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Abstract

In recent years, extensive efforts have been undertaken to establish a catalog of genetic aberrations involved in the formation and progression of common cancer types. These endeavors improved our ability to diagnose cancer and treat patients with drugs specifically targeting mutations responsible for driving malignancy. Although rare cancers account up to 25% percent of all cancer diagnoses, their genomic landscapes remain poorly characterized.

Targeted sequencing of patient samples from two rare skin cancer types, microcystic adnexal carcinoma (MAC) and desmoplastic trichoepithelioma (DTE), patient samples revealed a heterozygous hotspot mutation in the Ras homolog gene family member A (RhoA) gene with a prevalence of 65%, altering the amino acid sequence from glycine 17 to glutamic acid (G17E).

RhoA acts as a molecular switch and is involved in a multitude of cellular processes, including cell division and migration. The aim of this thesis was to dissect phenotypic changes induced by this mutation, identify its role in tumor formation and unravel the underlying molecular mechanism. The generation of several cell line models including overexpression and CRISPR/Cas9-mediated site-directed mutagenesis at the endogenous locus allowed us to study the phenotypes induced by RhoA-G17E. We detected severe morphological perturbations of the cytoskeleton and RhoA-G17E-dependent enhancement of invasiveness *in vitro*. We are currently investigating the tumor-initiating capacity of RhoA-G17E *in vivo*, by introducing the mutation via lentiviral *in utero* injections, which allows for stable integration and propagation of RhoA-G17E by targeting undifferentiated embryonic epithelium.

Using mass spectrometry, we detected that RhoA-G17E strongly binds to guanine nucleotide exchange factors (GEFs) required for GTP-binding and RhoA activation. We found that RhoA-G17E mutants act in a dominant-negative manner by sequestering GEFs within the cell, preventing RhoA-WT from binding GTP. Moreover, we uncovered a link between RhoA, a sequestered GEF and the tumor suppressor CYLD, which was previously implicated in trichoepithelioma (TE), histologically related to DTE and MAC. Although, further investigations are required, this link may provide new insights into the molecular mechanisms underlying these tumor types.

Zusammenfassung

Der Fortschritt im Bereich des „Next-Generation Sequencing“ über die letzten Jahre ermöglichte die Identifikation von genetischen Veränderungen, die mit der Entstehung von häufigen Krebsarten assoziiert sind. Obwohl 25% aller Krebserkrankungen von seltenen Krebstypen eingenommen werden, wurde die genetische Charakterisierung dieser Krebsarten weitgehend vernachlässigt und es ist wenig über deren Pathogenese bekannt.

Durch Sequenzieren von Patientenproben von zwei seltenen Hauttumoren (mikrozystisches Adnexalkarzinom (MAK) und desmoplastisches Trichoepitheliom (DTE)) identifizierten wir eine häufige, heterozygote Punktmutation im Gen der GTPase Ras homolog gene family member A (RhoA).

Die Herstellung von Modellsystemen *in vitro* erlaubte eine Charakterisierung der Phänotypen, die mit der Mutation assoziiert sind und das Identifizieren von den zugrundeliegenden molekularen Mechanismen. Wir zeigen, dass die RhoA-G17E-Mutation morphologische und funktionelle Eigenschaften von Zellen verändert und ihnen ein tumorerzeugendes Potential verleiht. Dieser Hypothese gehen wir gegenwärtig, mit der Generierung eines *in vivo* Mausmodels nach.

Mittels Immunpräzipitation und Massenspektrometrie identifizieren wir eine starke Bindung zwischen RhoA-G17E und guanine nucleotide exchange factors (GEFs). Damit assoziiert, fanden wir heraus, dass es sich bei RhoA-G17E um eine dominant negative Mutante handelt, indem es GEFs besetzt und diese an der Aktivierung des Wildtypprotein hindert.

Des Weiteren konnten wir eine Verbindung zwischen RhoA und dem Tumorsuppressor CYLD herstellen, der eine Rolle in der Tumorgenese von Trichoepitheliomen spielt, welche histologische Ähnlichkeiten mit MAK und DTE aufweisen. Obwohl weitere Untersuchungen notwendig sind um diese Hypothese zu verstärken, kann dieser Link zu einem besseren Verständnis der zugrundeliegenden, molekularen Mechanismen dieser Tumorsubtypen betragen.

1. Introduction

Although, prevention and treatments of cancer have undergone large improvements, cancer-caused mortality rates are predicted to rise up to 70% over the next two decades (WHO, 2018). Cancer is a genetic disease caused by mutations that drive a malignant behavior. International consortia, such as The Cancer Genome Atlas (TCGA) have undertaken extensive efforts to establish a catalog of genetic aberrations, involved in the formation and progression of various cancer types. Providing valuable information about the genetic basis of certain cancer types, these data sets allow us to improve the development of new drugs and innovative treatments, as well as diagnostic approaches and preventative measures. Despite great advancements regarding the investigation of common cancer types, the genomic landscape of rare cancers remains poorly characterized. Although, rare cancers account for about 25% of all cancer diagnoses worldwide, affecting individuals regardless of age and race, they receive less scientific deliberation and funding in contrast to frequent tumor types. Thus, clinical diagnoses remain challenging and error-prone due to the lack of scientific understanding of rare tumor types. Ultimately, this results in incorrect treatment approaches and poor patient outcome. There is an urgent need to improve diagnostic accuracy in order to develop precise treatments. Thus, investigations of the genomic landscapes of rare cancer types are required to gain a better understanding of the underlying molecular mechanisms which provide the basis for novel therapeutic approaches. Nevertheless, these studies are impeded by the lack of a sufficient number and the quality of tumor samples ¹.

Here, we focus on the characterization of desmoplastic trichoepithelioma (DTE) and microcystic adnexal carcinoma (MAC), two rare skin cancer types for which genetic drivers remain entirely unknown. A unique selection of MAC and DTE tumor samples we obtained in collaboration with a group of renowned dermatologists allowed us to perform a targeted sequencing assay to identify genetic aberrations and underlying molecular mechanisms.

1.1 Rare Skin Cancers

1.1.1 Microcystic adnexal carcinoma (MAC) and desmoplastic trichoepithelioma (DTE)

Microcystic adnexal carcinoma (MAC) is a rare, slow-growing, yet malignant skin appendage tumor localizing to the face, head and neck of middle-aged and older patients. In general, it is clinically represented by flesh-colored plaques or scar-like papules (Figure 1b). Its origin has been hypothesized to be pluripotent keratinocytes differentiating into hair follicle or sweat glands. This has been further supported by immunohistologic analysis suggesting both, pilar and eccrine differentiation ². The upper dermis exposes micronests filled with keratins and ductal structures are formed in addition to the occurrence of a desmoplastic reaction ³. While metastatic events are rare, MAC is locally aggressive with deep infiltration of the dermis reaching into subcutaneous tissues, skeletal muscle, bones, the vascular adventitia and perineural spaces ².

Desmoplastic trichoepithelioma (DTE) are rare, benign adnexal tumors occurring within the facial region of younger adults. Originating from basal cells in the hair follicles, these neoplasms are characterized by the superficial, centrally depressed lesions with an asymptomatic disease progression (Figure 1a,1d). Diagnosis poses a challenge, since DTE are difficult to distinguish from skin lesions accompanying other diseases ⁴.

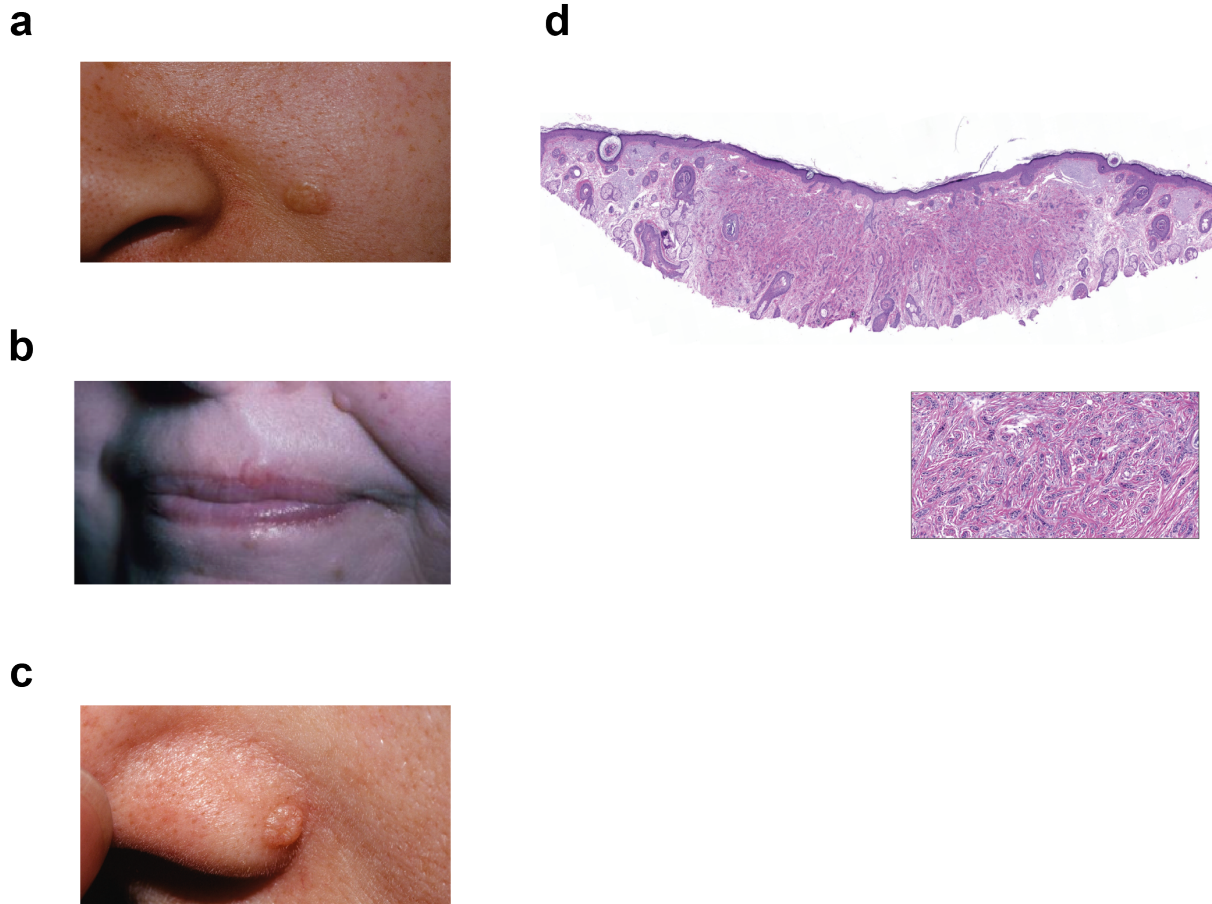


Figure 1 | Three types of rare skin cancers share histological similarities. Representative patients with **a**, Desmoplastic Trichoepithelioma - *Courtesy of Gary M. White, M.* **b**, Microcystic Adnexal Carcinoma - *Courtesy of Dirk M. Elston, MD.* **c**, Trichoepithelioma - *Courtesy of Gary M. White, MD.* **d**, H&E staining of desmoplastic trichoepithelioma whole tumor section.

1.1.2 Multiple Familial Trichoepithelioma (TE) and Brooke-Spiegler Syndrome (BSS)

The Brooke-Spiegler Syndrome (BSS) describes the autosomal-dominant inherited predisposition of affected individuals towards skin appendage neoplasms such as Multiple familial trichoepitheliomas (MFT), cylindromas and spiradenomas. Patients with BSS are born with a germ line mutation in one of the copies of the tumor suppressor gene Cylindromatosis (CYLD). The second, functional copy of the gene is sufficient to produce adequate amounts of the protein. Loss of heterozygosity (LOH) induced by the occurrence of additional mutations in the second copy of various cells in the skin results in formation of skin appendage tumors ⁵. Multiple familial trichoepithelioma (MFT) is a benign adnexal neoplasm that shares histological

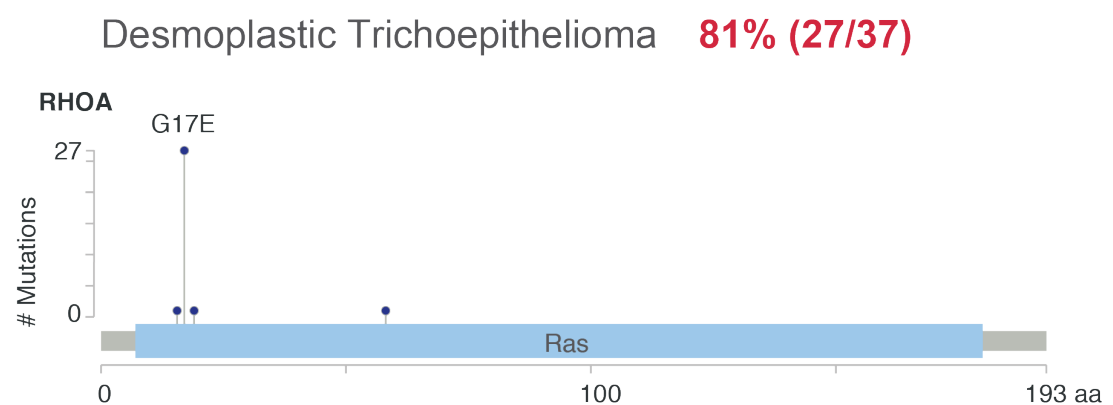
similarities with MAC, DTE, and with basal cell carcinoma (BCC) (Figure 1c). Its occurrence is confined to facial regions including scalp, neck and trunk and it is hypothesized to originate from immature hair follicles (NIH - refer to URL). The loss of CYLD has been described as an underlying molecular driver of MFT. CYLD belongs to the large family of deubiquitinating enzymes (DUBs) which are responsible for de-conjugating ubiquitin chains ⁶. In contrast to a multitude of DUBs, which cleave Lysin(K)48-linked polyubiquitin to target substrates for proteasomal degradation, CYLD exclusively removes K63-linked ubiquitin chains conferring other functional properties, such as cellular localization and signaling, but not degradation ⁷. Thus, CYLD has a major regulatory role in a variety of physiological processes including the immune response and inflammation, predominantly mediated by the regulation of the transcription factor NF- κ B, which ensures cell survival and promotes oncogenesis. The knockdown of CYLD was shown to lead to enhanced NF- κ B-signaling resulting in the translocation of the NF- κ B- co activator Bcl3 into the nucleus, driving the expression of cell cycle regulators (e.g., cyclin D1) or pro-inflammatory genes, which is associated with the development and progression of cancer. Most prominently, loss of CYLD has been identified as the main driver of cylindroma, multiple familial trichoepithelioma and related skin appendage tumors. Dysregulation of CYLD at the level of gene expression, post-translationally, or by loss of part of the chromosome, encoding this enzyme, has been associated with a variety of cancer types ⁸. In melanoma downregulation of CYLD was shown to be mediated by the transcription factor Snail ⁹. Another study elucidated the instability of CYLD-protein in head and neck cancer which indicated that CYLD is targeted for proteasomal degradation causing its decrease in protein levels ¹⁰. Especially its predominant role in the development of rare skin cancers as MFT, suggested CYLD as a putative driver of MAC and DTE.

1.2 Discovery of a novel mutation in rare skin cancers

In collaboration with a group of the dermatopathologists Klaus Busam (MSKCC, USA), Lorenzo Cerroni (Medical University of Graz, Austria), Dmitry Kazakov (University of Zurich, Switzerland) and Arno Rütten (Dermatopathology Friedrichshafen, Germany) we obtained a unique selection of tumor samples from patients suffering from DTE

and MAC. Using this collection of patient tumor material, we performed targeted sequencing of 341 cancer-related genes, which are part of the Memorial Sloan Kettering - integrated mutation profiling of actionable cancer targets (MSK-IMPACT) onco-panel. Deep-sequencing of these pre-selected genes allowed us to uncover a heterozygous missense mutation in the gene of Ras homolog gene family member A (RhoA) with a high prevalence. We detected RhoA-G17E in 81% of the DTE samples (27 out of 37 patients) and in 65% of the MAC samples (11 out of 17 patients), but failed to uncover additional mutations in other oncogenes, tumor suppressors or cancer associated genes, suggesting high functional importance of this mutation in tumor development and disease progression.

a



b

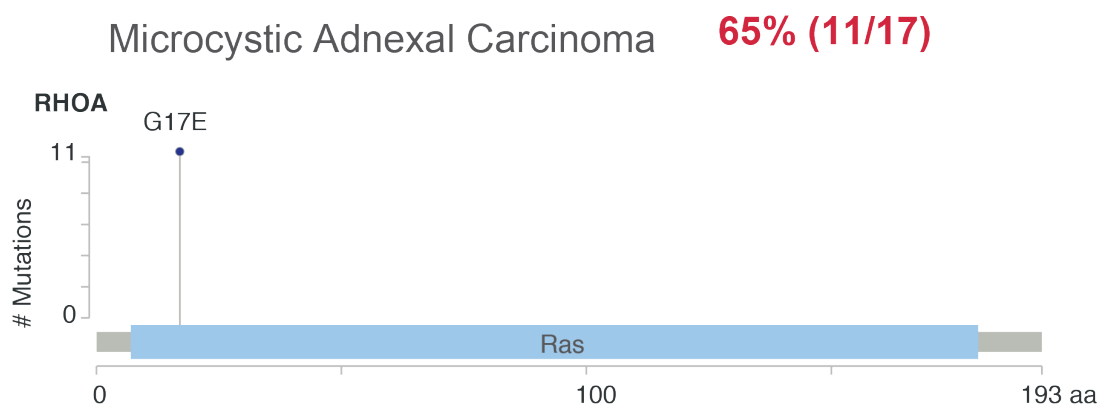


Figure 2| Prevalence of the RhoA-G17E mutation in **a**, desmoplastic trichoepithelioma **b**, microcystic adnexal carcinoma.

1.3 RhoA

As a member of the Ras GTPase superfamily, RhoA is a small monomeric GTPase with a molecular weight of 21 kDa. In response to extracellular stimuli such as the binding of growth factors or mechanical stimuli, RhoA binds and releases GDP and GTP thereby acting as a molecular switch by regulating intracellular signaling cascades upon interaction with downstream effectors. Influencing these signaling networks, RhoA is a crucial regulator of cytoskeleton organization; thus, it is involved in many cellular processes including migration, adhesion, cell division and transcription. Dysregulation of its function results in severe perturbation of cellular pathways, ultimately leading to the pathogenesis including developmental syndromes, vascular pathologies or cancer ¹¹. Thus, cycling between the active and inactive state requires stringent control mechanisms.

Three major groups of proteins control RhoA-activity by regulating GTP- or GDP-binding. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP and GTPase activating proteins (GAPs) hydrolyze the GTP returning RhoA to its inactive, GDP-bound state. Guanine nucleotide dissociation inhibitors (GDIs) represent another, yet passive group of RhoA-activity regulators by maintaining a cytoplasmic pool of inactive RhoA, protected from degradation ¹². GDIs sequester RhoA in its GDP bound state by binding its C-terminal switch region. The functional relevance of GDP-bound RhoA-sequestration to the cytoplasm remains largely unknown, although it has been hypothesized that the maintained inactive RhoA-pool serves the purpose of enhanced simultaneous RhoA-activation when required. Although, little is known about the process of GDI-release, it was suggested that this needs to happen prior to the interaction with GEFs, as these also bind and occupy the same switch regions ¹³. Adhesion- and growth factor receptors activate GEFs via G-coupled protein receptors located to the cell membrane upon binding of the respective ligands. GEF-binding induces a conformational change in the switch region of RhoA leading to a decreased affinity towards GDP. Due to a higher intracellular abundance of GTP, Rho GTPases have a higher affinity to GTP than GDP resulting in the rapid exchange for nucleotide triphosphate. Upon GTP-binding the affinity towards the GEF is weakened and subsequently displaced. RhoA-GTP then interacts with effector proteins. This interaction causes a disruption of an auto-inhibitory domain in the

effector resulting in the exposure of functional domains ¹⁴. Rho-associated protein kinase (ROCK) and mammalian homolog of *Drosophila* diaphanous -1 (mDia1) represent the two main RhoA-effector proteins mediating actin organization. Downstream signaling of ROCK leads to the activation of myosin which connects actin filaments resulting in the accumulation of actin bundles which induces cell contractility, whereas mDia-signaling confers actin and microtubule stability. To ensure cellular homeostasis, RhoA needs to be returned to its inactive GDP-bound state. Although, these proteins are termed “GTPases”, the initial GTP-hydrolysis is a slow reaction. Thus, GAPs are required, which accelerate the GTP-GDP exchange by facilitating a nucleophilic attack (Figure 3) ¹⁵.

Functional and structural data have provided evidence for GDP/GTP binding domains, an effector domain which allows the engagement with downstream targets (e.g., ROCK), hypervariable regions which differ between Rho family members and a CAAX motif for posttranslational modifications ¹⁶. The CAAX domain at the C-terminus is prone to methylation, lipid modifications and cleavage which determine the localization of the protein (e.g., geranylgeranyl prenylation allows anchorage to the cell membrane) or influence its activity ¹⁷. Constant binding of nucleotides requires defined nucleotide binding pockets. Switch-domains change their conformation depending the type of nucleotide (GDP or GTP) bound and the presence of Mg^{2+} enhances the affinity of nucleotide binding ¹³. GTP-binding further results in the conformational change of the effector domain allowing RhoA to interact with effectors in order to activate them and induce downstream signaling (Uniprot - refer to URL). Mutations within these nucleotide binding sites have previously been shown to have effects on GDP/GTP binding, altering the activity status of the GTPase, which results in dysregulation of downstream signaling pathways.

