^{N546K} transgene together with *MYCN* overexpression enabled induction of an aggressive, lethal neuroblastoma phenotype.⁵³

Survival of *R26-LSL-FGFR1-N546K*^{fl/wt};*Th-MYCN*^{tg/wt};*Th-IRES-Cre*^{tg/wt} mice was compared to that of *Th-ALK-F1174*^{tg/wt};*Th-MYCN*^{tg/wt} mice, which corresponds to a frequent genotype in human high-risk neuroblastoma.^{18,78} *Th-ALK-F1174*^{tg/wt};*Th-MYCN*^{tg/wt} (n = 15, see Section 7.8.) mice also developed neuroblastoma with 100% penetrance; these mice, however, survived 5-9 weeks (Figure 12B), which is two to three times longer than survival of mice bearing the *FGFR1*^{N546K} and *MYCN* transgenes.⁵³ As control cohorts, *R26-LSL-FGFR1-N546K*^{fl/wt};*Th-MYCN*^{tg/wt} (n = 10, see Section 7.9.), *Th-MYCN*^{tg/wt} (n = 40, see Section 7.10.), and *Th-ALK-F1174*^{tg/wt} (n = 34, see Section 7.11.) mice were analyzed. In these cohorts, only one mouse of the *Th-MYCN*^{tg/wt} and the *R26-LSL-FGFR1-N546K*^{fl/wt};*Th-MYCN*^{tg/wt} group each developed neuroblastoma leading to death at the age of 239 and 217 days, respectively, corresponding to the reported prevalence of neuroblastomas developing in heterozygous *Th-MYCN* transgenic mice (Figure 12B).^{18,78} Together, these results demonstrate that co-expression of a constitutively active tyrosine receptor kinase, i.e., *FGFR1*^{N546K} or *ALK*^{F1174L}, in combination with *MYCN* is required to robustly drive development of murine neuroblastoma with a lethal phenotype. In addition, the data suggest that *FGFR1*^{N546K} may be a stronger oncogenic driver in neuroblastoma than *ALK*^{F1174L}.⁵³

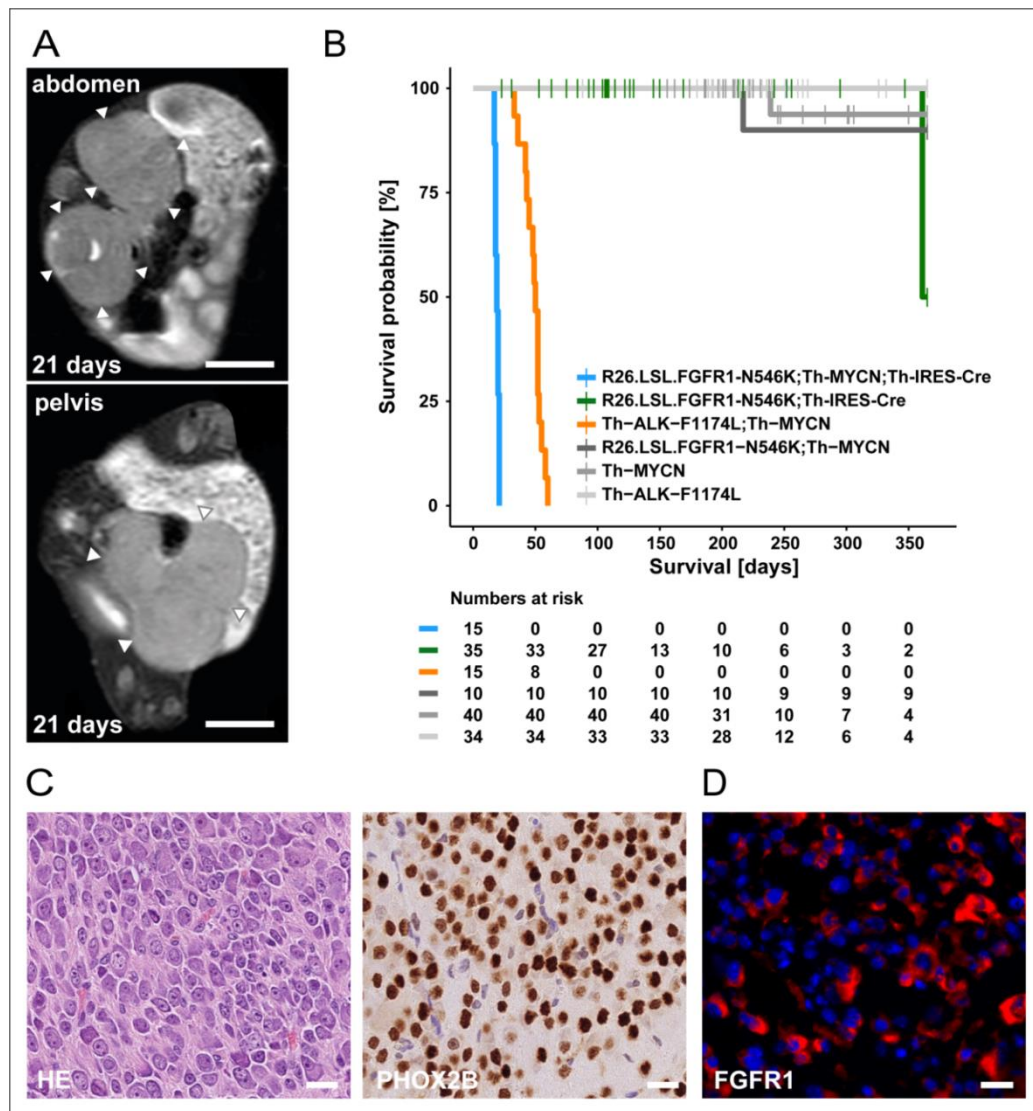


Figure 12: Neuroblastoma development in $FGFR1^{N546K};MYCN$ mice.

A: MRI of neuroblastomas (arrows) in the abdomen (top) and the pelvis (bottom) in a $FGFR1-N546K^{tg/wt};MYCN^{tg/wt}$ mouse. Scale bars, 0.5 cm.

B: Kaplan-Meier estimates of disease-specific survival of six mouse cohorts with distinct genotypes. $FGFR1^{N546K};MYCN$ (blue) vs. control (dark grey), $p < 0.001$; $FGFR1^{N546K};MYCN$ (blue) vs. $ALK^{F1174L};MYCN$ (orange), $p < 0.001$; $FGFR1^{N546K}$ (green) vs. control (dark grey), $p = 0.656$; all comparisons by log-rank test.

C: HE (left) and PHOX2B (right) staining of a tumor obtained from a $FGFR1-N546K^{tg/wt};MYCN^{tg/wt}$ mouse. The tumor was classified as differentiating neuroblastoma positive for PHOX2B (brown color). Scale bars, 20 μm .

D: Immunofluorescence staining of FGFR1 (red) of a tumor obtained from a $FGFR1-N546K^{tg/wt};MYCN^{tg/wt}$ mouse; cell nuclei were counterstained with DAPI (blue). Scale bar, 20 μm .

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

4.2.4. Treatment of *FGFR1*^{N546K};*MYCN* mice with futibatinib

The anti-tumor effect of futibatinib, which appeared to be more effective than erdafitinib in the *in vitro* experiments and previous studies⁷⁷, was evaluated in *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mice that developed aggressive neuroblastoma. MRI analysis was performed weekly, starting at the age of 13-15 days, to evaluate changes in tumor diameters over time. In all mice, a tumor was detected at first MRI analysis, and treatment was started at the same day. The mice were randomly split into either the treatment or the control group, which was treated with the solvent CMC-Na (n = 13, see Section 7.12.). Both futibatinib and control were administered daily by gavage, and drug holiday was started depending on the general condition, especially in case of more than 10% weight loss.⁵³

A dose of 30 mg/kg futibatinib (n = 4, see Section 7.13.) led to overall shrinkage of the tumor (Figure 13A/B), however, survival of treated mice did not differ from control mice (Figure 13C). Three mice had stable disease and one mouse had a partial response (Figure 13D) as best overall response according to RECIST (see Section 3.2.24). The mice thus did not die from tumor progression, but developed weight loss > 10% of the body weight and reduced general condition (according to parameters defined in the scoresheet, see Section 7.4.), presumably caused by diarrhea, which is a known adverse reaction to the drug, and increased stress levels due to the administration procedure.⁵³

The dose was therefore reduced to 5 mg/kg futibatinib (n = 14, see Section 7.13.) in an extended group. This reduction of futibatinib led to deceleration of the tumor growth (Figure 13A/B/D) and significantly prolonged survival (5-40 days versus 3-13 days of control mice treated with CMC-Na; Figure 13C). In 10/12 futibatinib treated mice, stable disease was observed as best response, while partial response and progressive disease occurred in one mouse each; in comparison, progressive disease was noted in 9/9 mice of the control cohort (Figure 13D). In the treatment cohort, 9/12 mice finally died due to progressive neuroblastoma, whereas stable disease was still found in 3/12 mice in the last MRI before sacrifice. One of these mice was sacrificed due to substantial weight loss despite absence of tumor progression, one mouse died spontaneously, and one mouse suffered from paralysis, likely caused by spinal compression by the tumor, albeit it was of small size. Thus, *FGFR1* inhibition by futibatinib at a reduced dose resulted in tumor growth control over a defined period of time and the lower dose showed less side effects. Taken together, futibatinib showed promising anti-tumor efficacy on neuroblastoma in mice co-expressing *FGFR1*^{N546K} and *MYCN*, which is in line with the *in vitro* results.⁵³

