MEDICAL UNIVERSITY OF VIENNA

Identification of essential protein complexes in cancer cell proliferation and leukemia drug resistance

Doctoral thesis at the Medical University of Vienna
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Doctor of Philosophy

Submitted by

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Declaration

The following doctoral thesis is compiled in a cumulative format and contains published results as well as work that has been submitted for publication. The author of this thesis (J.W.B) is first author on manuscript #2 and #3 as well as second author on manuscript #1. The work described in this thesis has been performed by the author in the research group of Univ.-Prof. Dr. Giulio Superti-Furga at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna, Austria, unless otherwise stated (detailed contributions are outlined below). The remaining parts of this thesis were written solely by the author himself with Giulio Superti-Furga providing scientific input and feedback for completion.

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V.A.B. and L.T.J. designed the research project, performed haploid-genetic screens, performed phenotypic validation experiments, analyzed and interpreted the data, and wrote the manuscript. P.M. performed bioinformatics analysis of screening as well as mass spectrometry data, and contributed to writing of the paper. J.N., J.S. and F.R.v.D. performed phenotypic validation experiments. J.W.B. and R.S. cloned expression constructs focusing on identified uncharacterized essential gene candidates, generated cell lines and performed affinity purification mass spectrometry experiments. N.O. assisted in cloning, cell line generation and affinity purifications of identified essential proteins. A.S. performed bioinformatics analysis of mass spectrometry data. C.M. and J.E.C. performed experiments and provided advice. H.J. assisted with electron microscopy experiments. K.L.B. supervised mass spectrometry experiments. J.C. performed and supervised bioinformatics analysis, and contributed to writing of the manuscript. G.S-F. designed research, supervised experiments focusing on mass spectrometry based characterization of novel essential proteins, analyzed and interpreted the data and contributed to writing of the manuscript. T.R.B. conceptualized the research project, supervised experimental design and conduct, data analysis and interpretation, and wrote the manuscript.

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J.W.B. conceptualized the research project, designed and cloned the retroviral expression vectors, generated inducible hematopoietic cell lines (K-562, KCL-22, Ba/F3), carried out phenotypic experiments characterizing the new expression vectors, performed affinity purification as well as phenotypic experiments focusing on NRAS G12D, analyzed and interpreted the data, and wrote the manuscript. A.F. conceptualized the research project, assisted in vector cloning, generated the inducible human colorectal adenocarcinoma cell line HT-29, carried out affinity purification and phenotypic experiments focusing on MLKL S358D, as well as subsequent HSP90-MLKL S358D directed validation experiments, analyzed and interpreted the data, and wrote the manuscript. M.R. assisted in experimental planning, data interpretation and manuscript writing. R.K.K performed bioinformatics analysis of mass spectrometry data. S.S. assisted in performing validation experiments. G.I.V. assisted in performing imaging experiments. A.C.M. performed mass spectrometry analysis of affinity purification samples. M.G. and J.Z. provided new reagents and advice for the development of new vector constructs. K.L.B. designed research, supervised mass spectrometry based sample analysis and provided feedback for the manuscript. G.S-F. conceptualized the research project, supervised data analysis and interpretation as well as contributed to writing of the manuscript.

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J.W.B. and G.S-F. conceptualized the study based on preliminary data and discussions with T.R.B. J.W.B. performed haploid genetic drug resistance screens, performed the majority of

phenotypic validation experiments, analyzed and interpreted the data, and wrote the manuscript. G.M.C. designed, performed and analyzed phenotypic experiments done in *Drosophila*. G.I.V. assisted in performing and analyzing imaging experiments. L.X.H. performed experiments, provided scientific insight and experimental advice. M.P. performed phenotypic validation experiments. F.S. performed bioinformatics analysis of haploid genetic screening data, generated circos plots and graphical display of insertion sites. A.F., M.R. and G.W. performed gene-trap mutagenesis of haploid CML cells. V.A.B. and T.R.B. provided reagents and gave experimental advice. R.K. supervised sequencing data analysis and gave experimental advice. M.M. designed and interpreted phenotypic experiments done in *Drosophila*, and contributed to writing of the manuscript. G.S-F. supervised experimental design, data analysis and interpretation, and wrote the manuscript.

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“One day, we imagine that cancer biology and treatment - at present, a patchwork quilt of cell biology, genetics, histopathology, biochemistry, immunology, and pharmacology - will become a science with a conceptual structure and logical coherence that rivals that of chemistry or physics.“

Taken from
D. Hanahan and R. A. Weinberg
The Hallmarks of Cancer
CELL, 2000

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Abstract

The mechanistic understanding of protein complexes in cancer cell proliferation and survival as well as in the response of malignant cells to targeted therapeutic perturbations is of great clinical importance. The development of tandem affinity purification (TAP) coupled to mass spectrometry (MS) and genetic screening technologies has fueled the phenotypic identification and functional dissection of protein-protein interactions on a large scale.

Haploid genetic screening has been instrumental for the identification of the repertoire of genes essentially required for suspension and adherent cancer cell proliferation under normal culture conditions. Interestingly, apart from well-studied gene sets this screening has revealed a collection of essential genes without any annotated biological function. Within this doctoral thesis I have employed TAP-MS technology to identify interacting proteins of a selected set of these essential candidates in order to understand in which biological processes they engage. Most importantly, this approach identified the transmembrane protein 258 (TMEM258/*C11orf10*) as an integral component of the oligosaccharyltransferase (OST) complex important for protein N-linked glycosylation.

In order to enable streptavidin-hemagglutinin (SH)-based TAP-MS approaches in a diverse compilation of cellular model systems, I furthermore developed a versatile inducible expression system named pRSHIC (retroviral expression of SH-tagged proteins for interaction proteomics and color-tracing). Using pRSHIC to study the NRAS G12D mutant protein recovered well-known interaction partners and cellular hallmark features of this well studied oncogene hence validating the functionality and utility of this novel vector tool. Moreover, pRSHIC-based TAP-MS analysis of the necroptosis-regulating MLKL S358D mutant protein revealed a critical functional HSP90 dependency for MLKL-induced cell death amenable to pharmacological interference by geldanamycin.

Chronic myeloid leukemia (CML) is characterized by expression of the *Philadelphia chromosome*-encoded BCR-ABL tyrosine kinase fusion oncoprotein. BCR-ABL engages numerous protein complexes leading to cellular leukemic transformation. Targeted inhibition of this oncoprotein using tyrosine kinase inhibitors (TKIs) has demonstrated durable clinical responses embodying the paradigmatic example for precision medicine. In order to understand the genetic requirements of cellular TKI therapy efficacy, I have performed genetic gene-trap-based screens of 1st, 2nd and 3rd generation BCR-ABL inhibitors in a haploid human CML cell line. These screens identified a common set of 6 resistance-inducing gene loss-of-functions demonstrating the critical importance of enhanced RAS/mitogen-activated protein kinase (MAPK) pathway activation and altered transcription regulation for the survival of CML cells upon TKI treatment. Most importantly, functional validation experiments on leucine zipper like transcription regulator 1 (*LZTR1*) led to the identification of the cullin (CUL) 3-LZTR1 E3 ligase complex as a novel rheostat of RAS signaling and hence MAPK activation. Moreover, these

findings provide the first mechanistic explanation for the involvement of identified *LZTR1* missense mutations in Noonan syndrome (NS), Schwannomatosis (SWNMT) and glioblastoma (GBM).

In summary, within this thesis I have employed MS-based interaction proteomic methodologies to delineate signaling complexes essential for cancer cell proliferation and used genetic screening approaches to uncover gene loss-of-functions contributing to resistance to targeted therapeutic agents.

Zusammenfassung

Das mechanistische Verständnis von Proteinkomplexen in ihrer Relevanz für das Krebszellenwachstum und die Entwicklung von Resistenzen gegenüber zielgerichteten medikamentösen Therapieformen ist von entscheidender klinischer Bedeutung. Die Etablierung von Tandem-Affinitäts-Aufreinigungsverfahren (TAP) in Verbindung mit Massenspektrometrie (MS)-basierter Analyse sowie genetische Screening-Technologien haben entscheidenden Anteil an der erfolgreichen funktionellen Aufarbeitung von zahlreichen zellulären Protein-Interaktionen.

Der Einsatz einer auf haploiden Zellen basierenden Screening-Methode hat es ermöglicht all diejenigen Gene zu identifizieren, die für das Wachstum von malignen Suspensionszellen und adhärenten Krebszellen unter Laborbedingungen unentbehrlich sind. Interessanterweise war die zelluläre Funktion für einen Teil dieser identifizierten Gene unbekannt. Im Rahmen dieser Doktorarbeit habe ich für eine ausgewählte Gruppe der unbekannten Proteine mittels TAP-MS Methode die Protein-Interaktionspartner identifiziert, um daraus Rückschlüsse auf die möglichen Genfunktionen ziehen zu können. Bedeutsam war vor allem die Identifikation des Transmembranproteins TMEM258 (*C11or10*) als integraler Bestandteil des Oligosaccharyltransferase (OST) Proteinkomplexes, der für die zelluläre aminoterminale Glykosylierung verantwortlich ist.

Des Weiteren habe ich ein vielfach einsetzbares, induzierbares, virales Vektorensystem (pRSHIC) etabliert, das die Bandbreite verfügbarer physiologisch relevanter Zelllinien für die Anwendbarkeit von Streptavidin-Hemagglutinin (SH)-basierter TAP-MS Methodik wesentlich erweitert. Die Identifizierung von bekannten Protein-Interaktionspartnern des Onkogens NRAS G12D mittels pRSHIC hat in einem ersten Schritt die experimentelle Validität und Verwendbarkeit dieses Systems erfolgreich demonstriert. Darüber hinaus hat die TAP-MS-gestützte Analyse der MLKL S358D Mutante, die bei zellulärer Expression zum Nekroptose Zelltod führt, Interaktionen mit HSP90 Proteinen identifiziert. Diese Assoziation ist entscheidend für die Funktion von MLKL und die Nekroptoseinduktion kann mittels pharmakologischer HSP90 Inhibition wie zum Beispiel Geldanamycin inhibiert werden.

Chronische myeloische Leukämie (CML) entsteht durch eine chromosomale Translokation, bei der das sogenannte *Philadelphia Chromosom* entsteht und dadurch das BCR-ABL Fusionsprotein mit Tyrosinkinaseaktivität generiert wird. BCR-ABL aktiviert verschiedene zelluläre Signaltransduktionswege und führt dadurch zu leukämischem Krebszellwachstum. Die pharmakologische Inhibierung der Tyrosinkinaseaktivität mittels zielgerichteten Arzneimitteln ist klinisch höchst erfolgreich und gilt als ein Paradebeispiel für personalisierte Präzisionstherapie. Um besser zu verstehen, welche Gene für diese exquisite zelluläre Sensitivität verantwortlich sind, habe ich im Rahmen dieser Doktorarbeit genetische Screens in einer haploiden CML Zelllinie unter Verwendung von BCR-ABL Inhibitoren der 1., 2. und 3.

Generation durchgeführt. Dabei habe ich sechs Geninaktivierungen identifiziert, die zur Resistenz gegenüber BCR-ABL Inhibitoren führen. Aus den Genfunktionen lässt sich schließen, dass die verstärkte Aktivierung des RAS/MAPK Signaltransduktionsweges und die veränderte Regulation der Gentranskription für das Zellüberleben nach BCR-ABL Inhibition eine entscheidende Rolle spielen. Darüber hinaus haben funktionelle Validierungsexperimente gezeigt, dass das Protein LZTR1 als Teil eines Cullin (CUL) 3 E3 Ubiquitinligase Proteinkomplexes ein entscheidender Regulator für die RAS Signaltransduktion und die Aktivierung des MAPK Signalweges ist. Durch diese Ergebnisse konnte ich eine erste mechanistische Erklärung für die Pathogenität der LZTR1 Mutationen erbringen, die in Patienten mit Noonan Syndrom, Schwannomatosis oder Glioblastom gefunden wurden.
Im Rahmen dieser Doktorarbeit habe ich in Kombination genetische Screening-Verfahren und MS-basierte Proteom-Analysemethoden verwendet um ein detaillierteres Verständnis der zellulären Signaltransduktionswege zu erhalten, die für das Wachstum von Krebszellen und die Entstehung von Arzneimittelresistenzen verantwortlich sind.

Abbreviations

aa	Amino acid
ABL1	Abelson Murine Leukemia Viral Oncogene Homolog 1
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP	Affinity purification
AP CML	Accelerated phase CML
APC	Adenomatous polyposis coli
BCL2	B-Cell CLL/Lymphoma 2
BCR	Breakpoint Cluster Region
BioID	Proximity-dependent biotin identification
BP CML	Blast phase CML
BRD4	Bromodomain-containing protein 4
CCLE	Cancer cell line encyclopedia
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
Cas9	CRISPR-associated protein 9
Cas9n	CRISPR-associated protein 9 nickase
CDK	Cyclin-dependent kinase
CETSA	Cellular thermal shift assay
CGH	Comparative genomic hybridization
CMR	Complete molecular response
CNV	Copy number variations
ComPASS	Comparative proteomic analysis software suite
COT	Serine/threonine-protein kinase COT (MAP3K8)
CP CML	Chronic phase CML
CR	Cytogenetic response
CRC	Colorectal cancer
CRKL	CRK like proto-oncogene
CRL	Cullin-RING E3 ligase complex
CSC	Cancer stem cell
CRISPR	Clustered, regularly interspaced, short palindromic repeats
CRISPRa	CRISPR transcriptional activation
CRISPRi	CRISPR transcriptional interference
crRNA	CRISPR RNA
CUL	Cullin
dCas9	nuclease inactive Cas9
dnAML	de-novo AML
DNMT	DNA methyltransferase
DSB	Double strand breaks
DUB	Deubiquitinating enzyme
DUSP	Dual-specificity phosphatase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERK	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
ET	Essential thrombocytosis
FACS	Fluorescent activated cell sorting
FLT3	Fms related tyrosine kinase 3
GAB2	GRB2 associated binding protein 2
GAP	GTPase activating protein
GBM	Glioblastoma

GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GPCR	G-protein coupled receptor
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HDR	Homology-directed repair
HECT	Homologous to the E6AP carboxyl terminus
HH	Hedgehog
HNSCC	Head and neck squamous cell carcinoma
HR	Hematological response
HRAS	Harvey sarcoma virus-associated oncogene
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSF1	Heat-shock factor 1
HSP90	Heat shock protein 90
HSPC	Hematopoietic stem and progenitor cell
IDH	Isocitrate dehydrogenase
IFN-α	Interferon-α
IMAC	Ion metal affinity chromatography
IMiD	Immunomodulatory drug
Indel	Insertion and deletion
iPSC	induced pluripotent stem cell
iTRAQ	Isobaric tags for relative and absolute quantitation
JAK	Janus kinase
JMML	Juvenile myelomonocytic leukemia
JNK	c-Jun N-terminal kinase
KO	knock-out
KRAB	Krüppel associated box
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC	Liquid chromatography
LUMIER	Luminescence-based mammalian interactome mapping
LZTR1	Leucine zipper like transcription regulator 1
MDR	Multidrug resistance protein
miRNA	microRNA
mir30	microRNA 30
miR-E	microRNA 30 based enhanced microRNA scaffold
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
Mb	Mega-base
MDS	Myelodysplastic syndrome
MEK	Dual specificity mitogen-activated protein kinase kinase
MLL	Mixed lineage leukemia
MLKL	Mixed lineage kinase domain-like protein
MM	Multiple myeloma
MMEJ	Micro-homology-mediated end-joining
MoA	Mechanism of action
MPN	Myeloproliferative neoplasms
MR	Molecular response
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
NF1	Neurofibromin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
NOA	Non-oncogene addiction
NOTCH	Neurogenic locus notch homolog protein
NRAS	Neuroblastoma RAS viral oncogene homolog
NS	Noonan syndrome
NSCLC	Non-small-cell lung carcinoma
nt	Nucleotide
OA	Oncogene addiction
OCT1	Organic cation transporter 1
OG	Oncogene
ORF	Open reading frame
OST	Oligosaccharyltransferase
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
Ph	Philadelphia chromosome
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
PMF	Primary myelofibrosis
PP2A	Protein phosphatase 2A
PPARγ	Peroxisome proliferator-activated receptor gamma
pRSHIC	retroviral expression of SH-tagged proteins for interaction proteomics and color-tracing
PTB	Phosphotyrosine-binding domain
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
PTPN1	Tyrosine-protein phosphatase non-receptor type 1
PTPN11	Tyrosine-protein phosphatase non-receptor type 11
PTPN12	Tyrosine-protein phosphatase non-receptor type 12
PV	Polycythemia vera
RAF	RAF proto-oncogene serine/threonine-protein kinase
RAS	Rat sarcoma virus protein
RB	Retinoblastoma-associated protein
RING	really interesting new gene domain
RIPK	Receptor-interacting serine/threonine kinase
RISC	RNA-induced silencing complex
RNAi	RNA interference
RTA	Replication and transcription activator
RTK	Receptor tyrosine kinase
SAINT	Significance analysis of interactome
SAM	Synergistic activation mediator
sAML	secondary AML
sgRNA	single guide RNA
SH	Streptavidin-hemagglutinin
SHC1	Src homology 2 domain-containing-transforming protein 1
shRNA	short hairpin RNA
shRNAmir	microRNA-embedded short hairpin RNA
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SILAC	Stable isotope labeling with amino acids in cell culture
siRNA	small interfering RNA
SLC	Solute carrier protein
SNP	Single-nucleotide polymorphism
SOS1	Son of sevenless homolog 1
SSL	Synthetic sickness/lethality

STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
SWNMT	Schwannomatosis
SYK	Spleen tyrosine kinase
TALEN	Transcription activator-like effector nuclease
TAP	Tandem affinity purification
TCGA	The Cancer Genome Atlas project
TF	Transcription factor
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitor
TMEM258	Transmembrane protein 258
TMT	Tandem mass tag
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TP53	Tumor suppressor p53
tracrRNA	trans-encoded small RNA
TSG	Tumor suppressor gene
TSS	Transcription start site
TyrK	Tyrosine kinase domain
VP64	VP16 tetrameric repeat
WT	wild type
WGS	Whole genome sequencing
WNT	Wingless-related integration site
WT1	Wilms tumor 1
ZNF	Zinc-finger nuclease

Gene and protein name abbreviations not listed here can be found in publicly available databases such as GeneCards, Ensembl, NCBI or UNIPROT.

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The long journey of doing a PhD undoubtedly presents a unique part in life and is filled with plentiful emotions, scientific and personal success stories as well as numerous mistakes and failures. The social environment created by the exceptional group of people, which CeMM is made of, presents a resourceful platform one can build upon especially in times when the light at the end of the tunnel gets out of sight. To develop the inner instinct of when to take the most fruitful decision at the hopefully most appropriate time point will always be to me the most unique skillset that I hopefully managed to acquire in finishing this PhD.

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1 Introduction

1.1 Molecular signal transduction networks in cancer

1.1.1 Cancer genomes

Cancer can be defined as a disease of altered cellular proliferation and identity states driven by aberrations in the genome and epigenome (Hanahan & Weinberg, 2000; Vogelstein *et al*, 2013; Garraway & Lander, 2013). The selective acquisition of mutations leading to either gain- or loss-of-function variants in the cancer genome induces manifold alterations in the wiring and regulation of signaling protein networks ultimately driving cellular transformation and cancer cell propagation. The description of gaining genomic alterations in a sequential way over time as the causal event for the development of malignancies has been pioneered by C. O. Nordling and A. G. Knudson in the formulation of the two-mutation hypothesis with an estimated need of 3 to 7 mutations for cancer formation postulated by D. J. B. Ashley (Nordling, 1953; Ashley, 1969; Knudson, 1971). The model of sequential mutation acquisition has been observed thereupon both from a genetic as well as histopathological viewpoint in many cancers and can be phenotypically exemplified by the step-wise developmental properties of colorectal cancer (CRC) or head and neck squamous cell carcinoma (HNSCC) (Vogelstein *et al*, 1988; Haddad & Shin, 2008; Vogelstein *et al*, 2013). In the development of CRC, the acquisition of adenomatous polyposis coli (*APC*) gene mutations in the colonic epithelium leads to the formation of small adenomas that upon acquisition of kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene mutations expand to large adenomas and with additional genetic or epigenetic alterations can transform into aggressive invasive carcinomas (Vogelstein *et al*, 2013).

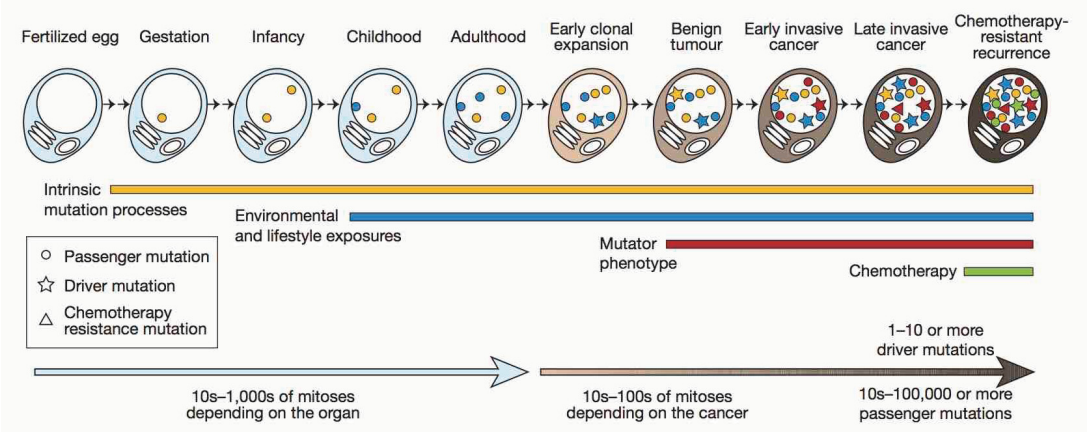

Figure 1: Acquisition of genetic alterations over time. Whereas non-pathogenic mutations will remain silent, pathogenic alterations will ultimately lead to clonal expansion and induce tumorigenesis and contribute to cancer drug resistance. *(Taken from Stratton et al, 2009. Reprinted with permission from Nature Publishing Group)*

The acquisition of mutations over time also implies that the probability for the development of malignancies increases with becoming older coining the phrase that cancer can be considered as the disease of an ageing society (Figure 1). Following this notion, it has been proposed that the risk for developing cancer can at least in a fraction of cases be linked to the amount of stem cell divisions happening over time (Tomasetti & Vogelstein, 2015). These results however have been considered highly controversial for the reason of omitting the substantial participation of exogenous (e.g. environmental) stimuli (Wu *et al*, 2016).

Interestingly, whereas most cancers follow the above mentioned sequential mutational pattern, the recent description of a phenomenon termed chromothripsis broadens the scope of how genetic perturbation events can lead to cancer development (Stephens *et al*, 2011; Maher & Wilson, 2012). Chromothripsis describes the observation of sudden large scale shattering and subsequent rearrangement of chromosomal fragments at a genome-wide scale within the range of only a few mutagenic events. It has been identified in various malignancies like melanoma, colorectal, thyroid, renal cell and up to 25% of bone cancer as well as neuroblastoma (Stephens *et al*, 2011; Molenaar *et al*, 2012).

Consequently, the acquisition of missense (amino acid (aa) changing), nonsense (premature stop codon) or frameshift (alteration of reading frame) mutations, shaped by the underlying mutational processes, can lead to activation, inactivation or property changes of the encoded protein. The distribution of mutations along the coding sequence of a given gene can already be informative about the potential phenotypic effect of the encoded protein on cellular transformation. Whereas oncogenic activating mutations tend to cluster in specific domains or even around specific aa residues, inactivating mutations are inclined to spread out through the entire gene body (Vogelstein *et al*, 2013). Additionally, it is the current view that not all mutations within one tumor cell do necessarily directly contribute to cancer development and progression, which led to the distinction between driver and passenger mutations as a means to categorize and prioritize functional impact of identified mutations (Vogelstein *et al*, 2013; Garraway & Lander, 2013). However, albeit being not directly involved in cell-autonomous signaling contributing to transformation, passenger alterations provide a pool of novel, “non-self” neo-epitopes possibly harnessable for cancer immunotherapy (Su & Fisher, 2016).

1.1.2 Deep sequencing approaches to decipher cancer genomes

Over the years, different complementary technologies have aided in the molecular characterization of cancer genomes with increasing resolution over time. The development of comparative genomic hybridization (CGH) methods has allowed for the detection of gene copy number variations (CNV), gains and losses of chromosomal fragments as well as loss of heterozygosity on a genome-wide level. This has enabled the identification of recurrently

deleted gene candidates characterizing specific disease subtypes (Mullighan *et al*, 2008), the identification of specific risk alleles for the development of leukemia predisposing precancerous disorders of the hematopoietic system (Olcaydu *et al*, 2009), as well as in pan-cancer CNV studies for the identification of commonly amplified regions targeting essential core survival pathways (Beroukhim *et al*, 2010).

The automated Sanger sequencing-based deciphering of the human genome until the year 2001 has revolutionized the understanding of the human genome and has provided a pronounced impulse for the development of new, next-generation sequencing (NGS) technologies (Lander *et al*, 2001; Venter *et al*, 2001; Metzker, 2010). This novel deep sequencing-based approaches have in the following 10 to 15 years allowed for the in-depth genomic characterization of numerous cancer types at unprecedented resolution (Stratton *et al*, 2009).

Various types of NGS-based sequencing technology like emulsion polymerase chain reaction (PCR), solid-phase bridge amplification or in-solution DNA nanoball generation-based platforms have been developed offering different read-length and sample through-put capabilities (Shendure & Ji, 2008; Meyerson *et al*, 2010; Mardis, 2011; Goodwin *et al*, 2016).

As a result, different consortia like The Cancer Genome Atlas project (TCGA) or genome sequencing centers like the Welcome Trust Sanger institute have collectively identified common and unique mutational patterns and novel tumor subtypes in lung cancer (Imielinski *et al*, 2012; Govindan *et al*, 2012; Cancer Genome Atlas Research Network, 2014), prostate cancer (Berger *et al*, 2011), HNSCC (Stransky *et al*, 2011; Cancer Genome Atlas Network, 2015), esophageal adenocarcinoma (Dulak *et al*, 2013), glioblastoma (Verhaak *et al*, 2010; Schwartzentruber *et al*, 2012), breast cancer (Curtis *et al*, 2012) and pediatric low-grade glioma (Zhang *et al*, 2013) beyond many others. The aggregate amount of cancer sequencing data has for the first time allowed to obtain a pan-cancer analysis at base resolution of the mutational diversity in human cancers (Lawrence *et al*, 2013; Kandoth *et al*, 2013; Alexandrov *et al*, 2013; Hoadley *et al*, 2014). Interestingly, these analyses have furthermore uncovered a strong inter-cancer diversity in the average somatic mutation frequency with pediatric cancer like rhabdoid tumor, Ewing sarcoma or medulloblastoma displaying 0.1-1 somatic mutations per mega-base (Mb) to lung squamous cell carcinoma and melanoma with an up to 1000 fold higher mutational burden and clear traces of carcinogen-induced mutagenic processes (Lawrence *et al*, 2013). Pathway level-based analysis has furthermore enabled the annotation of low-frequency mutations that otherwise would have been missed by single-gene-based testing. These include genes like lysine demethylase 1B (*KDM1B*) or lysine methyltransferase 2E (*KMT2E*/*MLL5*) both involved in histone methylation as well as the receptor protein encoding gene neurogenic locus notch homolog protein 3 (*NOTCH3*) or the cohesion subunit encoding stromal antigen 1 (*STAG1*) (Leiserson *et al*, 2015). These types of analysis will be

crucial in the future to estimate the number of additional cases to be sequenced to reach saturation level for the identification of almost all mutations per tumor entity. Additionally, the extension from performing exome (coding sequence only) sequencing analysis to whole genome sequencing (WGS) has started to reveal novel interesting mutational patterns in non-coding regulatory, enhancer or gene-adjacent regions. For instance, activating mutations in the telomerase reverse transcriptase (*TERT*) promoter region in familial and sporadic melanoma as well as mutations creating novel transcription factor binding sites in enhancer regions of the T-cell acute lymphocytic leukemia protein 1 (*TAL1*) have been identified (Horn *et al*, 2013; Huang *et al*, 2013; Mansour *et al*, 2014).

Moreover, multi-region sequencing in individual solid tumors has revealed a high degree of clonal evolution and intra-tumor mutational heterogeneity (Gerlinger *et al*, 2012; McGranahan & Swanton, 2015; Xu *et al*, 2012). Interestingly, bystander mutations potentially non-driver mutations themselves can function as valuable molecular barcodes to trace the cellular tree of origin and to delineate complex branched evolutionary patterns occurring within one tumor from initiation to therapeutic selection and adaptation.

NGS-based technologies have not only allowed deciphering the complex genomic make up but also enabled to quantify the transcriptome of human cancers at unprecedented resolution (Wang *et al*, 2009), allowing the additional detection of fusion oncogenes (Maher *et al*, 2009), identification of viral integrations contributing to tumorigenesis (Tang *et al*, 2013) as well as detection of circulating tumor cells and associated gene expression profiles (Yu *et al*, 2012).

1.1.3 Signaling pathway networks enabling malignant growth

Cells are complex biological systems and by operating within the context of a multicellular organism face the necessity to sense, transduce and integrate information supplied by external stimuli and convert them into appropriate and actionable responses (Hlavacek & Faeder, 2009). Cellular membrane-associated, cytosolic, organelle- or nuclear-residing signaling platforms formed by protein-complexes are the functional units of molecular signal transduction pathways. The careful orchestration of these protein networks in time and space is of crucial importance for cellular homeostasis (Kholodenko *et al*, 2010; Kolch *et al*, 2015). Genetic and epigenetic perturbations as describe previously are instigating deranged network organizations and signaling dynamics. Changes in protein activity states, loss of negative regulatory feedback circuits, or rewiring of nuclear transcriptional programs ultimately lead to altered cellular conditions and neoplastic transformation (Logue & Morrison, 2012).

The neoplastic mutational landscape has functionally and historically been categorized into two opposing gene groups: the activation of proto-oncogenes (OG), drivers of the transformation phenotype, and inactivation of tumor suppressor genes (TSG), safeguards of

normal cellular physiology. Early identification of OG has been tremendously fueled by investigating the cellular transformation capabilities of oncogenic (retro-) viruses, with the tyrosine protein kinase SRC being one of the first proto-oncogenes discovered by studying avian RNA Rous sarcoma virus (RSV) (Huebner & Todaro, 1969; Stehelin *et al*, 1976; Martin, 2004). On the contrary the tumor suppressor p53 (*TP53*) and retinoblastoma-associated protein (*RB*) genes both represent paradigmatic TSGs and act as protective security guards, maintaining genome integrity and cell cycle regulation, responding to plentiful intra- and extracellular elicited threats (Kinzler & Vogelstein, 1997). It has become clear that from the growing number of gene mutations identified by deep sequencing, not every gene can be outright classified as OG or TSG. This has lead that to the assumption that neoplastic cells contain a significant amount of bystander or passenger alterations. Being considered as non-functional in upholding the cellular transformed state, further work is needed to experimentally clarify whether these changes only represent silent passengers or actually constitute essential functional units (Stratton *et al*, 2009; Pon & Marra, 2015).