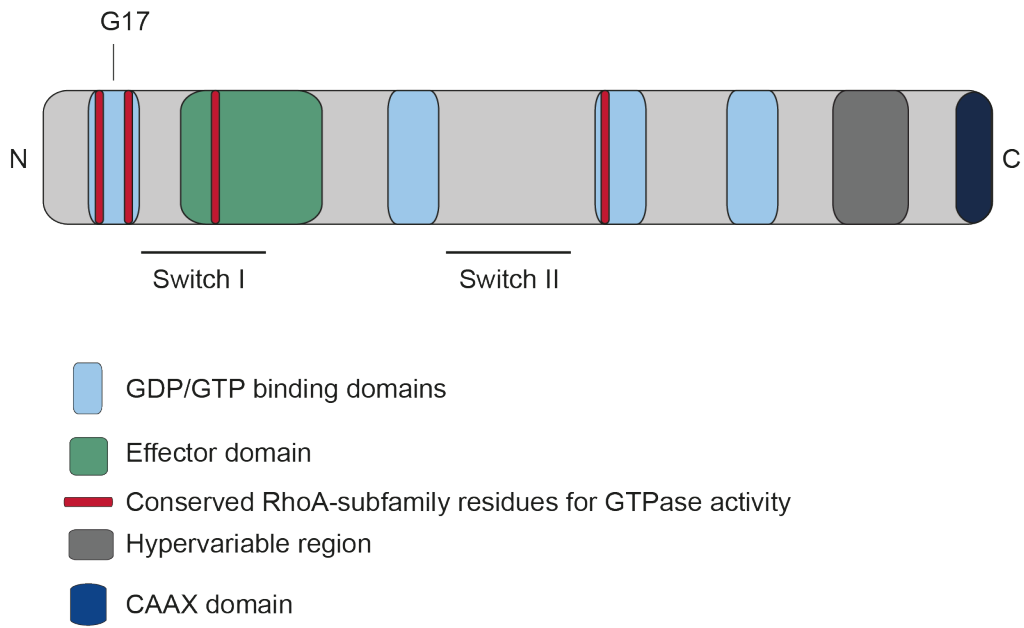
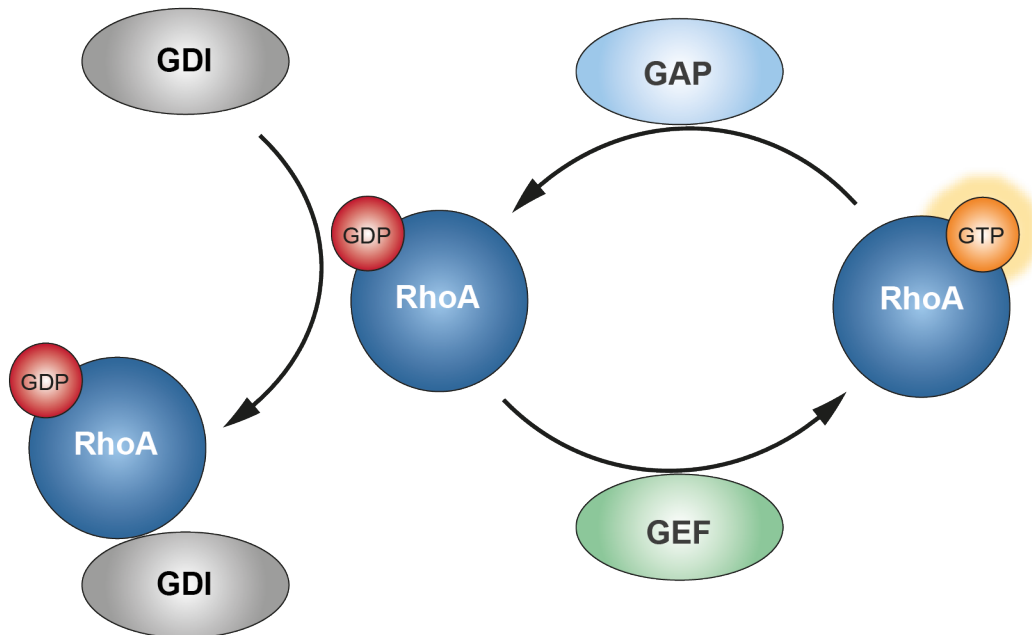
a**b**

Figure 3| RhoA functional domains and mode of action. **a**, schematic of RhoA functional domains. **b**, RhoA-activity cycle representing active GTP-bound RhoA (catalyzed by GEFs), inactive GDP-bound RhoA (GTP hydrolysis by GAPs) and sequestration of inactive RhoA by GDIs.

1.4 RhoA and cancer

RhoA is an important regulator of signaling pathways and thus, ensures tissue homeostasis. Altered expression or dysregulation of genes affecting proliferation, migration, survival or death promote the formation of primary tumors or disease progression invasion and activate metastatic processes. This suggests that dysregulation of RhoA plays a role in tumorigenesis ¹⁸. Enhanced RhoA expression has been detected in cancerous tissues, especially in epithelial tumors and at metastatic sites. Furthermore, it was shown that elevated RhoA levels conferred cancer cells with an invasive ability and increased cell motility *in vivo* ¹⁹. Other studies elucidated the contribution of inactive RhoA to colon cancer progression by activation of the WNT pathway ²⁰. Furthermore, the dysregulation of Rho-GEFs, GAPs and GDIs which define RhoA-activity in a direct or indirect manner have been implicated in tumorigenesis but also exert tumor suppressive effects ²¹. Overexpression of GEFs was shown to enhance RhoA-activity in neuroblastoma and was implicated in the invasion of a metastatic breast cancer cell line ^{22 23}. P190RhoGAP, a RhoA-GAP showed tumor suppressive effects as its overexpression correlated with the inhibition of glioma formation in mouse models ²⁴. RhoGDIs are capable of regulating a variety Rho-GTPases. Controversially, in some cancer types an upregulation, in others a downregulation of GDIs were associated with a specific cancer type ^{25 26}.

1.4.1 RhoA-mutations

In summary, both, overexpression and inactivation of RhoA in a direct or indirect fashion have been associated with the development and progression of cancer ²². The recent advancements in next-generation sequencing technologies led to the identification of RhoA-mutations in a variety of cancer types, which have been cataloged in TCGA databases ²⁷. These mutations seem to occur within distinct functional domains, most prominently they reside within GDP/GTP binding domains or the effector domain, responsible for the activity and interaction with downstream effectors, respectively. The RhoA-Y42C-mutations was found in diffuse-type gastric carcinoma but also germinal center B cell-derived lymphomas, located in the effector region ²⁸. Furthermore, this mutation was implicated in reducing GTP-binding which

was shown to confer the ability to avoid anoikis (apoptosis induced upon loss of cell-matrix interactions) ²⁹. A study on gastric adenocarcinoma samples revealed a multitude of novel RhoA mutations, also located in regions that allow RhoA to interact with its downstream effectors ³⁰.

1.4.2 Glycine 17 - a mutational hotspot in RhoA

TCGA data sets have elucidated a mutational hotspot at the amino acid position 17 in the RhoA gene. Mutations at this position have been reported in various cancer types (Figure 4). The malignant transformation of T-cells leads to the development of peripheral T-cell lymphomas (PTCL) representing a highly heterogeneous group of lymphoid tumors. An aggressive form of these tumors is angioimmunoblastic T-cell lymphoma (AITL), with poor patient outcome. In 70% of all AITL-cases, a recurrent mutation in the RhoA-gene was detected causing an amino acid exchange of glycine 17 to valine ³¹. The RhoA-G17A-mutation was also detected in breast and bladder cancer, as well as in lung carcinoma. This RhoA-mutant has been characterized by the preferential sequestration of active GEFs as it is not capable of binding neither GDP nor GTP, thus remaining trapped in an intermediate stage ³². Mutations altering the amino acid sequence from glycine 17 to glutamic acid (RhoA-G17E) have been reported in a variety of cancer types such as breast cancer, basal cell carcinoma, bladder cancer or colon cancer (cBioportal). Genetic analyses of diffuse gastric cancer (DGC) revealed a set of RhoA mutations, including G17E as novel drivers of this cancer type ³³. However, the question of whether RhoA acts as a tumor suppressor or oncogene is still under debate.

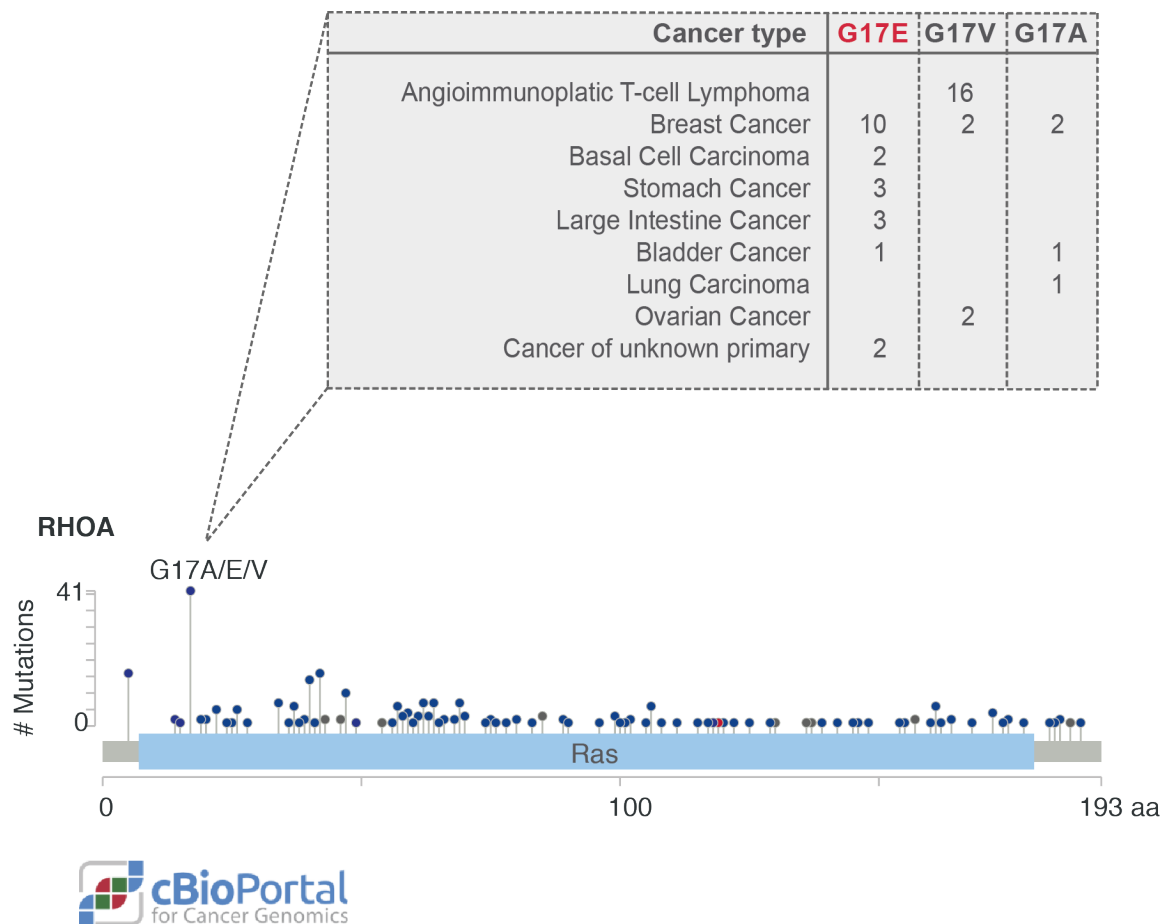


Figure 4| Mutational hotspots in the small GTPase RhoA.

1.4.3 RhoA - oncogene or tumor suppressor

Although RhoA has been considered an oncogene as studies have demonstrated that overexpression of RhoA-GTP is associated with tumor formation, extensive mutational analyses revealed that RhoA-mutations residing in defined regions of the gene may also confer an inactivating function¹⁸. The discrepancies on whether frequent hotspot mutations confer a gain- or loss of function have not been resolved in the field of RhoA-biology. The presence of these mutational hotspots advocates the hypothesis that these mutations confer a gain-of-function. The heterozygous RhoA-G17E mutation was shown to confer a proliferative effect in human breast cancer (BT474) and colon cancer (SW948) cell lines³⁴. Biochemical investigations on RhoA-G17V, which has been implicated as a driver of AITL, revealed this mutant to function in a dominant negative fashion. Overexpressed RhoA-G17V not only failed to bind GTP in rhotekin

pull-down assays but also severely reduced GTP-binding of the endogenous wildtype protein ³⁵. However, recent studies revealed that RhoA-G17V did not interfere with T-cell activation, whereas knock-out or inhibition of RhoA by *clostridium botulinum* C3 toxin resulted in diminished T-cell activation, suggesting that despite its dominant negative effect, RhoA-G17V imparts a neomorphic function ³⁶.

Furthermore, exchanging glycine 17 to alanine showed the same effect on GTP-binding. Both, RhoA-G17A- and RhoA-G17V-mutants exhibited strong interactions with GEFs depicted in immunoprecipitation experiments ^{32 37}. The RhoA-T19N mutant has previously been described to exert its dominant negative effect by the inactivation of remaining wildtype protein within the cell and has therefore found application as a valid control system. These studies suggest that the RhoA hotspot mutants in the GTP/GDP binding domain act in a dominant negative capacity, which suggests that the RhoA-G17E mutation in MAC and DTE may share this ability.

1.5 Aim

Over the past decade, tremendous advancements in Next-Generation Sequencing (NSG) technologies have allowed for extensive characterization of the genomic landscape of common cancer types leading to the development of novel drugs, which specifically target a genetic aberration. However, due to the lack of availability of high-quality patient samples, genetic aberrations of rare cancer types remain largely unknown, leading to miss-diagnoses and incorrect treatments in the clinics ¹. Therefore, it is important to gain a better understanding of the mutational landscape of rare cancer types to improve therapeutic approaches.

Having been provided with a unique selection of high-quality tumor samples from desmoplastic trichoepithelioma (DTE) and microcystic adnexal carcinoma (MAC) patients allowed us to perform targeted sequencing to identify genetic drivers of these rare, non-melanoma skin cancers. Thereby, we discovered a mutation in the RhoA gene altering its amino acid sequence from glycine 17 to glutamic acid. This mutation was highly prevalent across both tumor types, indicating its functional importance.

This thesis focuses on the phenotypic and mechanistic characterization of the RhoA-G17E mutation by addressing the following aims:

1. Overexpression and knock-in of RhoA-G17E at the endogenous locus of human epithelial cells will allow us to investigate changes of the cytoskeleton, the proliferative capacity and migration as well as invasion. Furthermore, these models are important to study the molecular mechanism underlying this mutation.
2. Detection of a single point mutation with a high prevalence in DTE and MAC patient samples suggests Rho-G17E's role in tumorigenesis. Addressing this role requires the generation of suitable *in vivo* models.
3. Providing evidence which supports the mechanistic link of RhoA-mutations, and mutations in Cyldromatosis (CYLD), a commonly mutated tumor suppressor in histologically similar skin cancer types.

2. Materials and Methods

2.1 Reagents

Reagent	Supplier	Product number
Plasmid Preparation		
One Shot® Stbl3™ Chemically Competent <i>E. coli</i>	Thermo Fisher Scientific	C737303
SOC (Super Optimal Broth) medium	Sigma Aldrich	S1797
LB medium	produced in- house	-
Nucleo Bond Xtra Midi Kit	Macherey Nagel	740410,50
Cell Culture		
DMEM (Dulbecco's Modified Eagle's medium) Powder	Thermo Fisher	52100047
MEM (Minimum Essential Medium Eagle) Powder	Thermo Fisher	61100-103
L-15m (Leibowitz 15 media)	Gibco, Life Technologies	11415-049
FBS (fetal bovine serum)	Sigma Life Science	F7524
L-Glutamine (200 mM)	Sigma Life Science	7513
Penicillin/Streptomycin (100x)	Sigma Life Science	PO781
Trypsin 1x (0,05%)	Gibco, Life Technologies	25300054
Trypsin 10x (0,5%)	Gibco, Life Technologies	15400-054
MG-132	Sigma Aldrich	M7449-200UL
Lenti-/ Retrovirus Generation		
Optimem®	Gibco, Life Technologies	31985-062
Lipofectamine 2000	Invitrogen	11668-019

Polybrene®	Santa Cruz Biotechnology	sc-134220
RNA Isolation/ Reverse Transcription/ qPCR		
RNeasy Mini Kit	Qiagen	74104
RNase-Free DNase Set	Qiagen	79254
SuperScript™ IV Reverse Transcriptase	Invitrogen	18090010
GoTaq® Master Mix	Promega	M7122
Oligo d(T)18 mRNA primer	NEB	S1316S
Immunoblotting		
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	23227
RIPA buffer, 10x	Cell Signaling Technology	9806S
Halt™ phosphatase inhibitor cocktail	Thermo Scientific	78427
Complete, EDTA-free protease inhibitor cocktail	Roche Diagnostics	11484100
NuPage® Loading dye (4X)	Thermo Fisher Scientific	NP0007
10x Bolt® sample reducing agent	Thermo Fisher Scientific	B0009
See Blue® Plus2 Standard 1x	Invitrogen	LC5925
Novex™ Sharp Pre-stained Protein Standard	Invitrogen	LC5800
Precast gels Bolt BisTris Plus 4-12%, 15-well/ 12 well	Thermo Fisher Scientific	NW04125BOX/ NW04122BOX
NuPAGE® MOPS SDS running buffer	Novex, Life Technologies	BT00061
Nitrocellulose membrane	Bio-Rad	162-0115
Hybond Blotting Paper	GE Healthcare Life Sciences	RPN6101M

Bolt® Transfer Buffer (20x)	Thermo Scientific	BT00061
Milk powder	Sigma Aldrich	M7409
Ponceau S, 1x	VWR	AFFY3281950G M
Amersham™ ECL™ Western Blotting Detection Reagent	GE Healthcare Life Sciences	RPN2106
Amersham Hyperfilm™ ECL	GE Healthcare Life Sciences	28906835
Odyssey® Blocking Buffer (PBS)	Li-Cor Biotechnologies	P/N 927-40000
Immunocytochemistry		
Gelatin solution Type B, 2% in H ₂ O, tissue culture grade	Sigma Aldrich	G1393-100ML
Normal goat serum (10%)	Thermo Fisher Scientific	50062Z
Hoechst 33258	Life Technologies	H3569
ProLong® Diamond Antifade Mountant	Thermo Fisher Scientific	P36970
Primary Antibodies		
Rhodamine phalloidin	Life Technologies	R415
Mouse anti-Occludin	Life Technologies	331500
Mouse anti-FAK	BD Transduction Laboratories	610087
Mouse anti-HA	produced in- house	-
Rabbit E-Cadherin	Abcam	ab15148
Rabbit anti-beta catenin	Abcam	ab32572
Rabbit anti-CYLD	Cell Signaling Technology	12797S
Mouse anti-Vinculin	Sigma Aldrich	V9131-100UL
Mouse anti-V5	Sigma Aldrich	V8012
Mouse anti-RhoA	Santa Cruz Biotechnology	sc-418
Rabbit anti-RhoA	Cell Signaling Technology	67B9

Monoclonal anti- α tubulin, clone 5	Sigma-Aldrich	T6074-200UL
Secondary Antibodies		
Rabbit IgG HRP Linked Whole Ab (from Donkey)	Sigma-Aldrich	GENA934-100UL
Mouse IgG HRP Linked Whole Ab (from Sheep)	Sigma-Aldrich	GENA931-100UL
F(ab') ₂ -Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 594 conjugate	Thermo Fisher	A-11020
IRDye® 800CW Goat anti-Rabbit IgG (H + L)	Li-Cor Biotechnologies	P/N 925-32210
IRDye® 680LT Goat anti-Mouse IgG (H + L)	Li-Cor Biotechnologies	P/N 925-68020
Proliferation/ Anoikis Assay		
CellTiter-Glo® 2.0 Assay	Promega	G9242
Boyden Chamber Assay		
Corning® FluoroBlok™ 24 Well Plate Permeable Support with 8.0 μ m Colored PET Membrane	Corning	351152
CellTracker Green CMFDA (5-chloromethylfluorescein diacetate)	Invitrogen	C7025
Antibiotics		
Blasticidin	Invivogen	ant-bl-1
Puromycin Dihydrochloride	Thermo Fisher Scientific	A1113803
G418 (Neomycin)	Invivogen	ant-gn-1
Immunoprecipitation		
GFP-Trap®	ChromoTek	gtm-20

HA-Tag Rabbit mAb	Cell Signaling Technologies	C29F4
Anti-V5 Agarose Affinity Gel antibody produced (mouse)	Sigma Aldrich	A7345
Mouse anti-FAK	BD Transduction Laboratories	610087
anti-HA	produced in- house	-
Pierce™ Silver Stain Kit	Thermo Fisher	24612
RhoA-Activation		
RhoA-G-LISA-kit	Cytoskeleton	BK124
In utero injections		
Ketamin	AniMedica	
Xylazine	AniMedica	
Capillaries for Nanojet II™ Injectors	Fisher Scientific	3000203G/X
Silkam® thread DS 16 - USP 4/0	Henry Schein	C0762130

2.2 Plasmid preparation

2.2.1 Transformation

1 µL of plasmid DNA (from Mini-Prep) was added to 15 µL of competent cells (Stbl3) and incubated 5 min on ice. Heat shock was performed for 45 seconds at 42°C and samples were again incubated on ice for 5 min. 1mL of SOC medium was slowly added and the samples were incubated at 37°C for 1h. Bacterial cells were centrifuged for 1 min at 5,000 rpm and the supernatant was largely removed. The pellet was carefully resuspended in the residual liquid and the cell suspension was plated on LB-plates containing antibiotics. The plates were incubated at 37°C overnight.