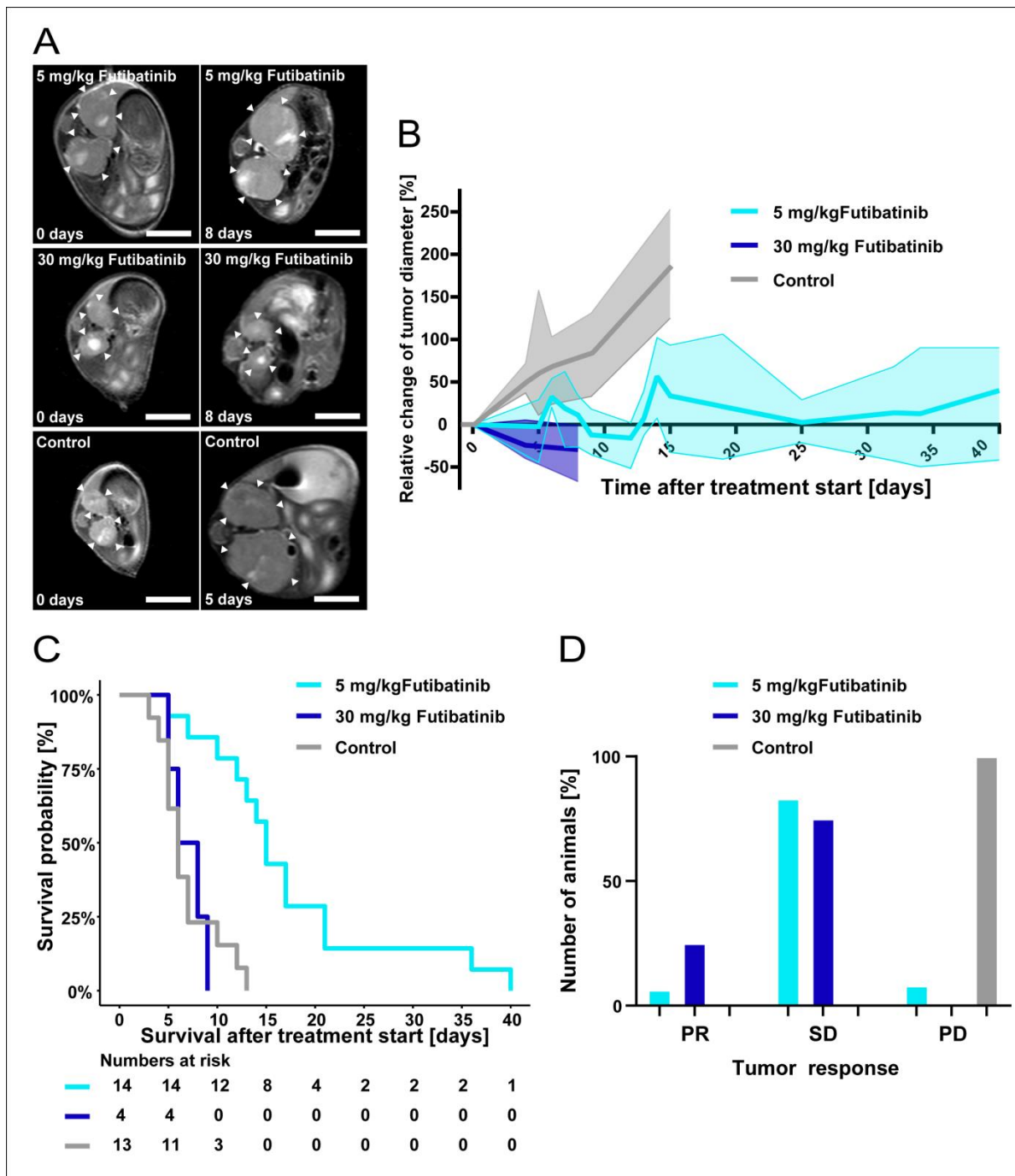


Figure 13: Survival and tumor growth dynamics upon treatment of $FGFR1^{N546K};MYCN$ mice with futibatinib.

A: Assessment of tumor growth dynamics by MRI of neuroblastomas (arrows) in $FGFR1-N546K^{tg/wt};MYCN^{tg/wt}$ mice under treatment with 5 mg/kg futibatinib (top), 30 mg/kg futibatinib (middle) and control solution (bottom). All tumors are shown at start day of treatment and 5 and 8 days after treatment start in the control and the futibatinib treated mice, respectively. Scale bars, 0.5 cm.

B: Relative change of tumor diameters in $FGFR1-N546K^{tg/wt};MYCN^{tg/wt}$ mice over time under treatment with 5 mg/kg futibatinib (n = 12), 30 mg/kg futibatinib (n = 4), and control solution (n = 9). The means and ranges of tumor diameters are illustrated. These parameters were calculated by using the diameters of the left and right adrenal glands, the space between the glands, and the pelvis in each mouse.

C: Kaplan-Meier estimates of overall survival after start of treatment of *FGFR1-N546K^{tg/wt};MYCN^{tg/wt}* mice with 5 mg/kg futibatinib (n = 14), 30 mg/kg futibatinib (n = 4), and control solution (n = 13). 5 mg/kg futibatinib vs. control, p < 0.001; 30 mg/kg futibatinib vs. control, p = 0.929; by log-rank test.

D: Relative numbers of mice with partial response (PR), stable disease (SD) and progressive disease (PD) under treatment with 5 mg/kg futibatinib (n = 12), 30 mg/kg futibatinib (n = 4), and control solution (n = 9). Best overall response was evaluated according to RECIST.

Figure based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

5. Discussion

Since treatment of high-risk neuroblastoma remains challenging, establishing new treatment options is required to improve patient outcome. Such novel therapies aim at improving the response rate and extending the time of remission, both at initial diagnosis and relapsed and refractory disease. Molecular characterization of the tumors by targeted or genome-wide sequencing is capable of identifying alterations in the cancer genome that may serve as therapeutic targets for pharmacological inhibition, representing a promising treatment option in appropriate patients.²⁷

FGFR inhibitors have been approved for treatment of *FGFR*-altered tumors in adult patients, but not in children yet. Mutant *FGFR1*^{N546K} has been recurrently found in neuroblastoma patients and were associated with poor outcome. In this project, the role of *FGFR1*^{N546K} in neuroblastoma pathogenesis and its potential significance as therapeutic target was assessed using *FGFR1*^{N546K}-mutant Ba/F3 cells and a genetically engineered mouse model expressing *FGFR1*^{N546K} in the presence or absence of *MYCN*. Our data support the hypothesis that *FGFR1*^{N546K} is an oncogenic driver in neuroblastoma. Additionally, *FGFR1*^{N546K} is sensitive to pharmacological FGFR inhibition and may thus be a promising therapeutic target.⁵³

5.1. *FGFR1*^{N546K} is an oncogenic driver in Ba/F3 cells

A p.N546K mutation was successfully introduced into the *pBABE-puro* vector containing *FGFR1* beta by site-directed mutagenesis, and this plasmid was stably transduced into Ba/F3 cells (Figure 5A/B).⁵³ Ba/F3 cells are a well-known *in vitro* model to assess the transforming capacity of tyrosine kinases and the effect of tyrosine kinase inhibitors.^{72,82} Ectopic expression of *FGFR1*^{N546K} transformed Ba/F3 cells from IL-3 dependent to *FGFR1*^{N546K}-driven cell growth after withdrawal of IL-3, thus supporting its oncogenic potential (Figure 6A). Consistent with the phenotypic effect, constitutive phosphorylation of FGFR1 signaling pathway components, including FGFR1 itself, FRS2-α, ERK, AKT, and STAT3, was observed.⁵³ In distinct replicates of *FGFR1*^{N546K} expressing Ba/F3 cells however, gradual differences in phosphorylation of down-stream components were noted, particularly in replicate 3, in which little downstream activation was observed. This finding might be due to spontaneous mutation in another gene that promoted IL-3 independent proliferation in these cells. Taken together, IL-3 independent *FGFR1*^{N546K} expressing Ba/F3 cells induced constitutive activation of the FGFR1 signaling pathway (Figure 6B). Our data are in line with immunohistochemical stainings on human pilocytic astrocytoma with *FGFR1*^{N546K} mutation showing strong positivity for phosphorylated FGFR1 and ERK.³³ Lew *et al.* found that the FGFR1 mutation altered the FGFR1 auto-phosphorylation and thus, caused increased kinase activity *in vitro*.⁸³ Additional-

ly, *in silico* modeling supported that the activating mutation is responsible for permanently converting the protein from its auto-inhibited in its active state.⁵⁶

5.2. Treatment of *Ba/F3 FGFR1^{N546K}* cells with *FGFR* inhibitors impairs cell viability and *FGFR1* pathway down-regulation