The acquisition of mutations in OG and TSG allows a cell to gain key capabilities and characteristics that in seminal work by D. Hanahan and R. A. Weinberg have been condensed into the *"Hallmarks of Cancer"* in the year 2000 (Hanahan & Weinberg, 2000), and further revised and extended in 2011 (Hanahan & Weinberg, 2011). They provide a phenotypic and pathway-level framework of crucial features and enabling characteristics that define the distinction between normal cells and their transformed counterpart embedded in the tissue environment (Figure 2). Undoubtedly, there is interdependence and crosstalk between these different characteristics. Exemplified, enhanced cellular proliferation and tissue invasion is supported by sustained growth stimulation, increased sensitivity to mitogenic signals and the inactivation of counterbalancing negative regulatory factors (Hahn & Weinberg, 2002). Concomitant support by dysregulated cell cycle progression will be required in concert with the adaptation of genomic DNA integrity and maintenance-sensing mechanisms, as well as altered regulation or inactivation of cell death mechanisms (e.g. apoptosis, necrosis). As neoplastic cells accumulate, the coordination of endogenous metabolic processes, the support by enhanced angiogenesis but also the failed recognition or adverse support by immune cells represent essential milestones in tumor development, maintenance and progression (Hanahan & Weinberg, 2000; 2011). As a consequence of NGS-based cancer genome sequencing campaigns, the frequent detection of alterations in epigenetic regulators has opened the question of whether these variations fuel the above mentioned processes or represent an independent hallmark characteristic. While further work will be required for clarification, undoubtedly altered transcriptional regulation and the role of epigenetic lineage memory represent important liabilities of cancer cells that distinguish them from their healthy counterparts (Bracken & Helin, 2009; Chi *et al*, 2010; Dawson & Kouzarides, 2012). The

intricate interplay between cell-autonomous and non-cell-autonomous mechanisms, further increases the experimental difficulty of interpreting the involvement of nucleotide variants on the cancer phenotype itself or bystander support especially in the case of germline variant-induced cancer predispositions (Miething *et al*, 2014; Dong *et al*, 2016).

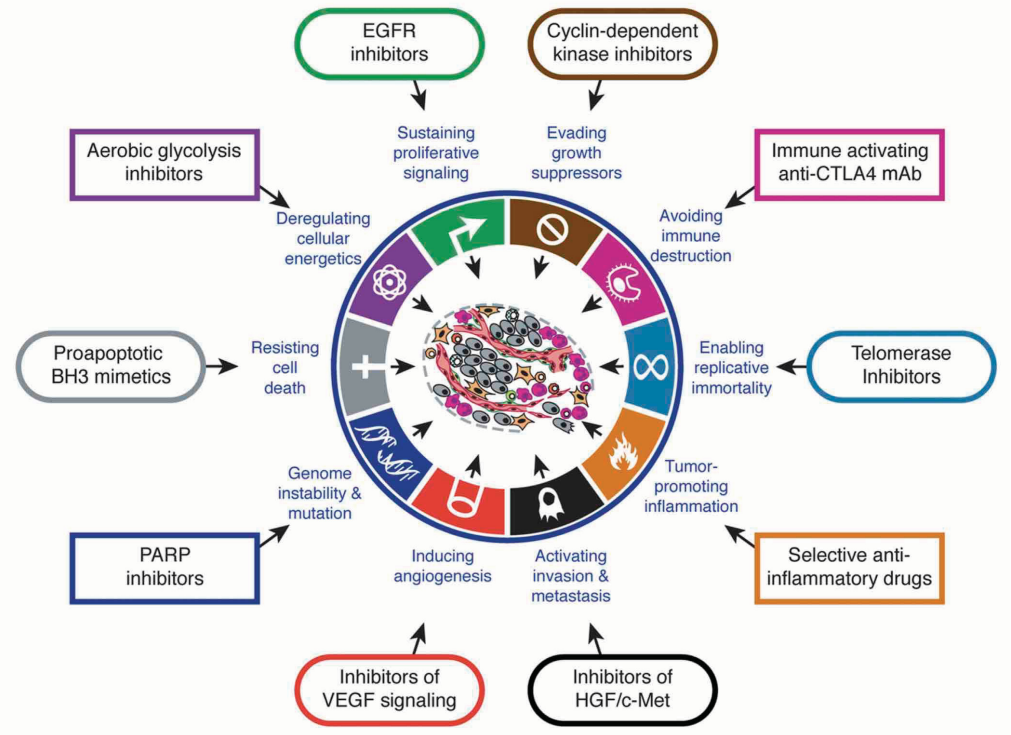

Figure 2: Core features associated with the acquisition of cancer phenotypic traits. Characteristic key pathway alterations are depicted, collectively referred to as the *"Hallmarks of cancer"* including selected hallmark-targeting therapeutic interventions. *(Taken from Hanahan & Weinberg, 2011. Reprinted with permission from Elsevier)*

The acquisition of cancer-associated traits is crucially dependent on the altered activation and regulation of key cellular signaling pathway programs like the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB)-AKT/mammalian target of rapamycin (mTOR), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), transforming growth factor (TGF)-β, wingless-related integration site (WNT)/β-catenin, neurogenic locus notch homolog protein (NOTCH), Hedgehog (HH), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Hippo signaling pathway.

The cell lineage developmental stage, in which initiating alterations originate have sparked the field of cancer stem cell research (Clevers, 2011). The search for the cell of origin represents an important aspect both for the basic understanding of cancer biology as well as for therapeutic strategies and clinical decision processes (Gilbertson, 2011; Wang, 2010). Nevertheless, it is more and more becoming clear that the heterogeneity within one tumor is greater than expected and the lineage plasticity granted by epigenetic alterations complicate

the unequivocal definition and identification of neoplasia-inducing cells (Jordan *et al*, 2006; Gupta *et al*, 2009a; Graf, 2011; Nguyen *et al*, 2012). Supporting evidence can be found in the considerable similarities that exist between pathways involved in organism development and being deregulated in cancer (Bellacosa, 2013). Numerous gene candidates, being somatically mutated in cancer also have been identified as germline variants leading to various forms of developmental syndromes with distinct cancer predispositions (see chapter 1.1.7). The cellular lineage plasticity observed in the seminal work of the discovery of induced pluripotent stem cells (iPSC) partially requiring the overexpression or activation of oncogenic transcription programs further illustrates this close relationship (Takahashi *et al*, 2007; Carette *et al*, 2010). The RAS/MAPK pathway represents a paradigm for molecular pathway functionality and regulation (Hunter, 1997). Due to its involvement in growth support, regulation of differentiation in development and cancer, as well as being a therapeutic target and mechanistic resistance factor this pathway therefore will be discussed in more detail in chapter 1.1.6.

1.1.4 Protein complexes executing gene function in forming signaling networks

The formation, steady state maintenance, as well as dynamic rearrangement of protein complexes upon transmission of cellular information represents the fundamental organizing principle of every cellular signal transduction pathway. The quaternary structural assembly of proteins into homomeric or heteromeric assemblies allows for the gathering of different sized multi-protein complexes (Marsh & Teichmann, 2015). The homo- and hetero-dimerization of STAT proteins (Miklossy *et al*, 2013), the association of catalytic (e.g. p100α) and regulatory (e.g. p85α) subunits of the PI3K complex (Thorpe *et al*, 2015) or the construction of large molecular machines like the cellular degradation apparatus, the proteasome containing over 30 protein subunits (Kish-Trier & Hill, 2013) represent diverse examples of how protein-protein interactions shape cellular physiology. Protein complexes as functional units are subject of intensive investigations ranging from yeast up to human cells (Gavin & Superti-Furga, 2003). Changes in protein abundance, post-translational modifications, protein activity states, and interaction propensities are important factors within the regulatory wiring of protein complexes (Lee & Yaffe, 2016). Additionally, the availability of proteins due to their compartmentalized subcellular distribution at the cell surface, in lipid bilayer membranes, the cytoplasm or within cellular organelles and the nucleus is important for differential complex assembly (Gavin & Superti-Furga, 2003; Hung & Link, 2011). Single-nucleotide polymorphisms (SNPs) and gene gain- or loss-of-functions impact on the assembly and composition of protein complexes thereby altering signal transduction and changing cellular behavior, contributing to human disease development (Ideker & Sharan, 2008; Hannum *et al*, 2009).

Protein interactions act as the driving forces to assemble individual proteins into cellular machines that provide the basis of forming signal transduction pathways allowing controlled and directed orchestration of gene function (Kuriyan & Eisenberg, 2007; Rebsamen *et al*, 2013). Protein complexes are therefore the ultimate pathway building blocks forming molecular networks and transducing information into and within the cell. Different technologies enable the experimental identification and characterization of signaling complexes in steady state conditions or upon phenotypic perturbation and will be discussed in more detail from chapter 1.5 onwards.

1.1.5 Post translational modifications in protein complex assembly and cellular signal transduction

Proteins themselves are built up out of one or multiple domains, usually spanning 50-400 amino acids (aa) (Lee & Yaffe, 2016; Letunic *et al*, 2012). Protein domains are the specification factors within proteins providing functional modules for signal exchange. PTMs act as molecular modifier codes being read by protein domains and thereby regulating nearly every cellular signaling process in a highly dynamic and controllable fashion (Hunter, 2007). These include phosphorylation, ubiquitination, acetylation, glycosylation, methylation and many more (Deribe *et al*, 2010). Until now over 200 different types of PTMs have been reported (Mann & Jensen, 2003). They assist in adapting and maintaining protein folding and stability, regulate subcellular transport and distribution as well as signal transmission. Target protein residues can be altered by different modification on the same acceptor site creating a second layer of complexity and possibility for PTM-induced protein and pathway regulation (Deribe *et al*, 2010). The occurring modifications can influence protein conformation, stability, activity, subcellular localization as well as protein-protein interactions. Therefore, the sole monitoring of DNA sequence alterations or modulations of RNA transcript levels only captures an incomplete picture of the state of protein signaling networks in health and disease (Lee & Yaffe, 2016).
The integration of cellular signaling information via PTMs, protein domains, and downstream assembly of protein complexes can be framed within the writer - reader - eraser mechanistic concept. Writer domains are catalytically active domains placing novel PTMs on proteins as for example a novel phosphorylation induced by a kinases or the ligation of ubiquitin molecules on destined target protein(s) by a designated E3 ligase (Lim & Pawson, 2010; Chi *et al*, 2010; Lee & Yaffe, 2016). Reader domains act as recognition devices of specific modification marks placed by writer domains. Classical examples include Src homology 2 (SH2) domains, binding to phosphotyrosine-containing residues, Src homology 3 (SH3) domains recognizing proline-rich sequences, polo-box domains interacting with phosphoserine or -threonine residue-containing motifs, Bromodomains recognizing acetyl-lysine-containing stretches or different

ubiquitin binding domains (e.g. UBA) recognizing single or multiple attached ubiquitin molecules (Pawson & Nash, 2003). Eraser domains close the regulatory circle instigated by writer and reader domains by removing again specific PTMs. Classical examples include the large families of phosphatases removing phosphorylation marks in a regulated manner as well as deubiquitinating enzymes (DUB) removing ubiquitin moieties from target proteins (Tonks, 2006; Shi, 2009; Komander *et al*, 2009; Nijman *et al*, 2005).

Phosphorylation is one of the first PTMs that has been discovered (Krebs & Fischer, 1955). Mainly three protein residues are targeted by phosphorylation, interestingly displaying a skewed distribution of around 85% serine, 15% threonine and only 0.4% tyrosine residues being modified (Lee & Yaffe, 2016). More recently the identification of histidine residue phosphorylation has gained interest with research tools just becoming available, enabling the detailed explorations of its biological importance (Fuhs *et al*, 2015). Protein kinases represent the writer element in phosphorylation driven signal transduction by attaching phosphate groups onto substrate proteins in an ATP-dependent manner (Ubersax & Ferrell, 2007). The human genome encodes over 500 different kinases with diverse effector functions in the regulation of cell growth, differentiation and cell death beyond many others (Manning *et al*, 2002). Due to their pleiotropic involvement in human diseases like cancer they have attracted attention to identify chemical inhibitors for therapeutic use. Phosphorylation-based signaling is counter regulated by the concerted action of eraser proteins, the protein phosphatases. They can be divided based on their phosphorylation specificity into the groups of tyrosine, serine-/threonine-specific, dual specificity and histidine phosphatases (Shi, 2009; Tonks, 2013; Patterson *et al*, 2009; Rigden, 2008). As a protein family they have overall received less attention compared to their writer counterparts, nevertheless the high frequency of cancer associated mutations as for example identified in tyrosine-protein phosphatase non-receptor type 11 (*PTPN11*) or phosphatase and tensin homolog (*PTEN*) clearly outline their importance as a critical regulatory step in the coordination of phosphorylation-driven cellular signaling (Julien *et al*, 2011). Extensive crosstalk exists in between different writer-reader-eraser systems with for example ubiquitin-mediated PTMs being in close interplay with phosphorylation (Hunter, 2007).

Ubiquitin is a small size protein of 76 aa, mostly conjugated onto lysine residues, in rare cases also onto cysteine, serine or threonine (Clague *et al*, 2015). Ubiquitin moieties can be attached as monomers, forming mono-ubiquitin modifications or as multiple ubiquitin molecules forming poly-ubiquitin chains (Rajalingam & Dikic, 2016). In the case of mono-ubiquitination, attachment is achieved via the C-terminal glycine residue of ubiquitin and a lysine residue on the target protein. In the case of poly-ubiquitination linkage at a lysine or the N-terminal methionine residue on ubiquitin itself leads to branched or linear chain formation. In contrast to phosphorylation, the placement of ubiquitin involves a coordinated enzymatic cascade of E1 enzymes leading to ATP-dependent ubiquitin activation, E2 enzymes for ubiquitin

conjugation and a broad repertoire of E3 enzyme proteins or complexes for ubiquitin ligation onto target proteins, providing modification specificity (Dikic & Robertson, 2012; Komander & Rape, 2012). E3 ligases represent the final writer step and can be categorized into three main groups: the RING E3 ligases contain either RING (really interesting new gene) or U-box domains (e.g. the monomeric RING domain-containing E3 ligase c-CBL or the family of cullin-RING ligase complexes) and present the largest subgroup, followed by HECT (homologous to the E6AP carboxyl terminus) E3 ligases (e.g. NEDD4, HERC and other HECT ligases) and RBR (RING-between RING-RING) E3 ligases (Berndsen & Wolberger, 2014; Dikic & Robertson, 2012). Marks placed by these enzymes will be read by ubiquitin-binding domain-containing proteins sensing the different structural shapes of mono- and poly-ubiquitin attachments (Dikic *et al*, 2009). The array of different types of deubiquitinating enzymes acting as eraser proteins remove these attached modifications (Nijman *et al*, 2005; Komander *et al*, 2009). Different PTMs similar to ubiquitin like small ubiquitin-like modifier (SUMO) or ubiquitin-like proteins (UBLs, e.g. NEDD8) exist, requiring similar cascades for activation and placement. The attachment of neural precursor cell expressed developmentally down-regulated protein 8 (NEDD8) is important for the activity of cullin E3 ligase complexes by recruiting E2 enzymes to the ligase complex (Hori *et al*, 1999; Kawakami *et al*, 2001). Many recent studies reveal the critical involvement of alterations in ubiquitin pathway members as crucial mediators in the pathophysiology of human diseases (Popovic *et al*, 2014). Moreover, approaches to therapeutically interfere or even harness this PTM using small molecule inhibitors are being developed (Nalepa *et al*, 2006; Winter *et al*, 2015).