2.2.2 Midi-Prep

200 mL of LB-medium (appropriate antibiotic added) were inoculated with a single colony, picked from the agar plate and incubated overnight at 37°C in a shaker at 200 rpm. The next morning, the cultures were transferred to centrifugation bottles and centrifuged for 30 min at 4°C and 5000 x g. After removing the supernatant, the pellet was resuspended in 8 mL Resuspension Buffer and transferred to a 50mL tube. 8 mL of the Lysis Buffer were added and the tubes were inverted a few times. After 5 min of incubation Neutralization Buffer was added. The filter columns were equilibrated with 12 mL of Equilibration Buffer. Subsequently, the lysate was added to the column and washed with 5mL of equilibration buffer. The filter was removed and the column washed with Wash Buffer. Plasmid-DNA was eluted in 5 mL Elution Buffer into 15 mL tubes containing 3.5mL Isopropanol. The samples were mixed by vortexing and DNA was precipitated by centrifugation at 4 °C and >5000 x g for 1h. After removing the supernatant, the DNA was washed with 70% Ethanol and allowed to dry for 15 minutes at RT. Plasmid DNA was resuspended in 300-400 µL H₂O and concentrations were measured using a DeNovix Spectrophotometer.

2.3 Cell lines and cell culture assays

16HBE cells and SW948 cells were cultured in MEM and Leibowitz media, respectively. MDA-MB-231 and BT474 were cultured in DMEM media. HEK Lenti-X™ 293T (Cloneteck) were used for lentivirus and retrovirus production and maintained in DMEM media. All media were supplemented with 10% FBS, 2mM L-glutamine, 100 IU mL⁻¹ penicillin/streptomycin. 16HBE, MDA-MB-231, BT474, HEK Lenti-X™ 293T were grown in a humidified incubator at 37°C and 5% CO₂. SW948 were maintained 0% CO₂. All cell lines were split every two to four days by aspirating the media, washing the cells with 1x PBS and detaching them with 5x Trypsin (0.25%) (Gibco, Life Technologies) (16HBE and SW948 cells) and 1x Trypsin (0.25%) (Gibco, Life Technologies) (HEK Lenti-X™ 293T, MDA-MB-231, BT474) for 5-10 minutes at 37°C.

2.3.1 Retro- and Lentivirus generation

To ensure stable overexpression of RhoA-mutants our cell lines were infected retro- or lentiviruses. Approximately 4×10^6 HEK Lenti-X™ 293T cells were plated on 10cm plates in full growth media and allowed to attach overnight. For lentivirus production 1328 µl Optimem (Thermo Fisher) were prepared in a polystyrene tube and 1.1 µg of psPAX2, 400 ng VSVG (pMDG) and 1.2 µg of the lentiviral vector of interest added. For retrovirus production 1328 µl Optimem (Thermo Fisher) were prepared in a polystyrene tube and 1.1 µg of psPAX2, 400 ng VSVG (pMDG) and 1.2 µg of the lentiviral vector of interest added. While incubating this mix for 5 minutes at RT, in a second polystyrene tube 52 µl Lipofectamine 2000 (Invitrogen) were added to 1328 µl Optimem. Subsequently, the content of the first tube was added to the second one in a dropwise manner, gently mixed by flicking and incubated at RT for 30 minutes. 7200 µl full growth medium without antibiotics were added to the mix and gently mixed. Full growth medium was aspirated from the HEK Lenti-X™ 293T cells and the transfection mix added in a dropwise manner. 12 hours post-transfection the medium was changed to full DMEM growth medium without antibiotics. Virus was harvested for the first time 24h post-transfection, followed by additional harvests every 12 hours for 2 days by collection of the supernatant and replacing it with fresh medium. Subsequently, the supernatant was spun down for 5 minutes at 400 x g to remove remaining cells and filtered through a 0.45 µm PVDF filter (Millipore). The virus was either immediately used for transduction or stored at -80°C.

2.3.2 Transduction

For successful transduction 400,000 cells of interest were plated in a 6-well plate in full growth media and allowed to attach. The media was aspirated and replaced by 0.5 mL of full growth media with 8 µg ml⁻¹ Polybrene® (Santa Cruz Biotechnology). 0.5 mL of virus were added and incubated at 37°C overnight. After 12 hours, the virus was replaced with fresh media. 48 hours post-infection, cells were selected with antibiotics.

2.3.3 Selection with antibiotics

48 hours post infection cells were selected by the addition of antibiotics for 3-7 days. Cells expressing constructs with a blasticidin resistance, were selected by adding 8 $\mu\text{g ml}^{-1}$ blasticidin (Invivogen) for three days. The cells receiving the construct containing the neomycin resistance were selected with 800 $\mu\text{g ml}^{-1}$ G418 (Invivogen) for at least 5 days. Selection with G418 requires frequent splitting of the cells in full growth medium without antibiotics to ensure efficacy of G418.

2.3.4 Fluorescent activated cell sorting

Cells transduced with constructs containing a fluorophore were additionally fluorescence activated cell sorted (FACS) which was conducted by the IMP/IMBA Biooptics Core Facility on a BD FACSAria III Cell Sorter (BD Biosciences). Before submitting the samples to the facility cells were trypsinized as described above. Cells were transferred to FACS-tubes by straining them through a 70 μm mesh to obtain single cells. Subsequently, cells were spun down and resuspended in 0.5 mL FACS-buffer consisting of 10% FBS in PBS and kept on ice.

2.3.5 Cryopreservation

Cells were trypsinized as previously described and centrifuged at 400 x g for 5 minutes at room temperature. The cell pellet was resuspended in freezing media (90% FBS and 10% DMSO sterile filtered through a 0.22 μm filter, Steriflip Millipore) and aliquoted into Nunc® CryoTubes®. The tubes were placed in CoolCell® LX (Biocision) ensuring a constant freezing rate of $-1^{\circ}\text{C}/\text{minute}$ when transferred to -80°C . When the respective temperature was reached the cells were stored in liquid nitrogen.

Rapid thawing was achieved by placing the tubes briefly in a water bath at 37°C . Subsequently, cells were taken up in 5mL full growth media and centrifuged 400 x g for 5 minutes to remove residual DMSO. Depending on the pellet size, cells were plated into a 6-well plate or a 10cm plate in full growth media.

2.3.6 Proliferation assay

Approximately 1,000-3,000 cells were plated in a 96-well plate in triplicates or quadruplets and allowed to adhere overnight. After 72h the number of living cells was determined by adding cell by adding the same volume CellTiterGlo 2.0 (Promega) to each well. Lysis of the cells was achieved by pipetting up and down vigorously and incubating the plate on an orbital shaker for 2 minutes at 400 rpm. The samples were transferred to an opaque plate and incubated for 10 minutes at room temperature protected from light to allow the signal to stabilize. The luminescent signal was detected by a Biotek Synergy 2 plate reader.

2.3.7 Anoikis assay

Approximately 1,000-3,000 cells were plated in a 96-well low-adhesion plate in triplicates or quadruplets. After 6 days, the number of living cells was determined by adding the same volume CellTiterGlo 2.0 (Promega) to each well. Lysis of the cells was achieved by pipetting up and down vigorously and incubating the plate on an orbital shaker for 2 minutes at 400 rpm. The samples were transferred to an opaque plate and incubated for 10 minutes at room temperature protected from light to allow the signal to stabilize. The luminescent signal was obtained using a Biotek Synergy 2 plate reader.

2.3.8 Boyden chamber trans-well migration assay

Serum-starved cells (0% FBS, 24h at 37°C) were labelled with cell tracker green (Invitrogen) for 15 minutes at 37°C and allowed to recover for 1h. Approximately 80,000 cells were seeded onto 8µm-pores FluoroBlok filters (Falcon) and placed in a 24-well plate. The lower compartment was supplied with media containing 10% FBS and the cells were allowed to migrate through the pores for 20-24h. The number of migrated cells was determined by manually counting ten fields of vision and pictures were taken on an Axioobserver Z1.

2.3.9 Gap closure assay

Approximately 150,000 16HBE cells were seeded on 24-well plates and allowed to form a confluent monolayer. Using a pipet tip a scratch was introduced in the middle of the wells. Cells were imaged at different time points using an inverted Axioobserver Z1. Furthermore, imaging was performed on a Celldiscoverer 7 (Zeiss) automated platform for live cell imaging (18h, image acquired every 30 min, 37°C, 5% CO₂). We used the FIJI software to measure the area of the scratch 10 min, 1 h, 5 h and 10 h.

2.3.10 Live cell imaging - single cell migration

Approximately 70,000 cells were seeded on a 24-well plate and allowed to attach. The cells were incubated with SiR-DNA dye for 1 h at 37°C and subsequently, images were acquired every 5 min for 15 hours with an inverted Axioobserver Z1 with a sCMOS camera and incubation chamber (maintaining a constant temperature of 37°C, phase contrast and far-red).

2.4 Gene expression analysis

2.4.1 RNA isolation

Approximately 600,000 cells were seeded in a 6-well plate in regular growth media allowed to attach. RNA was isolated according to the RNeasy® Mini Kit protocol (QIAGEN) with minor modifications. As a reducing agent, 10 µl β-mercaptoethanol (β-Me) were added to 1 ml of Buffer RLT lysis buffer. 350 µl of lysis buffer were directly added to the well. Subsequently, the lysate was harvested with a cell scraper and transferred into a 1.5 ml tube. The RNA was isolated according to the manufacturer's instructions and eluted with 40 µl RNase-free water. RNA concentrations were measured by Nanodrop.

2.4.2 Reverse transcription

For reverse transcription, the SuperScript IV Reverse Transcriptase Kit (Invitrogen) was used to set up the following reaction: 1 µg of total RNA, 1 µL Oligo dt Primer (50 µM) and 1 µL dNTPs (10 mM) were filled up to a volume of 14 µL with water. The reaction was heated up to 65°C for 5 min and, followed by a 1 min incubation on ice. 6 µL of the Superscript IV mix (1 µL SuperScript IV reverse transcriptase, 1 µL DTT (0,1 M), 4 µL SuperScript IV First Strand Buffer(5X)) were added and carefully mixed. The reaction was incubated at 50°C for 10 minutes and inactivated by increasing the temperature to 80°C for 10 minutes.

2.4.3 RT qPCR

RT-qPCR was performed using GoTaq® qPCR Master Mix (Promega). Therefore, 2 µL of diluted cDNA (1:10) were added to 5 µL GoTaq® qPCR SYBR Green Master Mix, 0.2 µL of forward and reverse primer and 2.6 µL water yielding a volume of 10 µL. The respective sample was prepared in quadruplets in a 384-well plate and run was performed at a Bio-Rad CFX 384 Real-Time Cycler according to the protocol represented in the table below.

Temperature	Time
95°C	2 min
95°C	15 sec
60°C	60 sec
60-95°C	

The Bio-Rad CFX Manager Software was used to analyze gene expression levels using RPL27, RPS13 and B2M as housekeeping genes. In addition to that expression levels were normalized to samples overexpressing the empty vector or shRenilla.

2.4.4 qPCR primers

qPCR primers were designed using the Primer Blast Tool from NCBI

[\(http://www.ncbi.nlm.nih.gov/tools/primer-blast/\)](http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

RHOA-FW: ATTCGTTGCCTGAGCAATGG

RHOA-RV: TCTTCAGGTTTCACCGGCTC

RPS13-FW: GCTCTCCTTTCGTTGCCTGA

RPS13-RV: ACTTCAACCAAGTGGGGACG

RPL27-FW: CATGGGCAAGAAGATCG

RPL27-RV: TCCAAGGGGATATCCACAGA

2.5 Immunoblotting

2.5.1 Cell lysis and sample preparation

1,000,000 cells were spun down, washed with 1xPBS and frozen at -80°C. The cell pellet was resuspended in 80-100 µL of 1x RIPA-Buffer substituted with Protease Inhibitor and Phosphatase Inhibitor. A short incubation step on ice for 5 minutes was followed by a 5 minutes sonication cycle (30 seconds on/30 seconds off). Subsequently, the samples were centrifuged for 10 minutes at 14,000 x g to remove cell remnants. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

2.5.2 SDS-Gel run and protein transfer

NuPAGE™ LDS Sample Buffer (4x) and NuPAGE™ Sample Reducing Agent (10x) were added to around 25-40 µg. Denaturation of the proteins was achieved by heating up the samples to 95°C. Thereafter, the samples were loaded onto a precast NuPAGE™ 4-12% Bis-Tris Protein Gels, which were run at 70-100V for 90-120 minutes in 1x NuPAGE™ MOPS SDS Running Buffer. Proteins were transferred to a

nitrocellulose membrane (Biorad) in 1x Bolt™ Transfer Buffer at 100V for 90 minutes using a wet-transfer system. Ponceau S (VWR) staining was performed to confirm successful protein transfer.

2.5.3 Blocking and antibody incubation

The membrane was blocked in 5% milk in TBS-T (1xTBS; 0,1% Tween) or Odyssey® Blocking Buffer (PBS)/TBS-T (1:1) for 1h at RT on an orbital shaker. Primary antibodies were diluted in 5% milk or Odyssey® Blocking Buffer (PBS)/TBS-T (1:1) to yield the concentration as displayed in table and shaken over night at 4°C. The following day, the membrane was washed in TBS-T four times for 5 min followed by the incubation of the secondary antibody, diluted in 5% milk or Odyssey® Blocking Buffer (PBS)/TBS-T (1:1) for 1h at RT. To remove unbound secondary antibody, the membrane was again washed four times 5 minutes and either developed on a ChemiDoc by adding the HRP-substrate or fluorescence was measured using Odyssey Clx (Li-Cor).

2.6 Immunocytochemistry

Coverslips were sterilized in 70% EtOH, washed with sterile MilliQ water and allowed to dry. 0.1% gelatin in PBS was used to coat the coverslips, which were placed in a 6-well plate. Approximately 250,000 16HBE cells were seeded on the coated coverslips and allowed to attach overnight. Cells were fixed with 4% PFA(VWR) or iced-cold Methanol (VWR) for 20 or 5 minutes respectively. The cell membranes were permeabilized with 0,25% TritonX 100 (Sigma Aldrich). To prevent the non-specific binding of antibodies samples were blocked 1x Normal Goat Serum (NGS) for 1h at RT. Primary antibodies were diluted in 1x NGS as indicated in the table below and incubated overnight at 4°C. Secondary antibodies (anti-mouse, anti-rabbit and anti-chicken Alexa Fluor 555, 594 (red), 488(green)) were diluted in 1:1000 in 1X NGS incubated for 1h at RT. Hoechst was diluted 1:5000 in 1X PBS and incubated for 5 minutes at RT. The coverslips were mounted using Prolong Gold and allowed to dry

prior to imaging on an inverted LSM 880 Axio Observer.

Phalloidin	1:100
Tubulin	1:100
Occludin	1:100
E-Cadherin	1:100
FAK	1:50
β -Catenin	1:100
Hoechst	1:5000

2.7 Immunoprecipitation (IP) and sample preparation for Mass Spectrometry (MS)

2.7.1 Co-immunoprecipitation anti-HA-coupled magnetic beads (CST)

18 x 10⁶ 16HBE cells transduced with pLVX-HA-RhoA-mutants (G17E/A/V and T19N) were plated on 15 cm plates in duplicates and allowed to attach. Subsequently, cells were washed with ice-cold 1x PBS and lysed with 0.5 mL Lysis Buffer. Lysates were pooled in ultra-low adhesion tubes (Sigma Aldrich) and centrifuged 10 minutes at 4°C and 15,000 x g. Meanwhile, 25 μ L of beads were equilibrated in low adhesion tubes by washing them 3x with 0.5 mL Lysis buffer. After centrifugation, the supernatant was added directly to the beads and 20 μ L were kept for Western Blot and Silver Staining quality control. Proteins were allowed to bind for 3 hours at 4°C on an orbital shaker. Using a magnetic rack beads were pulled down in order to remove the unbound fraction of protein. 20 μ L were stored as a control for immunoblotting. The beads were washed 4x 20 minutes at 4°C on an orbital shaker using the lysis buffer without protease and phosphatase inhibitors. Subsequently, final washes with wash buffer (no detergent) were performed to remove remaining detergent from the beads. Samples were submitted for mass spec analysis.

Components	Stock	Final conc (mM)	Lysis Buffer(mL)	Wash Buffer (mL)
KaOAc	1M	150M	7.5	7.5
HEPES	1M	30M	1.5	1.5
MgSO ₄	1M	5M	0.25	0.25
NP40	10%)	0.5%	2.5	
H ₂ O			37.75	40.75

2.7.2 Co-immunoprecipitation anti-GFP-coupled magnetic beads (Chromotek)

The same protocol as in 2.7.1 was applied with the exception that we used the 16HBE cell lines stably overexpressing a GFP-tagged version of the RhoA-mutants (pQXCIP_GFP-RhoA).

2.7.4 Co-immunoprecipitation anti-V5 (Sigma Aldrich)

4 x 10⁶ cells were seeded in 10cm plates and allowed to attach. Cells were transiently transfected with v5-tagged constructs as described in 2.3.1. 36 h post-transfection cells were washed with ice-cold PBS. To lyse the cells 1 mL of Low Salt Buffer (LSB), containing protease and phosphatase inhibitors, was added. The samples were centrifuged for 10 minutes 3000 rpm at 4°C. 20 µL of v5-coupled beads were equilibrated by washing them 3x in LSB. After centrifugation, the lysates were directly added to the beads and incubated for 1 h at 4°C on an orbital shaker. 20 µL of lysate were stored as a control for immunoblotting. The beads were briefly spun down, washed 3x in High Salt Buffer for 10 minutes at 4°C and stored in PBS.

Low Salt Buffer

Reagent	Stock	Final conc.
Tris-HCl pH8	1M	50mM
NaCl	5M	100mM
MgCl ₂	1M	10mM
EDTA	0,5M	1mM
DTT	1M	1mM
Glycerol	80%	10%
NP-40	10%	0,5%
Water		

High Salt Buffer

Reagent	Stock	Final conc.
Tris-HCl pH8	1M	50mM
NaCl	5M	300mM
MgCl ₂	1M	10mM
EDTA	0,5M	1mM
DTT	1M	1mM
Glycerol	80%	10%
NP-40	10%	0,5%
Water		

2.7.4 Silver Staining

To detect the amount of total protein (input), proteins bound to the beads (immunoprecipitation) and remnants in the unbound fraction, we performed silver staining of SDS-gels according to the Pierce™ Silver Stain Kit.

2.8 RhoA-activation assay

2.5×10^6 16HBE cells were plated in duplicates on 10cm dishes and allowed to attach. Cells were washed with 1xPBS to remove residual serum-containing media. Media was exchanged for MEM with 0% FBS and 0% glutamate for 24h hours. Cells were either treated with 0.1 mg 1 mL^{-1} Calpeptin in DMSO or vehicle (DMSO) for 30 min or 1 μM lysophosphatidic acid (LPA) for 5 min and then further processed using the RhoA-G-LISA kit according to the manufacturer's protocol. In brief, cells were lysed using 125 μL of lysis buffer and extracted proteins were snap-frozen in liquid nitrogen. Small amounts of protein were kept for protein concentration determination using Precision Red. Equal amounts of proteins (12.5 μg) were applied to anti-GTP-coated G-LISA plates (96-well) in duplicates or triplicates. Bound RhoA-GTP was detected with a primary antibody against RhoA. A secondary HRP-coupled antibody allowed for an absorbance-based read-out.

2.9 Genomic DNA extraction and genotyping

Single cell clones were grown to 80-90% confluence in 96-well plates. Cells were washed and 25 μL Quick Extract (EpiBio) were added to each well, mixed well by pipetting and incubated at RT for 10 minutes. Samples were transferred to 65°C for 15 minutes followed by vigorous pipetting. Inactivation of proteases was achieved by heating up the samples to 95°C for 5 minutes. 1-2 μL of genomic DNA were directly used for PCR.

Components	Volume in μL
10 mM dNTPs	0.4
100 μM primer fwd	0.1
100 μM primer rev	0.1
10 x KlenTaq Buffer	2
Template DNA	200 ng gDNA
KlenTaq (mix)	1
1% Triton X 100*	0.2
H ₂ O	x
Total	10

Step	Temperature in $^{\circ}\text{C}$	Duration	Cycles
Initial denaturation	96	2 min	1x
Denaturation	96	30 sec	35x
Annealing	X	30 sec	
Elongation	68	2 min/kb	
Final elongation	68	5 min	1x

0.5% Agarose Gels were run to confirm the correct size of the amplicon. DNA was purified using the gel extraction kit (IMP-MBS) according to the protocol. To purify PCR-products without gel extraction, Exonuclease I - Shrimp Alkaline Phosphatase digest was conducted (in-house IMP-MBS).

Exo-SAP mix	$\mu\text{L}/20 \mu\text{LPCR products}$
Exonuclease I (NEB M0293)	0.04
Shrimp Alkaline Phosphatase (M0371)	0.4
Storage Solution*	1.56

*Storage Solution: 10mM Tris-HCl (pH 7.5), 100mM NaCl, 5mM beta-mercaptoethanol, 0.5 mM EDTA, 50% Glycerol, 1 mM MgCl_2 ; pH 7.5, stored at 25°C

To screen for the presence of the mutation of interest $1\mu\text{L}$ of the purified DNA was sent for Sanger Sequencing.