The specific *FGFR* inhibitors erdafitinib and futibatinib effectively impaired cell viability in *FGFR1^{N546K}*-driven *Ba/F3* cells *in vitro* (Figure 7A-D). In line with this observation, concentration-dependent dephosphorylation of *FGFR1* pathway proteins, including *FGFR1*, *FRS2-α*, *ERK*, and *STAT3*, was found upon erdafitinib treatment, with some variation in distinct replicates (Figure 8).⁵³ In the cell viability assay futibatinib appeared to be more effective than erdafitinib, but this finding was limited by the lower sample size for futibatinib ($n = 4$) in comparison to erdafitinib (at least $n = 9$; Figure 7A/C). Mean IC_{50} values for both *FGFR* inhibitors ranged between 31 and 129 nM in IL-3 independent replicates 1 and 2, which is more than 45-fold lower than those in IL-3 dependent *Ba/F3* cells (Figure 7B/D).⁵³ By contrast, IL-3 independent replicate 3 was resistant, which was likely due to the fact that growth of these cells was not *FGFR1^{N546K}* dependent (see above; Figure 8). The IC_{50} observed in IL-3 independent replicates 1 and 2 are comparable to those found in *FGFR1*-amplified lung carcinoma *H1581* cells, which were sensitive to erdafitinib with an IC_{50} value of 14 nM.⁸⁴ Sensitivity of erdafitinib has been defined by IC_{50} values below 1,000 nM, and lack of sensitivity by IC_{50} values above 5,000 nM in various tumor cell lines.⁸⁵ Nakamura *et al.* also assessed the transforming capacity and sensitivity of various *FGFR* variants to *FGFR* inhibitors in *3T3* cells. In contrast to our data, this study reported resistance in *FGFR1^{N546K}* *3T3* cells to erdafitinib, with an IC_{50} of 365 nM, while the cells were comparatively sensitive to futibatinib, with an IC_{50} of 79 nM. However, a limited transforming potential of *3T3 FGFR1^{N546K}* cells was reported, as these cells proliferated less than *3T3* cells transduced with *FGFR1^{WT}*.⁷⁷ Furthermore, a recent study by Cimmino *et al.* found that the *FGFR* inhibitor AZD4547 was not effective in neuroblastoma cell lines overexpressing mutant *FGFR^{N546K}*.⁴⁷ While these results appear to be in contrast to our findings, it has to be noted that *3T3* cells and neuroblastoma cell lines – but not *Ba/F3* cells – are immortalized and derived from malignant cancer cells, respectively, which may hamper the assessment of the transforming capacity of *FGFR1^{N546K}* in these models. The transforming capacity of *FGFR1^{N546K}* was further supported by the development of neuroblastomas in a genetically engineered mouse model with conditional expression of *FGFR1^{N546K}* under the *Th* promoter.

5.3. Targeted expression of *FGFR1*^{N546K} in mice

A transgenic mouse model with conditional expression of *FGFR1*^{N546K} was successfully generated. Initially, we used genetically engineered mice in which mutant *FGFR1*^{N546K} was integrated randomly into the genome using a PiggyBac transposon approach. However, it was not possible to generate F1 generation mice bearing this mutation because F0 generation mice seemed to be infertile. This issue may be due to the random integration of transgenes, which might have caused variegated expression and inadvertent disruption of genes. The advantage of the *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} mouse model is that a *FGFR1*^{N546K} allele is specifically integrated in the *ROSA26* locus by CRISPR/Cas (Figure 9A) and activated under the control of the *Th* promoter by Cre recombinase, which minimizes potential effects during mouse embryogenesis by directing the expression to the sympathetic nervous system.^{53,79,80}

5.4. Expression of *FGFR1*^{N546K} in mice leads to spontaneously regressing neuroblastoma

All mice of the *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} cohort that were sacrificed at the ages of 7, 14, and 21 days (n = 9) had developed neuroblastoma (Figure 10A/B). Tumor sizes of mice that were sacrificed early were larger than those of mice that were sacrificed late, suggesting that spontaneous regression may occur in these animals (Figure 10C/D).⁵³ Long-term observation in *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} cohort (n = 35) supported that life expectancy was not limited due to neuroblastoma in 34/35 mice (Figure 12B).⁵³ This finding is in line with recent data which suggested that the presence of activating mutations in tyrosine kinase receptors or other RAS pathway alterations is compatible with spontaneous regression in neuroblastoma when alterations activating telomere maintenance mechanisms (TMM) are absent, whereas the later are required for full malignant transformation.^{19,44} TMM leads to immortal proliferative capacity and is induced either by induction of telomerase or the alternative lengthening of telomeres (ALT) pathway. Telomerase may be induced by various mechanisms, such as transcriptional induction through elevated *MYCN* expression.^{19,44} Consequently, our data suggest that *FGFR1*^{N546K} was sufficient to drive tumor development in *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-IRES-Cre*^{tg/wt} mice, however, that the tumors did not progress to full malignancy due to lack of TMM. Further experiments need to be conducted to confirm this hypothesis.

In addition, papilloma and sarcoma were observed in this mouse model, indicating that the transgene expression was not restricted to the sympathetic nervous system (Figure 11A-C). However, these tumors led to death mainly in older mice leaving sufficient time to allow neuroblastoma development.

5.5. Co-expression of *FGFR1*^{N546K} and *MYCN* in mice drives aggressive neuroblastoma progression

In contrast to mice expressing only the *FGFR1*^{N546K} transgene, *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-MYCN*^{tg/wt}; *Th-IRES-Cre*^{tg/wt} mice (n = 15) developed progressive neuroblastoma with 100% penetrance leading to death at the age of 17-21 days (Figure 12A/B).⁵³ This finding is consistent with the hypothesis that TMM in combination with mutations in genes of the RAS pathway drive fully malignant tumors,^{19,44} as the *FGFR1*^{N546K} transgene was expressed in this model together with *MYCN*, which might have induced telomerase. Further experiments need to be conducted to confirm the presence of TMM in these mice.

Comparing survival of *MYCN*; *FGFR1*^{N546K} mice with that of mice of the well-established *MYCN*; *ALK*^{F1174L} model revealed that the latter lived more than twice as long as the former (Figure 12B).⁵³ Recently published data by our group indicated that – while proliferation was increased in *MYCN*; *ALK*^{F1174L} tumors – apoptosis was impaired in *MYCN*; *FGFR1*^{N546K} tumors, which may explain the aggressive phenotype of the latter.⁵³

5.6. Futibatinib inhibits tumor growth and prolongs survival in *FGFR1*^{N546K}; *MYCN* mice

Futibatinib showed anti-tumor efficacy in *R26-LSL-FGFR1-N546K*^{fl/wt}; *Th-MYCN*^{tg/wt}; *Th-IRES-Cre*^{tg/wt} mice. Stable disease was observed in most of the mice upon treatment with 5 mg/kg and 30 mg/kg futibatinib (Figure 13A/B/D).⁵³ Overall survival was extended to 5-40 days in the treatment cohort using 5 mg/kg futibatinib, compared to 3-13 days in the control cohort (Figure 13C).⁵³

The early development and fast progression of tumors in juvenile mice, however, impeded determination of an appropriate starting time for the treatment. For practical reasons, a starting time of 13-15 days of age was chosen because it was the earliest possible time to conduct the stressful interventions of MRI and drug administration by gavage. At this age, however, the disease was already quite advanced with high tumor growth dynamics, which may have impaired the effect of the drug.

Dose finding of futibatinib was based on adverse events and effects on the tumor growth. After testing 30 mg/kg futibatinib, the dose was reduced to 5 mg/kg futibatinib daily to minimize side effects. Different treatment schedules for futibatinib in murine or nude rat xenograft models bearing *FGFR*-altered tumors have been tested previously.⁸⁶ A once daily administration showed the best results in comparison to longer intervals, because serum half-life was only between 0.25 and 1 hour.⁸⁶ However, inhibition of *FGFR2* phosphorylation was observed for 12 hours when using 5 mg/kg futibatinib.⁸⁶ Tumor size reduction was found at

doses between 0.5 mg/kg and 5 mg/kg futibatinib in a *FGFR2*-amplified gastric cancer model, at 5 mg/kg futibatinib in a *FGFR3*-fusion-positive multiple myeloma model, and between 12.5 mg/kg and 50 mg/kg in a *FGFR1/2*-amplified breast cancer model.⁸⁶ Thus, effective doses of futibatinib seem to depend on both cancer type and type of *FGFR* alteration.

Side effects of futibatinib were mainly gastrointestinal symptoms, like diarrhea, and consequently, weight loss and reduced condition (according to the scoresheet, see Section 7.4.) occurred.⁵³ This spectrum is in line with observations in clinical studies of adult patients, showing hyperphosphatemia, which can cause diarrhea, as the most common adverse event of futibatinib.⁶⁷ Additionally, drug administration by gavage was a stressful procedure and led to reduced food intake, also in the control cohort. Since the weight of mice at treatment start was only between about 5-10 g [compared to adult mice, e.g. at the age of 3 month, of about 27 g for male and 22 g for female]⁸⁷, weight loss had a high impact on the condition of the mice. Together, these facts limited long-term observation of mice treated with futibatinib. To cope with the issue of a low starting weight, our group recently re-implanted tumors from *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mice into immunocompromised adult mice and treated them with futibatinib. Results from this experiment supported the anti-tumor effect of futibatinib on *FGFR1^{N546K}* mutant neuroblastoma.⁵³

Together, our data suggest that FGFR inhibitors may be effective in patients with *FGFR1^{N546K}* mutant neuroblastoma, however, dose-finding studies in children are necessary to assess toxicity and potential efficacy. As a starting point, it has been established that adult patients with *FGFR*-altered tumors receive 20 mg futibatinib daily by oral administration.⁶⁷ If FGFR inhibitors show anti-tumor activity in *FGFR1^{N546K}* mutant neuroblastoma, combination therapies consisting of an FGFR inhibitor and other drugs should be considered in the future to eventually overcome the aggressiveness of *FGFR1^{N546K}*-mutated neuroblastoma.⁸⁸

5.7. FGFR inhibitors may be a promising treatment option for *FGFR*-altered pediatric malignancies despite recruitment challenges in clinical trials