As mentioned above, there is intricate cross-talk between different types of PTMs, exemplified by the role of phosphorylation and ubiquitination in the regulation of epidermal growth factor receptor (EGFR) signaling from initiation to attenuation and termination. Phosphorylation events upon ligand-induced receptor activation lead to the recruitment of adaptor proteins like growth factor receptor-bound protein 2 (GRB2) or Src homology 2 domain-containing-transforming protein 1 (SHC1) via phosphotyrosine-binding domain (PTB) or SH2 domain based interactions and son of sevenless homolog 1 (SOS1)-RAS-MAPK signaling cascade activation (Zheng *et al*, 2013). Signal attenuation and termination is subsequently achieved by the phosphorylation-induced CBL E3 ligase-mediated EGFR ubiquitination, clathrin-mediated endocytosis of ligand-receptor complex and eventual lysosomal degradation initiated upon recognition by the endosomal sorting complex required for transport (ESCRT) complex (Deribe *et al*, 2010). Finally, coordinated removal of ubiquitination moieties by dedicates deubiquitinating enzymes (DUBs), exemplified by the ubiquitin-specific peptidase 2 (USP2)-mediated delayed EGFR endocytosis, present negative regulatory feedback mechanisms for fine tuning signal duration and strength (Liu *et al*, 2013; Rose *et al*, 2016).

A second example of ubiquitination and phosphorylation collaboration on the cross-roads of cell survival, cytokine production and cell death initiation is the triggering of complex signaling wiring upon tumor necrosis factor alpha (TNF-α) binding to the tumor necrosis factor receptor 1 (TNFR1) (Vanden Berghe *et al*, 2014; Conrad *et al*, 2016). Sequential and coordinated placement of PTMs, predominantly linear and K63-linked poly-ubiquitination in the initiating step, lead to the formation of protein-complexes directing distinct cellular signaling reactions. The formation of the receptor-proximal complex I assembly induces subsequent canonical NF-κB as well as MAPK pathway activation. Alternatively, in a cell- or perturbation-dependent manner altered protein complex formation can be triggered leading to the induction of either apoptotic or necroptotic cell death. TRADD (TNFR1-associated death domain)-dependent complex IIa or RIPK1 (receptor-interacting serine/threonine kinase) 1-dependent complex IIb (the "ripoptosome") formation leads to TNF-α induced apoptosis whereas RIPK1/RIPK3/MLKL (mixed lineage kinase domain-like protein) complex IIc formation (the "necrosome") ultimately triggers execution of regulated necrosis (Vanden Berghe *et al*, 2014).

1.1.6 RAS - MAPK pathway activation in cancer

The MAPK pathway presents a paradigmatic example of protein complex-driven information transduction involving signal amplification, feedback regulation and diversification of the input information important in regulating cell growth, survival, and differentiation as well as the adaptation and appropriate integration of multiple stress stimuli (Johnson & Lapadat, 2002; Amit *et al*, 2007; Rauch *et al*, 2016). Their essential enrolment in cellular and organism physiology is reflected by the plentitude of mutations identified in developmental syndromes, cancer and disorders of the immune system (Shendure & Akey, 2015; Tidyman & Rauen, 2016; Twigg *et al*, 2013; Salzer *et al*, 2016).

The MAPK network forms a sequential kinase cascade, whereby MAPK kinase kinase (MAPKKK) phosphorylates its dedicated MAPK kinase (MAPKK) which by itself will upon phosphorylation-induced activation phosphorylate its dedicated MAPK therefore leading to a sequential signal amplification and extension (Plotnikov *et al*, 2011). These cascades can be classified based on their final MAPK into four main groups, the extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) 1/2/3, p38α/β/γ/δ and ERK5 (Johnson & Lapadat, 2002; Morrison, 2012).

The group of RAS GTPase proteins act as upstream activators of the canonical ERK1/2 MAPK pathway. They act as coordinating hub for the integration of external growth signals transduced via cell surface receptors and associated adaptor proteins. Apart from MAPK, they can in parallel induce multiple other pathways of which the PI3K, phospholipase C epsilon (PLCε), and Ral guanine nucleotide exchange factor (RalGEF) are most prominently studied due to

their importance for oncogenic transformation and cancer maintenance (Karnoub & Weinberg, 2008). There are four different main RAS gene isoforms *HRAS*, *NRAS*, and the two splice isoforms *KRAS4A* and *KRAS4B*. Activation of RAS is achieved via a set of diverse guanine nucleotide exchange factors (GEF), one of the most prominent ones being SOS1 or RAS guanyl-releasing protein 1 (RASGRP1). Structural conformation changes induced by the GEF interaction lead to the exchange of guanosine diphosphate (GDP) with guanosine triphosphate (GTP) and hence activation of RAS proteins. Inactivation is achieved via the interaction with a second set of GTPase activating proteins (GAP) that induce enhanced activation of the endogenous GTPase activitiy of RAS proteins (Vigil *et al*, 2010). One of the most prominent GAPs is Neurofibromin (NF1) that forms an important negative regulatory feedback circuit that is frequently found to be abrogated in cancer but also developmental syndromes (Ratner & Miller, 2015).

Oncogenic mutations in RAS have first been found in the Harvey sarcoma virus-associated oncogene (*HRAS*) leading to cellular transformation, (Parada *et al*, 1982; Santos *et al*, 1982). By now it is clear that mutations in the RAS gene family are one of the most common alterations found in human cancers with for example over 90% of pancreatic ductal adenocarcinoma cases displaying *KRAS* mutations (Pylayeva-Gupta *et al*, 2011; Cox *et al*, 2014). Residues G12, G13 and Q61 show the highest frequency of mutations interfering with the GTP>GDP exchange cycle maintaining RAS predominantly in an active state. The unequal distribution of activating mutations in the four main RAS isoforms in different cancer types might represent dosage dependent activation requirements in different tissues and developmental stages (Karnoub & Weinberg, 2008).

Active RAS signaling leads to the activation of members of the MAPKKK RAF proto-oncogene serine/threonine-protein kinase family (ARAF, BRAF and CRAF) which in turn induces phosphorylation of the MAPKKs dual specificity mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) and subsequent phosphorylation of the MAPKs ERK1/2. Whereas activating mutations in ARAF, CRAF and MEK1 can be found at low frequency across different types of cancer, BRAF V600E mutations are frequently found in melanoma, colorectal cancer and hairy cell leukemia (Holderfield *et al*, 2014; Arcaini *et al*, 2012). Activated ERK1/2 kinases induce the phosphorylation of target proteins both in the nucleus as well as the cytoplasm leading to the initiation of transcriptional programs as well as negative regulatory feedback loops (Plotnikov *et al*, 2011; Courtois-Cox *et al*, 2006; Avraham & Yarden, 2011). Dual-specificity phosphatases (DUSPs) play an important role for the negative feedback regulation of the MAPK signaling strength in a timely controlled fashion (Jeffrey *et al*, 2007; Shojaee *et al*, 2015). Moreover, different scaffolding proteins like kinase suppressor of Ras 1 (KSR1) have been identified assisting in the subcellular assembly of MAPK signaling complexes with a crucial

role in maintaining pathway activation (Kornfeld *et al*, 1995; Nguyen *et al*, 2002; Dhawan *et al*, 2016).

Due to its prominent role in growth factor induced and pro-survival signaling, as well as cancer cell proliferation and drug resistance, the RAS/MAPK pathway has been an area of intense research to develop therapeutic agents able to counteract enhanced activation (Steelman *et al*, 2011; Ward *et al*, 2012; Pritchard & Hayward, 2013; Samatar & Poulikakos, 2014).

1.1.7 RASopathies - germline genetic alteration of the MAPK pathway

The RASopathies denominate a heterogeneous group of developmental syndromes characterized by the presence of germline mutation alterations in members of the RAS/MAPK pathway affecting collectively around 1:1000 individuals and therefore represent one of the biggest group of malformation syndromes (Rauen, 2013). Based on their clinical-phenotypic presentation, they are subdivided into various subtypes, namely Noonan syndrome (NS), Noonan syndrome with multiple lentigines (NSML, previously named as LEOPARD syndrome), neurofibromatosis type 1, Legius syndrome (LS, previously named as neurofibromatosis 1-like), Costello syndrome (CS), hereditary gingival fibromatosis, capillary malformation-arteriovenous malformation (CM-AVM) and cardio-facio-cutaneous syndrome (CFC) (Tidyman & Rauen, 2009; Rauen, 2013; Hernández-Porras & Guerra, 2017). Their common nature in increasing MAPK pathway activation results in shared yet distinct phenotypic traits characterized by cardiovascular defects, facial dysmorphias, short stature, cutaneous lesions, neurodevelopmental defects and increased likelihood of developing cancer. Phenotypic similarities do exist in between subgroups as for example NS shares commonalities with CS and CFC (Tidyman & Rauen, 2009).

Commonly affected gene candidates include *PTPN11* (Tartaglia *et al*, 2001), *SOS1* (Roberts *et al*, 2007; Tartaglia *et al*, 2007), *SOS2* (Cordeddu *et al*, 2015), *HRAS* (Aoki *et al*, 2005), *KRAS* (Schubbert *et al*, 2006), *NRAS* (Cirstea *et al*, 2010), *RIT1* (Aoki *et al*, 2013; Chen *et al*, 2014), *RRAS* (Flex *et al*, 2014), *NF1* (Ratner & Miller, 2015), *RASA1* (Eerola *et al*, 2003), *RASA2* (Chen *et al*, 2014), *SPRED1* (Brems *et al*, 2007), *BRAF* (Niihori *et al*, 2006), *CRAF* (Pandit *et al*, 2007), *SHOC2* (Cordeddu *et al*, 2009), *CBL* (Brand *et al*, 2014), *MAP2K1* (Chen *et al*, 2014) and *LZTR1* (Yamamoto *et al*, 2015). However, NGS-based approaches are still expanding the field of mutations in known components of the MAPK pathway as well as identifying mutations in genes that have not yet been linked to function within this signaling cascade (Chen *et al*, 2014; Aoki *et al*, 2016; Tidyman & Rauen, 2016).

Importantly, a subset of the identified activating mutations represent a predisposition for cancer development (Ratner & Miller, 2015). Specifically, there is an increased likelihood for the development of juvenile myelomonocytic leukemia (JMML) in NS (Tartaglia *et al*, 2003; Aoki &

Matsubara, 2013; Niemeyer, 2014). However, on a functional biochemical level the identified mutations often display less pathway-activating potential compared to their, in cancer identified, counterpart mutations. This can be explained by the experimental finding that strong gain-of-function mutations for example in *KRAS* are associated with lethality during development indicative of a critical activity threshold level for germline variants in this genes (Schubbert *et al*, 2007). Indeed, the NS associated *KRAS* mutations KRAS V14I and T58I display milder increase in activity compared to cancer associated G12D variants (Schubbert *et al*, 2006). In addition, recent findings on germline variants in *NF1* and *PTPN11* have identified additional non-cell autonomous microenvironment-induced effects for the development of RASopathy associated cancers potentially counterbalancing for the milder activation propensity in cellular transformation (Yang *et al*, 2008; Dong *et al*, 2016).

1.2 Hematopoietic malignancies

Hematopoietic malignancies summarize the groups of heterogeneous cancer entities arising within the hematopoietic cell compartment. The oncogenic transformation of a hematopoietic stem or progenitor cell (HSPC) clone or population leads to regulated differentiation escape and uncontrolled expansion of transformed poorly-matured cells replacing normal, physiological hematopoiesis over time (Bonnet & Dick, 1997; Krivtsov *et al*, 2006; Valent *et al*, 2012). The number of hematopoietic malignancies is estimated at around 60 000 new leukemia cases as well as 24 000 estimated leukemia deaths in the United States in 2016 (leukemia statistic numbers excluding lymphoma (~81 000 new cases, 21 000 deaths) and myeloma (30 000 new cases and 12 500 deaths) cases) (Siegel *et al*, 2016). Comparable numbers can be observed in Europe (http://eco.iarc.fr/EUCAN/, November 2016) with a 5-year age-standardized overall survival for adult leukemia cases in Austria ranging from 39.8 to 45.8% (Allemani *et al*, 2015).

Hematopoietic cancers are categorized based on the remaining lineage-association of hyper proliferating cells, carrying either myeloid or lymphoid features as well as based on the disease-progression severity in chronic or acute disease types (Figure 3).

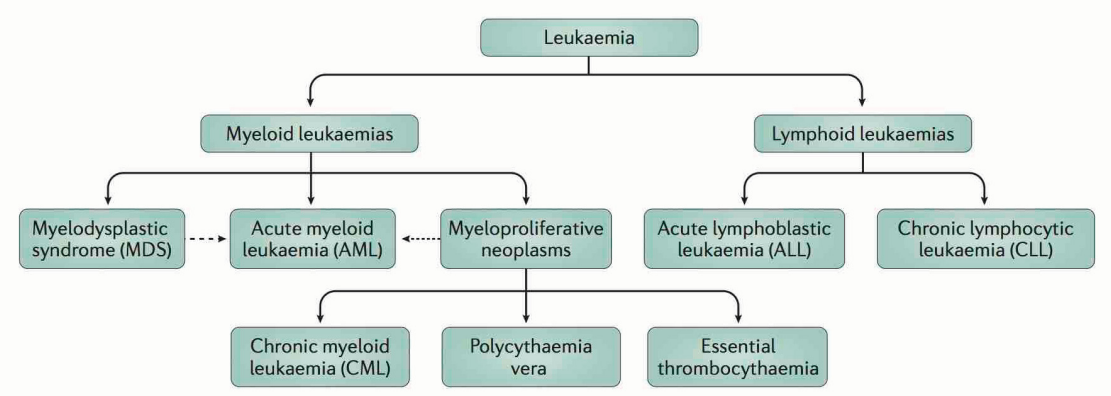

Figure 3: Tree view of subtype distribution of leukemias within the group of hematopoietic malignancies. *(Taken from Khwaja et al, 2016. Reprinted with permission from Nature Publishing Group)*

The clinical distinction of different leukemia subtypes is furthermore guided by classifications including histomorphological and cytogenetic characteristics vital for diagnosis, therapeutic decision making and risk stratification (Vardiman, 2012; Khwaja *et al*, 2016). The development of gene-expression and DNA methylation-based descriptions of malignant cells in acute myeloid leukemia (AML) or mixed lineage leukemia (MLL)-rearranged leukemia have provided first improvements in the refined definition of clinical disease states (Staudt, 2003; Valk *et al*, 2004; Armstrong *et al*, 2002; Stumpel *et al*, 2009). The application of deep sequencing studies has provided novel insights into the disease biology at unprecedented detailed resolution and

led to the recognition of known and novel disease-causing variants in AML (Mardis *et al*, 2009; Cancer Genome Atlas Research Network, 2013), T-cell acute lymphoblastic leukemia (T-ALL) (Zhang *et al*, 2012a), High-risk ALL (Roberts *et al*, 2012), hypo-diploid ALL (Holmfeldt *et al*, 2013), chronic lymphocytic leukemia (CLL) (Puente *et al*, 2011; Landau *et al*, 2015), multiple myeloma (MM) (Chapman *et al*, 2011), myelodysplastic syndrome (MDS) (Yoshida *et al*, 2011) beyond others. These findings are instructive for the improved understanding of the underlying disease biology but will undoubtedly in future years also be an irreplaceable additional layer of information for the identification and stratification of therapeutic interventions. Indeed, the NGS-based delineation of clonal variations in AML have started to shed light on the complex clonal selection processes elicited by disease progression itself as well as therapeutic interventions (Ding *et al*, 2012; Patel *et al*, 2012). These studies have clearly demonstrated that mutational alterations in multiple pathways important for growth regulatory processes (e.g. *FLT3*, *KRAS*, *NRAS*, *NF1*, *BRAF*, *PTPN11*), cytokine signaling (e.g. *JAK2*, *JAK3*, *IL7R*), cell death and cell cycle related pathways (*RB*, *TP53*, *CDKN2A/B*, *BCL2*, *BIM*) as well as genes involved in transcriptional and epigenetic regulation (*MYC*, *ETV6*, *RUNX1*, *EP300*, *EZH2*, *WT1*, *PHF6*, *MLL, NOTCH1*) ultimately lead to leukemic cell transformation. Interestingly, deep sequencing analysis have demonstrated that the increased susceptibility for developing myeloid or lymphoid leukemias due to aging hematopoiesis can be linked to gene mutations arising in epigenetic regulator proteins (e.g. *TET2*, *ASXL1*, *DNMT3A*) (Jaiswal *et al*, 2014; Shlush *et al*, 2014; Shih *et al*, 2012). Hence, the cell of origin and initiating transformation event strongly influence disease onset and biology (Huntly *et al*, 2004), progression as well as therapeutic response and prognosis (Krivtsov *et al*, 2006). Furthermore, it is becoming more and more clear that the influence of supportive signals elicited by the tumor microenvironment, leukemia initiating cells being within the bone marrow niche, are of vital importance especially with regards to potential curative treatment approaches (Valent & Deininger, 2008; Reagan & Rosen, 2016).