2.10 Cloning

2.10.1 sgRNA cloning

sgRNAs were designed using the UCSC Genome Browser. Overhangs were introduced, which were compatible with the target vector (CACC sequence to 5'-end of sgRNA sense strand and AAAC to 5'-end of sgRNA antisense strand).

sgCYLD1s	caccATATTCAAGATCGTTCTGTG
sgCYLD1as	aaacCACAGAACGATCTTGAATAT
sgCYLD2s	caccAAAGGCCTCCAAATAGACGT
sgCYLD2as	aaacACGTCTATTTGGAGGCCTTT
sgCYLD3s	caccGGATAACCCTATTGGCAACT
sgCYLD3as	aaacAGTTGCCAATAGGGTTATCC
sgCYLD4s	caccTTCAACTAAGGAACTCCGGG
sgCYLD4as	aaacCCCGGAGTTCCTTAGTTGAA

Vector: *pSGRNAUITEC_U6-sgRNA_Ef1as-Cas9-P2A-BFP* (designed by Julian Jude, PhD)

The vector was digested using 5-10 µg of plasmid DNA, 3µL of 10X NEB Buffer 3.1, 1 µL BsmBI enzyme filled up to 30 µL with ddH₂O for 2-3 hours at 55 °C. 1 µL of calf intestine phosphatase were added and incubated at 37 °C for 30 – 60 min to dephosphorylate the vector. After adding 6 µL of Gel Loading Dye, Purple (6X) (NEB) the full reaction was loaded onto an agarose gel and the correct band was cut out and purified (IMP-MBS kit). For annealing and phosphorylation the following reaction was prepared:

Vol in µL	Reagent
1	sense oligo (100 µM)
1	antisense oligo (100 µM)
1	10X T4-DNA-Ligation Buffer
6.5	water
0.5	T4 Polynucleotide Kinase (PNK) (NEB)

The reaction was transferred to a PCR thermocycler and incubated as follows: 30 min at 37 °C, 5 min at 95 °C, Ramp down to 25 °C by 5 °C per minute. Annealed and phosphorylated oligos were diluted 1:200.

For the ligation, the following reaction was prepared and incubated for 1 h at RT:

50 ng	cut, dephosphorylated and gel-purified vector
1 µL	annealed and phosphorylated oligos (1:200 dilution)
1 µL	10x T4-DNA Ligation buffer
Up to 9.5 µL	water
0.5	T4-DNA ligase

10 µL of the ligation reaction was transformed into 100 µL bacteria (Stbl3 for lentiviral constructs). Colonies were picked for Mini-Preps to verify the correct sequence.

2.10.2 Gibson Assembly

The Gibson assembly mix was prepared as follows: 100ng of the largest (pRRL-lentiviral expression vector), 8.8 ng of the second largest (P2A-GFP) and 4.4 ng of the smallest fragment (RhoA-WT/ RhoA-G17E) were combined, 10 µL of 2x Gibson assembly master mix (IMP-MBS) and up to 20 µL of water were added. 1 µL of was removed to serve as a re-ligation control. The reaction was incubated at 50 °C for 1 h. 5 µL of the product and 5 µL of the re-ligation control were transformed into Stbl3 bacteria as described in 2.2.1. The next day colonies were picked for Mini-Prep inoculation. Sequences were verified by sanger sequencing.

2.11 Animal studies

All animal experiments were performed in accordance to animal protocol approved by the IMBA/IMP animal service and the Austrian laboratory animal act. For all experiments, 5- to 8-week old female and male C57BL/6J or FVB/N mice were used.

2.11.1 *In utero* injections

2.11.1.1 *Mating of mice*

For timed pregnancies breeding pairs (two females and one male) were set up in the evening. The next day, vaginal plugs were checked to determine successful mating, this time point was considered E0.5. Subsequently, pregnant mice were removed from the cage and kept separately.

2.11.1.2 *Lentivirus generation*

Lentivirus was generated as described in 2.3.1 with the following exceptions: 4 x 10cm plates of 4 x 10⁶ HEK Lenti-X™ 293T cells per construct were used for virus generation. Lentivirus was eco-packaged using 1.1 µg of psPAX2, 400 ng EcoEnv and 1.2 µg of the lentiviral vector of interest added (Fig). 12 hours post-transfection the medium was changed to 5 mL full DMEM growth medium without antibiotics. Virus was harvested every 12h for 3 days, filtered and subsequently stored at 4°C. 1 mL of virus was kept for test transduction *in vitro*.

2.11.1.3 *Ultracentrifugation*

Virus concentration was performed by ultracentrifugation using Beckman Optima™ Ultracentrifuge with a SW-32 Rotor. Virus was equally distributed into 30 mL Beckman conical centrifugation tubes (precise balancing required) and centrifuged for 2.5h at 15,000 rpm 4°C. Excess medium was aspirated and pellet resuspended in 10 µL Hanks buffered saline solution (HBSS, in-house IMP/IMBA Media Kitchen). 1 µL of concentrated virus was kept for test transduction *in vitro*. 0.5 µL of blue food dye were added to visualize small amounts of virus, facilitating its uptake into the injection needle.

2.11.1.4 Test infections in vitro

400,000 YUMM 1.7 cells (murine cell line) were seeded on 6-well plates and allowed to attach. Cells were transduced with 1 μ L of concentrated virus or 1 mL of bulk virus and checked under a fluorescent microscope for a signal 24-48 h post transduction.

2.11.1.5 Needle preparation

Glass capillaries were pulled using a Flaming/ Brown micropipette puller (P-97) with the following protocol:

Heat = 530; Pull = 70; Velocity = 70; Time = 200

Subsequently, the needle was sharpened using a micro-grinder (Narishige Japan)

2.11.1.6 Ultrasound-guided injections

Injections were conducted according to a well-defined protocol by Beronja et al. changing the following parameters:

For anesthesia 150 mg kg⁻¹ ketamine per mouse were used. The peritoneal incision was closed using a non-absorbable suture and abdominal skin was stapled. After surgery one mouse was placed in a cage and allowed to recover under an infrared lamp. Mice were monitored for post-surgical bleedings.

2.11.1.7 Skin harvest and immunofluorescent staining

On day E 15.5 mice were euthanized and embryos were removed from the uterus, decapitated and placed in ice-cold PBS. Using forceps, the back skin was carefully removed and fixed in 4% PFA for 5-8 h. After fixation, skin was placed on glass slides and allowed to dry. Immunocytochemistry was performed as described in 2.6 using primary antibodies against GFP (chicken, Sigma Aldrich) and mCherry (Abcam) in 10% NGS-Buffer. Secondary Alexa Fluor 555 goat anti-chicken for GFP and anti-

rabbit mCherry were diluted 1:1000. Nuclear staining was performed using Hoechst and samples were mounted in Prolonged Gold after extensive washes. Images were acquired on an inverted LSM 880 Axio Observer.

3. Results

3.1 Human bronchial epithelial cells allow studies of RhoA functions

RhoA is one of approximately 20 Rho-GTPase family members which act as molecular switches by converting extracellular signals to induce changes in the cytoskeleton ³⁸. 16HBE-14o (further referred to as 16HBE) are SV-40 large T-antigen immortalized human bronchial epithelial cells previously used to study the effects of RhoA-regulation on epithelial apical junction formation ³⁹. Thus, we chose this model to investigate the morphological effects of the RhoA-G17E mutation *in vitro*. We stably overexpressed this mutation in addition to other previously described RhoA-mutations (G17A, G17V, T19N) using lenti- or retrovirally packaged mammalian expression vectors containing the RhoA-protein, neomycin as a selection marker, and IRES-GFP or ZsGreen1 as a fluorescent label, which stably integrate into the genome (Supplementary Figure 1a). Selection on antibiotics and FACS sorting were performed to enrich for transduced cells (Supplementary Figure 1a). qPCR and Immunoblotting were performed to confirm successful overexpression. To monitor the intracellular localization of the overexpressed RhoA-mutants, we used a construct cloned in our lab where RhoA was directly fused to GFP or tagged with the hemagglutinin(HA) peptide (Supplementary Figure 1a). This further allowed us to precipitate the RhoA-mutants using HA-/GFP-antibody coupled beads to ultimately identify putative binding partners by mass spectrometry. The overexpression model served us to mimic the environment of the heterozygous mutation which naturally occurs in DTE and MAC patients.

In addition to the lentiviral overexpression system, we aimed to introduce the RhoA-G17E mutation into the endogenous locus in 16HBE cells using CRISPR/Cas9. To achieve this, Cas9-expressing 16HBE cells were electroporated with a vector containing a small guide RNA that located Cas9 to the RhoA locus and a repair template containing the RhoA-G17E mutation. After single cell sorting, we extracted and Sanger-sequenced the genomic DNA to confirm positive RhoA-G17E knock-in clones (Supplementary Figure 1e). We only identified two clones carrying the RhoA-G17E mutation, which were both homozygous for RhoA-G17E (Supplementary Figure

1e). These clones were used for subsequent functional analysis. The generation of the CRISPR/Cas9 RhoA-G17E knock-in cell lines should allow us to prevent the introduction of overexpression biases.

3.2 RhoA-G17E mutations have been described in other cancer types

TCGA-data indicate that Glycine 17 in RhoA represents a mutational hotspot. RhoA-G17V was identified as a highly prevalent mutation in angioimmunoplastic T-cell lymphoma and RhoA-G17A was detected in breast cancer and lung carcinoma. The conversion from Glycine to Glutamic acid at this position has been identified in other cancer types as breast, bladder or stomach cancer but not in rare skin cancers (Figure 4). Moreover, the heterozygous RhoA-G17E mutation is present in the breast cancer cell line BT474 and the colon cancer cell line SW948. With these models, we aimed to perform rescue experiments where knockdown of endogenous RhoA using shRNAs against the 3'UTR of RhoA was performed followed by overexpression of empty vector (EV), RhoA-WT and RhoA-G17E. Successful knock-down and overexpression were validated by immunoblotting (Supplementary Figure 1b,1c). SW948 cells grow in sphere-like clusters preventing efficient infection as reflected by the immunoblot in supplementary figure 1b. Thus, we used the BT474 cell line, which we stably transduced with EV, RhoA-WT and RhoA-G17E, combined with a transient knock-down of endogenous RhoA using a small hairpin (Supplementary Figure 1c).

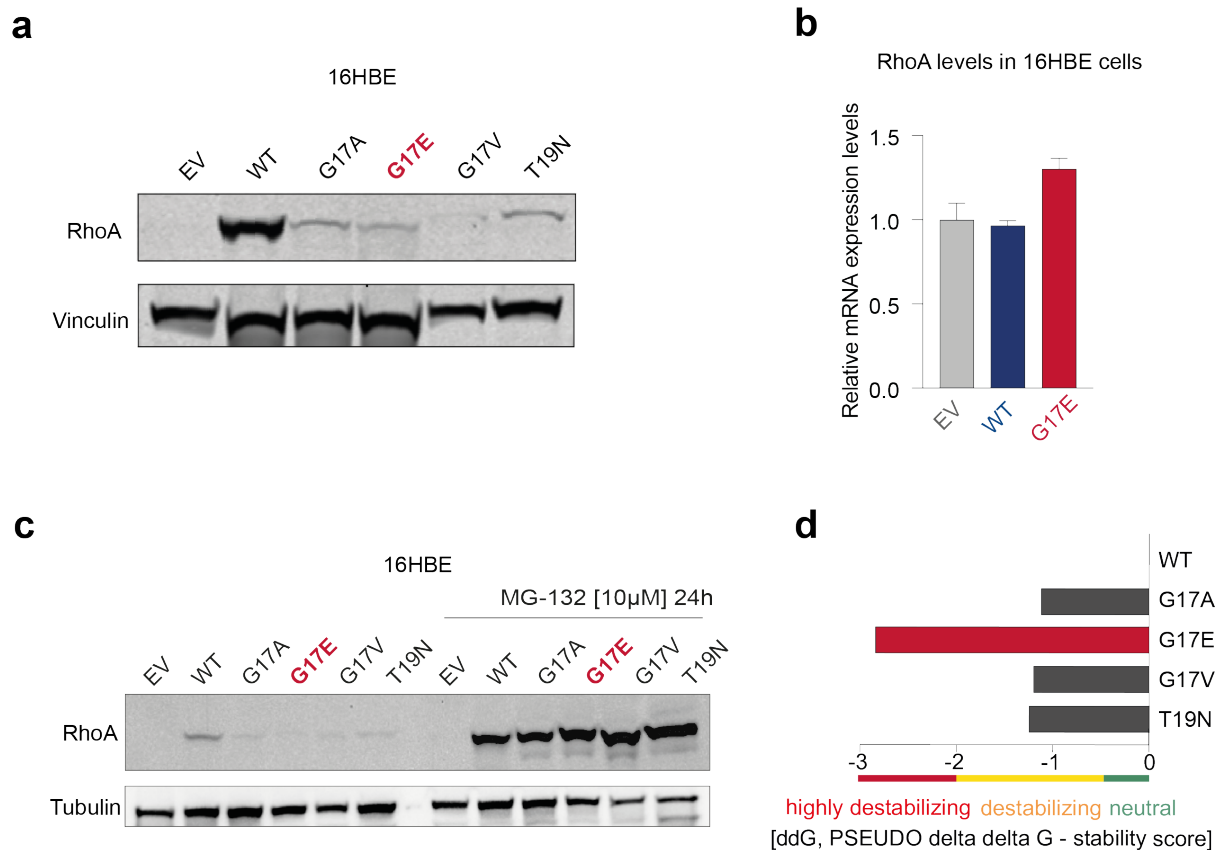


Figure 5| The RhoA-G17E-mutation has a highly destabilizing effect on the protein. a, Validation of overexpression of RhoA mutants in 16HBE by immunoblotting. **b,** Relative mRNA expression of RhoA in 16HBE stably overexpressing empty vector (EV), RhoA wildtype (WT), RhoA-G17E (G17E). qPCRs were performed in four replicates. **c,** 16HBE cells stably overexpressing RhoA mutants were treated with 10μM MG-132 for 24h. **d,** Protein stability of RhoA mutants assessed by Site Directed Mutator (SDM).

3.3 The G17E-mutation has a highly destabilizing effect on the protein stability of RhoA

To investigate phenotypic changes induced by the RhoA-G17E mutation we introduced the mutation by stable overexpression of a lentiviral vector or by Cas9-mediated knock-in in 16HBE cells as described in 3.3. Despite higher mRNA level of RhoA-G17E compared to RhoA-WT, immunoblotting revealed significantly lower protein levels of RhoA-G17E compared to the wildtype protein (Figure 5a, 5b). These contradictory results led us to explore whether the lower protein abundance of RhoA-G17E is due to reduction in translation or post-translational events, which affect protein stability. The regulation of translation initiation is highly dependent on the action of GTPases (e.g., eIF2) which are tightly controlled by GEFs (e.g., eIF2B). To address

whether dysregulation of translation results in lower protein levels, we first performed OPP-translation assays (Thermo Fisher), where we did not observe global differences in native protein translation between wildtype and RhoA-G17E mutated cell lines (data not shown), although this assay does not exclude that specifically RhoA translation is altered. This requires further analysis by SILAC and subsequent mass spectrometry which allows for quantitative analysis of protein synthesis. With the aim to investigate post-translational regulation of RhoA-G17E, we treated our 16HBE overexpression cell lines with 10 μ M of the proteasome inhibitor MG-132 for 24 hours and used immunoblotting as a read-out. Western blot (WB) analysis showed a strong increase of protein levels of RhoA-G17E by proteasome inhibitor treatment compared to untreated samples, whereas RhoA-WT levels were not as strongly affected by the proteasome inhibitor treatment (Figure 5c). To further explore the impact of RhoA-mutations on the stability we approached this question from a computational angle. Protein stability prediction tools, as Site Directed Mutator (SDM) or PoPMusic, were used to predict the stability of mutant proteins by assessing the $\Delta\Delta G$ (difference in free energy between native and mutated protein). Strikingly, we found that in comparison to other RhoA-mutations, the G17E has a highly destabilizing effect on the protein (Figure 1d). Despite, low protein levels of overexpressed RhoA-G17E and the endogenous wildtype protein still present in these cells, we observed severe morphological phenotypes upon closer investigations.

3.4 RhoA-G17E induces morphological perturbations of the cytoskeleton

RhoA is a major mediator of extracellular stimuli, which result in re-organization of the cytoskeleton, altered movement, and signaling in the cell, thus, we sought to investigate the impact of RhoA-G17E mutations on the cytoskeleton by immunocytochemistry. GTP-bound RhoA binds down-stream effector proteins, which then directly or indirectly affect filamentous actin assembly or disassembly ⁴⁰. Moreover, RhoA/ROCK signaling was shown to be involved in direct migration by affecting microtubule stability in various cell types ^{41 42}. To capture actin dynamics and tubulin stability, we again incorporated our transduced 16HBE epithelial cells as a model to perform immunofluorescence stainings. In general, all RhoA-mutants

exhibited a morphological perturbed cytoskeleton, represented by depolymerized actin (Figure 6 and Supplementary Figure 2). In particular, cells overexpressing RhoA-G17E lack an organized fibrillary actin network but rather form distorted granular structures (Figure 6). Immunofluorescent staining of α -tubulin was performed to visualize the cell morphology. While RhoA-WT and EV transduced cells maintained a round cell shape, forming connective clusters of cells, RhoA-mutants accumulated tubulin resulting in the formation of dendritic-like structures. Thereby, individual cells detached from the cell collective. Again, this phenotype was most prominent in the cells harboring RhoA-G17E (Figure 6).

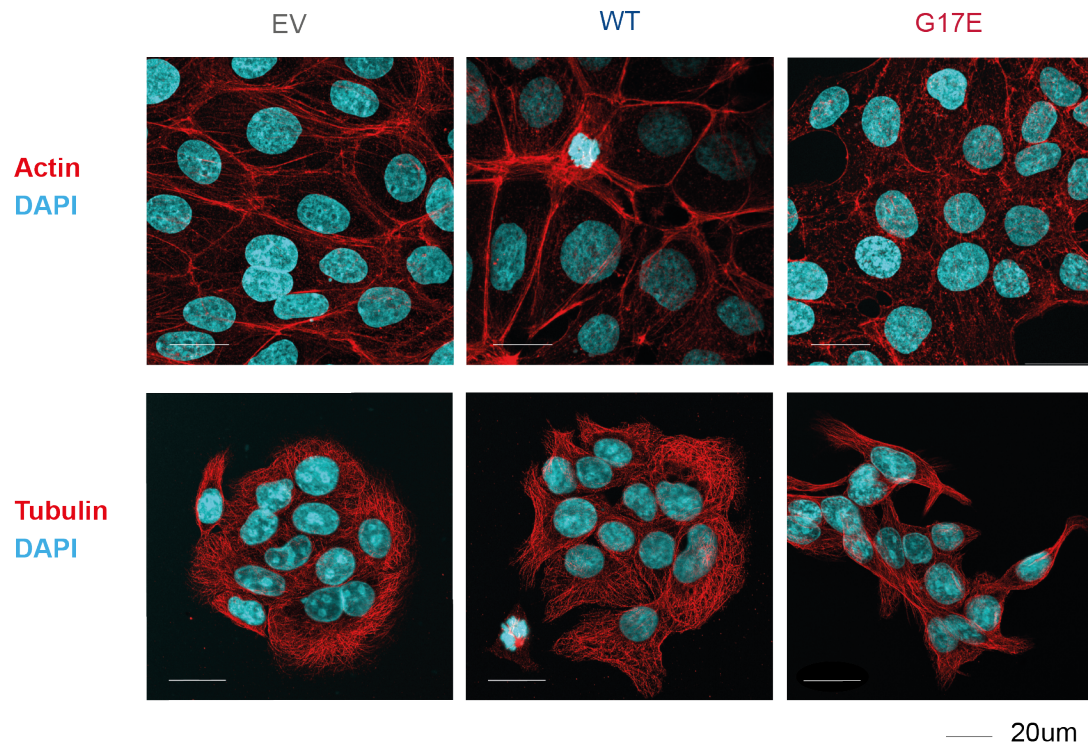


Figure 6| RhoA-G17E causes severe cytoskeletal perturbations. PFA-fixed 16HBE cells stably overexpressing EV, RhoA-WT and RhoA-G17E for actin (red) and tubulin (red): DAPI, 49,6-diamidino-2-phenylindole; scale bar 20nm

The formation of actin stress fibers has been reported to be tightly linked to plasma membrane focal adhesion. RhoA-inhibition was shown to reduce stress fiber assembly accompanied by loss of focal adhesion^{43 42}. As we observed severe actin depolymerization in our RhoA-G17E cell lines, we raised the question whether this has an impact on focal adhesion. We chose focal adhesion kinase (FAK) as a marker to investigate changes in focal adhesion as it is directly involved in the regulation of

RhoA-activity^{44 45}. Immunofluorescence staining of FAK revealed that RhoA-G17E leads to the loss of focal adhesion (Figure 7), recapitulating the phenotype observed upon RhoA-blockage. RhoA has been further implicated in to contribute to tight junction permeability by reorganizing the cytoskeleton. Thus, we investigated RhoA-G17E induced tight junction defects by immunofluorescence staining against occludin. In comparison to the RhoA-WT or EV transduced cells where we observed occludin localizing to the cell-cell borders, RhoA-G17E cells showed diminished occluding staining at the respective sites (Figure 7).

Our data suggest that RhoA-G17E causes severe morphological perturbations of the cytoskeleton, reflected by actin-depolymerization, reduced focal adhesions, and tight junction defects, which may affect essential cellular processes as cytokinesis or migration.