Currently, there are a few ongoing clinical trials assessing erdafitinib or futibatinib in *FGFR*-altered pediatric malignancies.²⁹ Within the pediatric MATCH phase 2 trial (ClinicalTrials.gov, Identifier: NCT03210714) only one neuroblastoma patient was recruited for the treatment with erdafitinib to date.⁸⁹ In two patients with low grade glioma or a glioneuronal tumor, including one with *FGFR1^{N546K}* mutation, partial responses were reported.⁸⁹ Additionally, the RAGNAR phase 2 trial (ClinicalTrials.gov, Identifier: NCT04083976) is analyzing the safety and efficacy of erdafitinib in pretreated patients with solid tumors bearing *FGFR* mutations or fusions at the age of 6 years and older. However, no neuroblastoma patient and no patient bearing a *FGFR1^{N546K}* mutant tumor has been included.⁹⁰ Patients older than 12 years with

advanced solid tumors bearing *FGFR1-4* fusions or mutations are currently recruited in the TAPUR phase 2 clinical trial (ClinicalTrials.gov, Identifier: NCT02693535) to assess the effect of futibatinib and other targeted drugs. However, enrolment of neuroblastoma patients is considerably impeded in this trial due to the age cutoff of 12 years. Another trial testing futibatinib in children is the phase 1/2 ESMART trial (ClinicalTrials.gov, Identifier: NCT02813135), but no *FGFR1*^{N546K} mutated neuroblastoma patients were recruited yet.⁹¹ The phase 1/2 FOENIX-CCA2 trial (ClinicalTrials.gov, Identifier: NCT02052778) has included one glioblastoma patient with *FGFR1*^{N546D} mutation and partial response was achieved after treatment with futibatinib.⁹² Overall, the low frequency of *FGFR1* mutations in neuroblastoma patients and age restrictions within the trials impair recruitment of an appropriate number of patients in such trials. Novel drug approval strategies need to be considered to determine the effect of FGFR inhibitors in children.⁵³ Preliminary data are promising since a patient with *FGFR1*^{N546K}-mutated pilocytic astrocytoma was successfully treated with the pan-FGFR inhibitor pemigatinib⁹³ and a patient with *FGFR1*^{N546K}-mutated neuroblastoma had a prolonged survival using futibatinib in combination with chemotherapeutic agents.⁵³

Together, results from this thesis and preliminary data from clinical trials support the notion that FGFR inhibitors may be a promising treatment option for children with *FGFR1*^{N546K}-mutated malignancies.

6. References

- 1 Maris JM. Recent advances in neuroblastoma. *N Engl J Med* 2010; **362**: 2202–11.
<https://doi.org/10.1056/NEJMra0804577>
- 2 Irwin MS, Park JR. Neuroblastoma: paradigm for precision medicine. *Pediatr Clin North Am* 2015; **62**: 225–56. <https://doi.org/10.1016/j.pcl.2014.09.015>
- 3 Mossé YP, Laudenslager M, Longo L, et al. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008; **455**: 930–35.
<https://doi.org/10.1038/nature07261>
- 4 Narod SA, Hawkins MM, Robertson CM, Stiller CA. Congenital anomalies and childhood cancer in Great Britain. *Am J Hum Genet* 1997; **60**: 474–85.
- 5 Chung C, Boterberg T, Lucas J, et al. Neuroblastoma. *Pediatr Blood Cancer* 2021; **68** **Suppl 2**: e28473. <https://doi.org/10.1002/pbc.28473>
- 6 Trahair T, Sorrentino S, Russell SJ, et al. Spinal Canal Involvement in Neuroblastoma. *J Pediatr* 2017; **188**: 294–98. <https://doi.org/10.1016/j.jpeds.2017.05.051>
- 7 Matthay KK, Blaes F, Hero B, et al. Opsoclonus myoclonus syndrome in neuroblastoma a report from a workshop on the dancing eyes syndrome at the advances in neuroblastoma meeting in Genoa, Italy, 2004. *Cancer Lett* 2005; **228**: 275–82.
<https://doi.org/10.1016/j.canlet.2005.01.051>
- 8 Strenger V, Kerbl R, Dornbusch HJ, et al. Diagnostic and prognostic impact of urinary catecholamines in neuroblastoma patients. *Pediatr Blood Cancer* 2007; **48**: 504–09.
<https://doi.org/10.1002/pbc.20888>
- 9 Matthay KK, Shulkin B, Ladenstein R, et al. Criteria for evaluation of disease extent by (123)I-metaiodobenzylguanidine scans in neuroblastoma: a report for the International Neuroblastoma Risk Group (INRG) Task Force. *Br J Cancer* 2010; **102**: 1319–26.
<https://doi.org/10.1038/sj.bjc.6605621>
- 10 Croteau N, Nuchtern J, LaQuaglia MP. Management of Neuroblastoma in Pediatric Patients. *Surg Oncol Clin N Am* 2021; **30**: 291–304.
<https://doi.org/10.1016/j.soc.2020.11.010>
- 11 Simon T, Hero B, Schulte JH, et al. 2017 GPOH Guidelines for Diagnosis and Treatment of Patients with Neuroblastic Tumors. *Klin Padiatr* 2017; **229**: 147–67.
<https://doi.org/10.1055/s-0043-103086>
- 12 Monclair T, Brodeur GM, Ambros PF, et al. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. *J Clin Oncol* 2009; **27**: 298–303.
<https://doi.org/10.1200/jco.2008.16.6876>

- 13 Brodeur GM, Seeger RC, Barrett A, et al. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. *J Clin Oncol* 1988; **6**: 1874–81. <https://doi.org/10.1200/jco.1988.6.12.1874>
- 14 Brisse HJ, McCarville MB, Granata C, et al. Guidelines for imaging and staging of neuroblastic tumors: consensus report from the International Neuroblastoma Risk Group Project. *Radiology* 2011; **261**: 243–57. <https://doi.org/10.1148/radiol.11101352>
- 15 Shimada H, Ambros IM, Dehner LP, et al. The International Neuroblastoma Pathology Classification (the Shimada system). *Cancer* 1999; **86**: 364–72.
- 16 Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet* 2007; **369**: 2106–20. [https://doi.org/10.1016/S0140-6736\(07\)60983-0](https://doi.org/10.1016/S0140-6736(07)60983-0)
- 17 White PS, Thompson PM, Gotoh T, et al. Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma. *Oncogene* 2005; **24**: 2684–94. <https://doi.org/10.1038/sj.onc.1208306>
- 18 Berry T, Luther W, Bhatnagar N, et al. The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. *Cancer Cell* 2012; **22**: 117–30. <https://doi.org/10.1016/j.ccr.2012.06.001>
- 19 Peifer M, Hertwig F, Roels F, et al. Telomerase activation by genomic rearrangements in high-risk neuroblastoma. *Nature* 2015; **526**: 700–04. <https://doi.org/10.1038/nature14980>
- 20 Hero B, Simon T, Spitz R, et al. Localized infant neuroblastomas often show spontaneous regression: results of the prospective trials NB95-S and NB97. *J Clin Oncol* 2008; **26**: 1504–10. <https://doi.org/10.1200/jco.2007.12.3349>
- 21 Matthay KK, Maris JM, Schleiermacher G, et al. Neuroblastoma. *Nat Rev Dis Primers* 2016; **2**: 16078. <https://doi.org/10.1038/nrdp.2016.78>
- 22 Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. *Nature Genetics* 2013; **45**: 279–84. <https://doi.org/10.1038/ng.2529>
- 23 Prapa M, Caldres S, Spano C, et al. A novel anti-GD2/4-1BB chimeric antigen receptor triggers neuroblastoma cell killing. *Oncotarget* 2015; **6**: 24884–94. <https://doi.org/10.18632/oncotarget.4670>
- 24 Foster JH, Voss SD, Hall DC, et al. Activity of Crizotinib in Patients with ALK-Aberrant Relapsed/Refractory Neuroblastoma: A Children's Oncology Group Study (ADVL0912). *Clin Cancer Res* 2021; **27**: 3543–48. <https://doi.org/10.1158/1078-0432.Ccr-20-4224>
- 25 Fischer M, Moreno L, Ziegler DS, et al. Ceritinib in paediatric patients with anaplastic lymphoma kinase-positive malignancies: an open-label, multicentre, phase 1, dose-escalation and dose-expansion study. *Lancet Oncol* 2021; **22**: 1764–76. [https://doi.org/10.1016/s1470-2045\(21\)00536-2](https://doi.org/10.1016/s1470-2045(21)00536-2)

- 26 Goldsmith KC, Park JR, Kayser K, et al. Lorlatinib with or without chemotherapy in ALK-driven refractory/relapsed neuroblastoma: phase 1 trial results. *Nat Med* 2023; **29**: 1092–102. <https://doi.org/10.1038/s41591-023-02297-5>
- 27 Qiu B, Matthay KK. Advancing therapy for neuroblastoma. *Nat Rev Clin Oncol* 2022; **19**: 515–33. <https://doi.org/10.1038/s41571-022-00643-z>
- 28 Brooks AN, Kilgour E, Smith PD. Molecular pathways: fibroblast growth factor signaling: a new therapeutic opportunity in cancer. *Clin Cancer Res* 2012; **18**: 1855–62. <https://doi.org/10.1158/1078-0432.Ccr-11-0699>
- 29 Touat M, Ileana E, Postel-Vinay S, André F, Soria J-C. Targeting FGFR Signaling in Cancer. *Clinical Cancer Research* 2015; **21**: 2684–94. <https://doi.org/10.1158/1078-0432.Ccr-14-2329>
- 30 Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer* 2010; **10**: 116–29. <https://doi.org/10.1038/nrc2780>
- 31 Dienstmann R, Rodon J, Prat A, et al. Genomic aberrations in the FGFR pathway: opportunities for targeted therapies in solid tumors. *Ann Oncol* 2014; **25**: 552–63. <https://doi.org/10.1093/annonc/mdt419>
- 32 Billerey C, Chopin D, Aubriot-Lorton MH, et al. Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. *Am J Pathol* 2001; **158**: 1955–59. [https://doi.org/10.1016/s0002-9440\(10\)64665-2](https://doi.org/10.1016/s0002-9440(10)64665-2)
- 33 Jones DT, Hutter B, Jäger N, et al. Recurrent somatic alterations of FGFR1 and NTRK2 in pilocytic astrocytoma. *Nat Genet* 2013; **45**: 927–32. <https://doi.org/10.1038/ng.2682>
- 34 Becker AP, Scapulatempo-Neto C, Carloni AC, et al. KIAA1549: BRAF Gene Fusion and FGFR1 Hotspot Mutations Are Prognostic Factors in Pilocytic Astrocytomas. *J Neuropathol Exp Neurol* 2015; **74**: 743–54. <https://doi.org/10.1097/nen.0000000000000213>
- 35 Reinhardt A, Stichel D, Schrimpf D, et al. Anaplastic astrocytoma with piloid features, a novel molecular class of IDH wildtype glioma with recurrent MAPK pathway, CDKN2A/B and ATRX alterations. *Acta Neuropathol* 2018; **136**: 273–91. <https://doi.org/10.1007/s00401-018-1837-8>
- 36 Kandoth C, Schultz N, Cherniack AD, et al. Integrated genomic characterization of endometrial carcinoma. *Nature* 2013; **497**: 67–73. <https://doi.org/10.1038/nature12113>
- 37 Taylor JGt, Cheuk AT, Tsang PS, et al. Identification of FGFR4-activating mutations in human rhabdomyosarcomas that promote metastasis in xenotransplanted models. *J Clin Invest* 2009; **119**: 3395–407. <https://doi.org/10.1172/jci39703>
- 38 Heist RS, Mino-Kenudson M, Sequist LV, et al. FGFR1 amplification in squamous cell carcinoma of the lung. *J Thorac Oncol* 2012; **7**: 1775–80. <https://doi.org/10.1097/JTO.0b013e31826aed28>