1.2.1 Myeloproliferative neoplasms

The main disease focus of this thesis has been on chronic myeloid leukemia (CML), which is part of the larger group of myeloproliferative neoplasms (MPN) (Arber *et al*, 2016). MPNs represent a clinically heterogeneous group of myeloid pre-leukemic disease entities. They can be divided into the set of classical MPNs, comprising *Philadelphia chromosome*-positive (Ph+) CML, and the *Philadelphia chromosome*-negative (Ph-) types polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF) (Campbell & Green, 2006; Arber *et al*, 2016). Genetic analysis have clarified associated driver mutations within the classical MPNs, namely mutations in *JAK2* leading to JAK2 V617F frequently found in PV, ET

and PMF (Kralovics *et al*, 2005; Campbell *et al*, 2005). Additionally, alterations in the thrombopoietin receptor gene (*MPL*) leading to MPL W515L have been found in ET and PMF (Pikman *et al*, 2006) and recently frameshift mutations in calreticulin (*CALR*) leading to the formation of an altered C-terminus partially utilizing 3' UTR sequences are associated with ET and PMF (Klampfl *et al*, 2013). The group of MPNs moreover comprises chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL) and mastocytosis associated with mutations in *CSF3R*, translocations involving *PDGFRA* and mutations in *KIT*, respectively (Maxson *et al*, 2013; Cools *et al*, 2003; Longley *et al*, 1996; Arber *et al*, 2016).

MPNs share the commonality of being clonal diseases with the initiating alteration(s) happening within the hematopoietic stem cell (HSC) fraction leading to dominant clonal hematopoiesis. Main hallmarks of these pre-leukemic diseases is their increased cellular expansion phenotype of mostly still terminally differentiated cells (Campbell & Green, 2006). Being chronic in the clinical presentation with long latencies and stable disease states over many years, all of the aforementioned nevertheless carry the imminent risk of progressing to an aggressive, difficult to treat secondary acute myeloid leukemia (sAML). Interestingly, these sAMLs also display distinct genetic abnormalities compared to primary *de-novo* AML (dnAML) and therefore especially present a clinically challenging disease subgroup (Milosevic *et al*, 2012).

1.2.2 Chronic myeloid leukemia

CML belongs to the group of classical MPNs, and originates from the so-called *Philadelphia chromosome* (Ph). It has been identified by P. C. Nowell and D. A. Hungerford in 1960 in Philadelphia and the formation of a balanced chromosomal translocation event between chromosome 9 and 22 (Ph+ t(9;22)(q34;q11)) has been discovered by J. D. Rowley in 1973 (Rowley, 1973; Nowell, 2007). There are about 8000 new CML cases and 1000 deaths related to CML per year in the United States (Siegel *et al*, 2016).

The in-frame fusion of parts of the Breakpoint Cluster Region (*BCR*) and Abelson Murine Leukemia Viral Oncogene Homolog 1 (*ABL1*) gene loci located on chromosome 22 and 9 respectively, leads to the formation of an oncogenic fusion protein, BCR-ABL, displaying constitutively active tyrosine kinase activity (Hantschel & Superti-Furga, 2004). Depending on the specific chromosomal breakpoint localization, three different fusion oncogenes have been identified with varying breakpoint locations and contributing length of the *BCR* gene part leading to the formation of either p185, p210 or p230 BCR-ABL isoforms, named according to their molecular weight (Melo, 1996). Whereas the p185 isoform predominates in Ph+ ALL, p210 represents the leading form in CML, but can also be found in Ph+ ALL (Ren, 2005; O'Hare *et al*, 2012). Additionally, a longer isoform, p230, has been identified in rare cases of chronic

neutrophilic leukemia (CNL) as well as CML and AML (Bertorelle *et al*, 2001; Boeckx *et al*, 2005). Beyond *BCR-ABL1*, different other *ABL1* gene fusion partners have been identified, which provide domains favoring dimerization, abrogate the autoinhibition state of WT ABL as well as activate additional downstream pathways giving rise to diverse cancer types (De Braekeleer *et al*, 2011; Hantschel, 2012).

The constitutive activity of this oncogenic kinase leads to the transformation of a hematopoietic stem cell, leading to enhanced survival signaling, proliferation and inhibition of apoptosis and terminal differentiation. The HSC-transformative capabilities of BCR-ABL have first been described in a murine model using retroviral infection of p210 BCR-ABL into HSPC. These mice developed a myeloproliferative syndrome demonstrating phenotypic features of chronic phase CML (Daley *et al*, 1990; Ren, 2005).

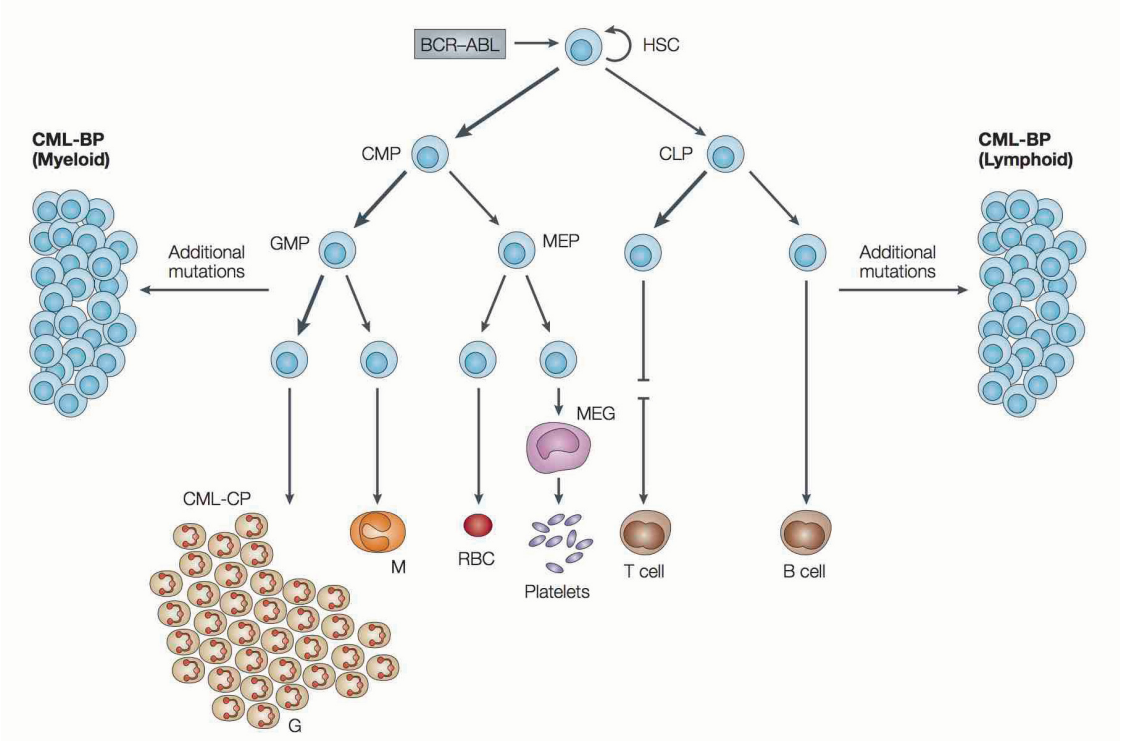

Figure 4: BCR-ABL-initiated hematopoietic cell lineage alterations leading to the development of CML. CMP - common myeloid progenitors, CLP - common lymphoid progenitors, GMP - granulocyte/macrophage progenitors, MEP - megakaryocyte/erythrocyte progenitors, G - granulocytes, M - macrophages, RBC - red blood cells, MEG – megakaryocytes. *(Taken from Ren, 2005. Reprinted with permission from Nature Publishing Group)*

Clinically CML can be categorized into three distinct stages: a chronic phase (CP) with expansion of still differentiated myeloid cells followed by, if untreated, an accelerated phase (AP) which represents a transition period into the final blast phase (crisis) (BP) characterized by massive proliferation of immature blasts and expansion of these into the peripheral circulation (Figure 4) (Melo & Barnes, 2007; O'Hare *et al*, 2012). Ph+ ALL does not progress

via the three distinct CML disease phases, can be phenotypically similar to CML lymphoid BP (Pui *et al*, 2008) and shows *ab initio* a more aggressive clinical progression with distinct additional genetic aberrations like *IKZF1*, *PAX5*, *EBF1*, *CDKN2A* and *CDKN2B* deletions rarely found in CML (Mullighan *et al*, 2008; Notta *et al*, 2011). Various different mechanisms and pathway alterations have been proposed and described leading to blast crisis progression, including differential expression of microRNAs (miRNAs), increase in DNA damage driven by BCR-ABL activity and epigenetic alterations (Perrotti *et al*, 2010; Bueno *et al*, 2008; Eiring *et al*, 2010; Makishima *et al*, 2011). Additionally, transcriptome analysis has highlighted that on a gene expression level AP and BP display very similar differential expression patterns yet clearly distinct from CP (Radich *et al*, 2006). However, much to the surprise and disappointment within the CML community, NGS-based approaches have yet failed to identify recurrent patterns of gene alterations strongly associated with CML BP development and failure of therapeutic responses (Soverini *et al*, 2015).

1.2.3 BCR-ABL driven signaling networks

Fusion of the *BCR* and *ABL1* gene loci leads to the combination of different domain features generating the hybrid oncoprotein BCR-ABL, a docking station for several SH2 and PTB domain-containing adaptors initiating the activation of multiple downstream signaling networks. As mentioned before, depending on the exact breakpoint location within the *BCR* gene, three different fusion proteins have been identified in patients, being p185, p210 and p230 (Figure 5). Whereas the ABL domain architecture remains constant in all three fusion proteins, in general only lacking the first *ABL1* coding exon, the BCR part varies marked in length and amount of contributed domains (Hantschel, 2012). The BCR portion in all three fusions consists of a coiled-coil domain (CC), a Dbl-homology (DH) and Pleckstrin-homology (PH) domain in the p210 isoform as well as an additional C2 and Rho GTPase-activating protein (RHOGAP) domain only found in the p230 fusion (Hantschel, 2012; Hantschel & Superti-Furga, 2004) (Figure 5). The ABL part encodes a tyrosine kinase (TyrK) domain amino-terminally flanked by an SH2 and SH3 domain and on the very carboxy-terminal end followed by an F-actin-binding domain (FABD) important for cytoskeletal association (Hantschel & Superti-Furga, 2004; Hantschel *et al*, 2005) (Figure 5). Negative regulatory interactions normally contributed by the ABL WT N-terminus, are replaced by the BCR-encoded CC domain that induces dimerization and oligomerization of BCR-ABL proteins leading to constitutive kinase activation and cellular transformation (Zhao *et al*, 2002; McWhirter *et al*, 1993). Furthermore, three different residues within BCR-ABL are of pronounced importance due to their contribution to kinase activity and to the initiation of downstream signaling pathways. The tyrosine (Y) 177 residue in the N-terminal BCR part has been demonstrated to critically function in the interaction with GRB2 upon phosphorylation, triggering GRB2-GRB2 associated binding

protein 2 (GAB2) binding, recruitment of SOS1 and hence RAS/MAPK pathway activation important for cellular transformation (Pendergast *et al*, 1993; Million & Van Etten, 2000; Sattler *et al*, 2002; Ren, 2005). The Y245 residue in ABL is located in the SH2 - TyrK domain linker region, displacing the second proline residue within a PxxP motif, leading to increased kinase activity upon phosphorylation (Nagar *et al*, 2003). The Y412 residue is located within the TyrK domain activation loop, is targeted via autophosphorylation leading to conformational switching between inactive and active states and therefore represents an indicative reporter of kinase activation (Dorey *et al*, 2001). Furthermore, a recent study has demonstrated that the SH2-TyrK domain interface is of critical importance for BCR-ABL and downstream STAT5 activation. Mutational or monobody-based disruption of the formed interface leads to reduced BCR-ABL activity *in vitro* and abrogates leukemogenic potential *in vivo* pinpointing a possible novel therapeutic targeting approach (Grebien *et al*, 2011). Additionally, the myristoyl-binding pocket in the ABL TyrK domain, usually in ABL WT bound by the myristoylated N-terminus important to keep the inactive state has been explored therapeutically, harnessing conformational changes upon small molecule myristoylation pocket binding (Adrián *et al*, 2006).

Whereas murine models have recapitulated some aspects of the BCR-ABL isoform specific phenotypic differences, leading to either more pronounced lymphoid or myeloid transformation, the underlying biochemical and cellular signaling differences are still until now only incompletely understood (Quackenbush *et al*, 2000). A recent comparative study has shown that BCR-ABL p185 and p210 differ in their signaling dynamics and differential abilities to activate downstream pathways like STAT3 and STAT5 as well as SRC kinases and MAPK pathways (Hantschel *et al*, 2016).