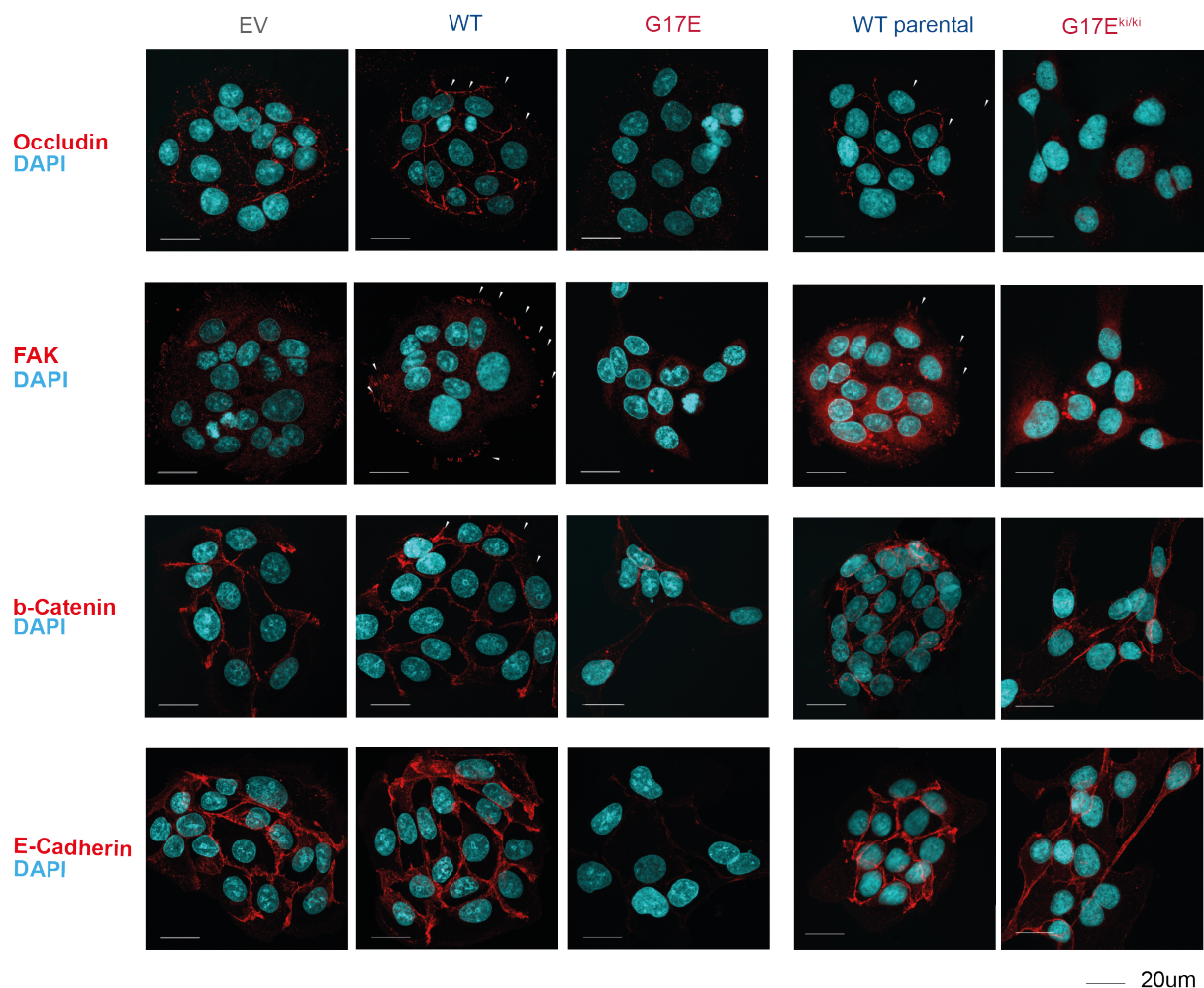


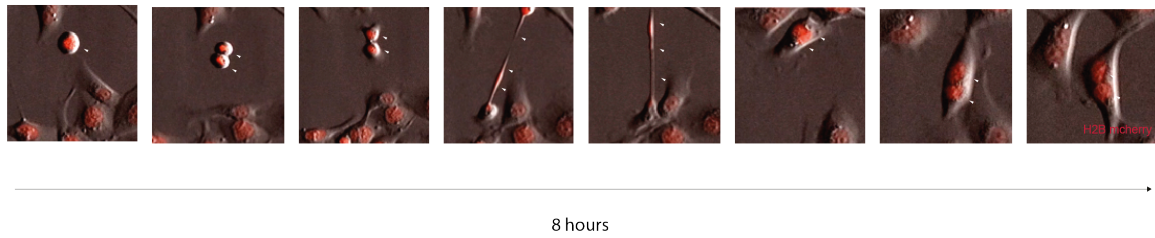
Figure 7| RhoA-G17E causes severe cytoskeletal perturbations. Immunofluorescent staining of PFA-fixed 16HBE cells stably overexpressing EV, RhoA-WT, RhoA-G17E and CRISPR-Cas9 engineered RhoA-G17E knock-in cell line and the respective WT-clone for occludin (red), FAK (red), b-Catenin (red) and E-Cadherin (red); DAPI; scale bar 20nm.

3.5 RhoA-G17E leads to cytokinesis defects and reduced proliferation rates

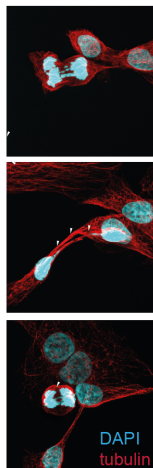
RhoA is involved in the re-organization of the cytoskeleton which is essential in cell division. Especially, during the last step of mitosis, cytokinesis, the accumulation of active RhoA at the midbody ring has been previously reported, elucidating RhoA's role in membrane abscission⁴⁶. To monitor cell behavior during mitosis, we performed time-lapse microscopy over 15 hours. Therefore, a clonal 16HBE CRISPR/Cas9 RhoA-G17E knock-in cell line was further transduced with a lentiviral vector to label the histone-protein H2B with mCherry which allowed us to visualize the nuclei. We followed a single cell undergoing mitosis (Figure 8a) using bright field and fluorescent microscopy (red=mCherry). Strikingly, we observed that the DNA-content was duplicated but the cells are challenged by not being able to complete membrane abscission, the last step of mitosis, leaving one cell with two nuclei. In addition, we performed immunofluorescent staining of 16HBE CRISPR/Cas9 RhoA-G17E knock-in against tubulin and used DAPI to for nuclear staining. Although, we did not synchronize the cells we observed that dividing cells were not able to complete successful chromosome segregation, represented by the formation of anaphase bridges. This adds further evidence to the obtained results from time-lapse experiments suggesting that the defects in chromosome segregation and cytokinesis lead to prolonged cell cycles. As a result, we expected lower proliferation rates of cells harboring RhoA-G17E. To test this hypothesis, we performed proliferation assays *in vitro* over 72 hours. Cells with a RhoA-G17E mutation showed a reduced proliferative capacity compared to RhoA-WT or EV (Figure 8b, 8c).

Our data indicated that cells harboring the RhoA-G17E mutation show increased chromosome miss-segregation which may be due to the destabilized actin and tubulin cytoskeleton (Figure 6). Ultimately, chromosome miss-segregation can lead to genome instability, a hallmark of cancer⁴⁷. Furthermore, we observed that these cells often failed to perform abscission during cell division which may result in aneuploidy and potentially causes the lower proliferation rates, recapitulating the phenotype of DTE and MAC, which have been characterized as slow-growing tumors.

a



b



c

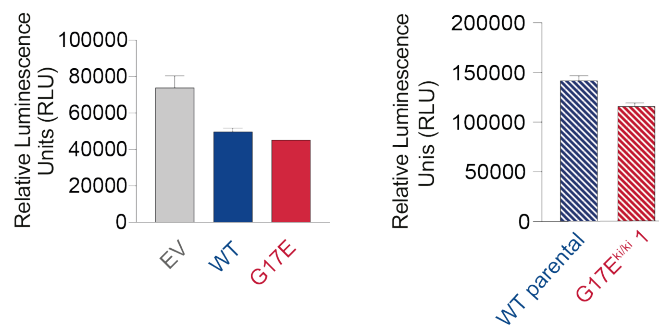


Figure 8| 16HBE cells harboring the RhoA-G17E mutation show cytokinesis defects and reduced proliferation rates *in vitro*. **a**, Time-lapse microscopy analysis of 16HBE CRISPR-Cas9 engineered RhoA-G17E knock-in cell line over 8 hours (red=H2BmCherry) **b**, Immunofluorescent staining of 16HBE CRISPR-Cas9 engineered RhoA-G17E knock-in, DAPI (blue) and tubulin (red). **c**, Proliferation Assay of 16HBE overexpressing empty vector (EV), RhoA wildtype (WT), RhoA-G17E (G17E) and CRISPR-Cas9 engineered RhoA-G17E knock-in cell line and the respective WT-clone. Viability was assessed by quantifying the present amount of ATP before and after 3 days of culture (n=4 replicates).

3.6 RhoA-G17E increases the direct migratory capacity but reduces collective movements *in vitro*

RhoA amongst other Rho family proteins influences cell migration and invasion by inducing dynamic re-organization of the cytoskeleton and changes in cell adhesion⁴⁸. Given that RhoA-G17E induces a loss of focal adhesion and strong actin depolymerization, our aim was to investigate the effects on migration. As RhoA is involved in diverse types of migration we were further interested to compare our cell lines with regard to their directed migratory capacity. Thus, we performed Boyden chamber assays to assess the invasiveness of our cell lines *in vitro*. Thereby, starved

cells are plated on a trans-well insert and allowed to migrate towards a chemokine rich (fetal bovine serum) environment. Prior to seeding the cells were stained with a green dye for detection. These assays revealed that RhoA-G17E shows an increased migratory capacity as we detected a significantly higher number of cells at the bottom of the insert in the nutrient-rich environment, where the migrated cells accumulate (Figure 9a). RhoA-WT cells lack this invasive phenotype and reside within the upper compartment of the chamber.

To further examine the changes in motility of the 16HBE RhoA-G17E cells we performed wound healing assays (Figure 9b). While RhoA-WT and EV transduced cells collectively migrated towards the induced scratch, cells harboring RhoA-G17E exhibited a highly distinctive migratory behavior (Figure 9c). Although the RhoA-G17E mutants migrated with an increased speed, they lost adhesion to the collective and individual cells moved aimlessly back and forth covering only short distances (Figure 9c). Hence, after ten hours EV and RhoA-WT transduced cells were capable of closing the gap with an efficiency of 45% and 35% respectively, whereas RhoA-G17E mutants yielded only 5% gap closure (Figure 9d). These data indicate that while epithelial cells harboring the RhoA-G17E mutation lose the capability to move as a collective, they increase their ability to migrate individually towards a chemo attractant.

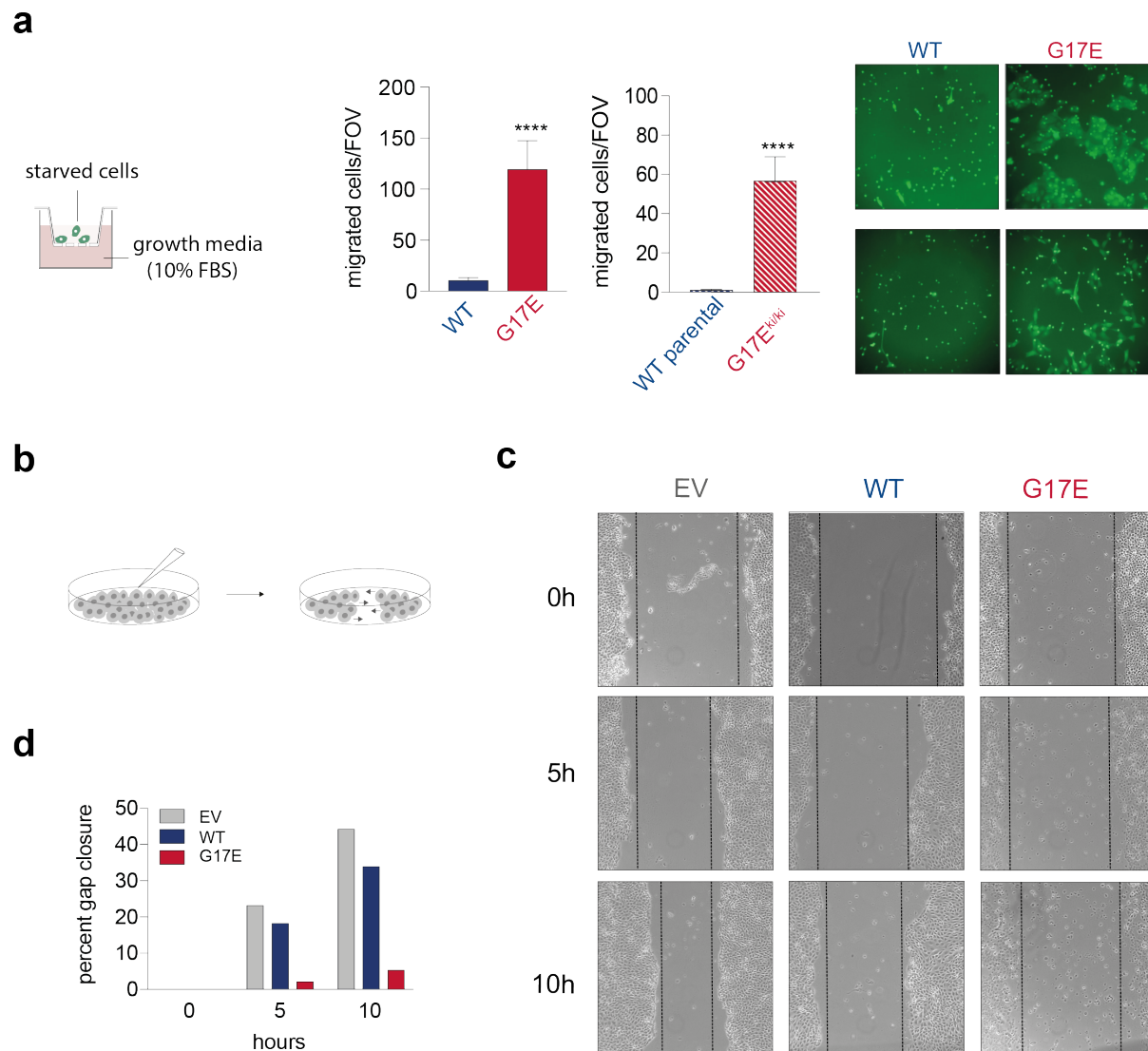


Figure 5| RhoA-G17E mutation enhances direct migration but reduces the potential to migrate in a collective. a, Schematic of the experimental set-up and number of starved, migrated cells towards full growth media. ($n = 10$ fields of vision (FOV)). **** $P < 0.0001$, two-tailed Mann-Whitney U test. **b,** Schematic of wound healing assay. A scratch was introduced after formation of a monolayer. Cells were allowed to migrate for 15h. **c,** Monolayer gap closure assay of 16HBE transduced with EV, RhoA-WT, RhoA-G17E. **d,** Images were taken at time point 0, after 5 and 10 hours using a brightfield microscope. **d,** Quantification of the gap closure assay.

3.7 RhoA-G17E confers cells with the ability to survive and proliferate upon low attachment

Most cells rely on continuous contact with the extracellular matrix (ECM) and undergo Anoikis, a type of apoptosis, when this interaction is lost. The ability to evade Anoikis is a hallmark of cancer and plays an important role during metastasis of cancer cells⁴⁹. Changes in the cytoskeleton and RhoA signaling contribute in sensing the loss of

cell-ECM interaction, thus we set out to investigate whether RhoA-G17E has an effect on the ability of cells to evade anoikis and grow on low-attachment plates. After 6 days of culture the luminescent signal was obtained which was proportional to the amount of ATP present. When normalizing the data to day 0 measurements, we obtained significantly more surviving cells in the G17E-mutant compared to the RhoA-WT or EV (Figures 10a). This was further recapitulated by conducting the experiment with our generated CRISPR/Cas9 endogenously mutated cell lines (Figures 10a).

Utilizing the BT474 breast cancer cell line which endogenously harbors the RhoA-G17E mutation, we performed rescue experiments where we knocked down the endogenous protein by transfecting the cells with a shRNA targeting the 3' UTR of RhoA. Prior to the knockdown, we transduced the cells with EV, RhoA-WT/G17E, which are not affected by the RhoA targeting shRNA. Thereafter, performed an Anoikis assay as described above. We showed that knockdown of endogenous RhoA in combination with EV-overexpression led to poor survival of the cells. This could be rescued by of the RhoA-WT overexpression (Figures 10b). As a consequence of RhoA-G17E overexpression the proliferation rates upon low attachment were significantly increased, suggesting that RhoA-G17E provides the cells with the ability to successfully evade Anoikis (Figures 10b).

Our data elucidates that the RhoA-G17E mutation allows the cells to proliferate upon low attachment, which further sheds light on the role of RhoA-G17E in tumorigenesis and invasion. To further capture the complexity of tumor initiation, the generation of suitable *in vivo* models is required.

a

6 days in low adhesion conditions

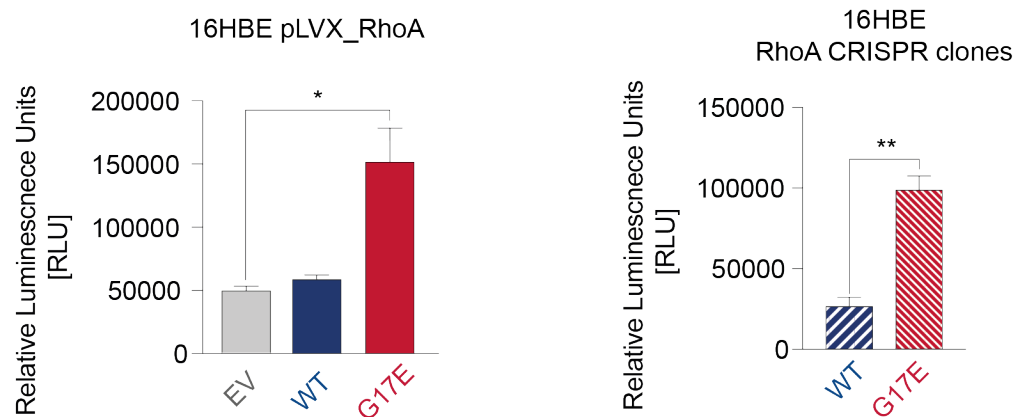
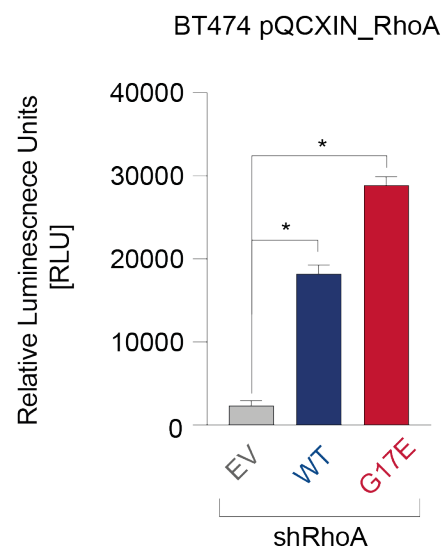
**b**

Figure 10| RhoA-G17E dependent evasion of anoikis. **a**, Anoikis assay showing living 16HBE cells transduced with EV, RhoA-WT and RhoA-G17E or endogenously mutated CRISPR clones after 6 days in low adhesion conditions. **b**, Anoikis assay showing living BT474 cells transduced with EV, RhoA-WT and RhoA-G17E and transiently expressing shRhoA to knock down endogenous levels of RhoA. RLU are proportional to amount of ATP present.

3.8 Investigation of the tumor-initiating capacity of RhoA-G17E *in vivo*

The high prevalence of the RhoA-G17E mutation in patient samples (81% in DTE and 65% in MAC) and the phenotypes we observed *in vitro* indicate that RhoA-G17E may contribute to tumor induction. It has been suggested that DTE originates from hair

follicle stem cells and MAC is derived from the sweat glands. Thus, we aimed at introducing this mutation into the undifferentiated cell population of the basal layer, which gives rise to upper layers of the skin epithelium.

Therefore, targeting of this layer directly posed a challenge as it is difficult to access. The laboratory of Elaine Fuchs optimized a technique which allows for stable integration of lentiviruses into the skin of mouse embryos⁵⁰. We designed and cloned RhoA-G17E or RhoA-WT coupled to GFP via a P2A peptide into a lentiviral vector represented in Figure 11b. To yield high lentiviral concentrations, I ultra-centrifuged the generated virus and used ultrasound-guided injections to introduce the virus into the amniotic cavity at E9.5.

This method allows for stable integration and propagation of the RhoA-G17E mutation. Therefore, I was required to establish this complex technique in our lab and make it suitable for our purposes. Thus, I first injected lentiviral constructs expressing GFP or mCherry under the control of a strong promoter (Figure 11a). Injection of the embryos at E9.5 allowed us to obtain GFP- and mCherry-positive cells as indicated in Figure. In contrast, we were not able to detect a fluorescent signal from embryos injected at E10.5 (data not shown), which is in line with a previously published protocol⁵⁰.

After successfully establishing this technique and having the reagents prepared we are aiming to inject animals with the RhoA-G17E and RhoA-WT lentivirus. We will harvest the skin at E15.5 or postnatally and perform immunofluorescence stainings to probe for successful integration and investigate morphological changes induced by the mutation. The pups will be monitored postnatally for tumor induction. Furthermore, we will subject the injected mice to dimethylbenz[a]anthracene (DMBA)-initiated and a 12-O-tetradecanoylphorbol-13-acetate (TPA) which promotes two-stage skin carcinogenesis. In this assay, we will assess whether the skin of RhoA-G17E transduced mice is more prone to tumor formation. These experiments are currently ongoing.