- 39 Kim S, Dubrovskaya A, Salamone RJ, et al. FGFR2 promotes breast tumorigenicity through maintenance of breast tumor-initiating cells. *PLoS One* 2013; **8**: e51671. <https://doi.org/10.1371/journal.pone.0051671>
- 40 Singh D, Chan JM, Zoppoli P, et al. Transforming fusions of FGFR and TACC genes in human glioblastoma. *Science* 2012; **337**: 1231–35. <https://doi.org/10.1126/science.1220834>
- 41 Williams SV, Hurst CD, Knowles MA. Oncogenic FGFR3 gene fusions in bladder cancer. *Hum Mol Genet* 2013; **22**: 795–803. <https://doi.org/10.1093/hmg/dds486>
- 42 Wang R, Wang L, Li Y, et al. FGFR1/3 tyrosine kinase fusions define a unique molecular subtype of non-small cell lung cancer. *Clin Cancer Res* 2014; **20**: 4107–14. <https://doi.org/10.1158/1078-0432.Ccr-14-0284>
- 43 Wu YM, Su F, Kalyana-Sundaram S, et al. Identification of targetable FGFR gene fusions in diverse cancers. *Cancer Discov* 2013; **3**: 636–47. <https://doi.org/10.1158/2159-8290.Cd-13-0050>
- 44 Ackermann S, Cartolano M, Hero B, et al. A mechanistic classification of clinical phenotypes in neuroblastoma. *Science* 2018; **362**: 1165–70. <https://doi.org/10.1126/science.aat6768>
- 45 Brady SW, Liu Y, Ma X, et al. Pan-neuroblastoma analysis reveals age- and signature-associated driver alterations. *Nature Communications* 2020; **11**: 5183. <https://doi.org/10.1038/s41467-020-18987-4>
- 46 Eleveld TF, Oldridge DA, Bernard V, et al. Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. *Nat Genet* 2015; **47**: 864–71. <https://doi.org/10.1038/ng.3333>
- 47 Cimmino F, Montella A, Tirelli M, et al. FGFR1 is a potential therapeutic target in neuroblastoma. *Cancer Cell Int* 2022; **22**: 174. <https://doi.org/10.1186/s12935-022-02587-x>
- 48 Lasorsa VA, Formicola D, Pignataro P, et al. Exome and deep sequencing of clinically aggressive neuroblastoma reveal somatic mutations that affect key pathways involved in cancer progression. *Oncotarget* 2016; **7**: 21840–52. <https://doi.org/10.18632/oncotarget.8187>
- 49 Schmelz K, Toedling J, Huska M, et al. Spatial and temporal intratumour heterogeneity has potential consequences for single biopsy-based neuroblastoma treatment decisions. *Nat Commun* 2021; **12**: 6804. <https://doi.org/10.1038/s41467-021-26870-z>
- 50 Padovan-Merhar OM, Raman P, Ostrovskaya I, et al. Enrichment of Targetable Mutations in the Relapsed Neuroblastoma Genome. *PLoS Genet* 2016; **12**: e1006501. <https://doi.org/10.1371/journal.pgen.1006501>
- 51 Bosse KR, Giudice AM, Lane MV, et al. Serial Profiling of Circulating Tumor DNA Identifies Dynamic Evolution of Clinically Actionable Genomic Alterations in High-Risk Neuro-

- blastoma. *Cancer Discov* 2022; **12**: 2800–19. <https://doi.org/10.1158/2159-8290.Cd-22-0287>
- 52 Nokchan N, Suthapot P, Choochuen P, et al. Whole-Exome Sequencing Reveals Novel Candidate Driver Mutations and Potential Druggable Mutations in Patients with High-Risk Neuroblastoma. *J Pers Med* 2024; **14**. <https://doi.org/10.3390/jpm14090950>
 - 53 Werr L, Boland J, Petersen J, et al. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026. <https://doi.org/10.1172/JCI189152>
 - 54 Li I, Huo Y, Yang T, Gunawan H, Alexandrov LB, Zage PE. Fibroblast growth factor receptor alterations and resistance mechanisms in the treatment of pediatric solid tumors. *Cancer Drug Resist* 2025; **8**: 28. <https://doi.org/10.20517/cdr.2024.208>
 - 55 Qaddoumi I, Orisme W, Wen J, et al. Genetic alterations in uncommon low-grade neuroepithelial tumors: BRAF, FGFR1, and MYB mutations occur at high frequency and align with morphology. *Acta Neuropathol* 2016; **131**: 833–45. <https://doi.org/10.1007/s00401-016-1539-z>
 - 56 Rivera B, Gayden T, Carrot-Zhang J, et al. Germline and somatic FGFR1 abnormalities in dysembryoplastic neuroepithelial tumors. *Acta Neuropathol* 2016; **131**: 847–63. <https://doi.org/10.1007/s00401-016-1549-x>
 - 57 Gessi M, Moneim YA, Hammes J, et al. FGFR1 mutations in Rosette-forming glioneuronal tumors of the fourth ventricle. *J Neuropathol Exp Neurol* 2014; **73**: 580–84. <https://doi.org/10.1097/nen.0000000000000080>
 - 58 Kitamura Y, Komori T, Shibuya M, et al. Comprehensive genetic characterization of rosette-forming glioneuronal tumors: independent component analysis by tissue microdissection. *Brain Pathol* 2018; **28**: 87–93. <https://doi.org/10.1111/bpa.12468>
 - 59 Bruzek AK, Zureick AH, McKeever PE, et al. Molecular characterization reveals NF1 deletions and FGFR1-activating mutations in a pediatric spinal oligodendroglioma. *Pediatr Blood Cancer* 2017; **64**. <https://doi.org/10.1002/pbc.26346>
 - 60 Rand V, Huang J, Stockwell T, et al. Sequence survey of receptor tyrosine kinases reveals mutations in glioblastomas. *Proceedings of the National Academy of Sciences of the United States of America* 2005; **102**: 14344–49. <https://doi.org/10.1073/pnas.0507200102>
 - 61 Dyson K, Rivera-Zengotita M, Kresak J, et al. FGFR1 N546K and H3F3A K27M mutations in a diffuse leptomeningeal tumour with glial and neuronal markers. *Histopathology* 2016; **69**: 704–07. <https://doi.org/10.1111/his.12983>
 - 62 Formisano L, Lu Y, Servetto A, et al. Aberrant FGFR signaling mediates resistance to CDK4/6 inhibitors in ER+ breast cancer. *Nat Commun* 2019; **10**: 1373. <https://doi.org/10.1038/s41467-019-09068-2>

- 63 Agelopoulos K, Richter GHS, Schmidt E, et al. Deep Sequencing in Conjunction with Expression and Functional Analyses Reveals Activation of FGFR1 in Ewing Sarcoma. *Clin Cancer Res* 2015; **21**: 4935–46. <https://doi.org/10.1158/1078-0432.CCR-14-2744>
- 64 Loriot Y, Necchi A, Park SH, et al. Erdafitinib in Locally Advanced or Metastatic Urothelial Carcinoma. *New England Journal of Medicine* 2019; **381**: 338–48. <https://doi.org/10.1056/NEJMoa1817323>
- 65 Hoy SM. Pemigatinib: First Approval. *Drugs* 2020; **80**: 923–29. <https://doi.org/10.1007/s40265-020-01330-y>
- 66 Kang C. Infigratinib: First Approval. *Drugs* 2021; **81**: 1355–60. <https://doi.org/10.1007/s40265-021-01567-1>
- 67 Goyal L, Meric-Bernstam F, Hollebecque A, et al. Futibatinib for FGFR2-Rearranged Intrahepatic Cholangiocarcinoma. *N Engl J Med* 2023; **388**: 228–39. <https://doi.org/10.1056/NEJMoa2206834>
- 68 Babina IS, Turner NC. Advances and challenges in targeting FGFR signalling in cancer. *Nature Reviews Cancer* 2017; **17**: 318–32. <https://doi.org/10.1038/nrc.2017.8>
- 69 New England Biolabs®. NEBaseChanger®. <https://nebasechangerv1.neb.com/> (accessed January 25th, 2025).
- 70 New England Biolabs®. Site Directed Mutagenesis. <https://www.neb.com/en/applications/cloning-and-synthetic-biology/site-directed-mutagenesis> (accessed January 25th, 2025).
- 71 Kunkel TA. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proceedings of the National Academy of Sciences of the United States of America* 1985; **82**: 488–92. <https://doi.org/10.1073/pnas.82.2.488>
- 72 Kong K, Ng PK, Scott KL. Ba/F3 transformation assays. *Oncotarget* 2017; **8**: 35488–89. <https://doi.org/10.18632/oncotarget.17828>
- 73 Malchers F, Dietlein F, Schöttle J, et al. Cell-Autonomous and Non-Cell-Autonomous Mechanisms of Transformation by Amplified *FGFR1* in Lung Cancer. *Cancer Discovery* 2014; **4**: 246–57. <https://doi.org/10.1158/2159-8290.Cd-13-0323>
- 74 Villaruz LC, Socinski MA. The clinical viewpoint: definitions, limitations of RECIST, practical considerations of measurement. *Clin Cancer Res* 2013; **19**: 2629–36. <https://doi.org/10.1158/1078-0432.Ccr-12-2935>
- 75 Huret J. FGFR1 (Fibroblast Growth Factor Receptor 1).
- 76 Ornitz DM, Itoh N. The Fibroblast Growth Factor signaling pathway. *Wiley Interdiscip Rev Dev Biol* 2015; **4**: 215–66. <https://doi.org/10.1002/wdev.176>
- 77 Nakamura IT, Kohsaka S, Ikegami M, et al. Comprehensive functional evaluation of variants of fibroblast growth factor receptor genes in cancer. *npj Precision Oncology* 2021; **5**: 66. <https://doi.org/10.1038/s41698-021-00204-0>