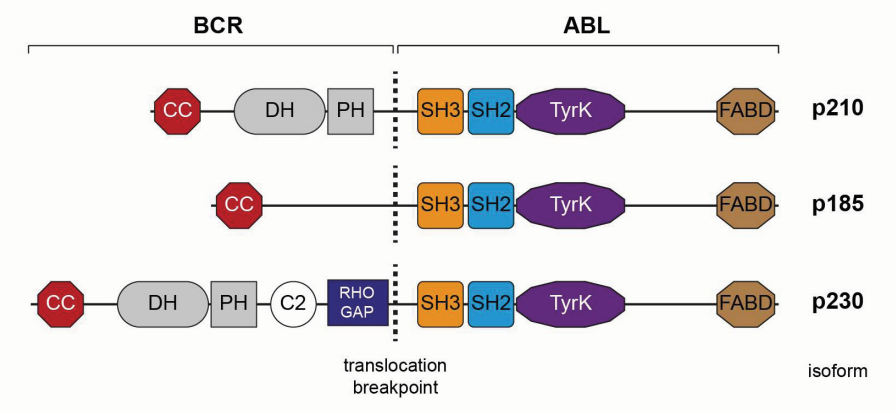

Figure 5: Protein domain arrangement present in the different BCR-ABL isoforms. CC - coiled-coil domain, DH - Dbl-homology domain, PH - Pleckstrin-homology domain, RHOGAP - Rho GTPase-activating protein domain, SH3 - Src homology 3 domain, SH2 - Src homology 2 domain, TyrK - tyrosine kinase domain, FABD - F-actin-binding domain. *(Adapted from Hantschel, 2012)*

In general, different genetic pathway requirements have been mapped over the years establishing clear evidence that activation of the RAS/MAPK (Afar *et al*, 1994; Goga *et al*, 1995), PI3K/AKT/mTOR (McCubrey *et al*, 2008) and JAK2/STAT5 (Bibi *et al*, 2014) pathways are essential for the development and maintenance of CML cells (Figure 6). Specifically, the assembly of BCR-ABL proximal adaptor proteins and phosphatases like GRB2 (Pendergast *et al*, 1993; Johnson *et al*, 2009), GAB2 (Sattler *et al*, 2002), CRK like proto-oncogene (CRKL) (Senechal *et al*, 1996; Johnson *et al*, 2009) and SHP2 (*PTPN11*) (Chen *et al*, 2007) represent essential non-redundant links for pathway activation. Interestingly, the three SRC family kinases LYN, HCK and FGR have been found to be essential for the development of Ph+ ALL but are dispensable for CML (Hu *et al*, 2004).

Activation of STAT5 represents a strong genetic dependency for the development and proliferation of CML cells (Hoelbl *et al*, 2006; Friedbichler *et al*, 2010; Walz *et al*, 2012; Hoelbl *et al*, 2010). There have been discussions in the field sparked by recent findings that BCR-ABL is capable to directly activate STAT5 neglecting JAK2 as the canonical prerequisite step (Hantschel *et al*, 2012). It is possible that STAT5 represents a convergence point of direct BCR-ABL- and cytokine-receptor JAK2-based activation fueling leukemic cell transformation and survival (Gallipoli *et al*, 2014). Interestingly, JAK2 demonstrates a limiting factor for the initiation and/or maintenance of Ph+ ALL but failed to do so in CML models, a feature shared with SRC family kinases and the *JUNB* proto-oncogene TF (Hantschel *et al*, 2012; Ott *et al*, 2007). Earlier reports have furthermore revealed that there are similarly overlapping and divergent roles of RAS/MAPK and STAT5 in Ph+ cell proliferation and drug resistance (Hoover *et al*, 2001). In summary, these insights provide first pathway-level genetic and biochemical explanations for the phenotypic differences observed in CML and Ph+ ALL disease courses as well as therapeutic responses.

Early studies based on murine models have shown that CML is a HSC-driven disease (Kavalerchik *et al*, 2008) and that these stem cells can actively contribute to reshape their bone marrow niche (Reynaud *et al*, 2011). The aberrant employment and activation of transcriptional programs driven by *MYC* (Abraham *et al*, 2016), *MYB* and *BMI1* (Waldron *et al*, 2012) represent limiting factors for the stem cell maintenance in CML but also AML and beyond (Zuber *et al*, 2011b). The concerted activation of HH signaling (Dierks *et al*, 2008), TGF-β (Naka *et al*, 2010), *PML* (Ito *et al*, 2008), *MSI1* (Ito *et al*, 2010) and *ALOX5* (Chen *et al*, 2009) in *in vivo* murine models provide evidence for the complex signaling wiring and interplay between the BCR-ABL oncogene and the cellular linage origin of CML. These requirements to maintain CML cancer stem cells provide potential explanations for the observed difficulties in long-term curative therapeutic efforts (Nicholson & Holyoake, 2009).

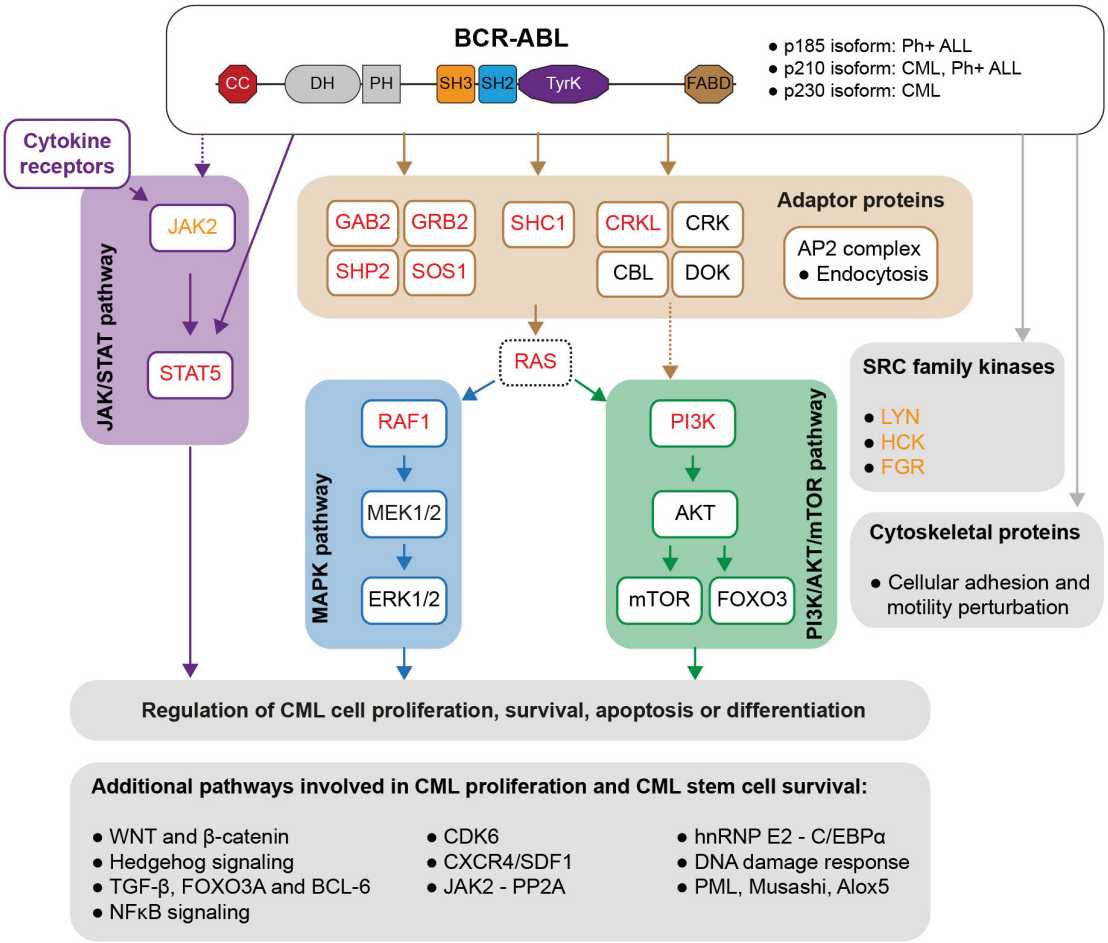

Figure 6: Signaling pathways initiated by the presence of the BCR-ABL fusion oncogene. BCR-ABL-proximal signaling proteins marked in red are essential for CML initiation and/or proliferation, proteins marked in orange are essential for Ph+ ALL initiation and/or proliferation. brown - adaptor proteins, violet - JAK/STAT pathway, blue - MAPK pathway, green - PI3K/AKT/mTOR pathway, grey - additional pathways directly involved in BCR-ABL driven signaling processes or CML stem cell survival independent of BCR-ABL. *(Adapted from Ren, 2005, Melo & Barnes, 2007, O'Hare et al, 2011, O'Hare et al, 2012, Cilloni & Saglio, 2012)*

1.3 Targeted cancer therapy

The treatment of cancer is a multidisciplinary process combining different therapeutic modalities to eliminate or prevent further growth of malignant cells leading to a potential cure or, if not achievable, prolongation of survival while preserving adequate quality of life. Surgery, radiation therapy, cancer-directed anti-neoplastic chemotherapy, hormonal therapy, modulation of the immune system to mount anti-tumor responses as well as transplantation-based approaches constitute a broad armamentarium of therapeutic tools (Savage *et al*, 2009; Chen & Mellman, 2013; Wyld *et al*, 2015; Schaue & McBride, 2015; Gharwan & Groninger, 2016; Baumeister *et al*, 2016; Khalil *et al*, 2016). Applied as single or combination regimens they are designed to combat the adaptive plasticity elicited by cancer cells upon disease evolution and during therapeutic intervention (Begg *et al*, 2011).

The unifying principle of pharmacological cancer treatment is to exploit vulnerabilities of neoplastic cells that are inherently connected to the transformed state and distinguishes them from their normal, non-transformed counter parts (Luo *et al*, 2009b). Functional signaling nodes and networks display selective importance in different cancer cell states therefore providing a therapeutic window of opportunity (Weinstein, 2002). Targeting rapid cell proliferation using conventional chemotherapy by inducing DNA damage, interfering with DNA metabolism, cytoskeletal dynamics and cell division, or radiation therapy can be cited as the first examples of targeting cancer by rationalizing on specific vulnerability traits (Luo *et al*, 2009b). The discovery of activating mutations in proto-oncogenes like *KRAS*, translocations like *BCR-ABL1* or amplification of TFs like *MYC* and their genetic requirement for cellular transformation has led to the conceptual framing of oncogene addiction (OA) (Weinstein, 2002; Felsher, 2004; Sharma & Settleman, 2007). Similarly, inactivation of TSG due to deletions, missense mutations or epigenetic silencing can represent a state of OA given the requirement that reactivation or reintroduction of the TSG WT sequence can revert the transformation phenotype (e.g. *TP53*, *APC*, *PTEN*) (Premsrirut *et al*, 2011; Dow *et al*, 2015).

On the contrary, multiple variations within the cancer genome lead to cell state and signaling network adaptations that reshuffle genetic dependencies and are referred to as non-oncogene addictions (NOA) (Solimini *et al*, 2007; Luo *et al*, 2009b). This comprises genes or whole pathways that are in comparison to classical oncogenes not or way less-frequently affected by mutational alterations themselves, however represent unsurpassable requirements for the survival and proliferation of cancer cells and less so for untransformed cells (e.g. *BRD4*, *MYB*, *STAT5*, *HSF1*) (Zuber *et al*, 2011c; 2011b; Hoelbl *et al*, 2006). NOA gene candidates can further be divided into two classes based on their mechanistic action, being tumor cell-autonomous or non-cell-autonomous involving the cellular microenvironment (Luo *et al*, 2009b).

Several OAs proved difficult to be tackled therapeutically. This can be due to either a missing or not yet identified enzymatic function or absence of any suitable pocket or cleft to tailor a small molecule for functional interference. Similarly, the oncogenic contribution of TSG deletions necessitating gene replacement for functional recovery, have led the focus on identifying potential NOA. The most extreme case of NOA-based targeted cancer therapy has been illustrated by therapeutically harnessing the genetic phenomenon of synthetic sickness/lethality (SSL) (Kaelin, 2005; Nijman, 2011; Chan & Giaccia, 2011). Whereas most of the mechanism-based therapeutic approaches aim to target within an evident therapeutic window, the genetic concept of SSL is based on the principle that the deletion of two genes is far more deleterious then the deletion of each individual gene candidate alone (Kaelin, 2005). It must be said that there is a long phenotypic gradient ranging from hard-wired genetic lethal interactions comprising core cellular machinery components to soft-wired contextual genetic lethal interactions merging with NOAs. Successful clinical application of the SSL concept has been exemplified by increased sensitivity of *BRCA1* and *BRCA2* mutant ovarian cancer (Fong *et al*, 2009) or metastatic DNA repair defect-associated prostate cancer (Mateo *et al*, 2015) to PARP1 inhibition using olaparip (Lynparza®). Moreover, the exploration of gene-deletion-induced loss of functionally redundant protein family members might create SSL-based therapeutic windows of opportunity (Muller *et al*, 2012; Nijhawan *et al*, 2012; Krönke *et al*, 2015). However, the high cellular context dependency of many discovered SSL interactions proved to be problematic in their translatability to broader clinical applications (Lord & Ashworth, 2013).

The exploitation of OA and NOA states for targeted cancer therapy follow the magic bullet concept proposed by Paul Ehrlich in a modern sense: to selectively target cancer cells in a personalized fashion with minimal side effects aiming to achieve durable responses and even cures. Such efforts have turned into reality for the first time by the development of TKI-based therapy in CML (Strebhardt & Ullrich, 2008; Druker *et al*, 1996; 2001b; Schiffer, 2007). Since then, a myriad of small molecule-based inhibitors of enzymatic functions or protein-interactions exemplified by the development of kinase inhibitors (Zhang *et al*, 2009; Dar & Shokat, 2011) or inhibitors of epigenetic regulators (Copeland *et al*, 2009; Kelly *et al*, 2010) have been identified and characterized in order to achieve cancer cell-selective induction of apoptosis, terminal differentiation and senescence, or triggering of alternate cell death pathways as the recently described forms of regulated necrosis (Labi & Erlacher, 2015; Rello-Varona *et al*, 2015).

Small molecule inhibitors for the pharmacological targeting of the cellular kinome can be categorized into five distinctive but related categories based on their molecular mode of action. ATP competitive type I inhibitors like the EGFR inhibitors erlotinib (Tarceva®) or gefitinib (Iressa®) bind to the ATP binding pocket of the kinase domain being in the active state (kinase

activation loop *"DFG-in"* conformation) whereas type II inhibitors like the BCR-ABL inhibitors imatinib (Gleevec®) or nilotinib (Tasigna®) favor the opposite condition by binding the inactive state (kinase activation loop *"DFG-out"* conformation) (Lamontanara *et al*, 2013; Wu *et al*, 2015b). In contrast, allosteric non-ATP competitive inhibitors are defined as molecules binding to the kinase domain outside of the ATP binding pocket leading to enzymatic inhibition. Depending on the distance of the compound binding site relative to the ATP binding pocket these inhibitors can be classified as type III and type IV acting as allosteric inhibitors like the MEK1/2 inhibitor trametinib (Mekinist®), or the BCR-ABL inhibitors rebastinib or GNF-2 (Wu *et al*, 2015a). Type V inhibitors utilize a combination of different binding modes (Wu *et al*, 2015b). These reversible kinase inhibitors are in contrast to the growing number of irreversible inhibitors, which attach in a covalent manner to a cysteine residue in the close proximity to the ATP binding site leading to continuous blockade of the enzyme as exemplified by the Bruton tyrosine kinase (BTK) inhibitor ibrutinib (Imbruvica®) or EGFR inhibitor afatinib (Giotrif®) (Wu *et al*, 2015b).