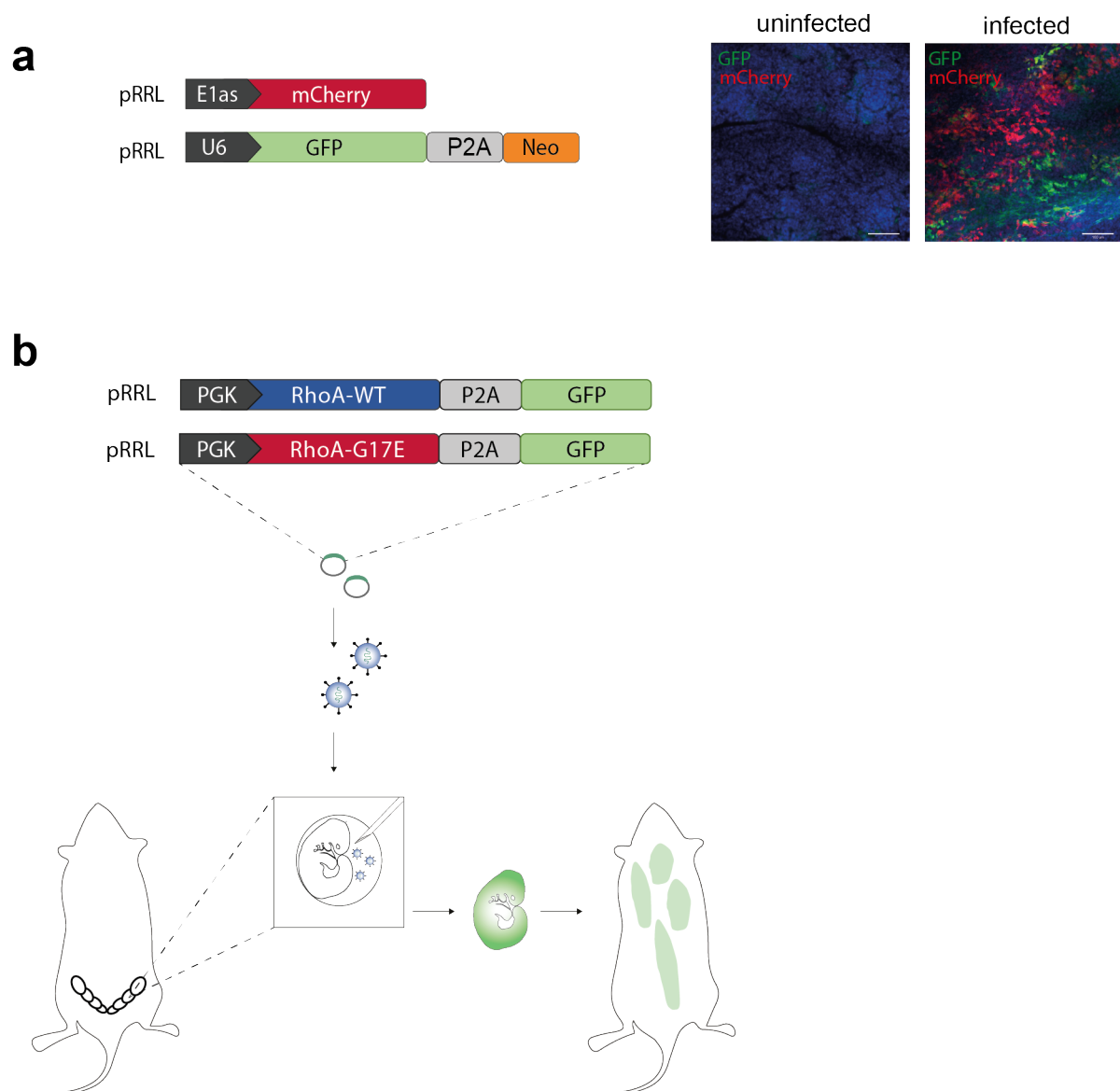


Figure 11| Investigation of the tumorigenic potential of RhoA-G17E. a, Lentiviral constructs used for initial test experiments and immunofluorescence staining of mouse embryonic skin sections analyzed at E15.5, stained for GFP (green) and mCherry (red), showing infected cells relative to non-infected control. Embryos were injected intra-amniotic on E9.5 with pooled LV-GFP/mCherry. **b,** Graphical summary of tumor initiation experiment using in utero injections.

3.9 RhoA-G17E is inactive and has a dominant negative effect on the WT-protein

Understanding the effects of RhoA-G17E on cellular signaling is key to elucidate its function in tumorigenesis. RhoA alternates between the inactive (GDP-bound) and active (GTP-bound) state. The amount of RhoA-GTP in a cell can be detected by an antibody and quantified using an ELISA assay. Using this assay, we investigated

whether the G17E mutation impacts the activation ability of RhoA in serum-starved 16HBE cells, that were treated with Calpeptin. Calpeptin indirectly activates RhoA by inhibition of SHP-2 tyrosine phosphatase which dephosphorylates RhoGEFs preventing their activation. Thus, inhibition of SHP-2 results in increased GEF-phosphorylation activating them to catalyze the GDP/GTP exchange. Another substrate of SHP-2 is p190GAP, a negative regulator of RhoA-activity. Calpeptin induced inhibition of SHP-2-mediated dephosphorylation of p190RhoGAP represents an additional manner to trap RhoA in its GTP bound form (Cytoskeleton - refer to URL). After 30 minutes, we extracted the proteins and used the ELISA-based assay to detect GTP-bound RhoA with an HRP-antibody (Figure 12a). We performed this assay in our overexpression cell lines where RhoA wildtype was expressed endogenously, in addition to the overexpressed RhoA-WT/G17E, or EV control, thus, we expected to see changes in GTP-bound RhoA upon stimulation with Calpeptin. In EV control cells we detected a small but consistent 1.2-fold difference in GTP-bound RhoA levels upon stimulation (Figure 12b). These levels could be further increased by overexpression of RhoA-WT (1.4-fold change from baseline). Interestingly, in the RhoA-G17E transduced cell line, we did not observe any increase in GTP-bound RhoA levels upon stimulation. This not only shows that RhoA-G17E fails to bind GTP upon Calpeptin treatment, but also indicates that it prevents the endogenous wildtype protein from activation (Figure 12b). Our data suggest that RhoA-G17E exerts its effects in a dominant negative manner. In addition, we repeated this assay using lysophosphatidic acid (LPA) to induce GTP-binding. LPA is a non-selective activator as it also activates other proteins (e.g., PI3K, Ras). By binding to growth factor receptors (e.g., EGF), signal transduction via G-coupled protein receptors leads to the activation of GEFs. LPA-induced GTP-binding of RhoA is rapid, thus, we incubated serum-starved cells for only 5 minutes with $1\mu\text{g mL}^{-1}$ LPA. We could recapitulate the results from the Calpeptin-induced assay showing that RhoA-G17E acts as a dominant negative mutant (Supplementary Figure 3).

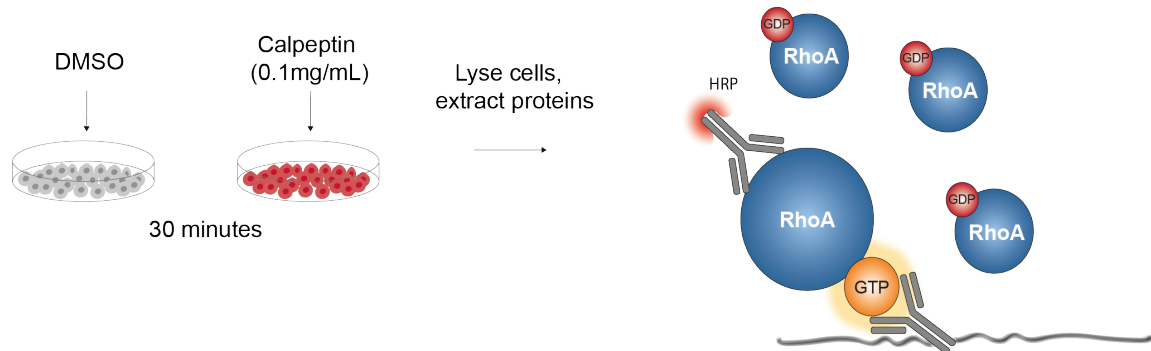
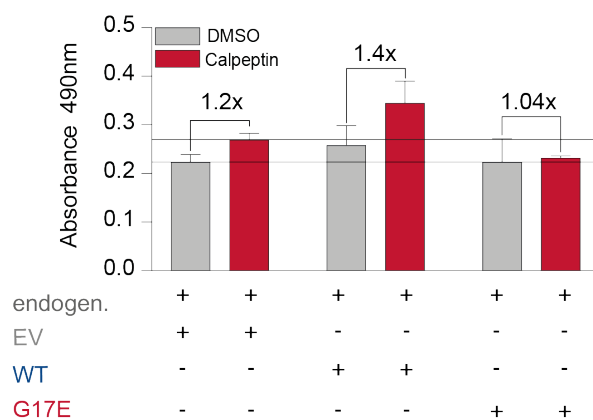
a**b**

Figure 12| Overexpressed RhoA-G17E fails to be activated and prevents activation of the endogenous protein. a, Schematic overview of ELISA-based RhoA-activation assay. Cells were stimulated with Calpeptin over 30 minutes followed by lysis and HRP-antibody based detection of GTP-bound RhoA. **b,** Levels of RhoA-activation in 16HBE cell lines stably overexpressing EV, RhoA-WT and RhoA-G17E. After a 30-minutes exposure to Calpeptin (0.1 mg mL⁻¹) or DMSO, the amount of GTP-bound RhoA was determined by obtaining the absorbance at 490nm.

3.10 RhoA-G17E preferentially binds guanine nucleotide exchange factors

The cycling between RhoA GTP- and GDP-bound states involves multiple protein interactions. We sought to investigate the RhoA-G17E interactome using co-immunoprecipitation coupled to mass spectrometry, to further study underlying molecular mechanisms of the dominant-negative action of RhoA-G17E. Glycine/glutamic acid 17 resides within a GTP-GDP binding site, thus we asked whether this amino acid exchange influences interactions with other proteins. To fully capture these interactions, we performed immunoprecipitation of HA-tagged and GFP-

tagged (data not shown) RhoA mutants from 16HBE cells (Figure 13a). Subsequently, co-immunoprecipitated proteins were analyzed by mass spectrometry (Figure 13a). A total of 3833 proteins were detected and quantified. With a FDR of 0.05 and a FC cut-off of 10 we identified a variety of GEFs as differentially and strongly bound by RhoA-G17E, compared to RhoA-WT (Figure 13b). Among the analyzed mutants (G17A/E/V) we obtained a redundant assembly of GEFs, including ARHGEF12 (LARG) (Figure 13c, 13d, Supplementary Figure 4). This data suggests that RhoA-G17E exerts its dominant negative effect by sequestration of GEFs, thereby preventing the wildtype protein from being activated. A similar mechanism has been described for RhoA-G17A and -G17V^{51 32}.

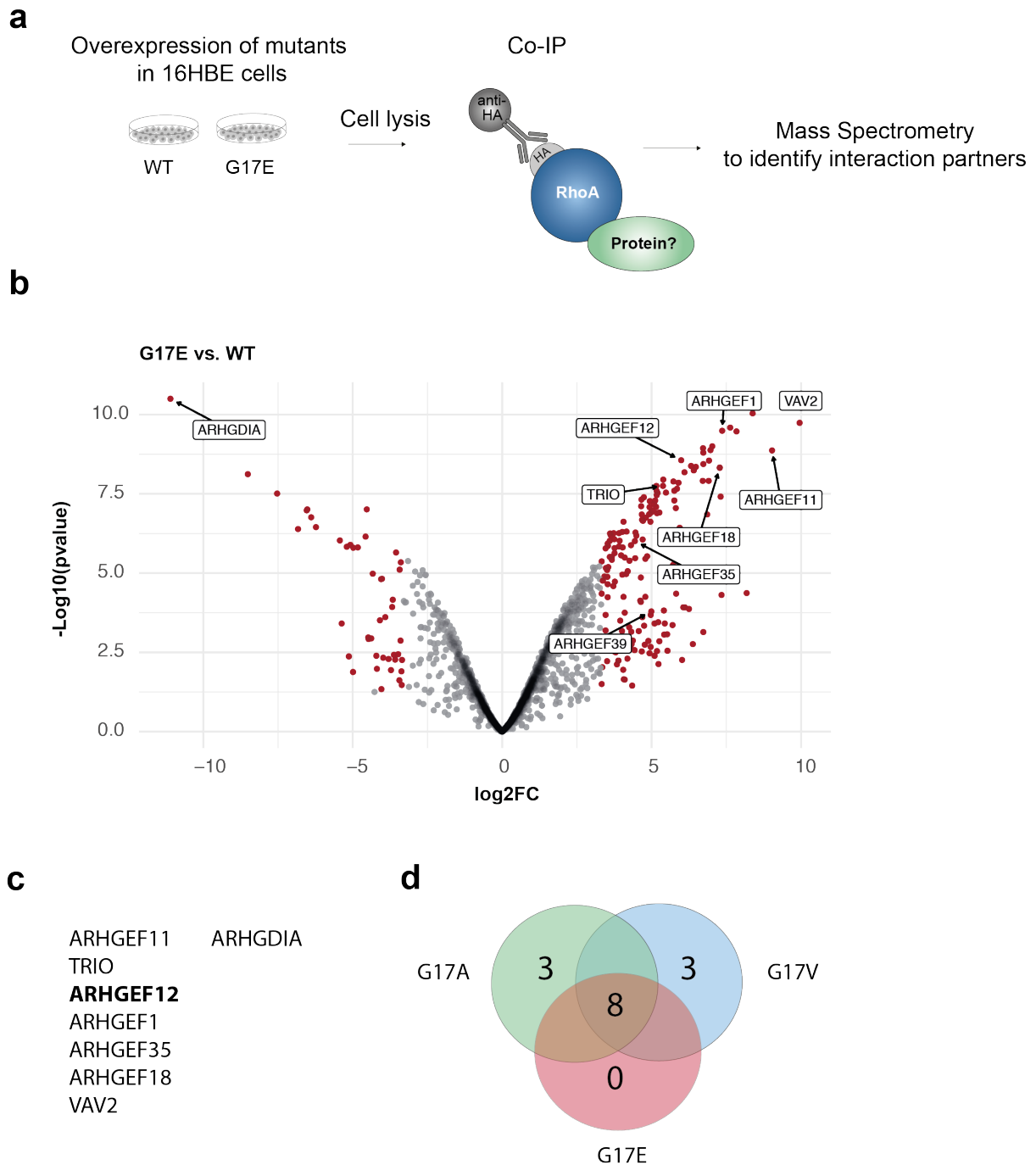


Figure 13| RhoA-G17E preferentially binds Guanine Nucleotide exchange factors (GEFs). **a**, Schematic overview showing immunoprecipitation of overexpressed HA-tagged RhoA-mutants using HA-antibody-coupled magnetic beads. **b**, Volcano blot representing differential bound proteins of RhoA-WT versus RhoA-G17E. GEFs are indicated by arrows (cut-offs FC=10, FDR = 0.05) red dots indicate significant hits. **c**, **d**, Overview of differentially bound GEFs and by RhoA-G17A, RhoA-G17E, RhoA-G17V vs. RhoA-WT and differentially bound GDIs by WT vs RhoA-G17E/V/A (cut-offs FoldChange=10, FDR = 0.05).

3.11 RhoA-G17E but not RhoA-WT strongly binds Arhgef12 (LARG)

By mass spectrometry we identified a number of GEFs strongly bound to RhoA-G17E but not to RhoA-WT, LARG (Arhgef12) was among our top hits. Thus, we further validated the interaction between LARG and RhoA-G17E by immunoprecipitation. We co-overexpressed v5-tagged LARG with RhoA-WT and RhoA-G17E in HEK293T cells and performed two independent experiments in which we precipitated v5-LARG or HA-RhoA, respectively (Figure 14a). Western blot analysis confirmed the strong interaction of LARG with RhoA-G17E, which was much lower with RhoA-WT (Figure 14b). Despite, lower protein levels of RhoA-G17E compared to RhoA-WT, we observed decreased levels of LARG when co-overexpressed with RhoA-G17E, compared to co-overexpression with RhoA-WT (Figure 14b). These results led us to hypothesize that not only RhoA-G17E may be a highly instable protein, but also affects protein levels of associated proteins. However, further studies are warranted to provide insights into the nature and mechanism underlying this observation.

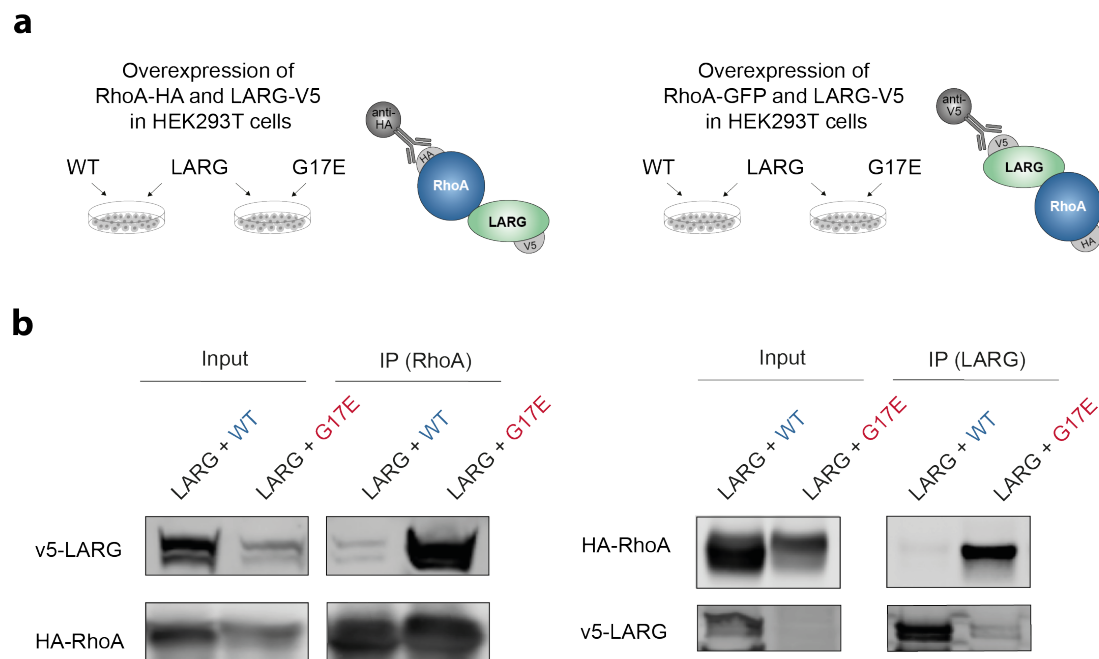


Figure 14| RhoA-G17E preferentially binds Guanine Nucleotide exchange factors (GEFs). **a**, Schematic overview of immunoprecipitation experiments: v5-LARG was co-overexpressed with RhoA-WT and RhoA-G17E and subsequent IP of either tagged RhoA or LARG. **b**, Validation of the interaction between RhoA-G17E and LARG (Arhgef12).

3.12 The tumor suppressor CYLD mediates RhoA-activity via LARG

Next, we set out to investigate whether there is a molecular link between RhoA mutations, which we identified in samples from DTE and MAC, and CYLD mutations, found in Brooke Spiegler syndrome, which predispose patients to skin appendage tumors as trichoepithelioma, highly related to DTE ⁵². CYLD is a deubiquitinase and thus interacts with an abundance of proteins in the cell. The CYLD-interactome has been previously investigated by mass spectrometry ⁵³. By intersecting the CYLD and RhoA-G17E interactomes, we identified one common hit: LARG (Figure 15a). LARG is a RhoA-GEF that has previously been implicated in the development of acute myeloid leukemia by generating a fusion protein with MLL (mixed lineage leukemia) ⁵⁴. Therefore, we sought to further investigate the link between CYLD, LARG, and RhoA. The generation of stable CYLD knock-down and knock-out (CYLD-KO) 16HBE cell lines (clonal) (Supplementary Figure 5a, 5b, 6a) and subsequent investigation of their effects on proliferation, anoikis and migration should allow us to assess whether CYLD-loss and our RhoA-G17E mutation share functional similarities, however CYLD-KO clones displayed varying phenotypes (Supplementary Figures 6b-e). We did not observe strong effects on direct migration or the evasion of anoikis (Supplementary Figures 5c, 6b, 6e) upon CYLD-loss. Nevertheless, we could show that the depletion of CYLD affects RhoA-activity (Figures 15b, 15c). By co-overexpressing RhoA-WT with a small hairpin against CYLD we revealed decreased levels of RhoA-GTP upon Calpeptin treatment compared to control samples lacking shCYLD overexpression (Figure 15b). We could recapitulate these results by performing the RhoA-Activation assay in CRISPR-Cas9 generated CYLD-KO cell lines (Figure 15c). This may support previous findings on the connection between CYLD, LARG and RhoA but may also provide new insights into the molecular mechanisms associated with CYLD-loss in the Brooke-Spiegler Syndrome.

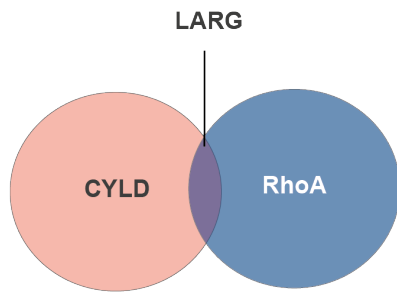
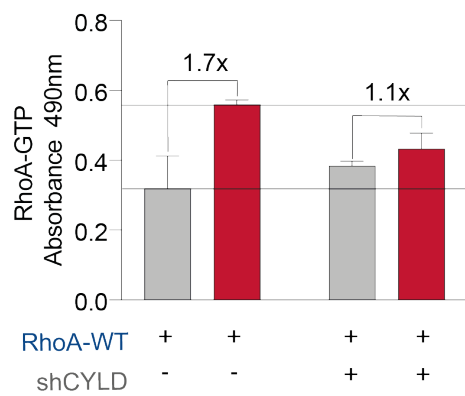
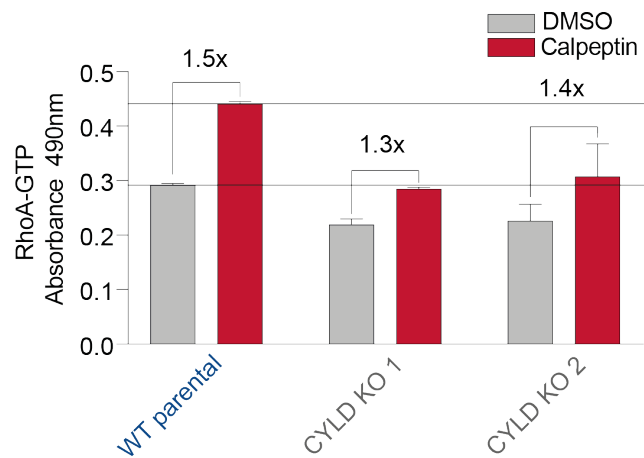
a**b****c**

Figure 15| CYLD loss results in reduced RhoA-activity. **a**, Schematic of CYLD and RhoA interactomes. **b**, Levels of RhoA-activation in 16HBE transiently overexpressing RhoA-WT + shCYLD or RhoA-WT + EV. **c**, Levels of RhoA -activation in 16HBE CYLD-KO clones relative to the parental WT cell line. After a 30-minutes exposure to Calpeptin (0.1mg/mL) or DMSO the amount of GTP-bound RhoA was determined by obtaining the absorbance at 490nm (b,c).