- 78 Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop J. Targeted expression of MYCN causes neuroblastoma in transgenic mice. *The EMBO Journal* 1997; **16**: 2985–95. <https://doi.org/10.1093/emboj/16.11.2985>
- 79 Bouabe H, Okkenhaug K. Gene targeting in mice: a review. *Methods Mol Biol* 2013; **1064**: 315–36. https://doi.org/10.1007/978-1-62703-601-6_23
- 80 Lindeberg J, Usoskin D, Bengtsson H, et al. Transgenic expression of Cre recombinase from the tyrosine hydroxylase locus. *Genesis* 2004; **40**: 67–73. <https://doi.org/10.1002/gene.20065>
- 81 Baldarelli RM, Smith CM, Finger JH, et al. The mouse Gene Expression Database (GXD): 2021 update. *Nucleic Acids Res* 2021; **49**: D924-D931. <https://doi.org/10.1093/nar/gkaa914>
- 82 Warmuth M, Kim S, Gu XJ, Xia G, Adrián F. Ba/F3 cells and their use in kinase drug discovery. *Curr Opin Oncol* 2007; **19**: 55–60. <https://doi.org/10.1097/CCO.0b013e328011a25f>
- 83 Lew ED, Furdui CM, Anderson KS, Schlessinger J. The precise sequence of FGF receptor autophosphorylation is kinetically driven and is disrupted by oncogenic mutations. *Sci Signal* 2009; **2**: ra6. <https://doi.org/10.1126/scisignal.2000021>
- 84 Giacomini A, Taranto S, Rezzola S, et al. Inhibition of the FGF/FGFR System Induces Apoptosis in Lung Cancer Cells via c-Myc Downregulation and Oxidative Stress. *Int J Mol Sci* 2020; **21**. <https://doi.org/10.3390/ijms21249376>
- 85 Perera TP, Jovcheva E, Mevellec L, et al. Discovery and Pharmacological Characterization of JNJ-42756493 (Erdafitinib), a Functionally Selective Small-Molecule FGFR Family Inhibitor. *Molecular Cancer Therapeutics* 2017; **16**: 1010–20. <https://doi.org/10.1158/1535-7163.Mct-16-0589>
- 86 Sootome H, Fujita H, Ito K, et al. Futibatinib Is a Novel Irreversible FGFR 1–4 Inhibitor That Shows Selective Antitumor Activity against FGFR-Deregulated Tumors. *Cancer Research* 2020; **80**: 4986–97. <https://doi.org/10.1158/0008-5472.Can-19-2568>
- 87 Inotiv. C57BL/6 inbred mice. Growth curve (accessed Feb 03, 2025).
- 88 Monika Lukoseviciute, Emma Need, Stefan Holzhauser, Tina Dalianis, Ourania N. Kostopoulou. Combined targeted therapy with PI3K and CDK4/6, or FGFR inhibitors show synergistic effects in a neuroblastoma spheroid culture model. *Biomedicine & Pharmacotherapy* 2024; **177**: 116993. <https://doi.org/10.1016/j.biopha.2024.116993>
- 89 Alice Lee, Alexander J. Chou, P. Mickey Williams, et al. Erdafitinib in patients with FGFR-altered tumors: Results from the NCI-COG Pediatric MATCH trial arm B (APEC1621B).
- 90 Pant S, Schuler M, Iyer G, et al. Erdafitinib in patients with advanced solid tumours with FGFR alterations (RAGNAR): an international, single-arm, phase 2 study. *Lancet Oncol* 2023; **24**: 925–35. [https://doi.org/10.1016/s1470-2045\(23\)00275-9](https://doi.org/10.1016/s1470-2045(23)00275-9)

- 91 Geoerger B, Paoletti X, Bautista F, et al. AcSé-ESMART, a European precision cancer medicine proof-of-concept platform trial. *Nature Medicine* 2023; **29**: 2985–87. <https://doi.org/10.1038/s41591-023-02580-5>
- 92 Bahleda R, Meric-Bernstam F, Goyal L, et al. Phase I, first-in-human study of futibatinib, a highly selective, irreversible FGFR1-4 inhibitor in patients with advanced solid tumors. *Ann Oncol* 2020; **31**: 1405–12. <https://doi.org/10.1016/j.annonc.2020.06.018>
- 93 Capone S, Ketonen L, Weathers SP, Subbiah V. Activity of Pemigatinib in Pilocytic Astrocytoma and FGFR1(N546K) Mutation. *JCO Precis Oncol* 2022; **6**: e2100371. <https://doi.org/10.1200/po.21.00371>

7. Appendix

7.1. List of Figures

Figure 1: *FGFR* structure, network, and dysregulation in cancer.

Figure 2: Workflow of site-directed mutagenesis.

Figure 3: Scheme of treatment with *FGFR* inhibitors in 96-well plate.

Figure 4: Scheme of treatment with futibatinib or control solution.

Figure 5: *pBABE-puro* vector with integrated *FGFR1*^{N546K}.

Figure 6: Transforming potential and *FGFR1* signaling pathway in *Ba/F3 FGFR1*^{N546K} cells.

Figure 7: Cell viability after treatment of *Ba/F3* cells with erdafitinib or futibatinib.

Figure 8: *FGFR1* signaling pathway after treatment of *Ba/F3 FGFR1*^{N546K} cells with erdafitinib.

Figure 9: Mating and genotyping of transgenic mice expressing *FGFR1*^{N546K} and/or *MYCN*.

Figure 10: Neuroblastoma development in *FGFR1*^{N546K} mice.

Figure 11: Papilloma of the tail in *FGFR1*^{N546K} mice.

Figure 12: Neuroblastoma development in *FGFR1*^{N546K}; *MYCN* mice.

Figure 13: Survival and tumor growth dynamics upon treatment of *FGFR1*^{N546K}; *MYCN* mice with futibatinib.

7.2. List of Tables

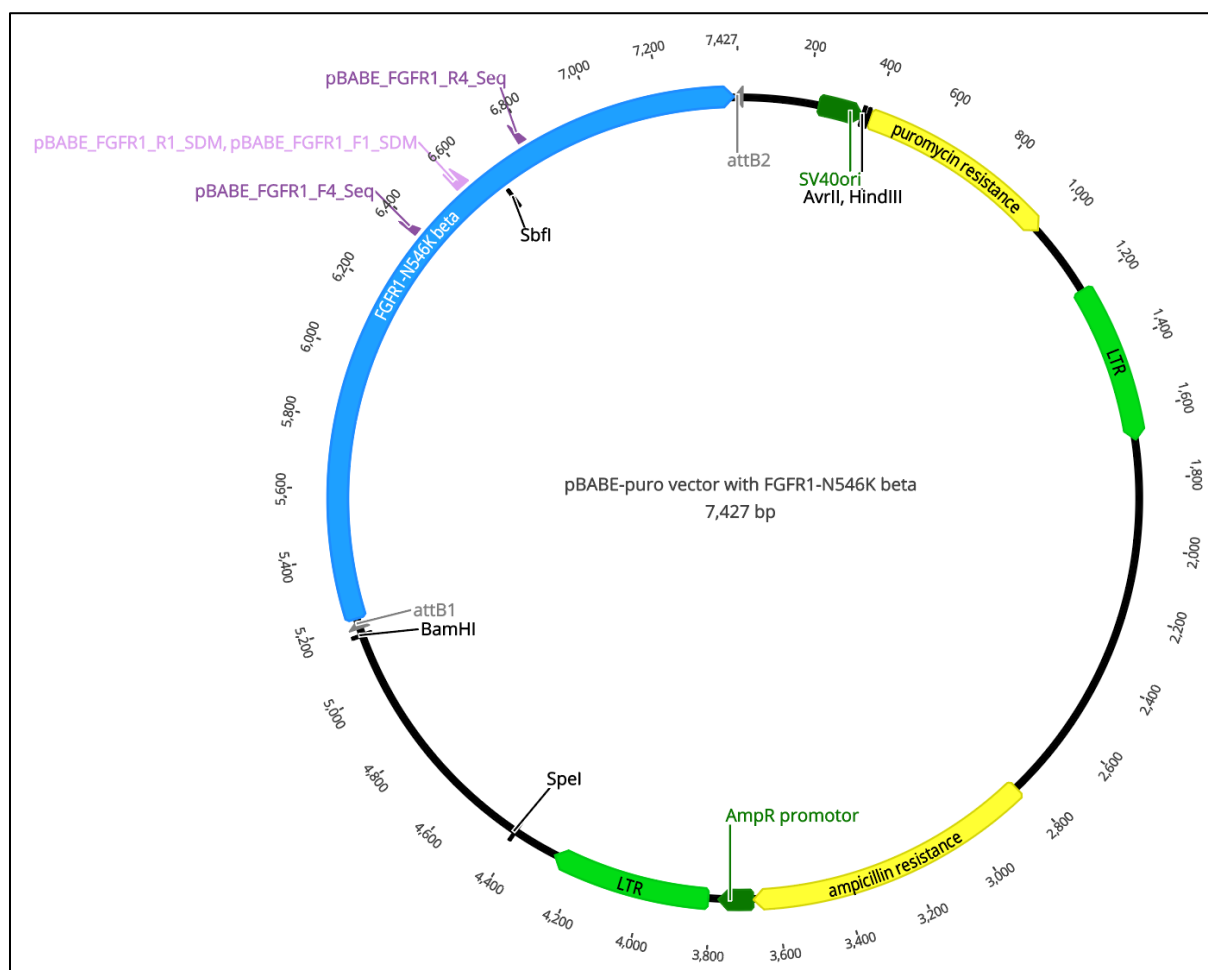
Table 1: Reagents used for experiments.