Advancements in the field for targeting OA addiction candidates have led to the successful clinical development of Fms related tyrosine kinase 3 (FLT3) inhibitors like midostaurin or quizartinib in AML and mastocytosis (Smith *et al*, 2012; Gotlib *et al*, 2016), JAK2 inhibitors like ruxolitinib (Jakafi®) in MPN (Verstovsek *et al*, 2010; Harrison *et al*, 2012), EGFR and Erb-B2 receptor tyrosine kinase 2 (ERBB2) inhibitors like gefitinib (Lynch *et al*, 2004), erlotinib (Shepherd *et al*, 2005) or lapatinib (Tykerb®) (Geyer *et al*, 2006; Arteaga & Engelman, 2014; Chong & Janne, 2013) for the treatment of non-small-cell lung carcinoma (NSCLC) and breast cancer, anaplastic lymphoma kinase (ALK) inhibitors like crizotinib (Xalkori®) (Kwak *et al*, 2010; Roberts *et al*, 2014; Bresler *et al*, 2014) in NSCLC, lymphoma and neuroblastoma displaying ALK activating mutations or translocations, as well as BRAF inhibitors like vemurafenib (Zelboraf®) (Flaherty *et al*, 2010) for the treatment of BRAF V600E mutant melanoma. More recently, kinase-focused targeting of the cell cycle machinery, exemplified by using the cyclin-dependent kinase (CDK) 4/6 inhibitor palbociclib (Ibrance®) in breast cancer has shown encouraging clinical results (Turner *et al*, 2015; Hortobagyi *et al*, 2016). It is furthermore worth mentioning that kinase inhibitors are increasingly utilized in non-malignant diseases such as pulmonary hypertension or in the prevention of in-stent restenosis after percutaneous coronary intervention (PCI) (Grimminger *et al*, 2010).

In contrast to the growing field of identified kinase driver oncogenes and associated inhibitors, alternative approaches have been developed by selectively targeting whole cellular processes or specific cell states altered in cancer cells reminiscent of the classical examples of anti-neoplastic chemotherapy. This includes the selective targeting of the cellular protein degradation machinery in multiple myeloma by using proteasome inhibitors like bortezomib (Velcade®) (Richardson *et al*, 2006; San Miguel *et al*, 2008), targeting protein folding using

heat shock protein 90 (HSP90) inhibitors (Whitesell & Lindquist, 2005; Ramalingam *et al*, 2015; Johnson *et al*, 2015), autophagy (Bellodi *et al*, 2009) or targeting mitochondrial translation (Škrtić *et al*, 2011) for potential NOA induced therapeutic windows of opportunities.

In the recent years, targeted or NGS-based sequencing campaigns and compound or functional genetic screening-focused approaches have uncovered the importance and opportunity for targeting proteins involved in the regulation of the epigenome as well as protein complexes involved in transcriptional regulation. This has so far led to the clinical and preclinical development of histone deacetylase (HDAC) inhibitors like panobinostat (Farydak®) (Giles *et al*, 2006; Tan *et al*, 2015) or belinostat (Beleodaq®) (Kirschbaum *et al*, 2014; Steele *et al*, 2008), DNA methyltransferase (DNMT) inhibitors like azacitidine (Vidaza®) (Fenaux *et al*, 2009; Sekeres *et al*, 2010) or decitabine (Dacogen®) (Lübbert *et al*, 2011) and mutant isocitrate dehydrogenases (IDH) 1 and 2 specific inhibitors (Rohle *et al*, 2013; Wang *et al*, 2013a). Furthermore, the clinical development of inhibitors targeting bromodomain-containing protein 4 (BRD4) (Filippakopoulos *et al*, 2010; Zuber *et al*, 2011c; Dawson *et al*, 2011; Berthon *et al*, 2016), CDK7 (Chipumuro *et al*, 2014), the histone methyltransferase DOT1L (Daigle *et al*, 2013) or enhancer of zeste homolog 2 (EZH2) (McCabe *et al*, 2012) will provide important insights whether targeting of epigenetic regulators can provide clinical benefit as single agents or need to be applied in combination.

The upcoming advancements in the field of cancer immunotherapy raise the question to which extent small molecule-based therapeutic agents can contribute to enhance the efficacy of currently developed treatment regiments in a synergistic manner (Seliger *et al*, 2010). Moreover, the attempt to engaging protein-complexes as therapeutic targets beyond enzymatic inhibition or interference with protein interactions has led to the design of a novel strategy harnessing E3 ligase protein-complexes for immunomodulatory drug (IMiD) small-molecule induced target protein degradation (Winter *et al*, 2015; Bondeson *et al*, 2015). Whereas proof of concept experiments in murine models have demonstrated the *in vivo* feasibility of this approach for different targets, the clinical applicability has still to be demonstrated.

1.3.1 Targeted therapy in chronic myeloid leukemia

Despite the fact that CML in most of the cases is diagnosed in CP with patients being asymptomatic or only demonstrating mild symptoms, CP disease without treatment inevitably progresses into AP and BP with a 3-6 months median survival (Sacchi *et al*, 1999). Initially, treatment of CML CP and AP or BP was limited to conventional chemotherapy regiments including hydroxyurea and busulfan with a 5-year survival of 38% and 44% respectively (Hehlmann, 2015). The sole curative approach consisted of chemotherapy followed by

hematopoietic stem cell transplantation (HSCT) but only available to a small, preselected and risk-stratified cohort (Pavlu *et al*, 2011). A first improvement in overall survival was the introduction of interferon-α (IFN-α)-containing treatment regimens, yet displaying only a 15-30% overall response rate and containing substantial side effects limiting the broad applicability of this modality (Hehlmann *et al*, 2003). The turning point in the standard of care for CML patients and beyond has been the development of STI-571 (imatinib, (Gleevec®)) as a first-in-class selective ABL inhibitor (Druker *et al*, 1996) (Table 1). Subsequent first clinical trials demonstrated unexpected and overwhelming therapeutic efficacies dramatically outperforming current standard treatments with even demonstrable transient activity in BP (Druker *et al*, 2001b; 2001a; Kantarjian *et al*, 2002). In contrast to prior treatments, the 5-year and 10-year survival within the randomized CML IV study on imatinib increased to 90% and 84%, respectively (Kalmanti *et al*, 2015). Treatment success has been classically assessed by overall survival but is increasingly supported by the use of prognostic scores demonstrating that age is one of the most critical prognostic factors (Sokal *et al*, 1984; Höglund *et al*, 2013). The introduction of different TKI-based therapies increasingly utilizes hematological response (HR), cytogenetic response (CR) and molecular response (MR) parameters for monitoring treatment efficacy and prognostic estimation on a phenotypic cellular level (Hughes *et al*, 2016). The achievement of complete MR (CMR) has proven to be predictive of survival and can be improved with high-dosage imatinib (Hehlmann *et al*, 2014). Long-term follow up results have demonstrated that patients treated with TKI therapy in CP have by now an overall survival rate close to the normal life expectancy (Höglund *et al*, 2013; Hehlmann, 2015). TKI-based therapy in CML nevertheless requires the administration of imatinib on a daily basis to maintain durable disease control. The stop imatinib (STIM)-1 study has assessed the consequences of treatment discontinuation in patients with undetectable minimal residual disease for 2 years and deep MR. Interestingly, with a molecular recurrence-free 5-year survival of 38% the obtained results indicated that in patients with first line deep MR to TKI therapy discontinuation can be an option under close surveillance (Etienne *et al*, 2017).

The subsequently developed 2nd generation inhibitors nilotinib (Tasigna®) (Kantarjian *et al*, 2006), dasatinib (Sprycel®) (Talpaz *et al*, 2006) and bosutinib (Bosulif®) (Puttini *et al*, 2006) have demonstrated within phase III trials a superior efficiency compared to imatinib (ENESTnd trial, (Saglio *et al*, 2010; Hochhaus *et al*, 2016)) (DASISION trial, (Kantarjian *et al*, 2010; Cortes *et al*, 2016b)) (Cortes *et al*, 2012) (Table 1). Additionally, nilotinib has further demonstrated to reduce the incidence of BCR-ABL mutations when used as first-line agent (Hochhaus *et al*, 2013). Unfortunately, no comparative trial between nilotinib and dasatinib or bosutinib for first line therapeutic choice has been performed to date.

Whereas nilotinib, dasatinib and bosutinib succeeded in combating some of the imatinib resistance conferring kinase domain mutations, both 1st (imatinib) and 2nd (nilotinib, dasatinib,

bosutinib) generation inhibitors fail in inhibiting the BCR-ABL T315I gatekeeper mutant isoform. Henceforth, ponatinib (Iclusig®) (O'Hare *et al*, 2009; Cortes *et al*, 2013) and rebastinib (DCC-2036) (Chan *et al*, 2011) have been developed as 3rd generation inhibitors specifically destined to combat resistance due to gatekeeper mutations (Table 1). Ponatinib has demonstrated clinical efficiency in CP, AP and BP, and especially in cases with BCR-ABL T315I-associated resistance as well as selected cases of Ph+ ALL (Cortes *et al*, 2013). The phase III trial assessing ponatinib as first line treatment in comparison to imatinib has been terminated due to life-threatening arterial occlusive events (Lipton *et al*, 2016). An additional phase II trial has established the efficacy of ponatinib as first line agent, however due to observed side effects its clinical application is currently limited to second line application subsequent to first line TKI failure following careful clinical evaluation and/or identification of a BCR-ABL T315I mutation (Jain *et al*, 2015). The development of rebastinib has been ended due to only modest clinical efficacy during the initial phase I trial (Cortes *et al*, 2016a).

In contrast to ponatinib, 1st and 2nd generation BCR-ABL TKIs showed on average mild and tolerated side effect profiles in many patients not interfering with the continuation of the respective clinical trials. Different degrees of myelosuppression have been observed with all four TKIs contributing to treatment interruption or discontinuation. Some side effects have solely been observed with one but not the other TKIs, like pleural effusions associated with dasatinib or pancreatitis associated with nilotinib treatment and most likely are rooted in their non-overlapping off-target profiles (Deininger & Manley, 2012).

	Compound name	Previous name	Market name	Company	Clinical trial status	CML clinical indication	Mechanism of inhibition	T315I gatekeeper mutation
1st generation	Imatinib	STI571	Gleevec®	Novartis	Approved	CP - 1st line AP, BP	ATP competition, Type II	Insensitive
2nd generation	Nilotinib	AMN107	Tasigna®	Novartis	Approved	CP - 1st line AP with resistance or intolerance to prior therapy	ATP competition, Type II	Insensitive
	Dasatinib	BMS354825	Sprycel®	Bristol-Myers Squibb	Approved	CP - 1st line AP, BP with imatinib resistance or intolerance	ATP competition, Type I	Insensitive
	Bosutinib	SKI606	Bosulif®	Pfizer	Approved	CP, AP, BC with resistance or intolerance to prior therapy	ATP competition, Type I	Insensitive
3rd generation	Ponatinib	AP24534	Iclusig®	Ariad	Approved	CP, AP, BP with T315I mutation mediated resistance	ATP competition, Type II	Sensitive
	Rebastinib	DCC2036		Deciphera	Discontinued		"switch pocket", ATP competition, Type II (?)	Sensitive
Myristoylation pocket	GNF2/GNF5			Novartis	Pre-clinical		Allosteric myristate pocket binders	Insensitive
	ABL001			Novartis	Phase I		Allosteric myristate pocket binders	Insensitive
Others	Tozasertib	VX680		Merck	Discontinued		ATP competition, Type I	Sensitive
	Danusertib	PHA739358		Nerviano Medical Sciences	Discontinued		ATP competition, Type I	Sensitive
	Axitinib	AG013736	Inlyta®	Pfizer	Phase I		ATP competition, Type I	Sensitive

Table 1: Different BCR-ABL TKIs in clinical use or in preclinical development. *(Adapted from Lamontanara et al, 2013)*

Apart from failures of TKI based therapy due to the development of resistance, non-drug adherence or TKI intolerance, the inherent insensitivity of CML stem cells albeit sustained and

efficient BCR-ABL inhibition presents a substantial clinical concern with regards to treatment duration and potential curative therapeutic intent (Wong *et al*, 2004; Corbin *et al*, 2011). Different preclinical and clinical studies are currently pursued to identify combination treatments in order to target the CML stem cell compartment. Interesting observations have been made by the combinatorial application of imatinib + IFN-α which led to more durable responses compared to single agent treatment (Preudhomme *et al*, 2010; Burchert *et al*, 2010). Preclinical observations and *in vivo* murine models have revealed diverging results with BCR-ABL signaling being involved in the differential regulation of the IFN-α surface receptor expression (Bhattacharya *et al*, 2011) as well as IFN-α playing an important role in regulating the cycling of dormant HSC within their niche (Essers *et al*, 2009). Additionally, potential immunomodulatory effects of IFN-α cotreatment cannot be excluded at the current state and further mechanistic experiments will be needed to identify the molecular basis of the observed clinical efficacies.

The use of TKI-based therapy in the treatment of Ph+ ALL cases has led to encouraging clinical trial results however not comparable to the outstanding responses observed in CML. Imatinib, nilotinib, dasatinib and ponatinib are currently used as first and second line agents in combination treatments with corticosteroids or dose-adapted chemotherapy. Whereas HR is initially achieved at high frequency, complete CR or MR is seldom reached and relapse over time occurs in many patients. Combination therapy involving BCR-ABL TKIs therefore represents an initial bridging regiment to HSCT (Malagola *et al*, 2016).

1.4 Cancer drug resistance

The development of cancer drug resistance - the unresponsiveness of neoplastic cells towards a specific therapeutic perturbation - is one of the main causes of cancer progression and associated mortality. The molecular understanding of the underlying mechanistic principles governing drug resistance is of importance for the identification of patients at risk as well as to instruct the design of combination approaches circumventing or preventing further outgrowth of malignant cells and disease progression. Each and every treatment modality, be it radiation therapy, hormonal therapy or immunomodulation has its associated mechanisms of resistance and the emphasis of the upcoming chapter will be according to the focus of this thesis on kinase targeted cancer therapy related types (Janne *et al*, 2009; Holohan *et al*, 2013).

Tumor cell populations are comprised of a complex mixture of different cell types with a mutational make up within the neoplastic fraction that is shaped following Darwinian selection processes, enriching for the most well-adapted subpopulation during disease evolution and especially upon drug treatment over time (Hanahan & Weinberg, 2000; Lambert *et al*, 2011; Valent *et al*, 2012; Stratton, 2013). The way in which tumor cells react to pharmacological perturbations can be categorized into displaying intrinsic or primary *de novo* resistance, whereby cancer cells do not respond to a given agent from the very beginning in contrast to acquired and secondary resistance, whereby neoplastic cells adapt and become insensitive over the treatment time course (Garraway & Janne, 2012; Holohan *et al*, 2013). Moreover, drug resistance can be driven by cell autonomous mechanism within the cancer cell population opposed to non-cell autonomous microenvironment-mediated support of malignant growth which also includes potential germline variants as contributing factors (Corso & Giordano, 2013; Gottesman *et al*, 2016).

Cancer cells display a high degree of plasticity in order to adapt to targeted cancer drugs to maintain cellular survival and proliferation. Different mechanisms for adjusting to pharmacological perturbation can be distinguished. First, the exposure of neoplastic cells to target-directed agents can trigger adaptive target upregulation or the development of additional mutation within the target itself, reducing inhibitor efficiency or even preventing drug binding (Lamontanara *et al*, 2013). Especially the development of mutations within the kinase domain and more specifically at the gatekeeper residue which can be found in several serine/threonine and specific tyrosine kinases. Within BCR-ABL, the threonine 315 (T315) residue forms a hydrogen bond with imatinib allowing proper inhibitor-binding in the inactive conformation. Mutation of T315 to isoleucine (I) leads to reduced spacing due to the increased side-chain bulkiness and prevents correct inhibitor binding and hence enzymatic inhibition (Lamontanara *et al*, 2013; Gorre *et al*, 2001). In general, mutations in gatekeeper residues leading to apparent clinical resistance have been identified for BCR-ABL as T315I in CML (Gorre *et al*, 2001) or for KIT as T670I in gastrointestinal stromal tumor (GIST) (Serrano *et al*, 2015) mediating

resistance towards imatinib, as well as the EGFR T790M mutation leading to gefitinib, erlotinib and lapatinib resistance (Kobayashi *et al*, 2005).