4. Discussion and Outlook

MAC and DTE are two types of rare non-melanoma skin cancer for which genetic aberrations are largely unknown. Targeted sequencing on a selection of high-quality patient samples allowed us to identify a mutation in the RhoA-gene, leading to an amino acid exchange from glycine to glutamic acid at position 17. The small GTPase RhoA is a regulator involved in a multitude of cellular processes such as cell division, migration or transcription. Therefore, the aim of this thesis was to identify the role of RhoA-G17E as a driver of tumorigenesis by investigating mutation-associated changes on a phenotypic and a molecular level. This required the establishment of cell culture-derived model systems using lenti- and retroviral overexpression and CRISPR/Cas9 in a human epithelial cell line (16HBE) (Supplementary Figure 1a).

To mimic the conditions of the heterozygous RhoA-G17E mutation in MAC and DTE patients, we transduced 16HBE cell lines with lenti- or retroviral vectors harboring G17E-mutant fused to GFP or hemagglutinin, which allowed for overexpression of the mutant in a wildtype background. In addition, we overexpressed RhoA-G17A and RhoA-G17V which have been described in association with other cancer types (Figure 4) and T19N, a dominant negative mutant used for functional studies^{55 56}. Transduction with RhoA-WT and the empty vector (EV) served as controls. We confirmed successful overexpression by western blot (Figure 5a, Supplementary Figure 1b, 1c). However, overexpression of RhoA-mutants in 16HBE cell lines consistently resulted in low protein levels. We also observed this phenotype when transducing other cell lines such as MDA-MB-231, BT474 or SW948 (Figure 5a, Supplementary Figure 1b, 1c). To rule out that a decrease in translation caused this phenotype we performed OPP-assays, which allow for fast detection of protein synthesis. These assays indicated no significant changes in global translation (data not shown), although this does not exclude that translation of RhoA itself is affected. By treating these cell lines with the proteasome inhibitor MG-132, we were able to restore protein concentrations up to WT levels (Figure 5c), suggesting that these mutants are highly unstable. The destabilizing effect of RhoA-G17E was further supported by computational analysis predicting the stability of proteins. By assessing

the differences in free energy between native and mutated protein ($\Delta\Delta G$), we identified RhoA-G17E as highly instable (Figure 5d) compared to the other RhoA-mutants.

To avoid overexpression biases, we engineered the 16HBE cells line for endogenous expression of RhoA-G17E using CRISPR/Cas9. We identified two clones homozygous for the RhoA-G17E mutation (Supplementary Figure 1e). Nevertheless, the homozygous clones may not exclusively represent the conditions in the DTE and MAC skin cancer patients, where the RhoA-G17E mutation is only present in one allele.

The establishment of these models allowed us to perform functional assays to investigate effects on migration, cell division or survival.

RhoA is a major regulator of cytoskeleton organization by modulating actin and microtubule stability ³⁸. We used the engineered 16HBE cell lines to perform immunofluorescence stainings of cytoskeletal components and proteins maintaining cell-cell contacts. This cell line has been previously used to study RhoA in association with epithelial apical junction formation. Thus, it represents a suitable model to investigate the effects of RhoA-G17E on the cytoskeleton. Over- or endogenous expression of RhoA-G17E showed severe morphological perturbations of the cytoskeleton. This was reflected by the depolymerization of actin and distorted tubulin networks (Figure 6). Although, we also observed these effects in RhoA-G17A, -G17V and -T19N they were more pronounced in G17E mutants (Figure 6, Supplementary Figure 2). Disturbed actin polymerization was an expected phenotype, as RhoA has been implicated as a key mediator of cytoskeleton rearrangements in response to extracellular signals ⁴⁰. Upon GTP-binding RhoA interacts with downstream effectors, such as ROCK which phosphorylates LIM-Kinase (LIMK), which phosphorylates and thereby inhibits cofilins, a family of actin-binding proteins, which sever actin filaments leading to their depolymerization. Thus, phosphorylation of cofilin results in stabilization of actin filaments ⁵⁷. Upon interaction with RhoA-GTP, ROCK further phosphorylates myosin light chain (MLC) causing the contraction of actin fibers ⁵⁸. RhoA-GTP also interacts with the effector mDia, a member of the formin family which leads to acceleration of actin nucleation, stabilization of microtubules and was shown to locate to the mitotic spindle and midbody ⁵⁹. Thus, the phenotypes we observed in RhoA-G17E expressing cells are indicative for inactive ROCK and mDia-signalling which in turn suggests that the RhoA-G17E mutation does not just render the protein

inactive, but also affects the wild-type RhoA protein in the cell, by acting in a dominant negative manner, which we could confirm by RhoA-activation assays (Figure 12b). Early studies on RhoA already demonstrated that the active GTPase not only increases actin polymerization and integrin clustering but also promotes coordinated assembly of focal adhesion complexes in response to growth factors ⁶⁰. Focal adhesion kinase (FAK) was shown to phosphorylate p190RhoGEF activating it and catalyzing the switch from RhoA-GDP to RhoA-GTP ⁶¹. Inversely, RhoA-activity correlated with FAK turnover ⁴⁵. Immunofluorescence stainings of FAK in RhoA-G17E cells display highly diminished levels of the protein compared to wildtype. This reduction was also recapitulated in the other mutants (G17A/V and T19N) (Supplementary Figure 2), yet less prominently than in RhoA-G17E. In addition, we observed a vast decrease of e-cadherin, β -catenin (data not shown) and occludin at the cell borders, suggesting diminished cell-cell-contacts in the G17E-mutants (Figure 6). Epithelial cells, such as 16HBEs exhibit a cohesive growth pattern, relying on attachment to each other and to the extracellular matrix. In line with the reduction in FAK and occluding, we observed that our RhoA-G17E-16HBE cells were capable of abandoning the collective suggesting that they might have an altered migratory capacity.

Cell migration is an essential process during development, immune surveillance and wound healing. The spatiotemporal regulation of Rho-GTPases is required to assure coordinated cell movements ⁶². Thus, dysregulation of Rho-GTPases is associated with cell migration and metastasis. Having demonstrated that cells harboring the RhoA-G17E mutation not only exhibit severe effects on actin and microtubule stability but also on the tight- and adherence junction formation, we sought to investigate whether these phenotypes correlated with a change in motility. Moreover, studies in cervical cancer cells elucidated that RhoA-overexpression is associated with increased migration *in vitro* reflected by the enhanced ability of the cells to perform gap-closure ⁶³. Previously, our lab showed that overexpression of RhoA-G17E enhances the migration speed of 16HBE cells (data not shown). Epithelial cells are characterized by their distinct collective migratory behavior. While cells transduced with EV and RhoA-WT migrated in a collective cell cluster as depicted in the gap closure assays, cells harboring the RhoA-G17E mutation exhibited a decreased capacity to perform wound closure (Figure 9c). In contrast, the loss of cell-cell-contacts

in cells harboring RhoA-G17E contributed to enhanced migration towards a nutrient-rich environment, as displayed in the results of our Boyden chamber assays (Figure 9a). Direct migration is an important feature of cancer cells which allows them to invade the vasculature and extravasate into distant organs ⁶⁴. These data provide evidence that RhoA-G17E alters the migratory capacity of epithelial cells contributing to an invasive phenotype, which further recapitulates the locally invasive character of microcystic adnexal carcinoma (MAC). To add further confidence to this hypothesis, we are conducting metastasis studies using *in vivo* mouse models.

Increased proliferation is a major hallmark of cancer ⁶⁵. RhoA has been implicated in the process of cell division by localizing to the cleavage furrow during cytokinesis, the final stage of mitosis. Live imaging of 16HBE cells harboring the RhoA-G17E mutation revealed that these cells often failed to perform complete abscission, leading to the formation of binucleated cells and slower proliferation rates due to the dividing cells remaining in an unresolved state (Figure 8a). FACS-analysis is warranted in order to quantitatively assess the amount of DNA and thus polyploidy, by DNA-staining.

Immunofluorescence staining of our CRISPR-Cas9 engineered epithelial cell line further revealed the increased occurrence of anaphase bridges indicative for lagging chromosomes (Fig 8b) in RhoA-G17E mutant lines. Since RhoA is a major regulator of the actin-cytoskeleton and promotes microtubule stabilization, RhoA downstream-signaling is critical during mitosis. Thus, our data indicate that cells harboring the RhoA-G17E mutation exhibit difficulties to perform chromosome segregation, potentially caused by microtubule instability and depolymerized actin fibers (Figure 6). Miss-segregation of chromosomes may result in genome instability, a hallmark of cancer. To provide further evidence to this data, the synchronization of our cell lines in mitosis is required. Since the synchronization of 16HBE cells has been challenging (data not shown), for further analysis we could use HeLa cells (cervical cancer), a well-established model to study the cell cycle ⁶⁶. Finally, performing metaphase spreads and karyotyping analysis is currently performed and will provide insights into numerical or structural chromosomal aberrations.

The evasion of anchorage dependent cell death (anoikis), represents a hallmark of cancer and is associated with metastasis. A previous study with a colon cancer cell line demonstrated that the RhoA-G17E mutation provided the cells with the ability to

survive in low-attachment conditions, whereas overexpression of the wildtype failed to accomplish this, although the paper attributed this to a general growth phenotype, rather than to the ability to evade anoikis³⁴. We recapitulated the results of this paper using another model, the BT474 breast cancer cell line, which endogenously harbors the RhoA-G17E mutation (Figure 10b). Moreover, we performed *in vitro* anoikis assays with our epithelial cell lines where we could demonstrate that the G17E-mutation confers the ability of the cells to survive and proliferate without proper attachment, whereas the WT and EV-overexpressing cells undergo anoikis (Figure 10a). These data demonstrated that although 16HBE is not a fully transformed cancer cell line, the introduction of the RhoA-G17E mutations provided these cells with cancer-related properties. Thus, the investigation of the tumor-initiating capacity of this mutation *in vivo* is required.

For cancer research the generation of genetically engineered mouse models (GEMMs) has been a significant advancement, enabling us to study the role of a mutation in a particular cell type during tumorigenesis. In order to establish a skin-restricted RhoA-G17E GEMM, an inducible (TetO-rtTA) epithelial specific Cre-LoxP system would have been required to ensure controlled onset of RhoA-G17E-expression in the skin epithelium. The establishment of a GEMM is a laborious procedure, thus we exploited accessibility of the basal layer of the mouse skin during embryogenesis to directly introduce our mutation into proliferating epithelial progenitors. To establish the technique, I performed ultrasound-guided injections of lentivirus expressing GFP or mCherry into the amniotic cavity of a pregnant mouse at E9.5, which has been reported to be the ideal time point to yield efficient viral integration into the embryo's skin⁵⁰. By harvesting the embryos skin at E15.5 and immunofluorescence stainings we confirmed successful viral integration (Figure 11a). Experiments where we inject the generated RhoA-WT/G17E constructs are currently ongoing.

Dissection of the molecular mechanism underlying the RhoA-G17E mutation is essential to understand its role in tumorigenesis. We showed that the introduction of the mutation caused severe morphological perturbations on the cellular level, thus our aim was to investigate the functionality of this mutant *in vitro*.

Previous investigations of RhoA-G17 hotspot mutations have elucidated that they exert a dominant negative effect. To examine the activation status of the RhoA-G17E mutant we used our overexpression system in 16HBE cells to perform ELISA-based RhoA-activation assays (G-LISA). The RhoA-activator Calpeptin indirectly activates the GTPase via inhibition of the SHP-2 tyrosine phosphatase which in turn inhibits GEFs by dephosphorylation. Calpeptin treatment allows for higher levels of phosphorylated, active GEFs, thus activation of RhoA. To ensure equally low RhoA basal levels the cells were starved prior to Calpeptin treatment (Figure 12a). The assay performed elucidated that in cells overexpressing the EV we obtained a small but consistent increase in RhoA-GTP- levels upon stimulation. In RhoA-WT transduced cells the difference in GTP-bound RhoA between untreated and treated samples was larger due to the presence of higher RhoA-WT levels in addition to endogenous RhoA expression. Strikingly, our RhoA-G17E mutant appeared to have a reduced capacity to bind GTP upon stimulation (Figure 12b). We further showed that overexpression of the mutant prevented the endogenous WT protein from its activation as we failed to detect an increase of RhoA-GTP by Calpeptin-treatment (Figure 12b). As a result, we suggest that RhoA-G17E exerts its function in a dominant negative manner. To add evidence to this hypothesis we performed this type of assay using a different RhoA-activator, namely lysophosphatidic acid (LPA). The non-selective ligand binds to cell surface receptors and activates GEFs via G-coupled protein receptor signaling. As a consequence, we were able to recapitulate the results from the Calpeptin-assay, elucidating the dominant negative effect of RhoA-G17E (Supplementary Figure 3). RhoA-activation assays using Rhotekin-beads to immunoprecipitate GTP-bound RhoA allow for a better quantitative readout as the total RhoA-protein levels can be assessed by immune blotting. The optimization of a suitable protocol is currently ongoing.

Functional and proteomic analysis on dominant-negative RhoA mutants revealed their high affinity towards GEFs. To identify putative differential binding of RhoA-G17E in comparison to RhoA-WT we performed immunoprecipitation and subsequently mass spectrometry. Our data indicated that RhoA-G17E exhibited increased GEF-binding compared to its wildtype counterpart. In addition to RhoA-G17E, we included RhoA-G17A and G17V in our proteomic analysis (Supplementary Figure 4) and identified a number of recurrent GEFs favored to be bound by RhoA-mutants. With this substantial body of data generated, we propose now the following model: RhoA-G17E exerts its

dominant-negative function by sequestering a GEFs within the cell, thereby preventing the wildtype protein from becoming activated (Figure 16).

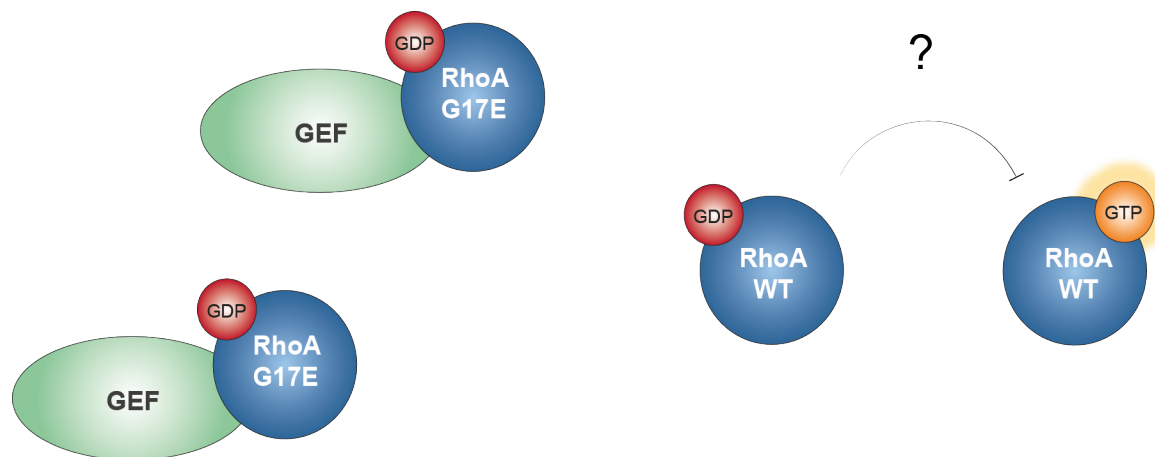


Figure 16| Working model

To further assess the role of RhoA-G17E in tumorigenesis we drew our attention to the group of tumors, where we had identified this mutation with high prevalence: MAC and DTE patient samples. Interestingly, trichoepithelioma, which is histologically highly similar to MAC and DTE is associated with mutations in the tumor suppressor CYLD. Thus, we aimed at investigating a potential link between CYLD and RhoA by examining their interaction partners. The identification CYLD-interaction partners, in addition to members of NF- κ B-signaling, by mass spectrometry provided novel mechanistic insights into the tumor suppressive role of CYLD. Previous studies indicated that it binds cell cycle regulators such as Polo-like kinase 1(Plk1) thereby modulating entry into mitosis and cytokinesis, elucidating its NF- κ B-signaling-independent function ⁵³.

Although RhoA and CYLD appeared to not interact with each other directly, proteomic analysis of the CYLD-interactome revealed that it binds Leukemia-associated RhoGEF (LARG), which was a recurrent binding partner of RhoA-G17E in our IP-MS experiments ⁵³ (Figure 15a). We successfully validated the strong interaction of RhoA-G17E and LARG by IP, where we either precipitated HA-RhoA or v5-LARG and probed for both proteins by immunoblotting (Figure 14b). Furthermore, Yang et al. identified a mechanistic link between these three proteins demonstrating that the

deubiquitinase CYLD removes ubiquitin chains from lysine at the amino acid position 63 (K63) of LARG. Thereby, the GEF becomes active and can catalyze the exchange of GDP for GTP ⁶⁷ (Figure 17). To assess the ability of CYLD to modulate RhoA-activity we generated clonal CRISPR-Cas9 knock-out (CYLD-KO) cell lines using CRISPR-Cas9. We showed that RhoA-GTP levels were reduced in the CYLD-KO clones compared to the parental wildtype clone upon Calpeptin treatment (Figure 15 b, 15c). To further elaborate on this hypothesis, we are currently repeating these experiments, where we transfect 16HBE cells that stably express shCYLD with a catalytically inactive mutant (C601A) or CYLD-WT. Thereby, we expect lower levels of RhoA-GTP in the C601A-mutant. To investigate whether this modulation of RhoA-activity is mediated via LARG, we can assess the K63-ubiquitin status of LARG in the generated cell lines, by immune precipitation of overexpressed LARG and subsequent analysis by western blot. The establishment of this relation is the first indication towards a mechanistic link between the morphologically similar tumor entities TE, DTE and MAC. However, the phenotypes we observed in the CYLD-KO cell lines, regarding evasion of anoikis, migration or proliferation were less prominent and differed from the ones we obtained in our RhoA-G17E mutants (Supplementary Figures 5,6). This indicates that the G17E-mutations may provide the protein with novel functional properties which confer the cells with the ability to evade anoikis and enhance their migratory behavior.