Table 2: Primers used for PCR.

Table 3: Antibodies used for experiments including application-specific dilutions.

Table 4: Devices used for experiments.

7.3. Appendix Figure 1: Scheme of *pBABE-puro* vector with *FGFR1*^{N546K} beta



Appendix Figure 1: Scheme of *pBABE-puro* vector with *FGFR1*^{N546K} beta.

FGFR1^{N546K} beta (blue) between the restriction sites attB1 and attB2 (grey) with the used primers for SDM (light purple) and dideoxy sequencing (dark purple) is illustrated. The ampicillin and puromycin resistance genes (yellow) with promoters (dark green) are marked. LTRs (Long Terminal Repeats; light green) regulate the expression of genes. The positions of the restriction enzymes (AvrII, HindIII, SpeI, BamHI, SbfI; black) are shown. The vector is 7,427 bp long. The illustration was generated by Geneious using the sequence of *pBABE-puro* with *FGFR1*^{N546K} beta.

7.4. Appendix Table 1: Scoresheet

A. General criteria (not experiment specific)	Score
I. Body weight	
- Unchanged	0
- Weight loss 5%-10%	5
- Weight loss 11%-19% or vertebrae and pelvic bones palpable	10
- Weight loss > 20% or vertebrae and pelvic bones palpable	20
II. General condition	
- Smooth and shiny fur, clean orifices, clear and shiny eyes	0
- Defects of fur (reduced or excessive body care)	1
- Dull fur, unkempt orifices, dull eyes, increased muscle tone, mild skin lesions (no inflammation)	5
- Dirty fur, sticky or moist orifices, dull eyes, high muscle tone, curved back, head tilt, moderate skin lesions (mild redness and swelling)	10
- Cramps, paralysis, severe skin lesions (severe redness and swelling, purulent discharge)	20
III. Spontaneous behavior	
- Normal behavior (e.g. sleep, reaction to touch, social contact)	0
- Minor deviation from behavior (e.g. reduced reaction)	1
- Uncommon behavior (e.g. reduced or increased motor function)	5
- Self-isolation, lethargy, apathy, hyperkinesia, coordination disorder	10
- Painful noises when grasped, automutilation	20
IV. Clinical findings	
- Normal breathing	0
- Minor deviation from normal situation (e.g. slight increased breathing)	1
- Moderate temperature deviation, increased abdominal breathing	10
- Severe temperature deviation (animal feels hot or cold), breath sounds, dyspnea, cyanosis	20

B. Mouse line specific criteria	
<ul style="list-style-type: none"> - Normal breathing, no lymphadenopathy - Tumor diameter < 0.5 cm 	0
<ul style="list-style-type: none"> - Normal breathing, minor deviation from normal situation (mild increase in abdominal circumference, good general condition: normal behavior, movements appropriate to age, smooth and shiny fur, normal feeding behavior) 	1
<ul style="list-style-type: none"> - Mild coordination disorder - Palpable tumor: diameter > 0.5 cm - Focal periodic seizures < 2 min - Lymphadenopathy in abdominal area, palpable spleen, starting worsening of general condition: movement progressively slows down, smooth and shiny fur, normal feeding behavior) 	5
<ul style="list-style-type: none"> - Increased breathing, diarrhea (< 2 days), constipation (< 1 days) - Coordination disorder - Palpable tumor: diameter > 1 cm - One-time cramp > 20 seconds or multiple cramps < 20 seconds with complete recovery between episodes and no further impairment - Strong increase in abdominal circumference, palpable spleen, worsening of general condition: dull fur, abnormal posture, limited movement activity 	10
<ul style="list-style-type: none"> - Lymphadenopathy (abdominal swelling), severely enlarged spleen (> 3.5x volume), leucocyte counts > 150,000/μL blood, breathing difficulties, ascites, bleedings, persistent diarrhea, persistent constipation, rectal prolapse, infectious diseases, apathy, lack of flight reflex - Palpable tumor: diameter > 1.5 cm - Neurological deficits: paralysis - Cramps on two consecutive days including one cramp > 20 seconds or one-time cramp > 1 minute with further impairment 	20

Appendix Table 1: Scoresheet.

Possible symptoms of the mice to determine the condition within the experiments are listed.

7.5. Appendix Table 2: Early sacrifices of *R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt}* mouse cohort

Mouse ID	Age in days	Histology of NB	PHOX2B staining
T1-7d	7	Differentiating NB	Positive
T8-7d	7	Poorly differentiated NB	NA
T9-7d	7	Poorly differentiated NB	NA
T4-14d	14	Poorly differentiated NB	Positive
T16-14d	14	Differentiating NB	NA
T17-14d	14	Differentiating NB	NA
FIM-200	21	Differentiating NB	NA
FIM-203	21	Poorly differentiated NB	NA
FIM-207	21	Poorly differentiated NB	NA

Appendix Table 2: Early sacrifices of *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYC^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort.

Mouse ID, age in days, histology of neuroblastoma according to INPCC and PHOX2B results are listed for mice sacrificed at the age of 7, 14 and 21 days. NB, neuroblastoma; NA, not available.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.6. Appendix Table 3: *R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt}* mouse cohort

Mouse ID	Survival in days	Stop criteria	Histology of NB	PHOX2B staining
FISCH-2066	104	Papilloma	NA	NA
FISCH-2068	347	Sarcoma	Poorly differentiated NB	NA
FISCH-2091	122	Papilloma	NA	NA
FISCH-2095	106	Papilloma	NA	NA
FISCH-2096	295	Sarcoma	NA	NA
FISCH-2148	63	Papilloma	NA	NA
FISCH-2151	126	Papilloma	NA	NA
FIM-2	189	Papilloma	NA	NA
FIM-5	23	Weight loss	NA	NA
FIM-7	104	Papilloma	Differentiating NB	Positive
FGFR1-11	107	Papilloma	NA	NA
FGFR1-12	75	Weight loss	NA	NA
FGFR1-14	107	Papilloma	Adrenal glands	NA
FGFR1-16	150	Papilloma	Differentiating NB	Positive
FGFR1-17	242	Sarcoma	Ganglioneuroblastoma	NA
FGFR1-20	129	Papilloma	Poorly differentiated NB	NA
FIM-19	109	Preservation of regressing NB	Poorly differentiated NB	NA
FIM-24	108	Papilloma	NA	NA
FIM-26	108	Papilloma	Differentiating NB	NA
FIM-32	31	Spontaneous	NA	NA
FIM-33	106	Papilloma	Differentiating NB	NA

FGFR1-32	252	Papilloma	NA	NA
FGFR1-34	97	Papilloma	Differentiating NB	Positive
FGFR1-36	256	Sarcoma	Differentiating NB	NA
FGFR1-37	84	Papilloma	Poorly differenti- ated NB	NA
FGFR1-38	242	Sarcoma	NA	NA
FGFR1-39	93	Papilloma	Differentiating NB	NA
FGFR1-40	217	Sarcoma	Differentiating NB	NA
FGFR1-41	169	Sarcoma	Differentiating NB	NA
FGFR1-42	365	Sarcoma	NA	NA
FGFR1-43	114	Papilloma	NA	NA
FGFR1-44	361	Progressing NB	Poorly Differenti- ated NB	Positive
FIM-83	53	Papilloma	NA	NA
FIM-90	213	Papilloma	Differentiating NB	NA
FIM-101	145	Papilloma, Sar- coma	Differentiating NB	Positive

Appendix Table 3: *R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt}* mouse cohort.

Mouse ID, survival in days, stop criteria, histology of NB according to INPCC and PHOX2B results are listed for mice expressing *FGFR1^{N546K}*. NB, neuroblastoma; NA, not available.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.7. Appendix Table 4: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort

Mouse ID	Survival in days	Stop criteria	Histology of NB	PHOX2B staining
BFISCH-248	19	Progressing NB	Differentiating NB	NA
FISCH-2138	21	Progressing NB	Differentiating NB	Positive
FISCH-2149	21	Progressing NB	Differentiating NB	Positive
BFIM-2	18	Progressing NB	NA	NA
FIM-15	20	Progressing NB	Differentiating NB	Positive
FIM-17	20	Progressing NB	Poorly differentiated NB	NA
FIM-18	18	Progressing NB	Poorly differentiated NB	Positive
FIM-23	21	Progressing NB	Poorly differentiated NB	Positive
FIM-36	20	Progressing NB	Differentiating NB	Positive
FIM-84	19	Progressing NB	Differentiating NB	NA
FIM-157	17	Progressing NB	NA	NA
FIM-160	17	Progressing NB	Differentiating NB	NA
FIM-163	21	Progressing NB	NA	NA
FIM-184	18	Progressing NB	Differentiating NB	Positive
FIM-186	18	Progressing NB	NA	NA

Appendix Table 4: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort.