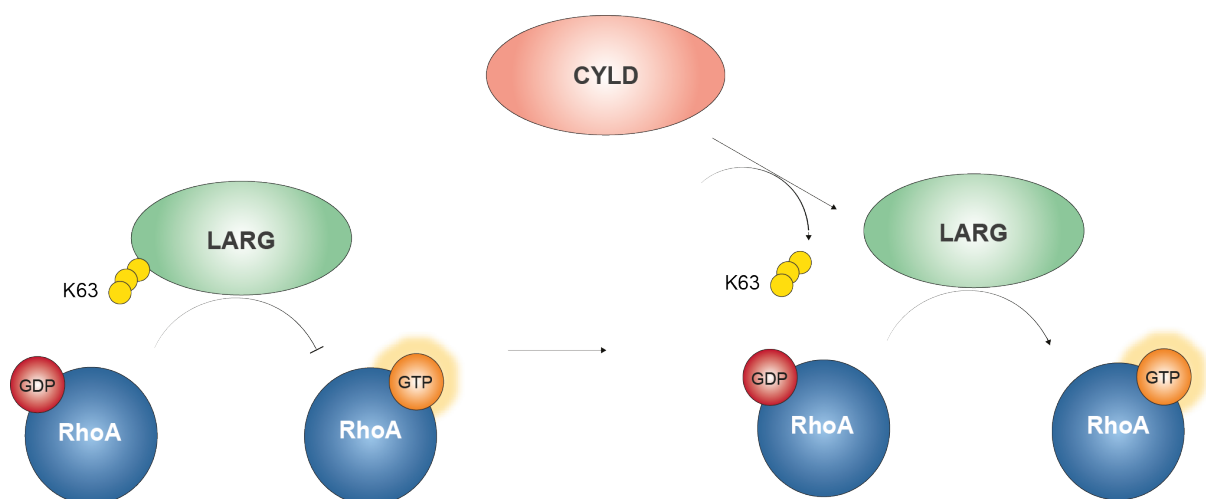


Figure 17| CYLD modulates RhoA-activity via LARG, Yang et al., 2013

5. Abbreviations

DTE - Desmoplastic trichoepithelioma

MAC - Microcystic adnexal carcinoma

TE - Trichoepithelioma

DMSO - Dimethyl sulfoxide

EDTA - Ethylenediaminetetraacetic acid

FBS - fetal bovine serum

h - hour

LB - Lysogeny broth media

min - minute(s)

sec - seconds

ON - overnight

x g = rcf - relative centrifugal force

rpm - rotations per minute

RT - room temperature

°C - degree Celsius

PBS - Phosphate Buffered Saline

TBS-T - Tris-buffered saline with Tween

GFP - Green Fluorescent Protein

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

NF- κ B - nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

Cre - cyclization recombination

TetO - Tetracycline-Controlled Transcriptional Activation

H&E - Hematoxylin and eosin staining

FC - Fold Change

FDR – False Discovery Rate

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URLs

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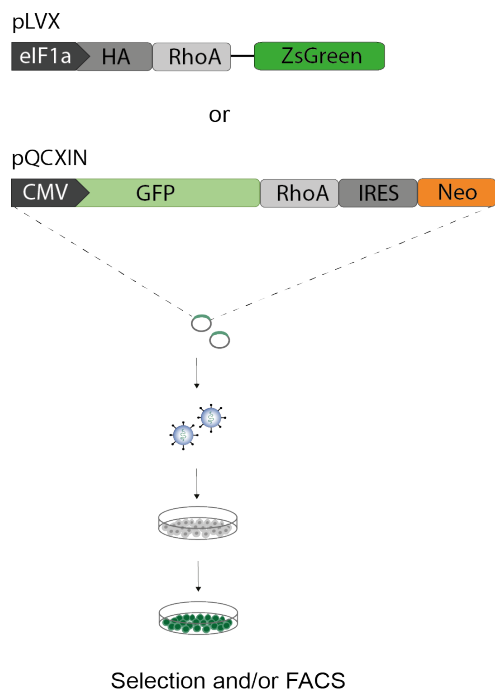
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cBioportal

Cytoskeleton <https://www.cytoskeleton.com/g-switch-about>

Supplementary Data

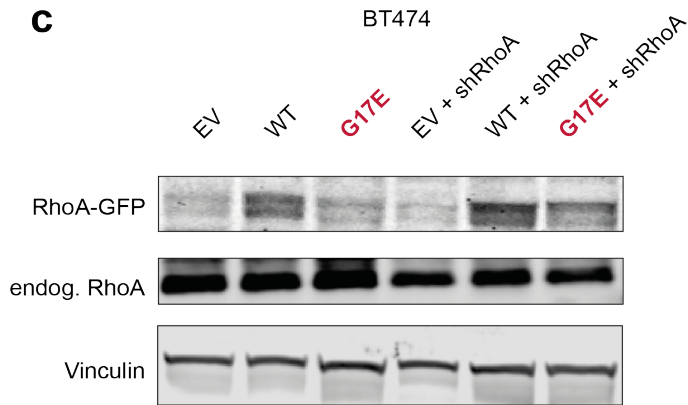
a



b



c



d

Cell Line	Tissue	Species	Experimental Purpose
16HBE14o-	human bronchial epithelial cells	human	functional and phenotypic analysis of RhoA mutations
HEK293T	human embryonic kidney cells	human	overexpression experiments
HEK LentiX	subclone of HEK293T	human	overexpression experiments lentivirus production
MDA-MB-231	breast cancer	human	metastasis studies
SW948	colon cancer	human	harbor endogenous RhoA-G17E mutation
CAL148	breast cancer	human	harbor endogenous RhoA-G17E mutation
BT474	breast cancer	human	harbor endogenous RhoA-G17E mutation
NIH 3T3	embryonic fibroblasts	mouse	tumor induction

e

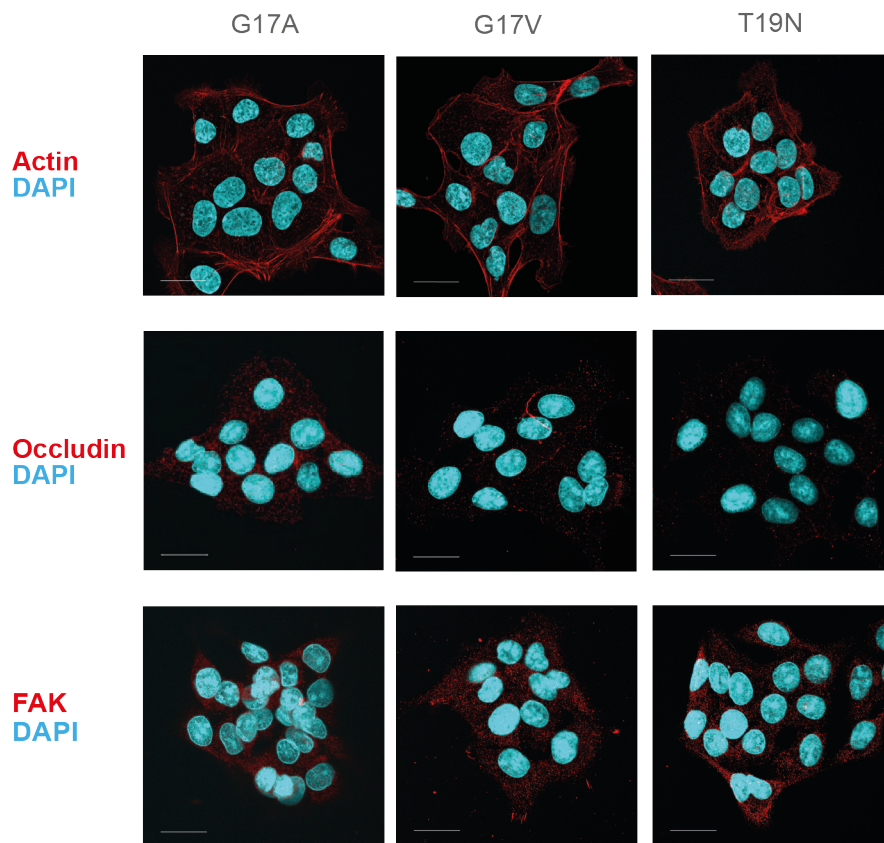
PAM G17E

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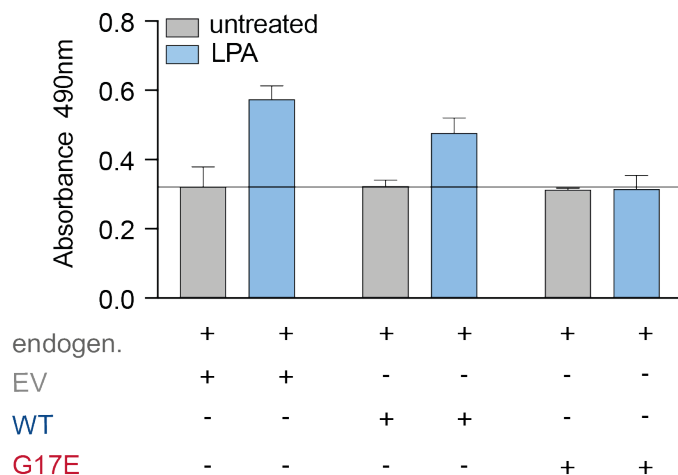
clone 2 ATTGTTGGTGATGGAGCTTGTGAAAAGACATGCTTGCTCATAGTCTTCAGCAAGGACCAGTTCC

RHOA-WT ATTGTTGGTGATGGAGCCTGTGGAAAGACATGCTTGCTCATAGTCTTCAGCAAGGACCAGTTCC

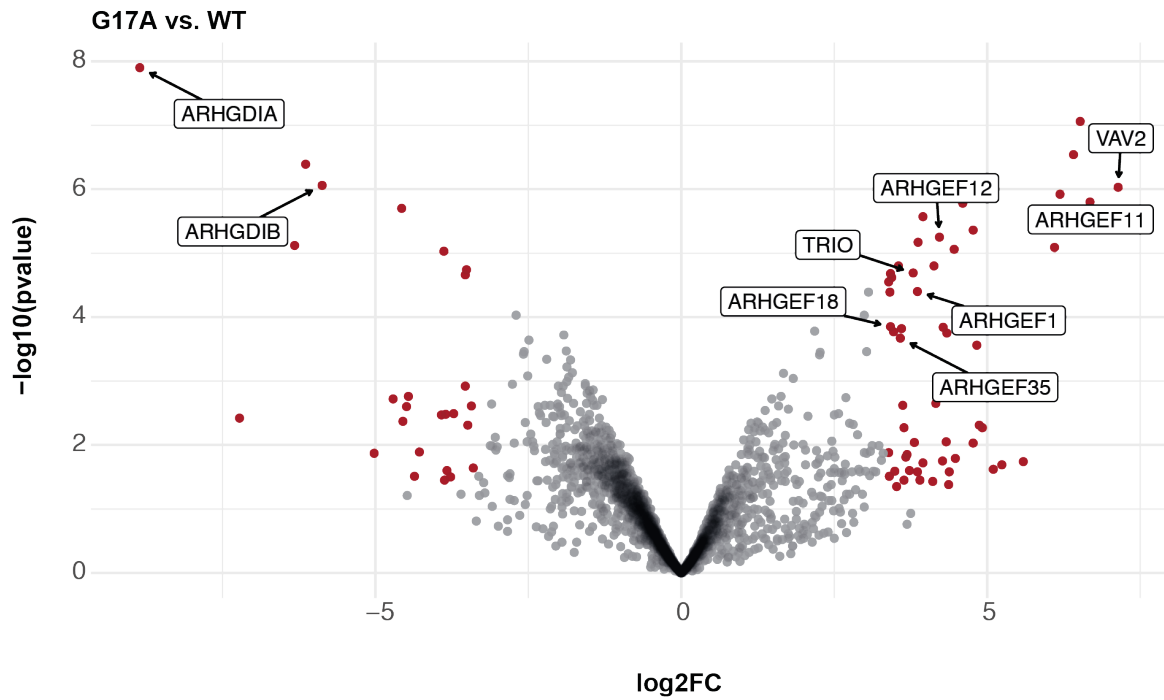
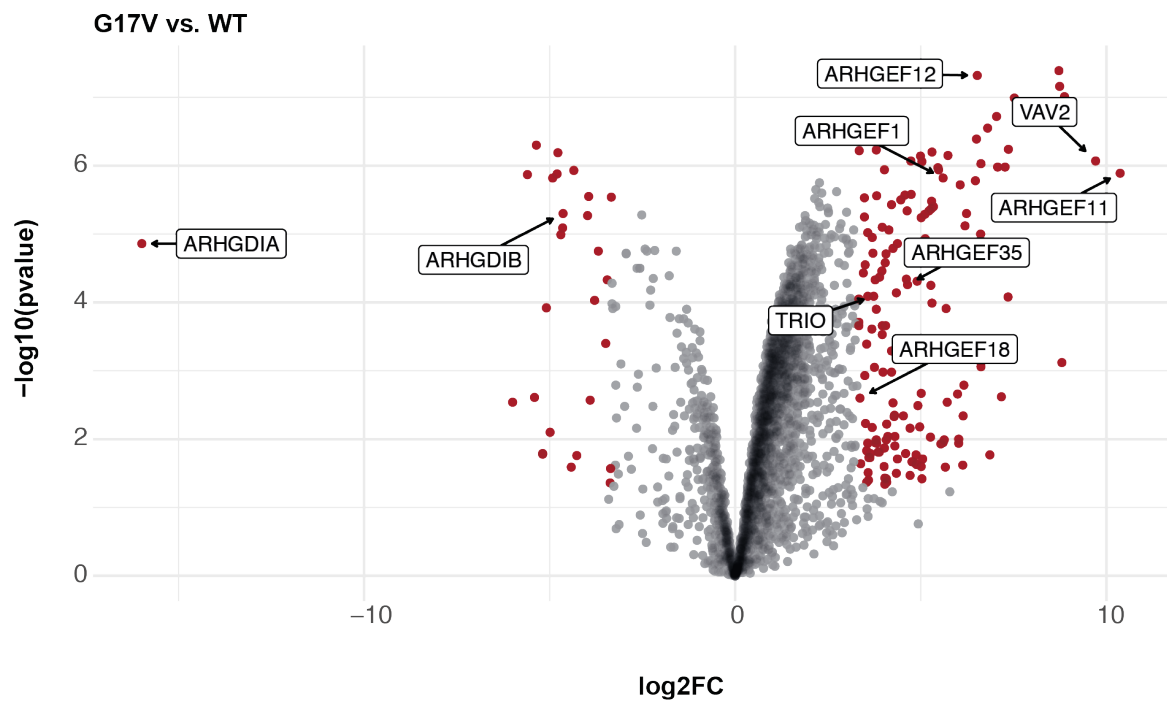
Supplementary Figure 1| RhoA cell line generation **a**, Retro- and Lentiviral constructs. **b**, Overexpression of RhoA-mutants in SW948 cell lines. **c**, Overexpression of RhoA-mutants in BT474 cell lines. **d**, Generated cell lines. **e**, Identification of RhoA-G17E knock-in clones by Sanger Sequencing



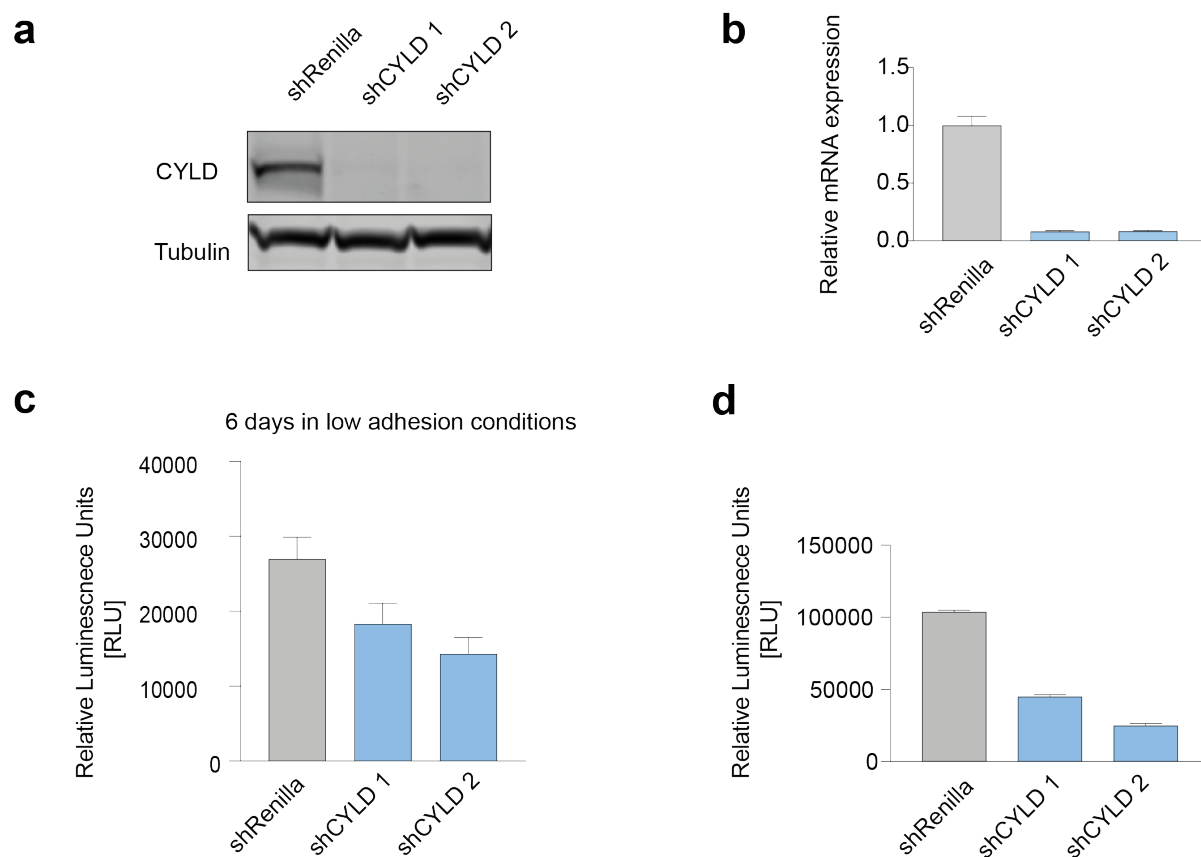
Supplementary Figure 2| RhoA-G17 mutants affect the cell morphology. Immunofluorescence staining of PFA-fixed 16HBE cells stably overexpressing RhoA-G17A,V and -T19N for actin, occludin (red),FAK (red); DAPI; scale bar 20nm.



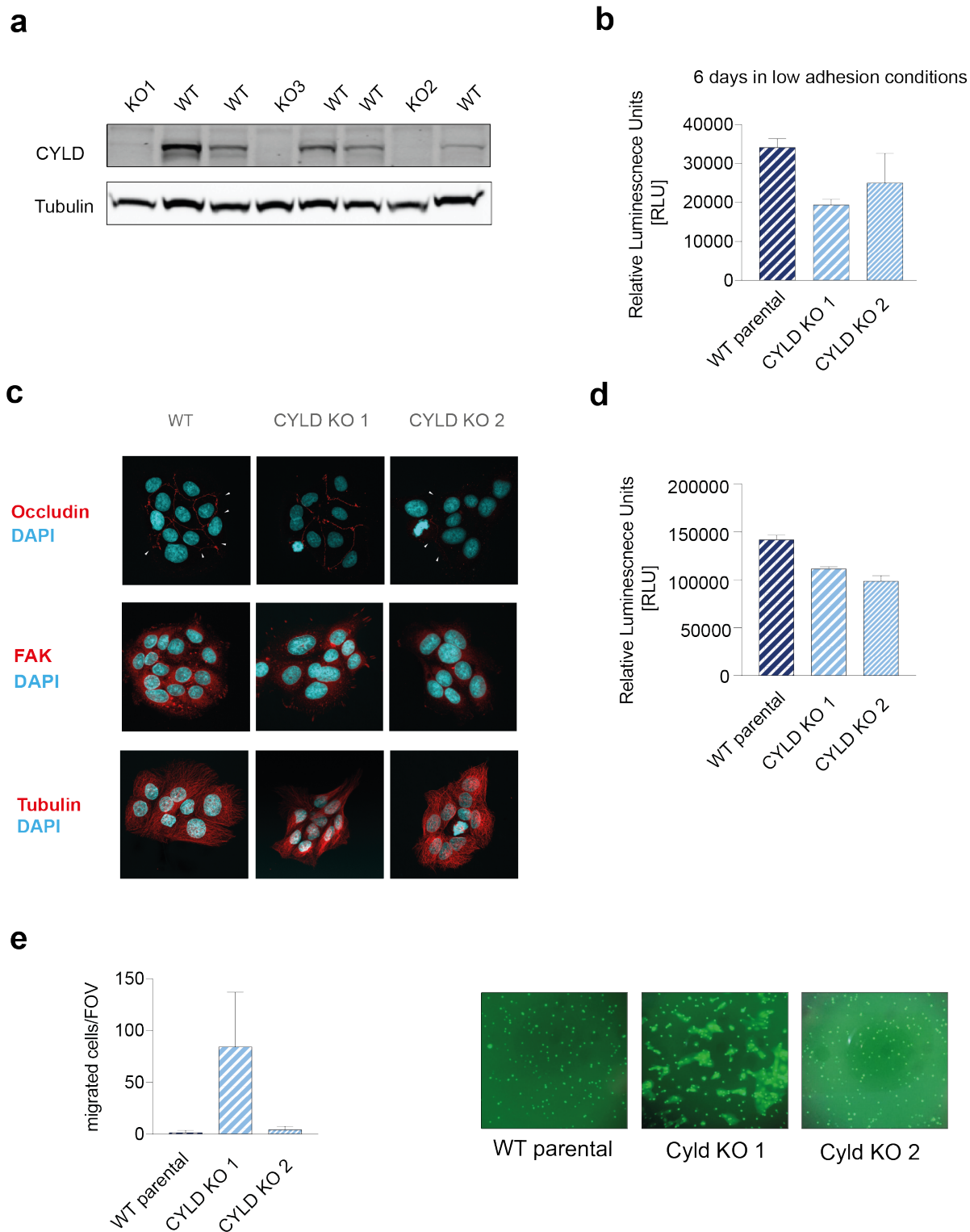
Supplementary Figure 3 | LPA-stimulated RhoA-G17E mutant fails to bind GTP. Levels of RhoA-activation in 16HBE cell lines stably overexpressing EV, RhoA-WT and RhoA-G17E. After a 5-minutes exposure to LPA (1μM) the amount of GTP-bound RhoA was determined by obtaining the absorbance at 490nm.

a**b**

Supplementary Figure 4 | RhoA-G17A and -G17V are GEF-traps. a, Volcano blot representing differential bound proteins of RhoA-WT versus RhoA-G17A, GEFs are indicated by arrows (cut-offs $\text{FC}=10$, $\text{FDR} = 0.05$). **b**, Volcano blot representing differential bound proteins of RhoA-WT versus RhoA-G17V, GEFs are indicated by arrows (cut-offs $\text{Log}_2\text{FoldChange}=2$, $\text{FDR} = 0.01$); red dots indicate significant hits



Supplementary Figure 5| CYLD knockdown phenotypes differ from RhoA-G17E **a**, Validation of CYLD knockdown by immunoblotting. **b**, Validation of CYLD knockdown by qPCR. **c**, Anoikis assay of CYLD knockdown cell lines after 6 days in low adhesion conditions. **d**, Proliferation assay of CYLD knockdown cell lines. Viability was assessed by quantifying the present amount of ATP before and after 3 days of culture. RLU are proportional to amount of ATP present.



Supplementary Figure 6 | CRISPR-Cas9 CYLD-KO in 16HBE cell lines. **a**, Validation of CYLD knock out by immunoblotting. **b**, Anoikis assay of CYLD knock out clones and WT-clone after 6 days in low adhesion conditions. **c**, Proliferation assay of CYLD knock out clones and WT-clone. Viability was assessed by quantifying the present amount of ATP before and after 3 days of culture. RLU are proportional to amount of ATP present. **d**, Immunofluorescence staining of PFA-fixed 16HBE CYLD knock out clones and WT-clone for occludin (red),FAK (red), tubulin (red); DAPI; scale bar 20nm. **e**, Boyden Chamber Migration Assay of CYLD KO clones: number of starved, migrated cells towards full growth media. (n = 10 fields of vision (FOV)). ****P<0.0001, two-tailed Mann-Whitney U test.