Mouse ID, survival in days, stop criteria, histology of neuroblastoma according to INPCC and PHOX2B results are listed for mice expressing the *FGFR1^{N546K}* and *MYCN* transgenes. NB, neuroblastoma; NA, not available.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.8. Appendix Table 5: *Th-ALK-F1174L^{tg/wt};Th-MYCN^{tg/wt}* mouse cohort

Mouse ID	Survival in days	Stop criteria
FISCH-853	52	Progressing NB
FISCH-932	58	Progressing NB
FISCH-935	33	Progressing NB
FISCH-1101	42	Progressing NB
FISCH-1248	43	Progressing NB
FISCH-1255	53	Progressing NB
FISCH-1312	52	Progressing NB
FISCH-1317	48	Progressing NB
FISCH-1378	49	Progressing NB
FISCH-1379	60	Progressing NB
FISCH-1380	36	Progressing NB
FISCH-1381	50	Progressing NB
FISCH-1384	55	Progressing NB
FISCH-1391	52	Progressing NB
FISCH-1773	45	Progressing NB

Appendix Table 5: *Th-ALK-F1174^{tg/wt};Th-MYCN^{tg/wt}* mouse cohort.

Mouse ID, survival in days and stop criteria are listed for mice expressing the *ALK^{F1174L}* and *MYCN* transgenes. NB, neuroblastoma.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.9. Appendix Table 6: *R26-LSL-FGFR1-N546K^{tg/wt};Th-MYCN^{tg/wt}* mouse cohort

Mouse ID	Survival in days	Stop criteria	Histology of NB	PHOX2B staining
FISCH-2140	365	End of observation period	NA	NA
FISCH-2141	365	End of observation period	NA	NA
FISCH-2150	365	Weight loss, end of observation period	NA	NA
FISCH-2163	217	Progressing neuroblastoma	Poorly differentiated NB	Positive
FISCH-2164	365	Spleen abscess, end of observation period	No NB	Negative
FIM-3	365	End of observation period	No NB	Negative
FIM-4	365	End of observation period	NA	NA
FIM-6	365	Weight loss, end of observation period	No NB	NA
FIM-20	365	End of observation period	NA	NA
FIM-25	365	End of observation period	No NB	NA

Appendix Table 6: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt}* mouse cohort.

Mouse ID, survival in days, stop criteria, histology of neuroblastoma according to INPCC and PHOX2B results are listed for the control cohort. The observation period ended after 365 days. NB, neuroblastoma; NA, not available.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.10. Appendix Table 7: *Th-MYCN*^{tg/wt} mouse cohort

Mouse ID	Survival in days	Stop criteria
EMFIS-35	203	Overage
EMFIS-36	203	Overage
EMFIS-38	203	Overage
FISCH-108	301	Overage
FISCH-111	365	Overage
FISCH-135	239	Progressing NB
FISCH-137	238	Overage
FISCH-164	302	Overage
FISCH-173	350	Overage
FISCH-177	350	Overage
FISCH-263	345	Overage
FISCH-264	345	Overage
FISCH-270	231	Overage
FISCH-272	231	Overage
FISCH-307	186	Overage
FISCH-329	156	Overage
FISCH-504	306	Overage
FISCH-534	283	Overage
FISCH-599	365	Overage
FISCH-801	174	Overage
FISCH-803	209	Overage
FISCH-829	201	Overage
FISCH-830	162	Overage
FISCH-881	222	Overage
FISCH-994	162	Overage

FISCH-1191	197	Overage
FISCH-1192	197	Overage
FISCH-1249	225	Overage
FISCH-1257	225	Overage
FISCH-1258	225	Overage
FISCH-1509	187	Overage
FISCH-1523	186	Overage
FISCH-1524	247	Overage
FISCH-1525	247	Overage
FISCH-1557	265	Overage
FISCH-1558	265	Overage
FISCH-1604	210	Overage
FISCH-1605	210	Overage
FISCH-1606	210	Overage
FISCH-1713	245	Overage

Appendix Table 7: *Th-MYCN*^{tg/wt} mouse cohort.

Mouse ID, survival in days and stop criteria are listed for mice expressing the *MYCN* transgene. NB, neuroblastoma.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.11. Appendix Table 8: *Th-ALK-F1174L^{tg/wt}* mouse cohort

Mouse ID	Survival in days	Stop criteria
THALK-22	180	Scratch marks
THALK-23	365	Scratch marks
FISCH-159	365	Overage
FISCH-161	365	Overage
FISCH-162	365	Overage
FISCH-178	189	Overage
FISCH-202	326	Overage
FISCH-203	332	Overage
FISCH-271	236	Overage
FISCH-273	236	Overage
FISCH-888	221	Overage
FISCH-889	221	Overage
FISCH-890	114	Overage
FISCH-931	221	Overage
FISCH-966	240	Overage
FISCH-967	232	Overage
FISCH-969	261	Overage
FISCH-1024	215	Overage
FISCH-1027	215	Overage
FISCH-1028	215	Overage
FISCH-1029	215	Overage
FISCH-1030	215	Overage
FISCH-1032	215	Overage
FISCH-1200	223	Overage
FISCH-1201	265	Overage

FISCH-1202	265	Overage
FISCH-1320	251	Overage
FISCH-1484	211	Overage
FISCH-1485	193	Scratch marks
FISCH-1764	269	Overage
FISCH-1765	269	Overage
FISCH-1778	192	Overage
FISCH-1984	198	Overage
FISMA-293	88	Scratch marks

Appendix Table 8: *ALK-F1174L*^{tg/wt} mouse cohort.

Mouse ID, survival in days and stop criteria are listed for mice expressing the *ALK*^{F1174L} transgene.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.12. Appendix Table 9: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort after treatment with CMC-Na

Mouse ID	Treatment	Survival after treatment start in days	Best overall response	Stop criteria
FIM-245	CMC-Na	5	PD	Progressing NB
FIM-318	CMC-Na	6	PD	Progressing NB
FIM-334	CMC-Na	3	PD	Spontaneous, progressing NB
FIM-339	CMC-Na	5	PD	Progressing NB
FIM-340	CMC-Na	5	PD	Progressing NB
FIM-347	CMC-Na	10	PD	Spontaneous, progressing NB
FIM-349	CMC-Na	7	PD	Progressing NB
FIM-350	CMC-Na	6	PD	Paralysis, progressing NB
FIM-351	CMC-Na	13	PD	Spontaneous, progressing NB
FIM-361	CMC-Na	7	PD	Progressing NB
FIM-364	CMC-Na	12	PD	Progressing NB
FIM-391	CMC-Na	6	PD	Spontaneous, progressing NB
FIM-419	CMC-Na	4	PD	Progressing NB

Appendix Table 9: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort after treatment with CMC-Na.

Mouse ID, treatment, survival in days, best overall response based on RECIST and stop criteria are listed for mice treated with CMC-Na as control solution. PD, progressive disease; NB, neuroblastoma.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

7.13. Appendix Table 10: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYCN^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort after treatment with futibatinib

Mouse ID	Treatment	Survival after treatment start in days	Best overall response	Stop criteria
FIM-240	5 mg/kg Futibatinib	15	SD	Progressing NB
FIM-242	5 mg/kg Futibatinib	21	SD	Progressing NB
FIM-246	5 mg/kg Futibatinib	15	SD	Spontaneous, progressing NB
FIM-248	5 mg/kg Futibatinib	17	SD	Progressing NB
FIM-251	5 mg/kg Futibatinib	13	SD	Weight loss
FIM-252	5 mg/kg Futibatinib	10	SD	Spontaneous
FIM-253	5 mg/kg Futibatinib	17	SD	Weight loss, progressing NB
FIM-330	5 mg/kg Futibatinib	7	SD	Spontaneous, progressing NB
FIM-332	5 mg/kg Futibatinib	21	SD	Progressing NB
FIM-333	5 mg/kg Futibatinib	14	SD	Paralysis
FIM-342	5 mg/kg Futibatinib	5	SD	Spontaneous, progressing NB
FIM-343	5 mg/kg Futibatinib	12	PD	Spontaneous, progressing NB
FIM-415	5 mg/kg Futibatinib	36	SD	Progressing NB

FIM-418	5 mg/kg Futibatinib	40	PR	Progressing NB
FIM-199	30 mg/kg Futibatinib	9	SD	Weight loss and other side effects
FIM-201	30 mg/kg Futibatinib	8	PR	Weight loss and other side effects
FIM-202	30 mg/kg Futibatinib	6	SD	Weight loss and other side effects
FIM-206	30 mg/kg Futibatinib	5	SD	Weight loss and other side effects

Appendix Table 10: *R26-LSL-FGFR1-N546K^{fl/wt};Th-MYC^{tg/wt};Th-IRES-Cre^{tg/wt}* mouse cohort after treatment with futibatinib.

Mouse ID, treatment, survival after treatment start in days, best overall response based on RECIST and stop criteria are listed for mice treated with 5 mg/kg or 30 mg/kg futibatinib. SD, stable disease; PR, partial response; PD, progressive disease; NB, neuroblastoma.

Table based on Werr L, Boland J, Petersen J. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026.⁵³

8. Pre-publication of Results

A part of the results were published in:

Werr L, **Boland J**, Petersen J, et al. Mutated FGFR1 is an oncogenic driver and therapeutic target in high-risk neuroblastoma. *J Clin Invest* 2026. <https://doi.org/10.1172/JCI189152>