The identification of the EML4-ALK L1196M gatekeeper mutation which elucidates resistance to crizotinib has led to the development of gatekeeper-targeting alternatives. Second line treatment with alternative TKIs like lorlatinib can nevertheless trigger the development of additional resistance mutations, in this case L1198F. Interestingly, selectivity profiling has uncovered that the combination of both mutations in a unique constellation leads to crizotinib resensitization illustrating the increased complexity and adaptability of cancer cells which in some cases can be harness again therapeutically (Shaw *et al*, 2016).

Second, the activation of pathways acting downstream of the initial drug target, reactivation of upstream signaling pathways or compensatory parallel pathway activations have been identified in various cancers. Examples include the amplifications of the MET receptor tyrosine kinase (RTK) leading to EGFR inhibitor resistance in lung cancer patients as well as mutational activation of KRAS closing the therapeutic window for EGFR inhibition in CRC (Engelman *et al*, 2007; Misale *et al*, 2012). Furthermore, the upregulation of platelet-derived growth factor receptor (PDGFR), serine/threonine-protein kinase COT (COT/MAP3K8) and G-protein coupled receptors (GPCRs) or acquisition of NRAS activating mutations upon vemurafenib treatment in BRAF mutant melanoma is able to maintain MAPK pathway activation and therefore reduced inhibitor sensitivity (Nazarian *et al*, 2010; Johannessen *et al*, 2010; 2013). Globally, the integrated redundancy within signaling pathways provides cancer cells with an armamentarium to adapt appropriately to kinase inhibitors by differentially regulating alternative kinases, adaptor proteins, growth factor receptors as well as proteins involved in negative regulatory feedback loops (Prahallad *et al*, 2012; Huang *et al*, 2012; Wilson *et al*, 2015).

Third, recent years have highlighted the importance of cell lineage identity, epigenetic modifications and transcriptional regulatory processes in the adaptation towards targeted cancer drugs (Sharma *et al*, 2010). The deletion of chromatin factors like *SOX10* or *SMARCE1* have been shown to be critical nodes in the reactivation of growth factor receptor signaling via EGFR upregulation (Sun *et al*, 2014; Papadakis *et al*, 2015). Moreover, two elegant studies have demonstrated that differential WNT pathway regulation and subsequent alternate enhancer regulation can drive *MYC* activation leading to persistent *MYC* transcription and hence resistance towards JQ1 mediated BRD4 inhibition in murine models of acute myeloid leukemia (Rathert *et al*, 2015).

Fourth, alterations in drug import and export transporter pumps have long been studied in the field of resistance towards antineoplastic agents. Numerous examples exist that have implicated the family of multidrug resistance proteins (MDRs) like ABCB1 or ABCG2 as important cellular safe-guard mechanism enabling regulated export of toxic entities from within

the cell (Szakács *et al*, 2004; Mo & Zhang, 2012). More recent findings have also started to highlight the importance of the solute carrier protein family (SLCs) as limiting factors in the uptake of cancer drugs and hence therapeutic efficiency (César-Razquin *et al*, 2015). For example, genetic screening has identified a crucial role for *SLC35F2* in the cellular uptake of the DNA damage-inducing anticancer drug YM155. Diverse expression patterns of *SLC35F2* in different cancer tissues might offer a first mechanistic explanation for the poor performance of this novel compound in early stage clinical trials (Winter *et al*, 2014).

Fifth, several studies have highlighted the relevance of non-cell autonomous factors in the development of resistance towards targeted agents by providing various signals within the cancer cell microenvironment sustaining proliferation and tumor cell survival (Lovly & Shaw, 2014; Lee *et al*, 2014). The secretion of extracellular messenger factors like phosphatidylinositol-glycan biosynthesis class F protein (PIGF) (Schmidt *et al*, 2011), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) (Straussman *et al*, 2012; Wilson *et al*, 2012) or Insulin-like growth factor (IGF) (Obenauf *et al*, 2015) can lead to enhanced MAPK and PI3K pathway activation as well as cytokine secretion leading to increased JAK/STAT activation (Lee *et al*, 2014) enabling cancer cell growth.

1.4.1 Tyrosine kinase inhibitor drug resistance in chronic myeloid leukemia

The limiting factor to the long-term success of targeted therapy in CML is the inherent and acquired resistance to BCR-ABL inhibiting TKIs. In general, direct BCR-ABL-affecting, BCR-ABL-dependent and BCR-ABL-independent modes of resistance can be distinguished (Milojkovic & Apperley, 2009; Lamontanara *et al*, 2013; Soverini *et al*, 2015) (Figure 7).

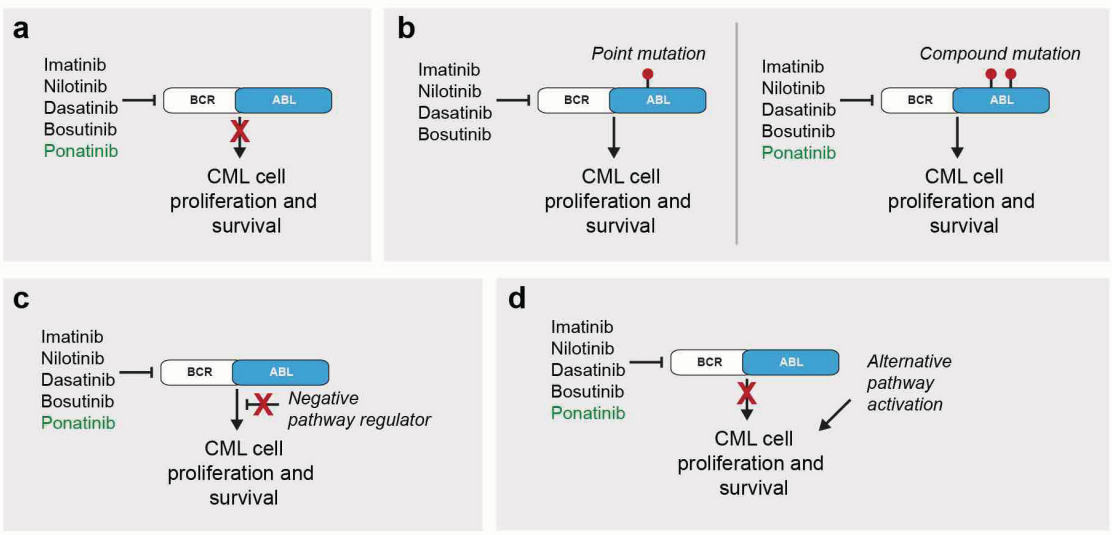

Figure 7: Different mechanisms leading to BCR-ABL TKI drug resistance. *(Adapted from O'Hare et al, 2011, O'Hare et al, 2012 and Zabriskie et al, 2014)*

The development of single point mutations in proximity to the ATP binding pocket of the ABL TyrK domain, but most prominently the T315I gatekeeper mutation, as discussed previously, have been hampering the clinical success of TKI based therapy in CML. Apart from the gatekeeper residue additional prominent spots of mutations cluster within the activation loop as well as the glycine-rich loop (Lamontanara *et al*, 2013). The head to head phenotypic characterization of recovered point mutations leading to reduced enzymatic inhibition and cellular sensitivity has propelled the assembly of selectivity-sensitivity charts like the ABLogram (O'Hare *et al*, 2007). These data demonstrate unifying resistances mediated by the T315I mutation. But, whereas glycine-rich loop residing Y253F/H and E255K/V mutations are insensitive to imatinib they still retain full to intermediate sensitivity to dasatinib treatment (O'Hare *et al*, 2007). This allows for the clinical selection of suitable second line TKIs for therapy continuation on a mechanistic basis. It is noteworthy, that biochemical evaluations have further revealed that some of the identified mutations not only reduce or prevent inhibitor binding but also increase intrinsic kinase activity with potential implications for disease progression and prognosis (Griswold *et al*, 2006).

The development of ponatinib and rebastinib as potent inhibitors of T315I mutant BCR-ABL has provided new therapeutic possibilities and novel biochemical insights into the mutational adaptability of kinase targets. In the case of ponatinib structure-based guidance has led to the incorporation of a carbon-carbon triple bond connection allowing to achieve binding in the presence of the isoleucine 315 residue, otherwise mimicking some of the binding contacts of imatinib (O'Hare *et al*, 2009). In contrast, rebastinib has been designed as a “switch-control” small molecule promoting ABL into a type II inactive conformation (Chan *et al*, 2011). Both agents have demonstrated activity against the T315I gatekeeper mutant with ponatinib being available for second line clinical use in resistant CML and Ph+ ALL patients. Furthermore, *ex vivo* patient profiling campaigns have identified the kinase inhibitor axitinib (Inlyta®) being able to selectively inhibit the gatekeeper mutant form of BCR-ABL with *in vivo* therapeutic efficacy (Pemovska *et al*, 2015). However, concerns about the clinical utility attributed to the extreme mutational selectivity creating the necessity for TKI combinations have been raised (Zabriskie *et al*, 2016).

The strong selective pressure elicited upon treatment with BCR-ABL T315I targeting TKIs like ponatinib has further triggered the development of compound mutations-based resistances, whereby two independent point mutations can be found on one kinase domain encoding allele within the *ABL1* gene (Khorashad *et al*, 2013; Zabriskie *et al*, 2014). Similar ABLogram-like functional mutation profiling efforts have revealed that non-T315I containing compound mutations do show selected sensitivity towards other 1st and 2nd generation TKIs whereas T315I-inclusive mutants remain fully resistant requiring alternative salvage therapeutic approaches (Zabriskie *et al*, 2014).

Interesting biochemical and potentially therapeutic valuable insights have been obtained by the development of allosteric inhibitors like GNF-2 and GNF-5 that are binding the myristoylation pocket present within the ABL TyrK domain, leading to conformational changes upon compound binding and hence enzymatic inhibition (Adrián *et al*, 2006). Whereas BCR-ABL T315I remains resistant to single agent GNF-2 or GNF-5 treatment, coadministration with nilotinib can lead to resensitization effects with a potential clinical implication as alternative strategy to ponatinib (Zhang *et al*, 2010b).

Besides mutational alterations, drug target upregulation mediated by enhanced transcriptional activity or locus amplification can lead to increased BCR-ABL expression levels (Gorre *et al*, 2001). Dosage adaptations or switching of TKI can be used in an attempt to counteract this kind of resistance (Rudzki & Wolf, 2011).

Deletion of negative feedback regulators as well as the upregulation or mutational activation of alternative growth and cell survival supporting factors are hallmarks of yet BCR-ABL-dependent or completely independent resistance mechanism. Early studies that have utilized CML cell lines continuously grown in increasing amounts of TKI and subsequent genomic and phosphoproteomic characterization of obtained resistant cell lines have identified LYN (Donato *et al*, 2003; Gioia *et al*, 2011), spleen tyrosine kinase (SYK) (Gioia *et al*, 2011), GAB2 (Wöhrle *et al*, 2013) and AXL RTK (Gioia *et al*, 2011) upregulation as contributing factors to reduce inhibitor sensitivity. Interestingly, AXL activation has also been observed in the resistance development towards EGFR inhibition in lung cancer (Zhang *et al*, 2012b). Moreover, increased levels of STAT5 have been identified in a murine model of BCR-ABL-mediated leukemia leading to enhance pro-survival signaling due to increased STAT5 target gene activation like *MYC*, *BCL2* and *PIM1* (Warsch *et al*, 2011). In an alternative murine model by utilizing an RNA interference (RNAi) knock-down approach, reduced expression of *TP53* has been implicated in reduced TKI sensitivity (Wendel *et al*, 2006) potentially contributing to CML BP progression in a proportion of patients (Perrotti *et al*, 2010). Gene deletions and polymorphisms have been found in the apoptosis regulatory genes *BAD* and *BIM* that correlated with reduced TKI therapy response rates (Kuroda *et al*, 2006; Ng *et al*, 2012; Faber *et al*, 2011).

Importantly, similar to many other antineoplastic agents imatinib and other BCR-ABL TKIs can be substrates of the ABCG2 drug export pump leading to reduced intracellular inhibitor concentrations and incomplete BCR-ABL inhibition (Ozvegy-Laczka *et al*, 2004; Balabanov *et al*, 2011). Additionally, a long standing controversy has been around the importance of organic cation transporter 1 (OCT1/*SLC22A1*) in the uptake of imatinib into leukemic cells thereby influencing TKI sensitivity. Whereas studies focused on cell-intrinsic mechanisms have so far failed to pinpoint an unequivocal association, clinical studies have revealed an association between the expression levels of *SLC22A1* and the response to imatinib treatment, implying

potential secondary, drug metabolizing factors or an absorption-based mechanism as explanatory models (Watkins *et al*, 2015).
Single cases have been reported to develop resistance due to the acquisition of BCR-ABL-independent single-nucleotide mutational alterations or genomic aberrations. However, no recurrent candidate gene or region has yet been identified by recent NGS-based sequencing efforts (Nadarajan *et al*, 2011; Soverini *et al*, 2015).
The bone marrow micro-environment undoubtedly represents an important support for the maintenance and survival of normal as well as leukemic stem cells and contributes inevitably to the reduced sensitivity of CML stem cells to TKI therapy (Valent & Deininger, 2008; Corbin *et al*, 2011; Chen *et al*, 2010).

Combinatorial administration of therapeutic agents has been established with the aims to enhance treatment efficiency, delay or prevent the development of resistance by perturbing alternate critical signaling pathways and at the same time diminish side-effect profiles by reduced dosing schedules of individual inhibitors (Borisy *et al*, 2003; Kummar *et al*, 2010; Knight *et al*, 2010). One of the first combination trials has been conducted in acute leukemia cases with the aim to improve therapeutic success and reduce dismal adverse events (Frei *et al*, 1958). The concept of synergistic drug combinations has thereof been explored in great detail and has proven to be a fruitful strategy in areas beyond cancer, including anti-microbial therapy and the treatment of persistent viral infections (Cokol *et al*, 2011; Bock & Lengauer, 2012). Numerous combinatorial, polypharmacology approaches are currently being tested in CML to circumvent resistance and eradicate TKI resilient CML stem cells by concomitant administration of B-Cell CLL/Lymphoma 2 (BCL2) (Goff *et al*, 2013) or HDAC inhibitors (Zhang *et al*, 2010a) as well as protein phosphatase 2A (PP2A) activation (Neviani *et al*, 2013) or activation of peroxisome proliferator-activated receptor gamma (PPARγ) (Prost *et al*, 2015). Furthermore, interference with cytokine-induced signaling is being tested by combining nilotinib and the JAK2 inhibitor ruxolitinib (Gallipoli *et al*, 2014; Traer *et al*, 2012). Moreover, a candidate approach is related to the observation that CML cell subpopulations activate autophagy as a cell survival program and hence pharmacological targeting of autophagy to enhance TKI action is currently being evaluated (Bellodi *et al*, 2009; Helgason *et al*, 2011). The development of functional genetic screening systems allows the unprecedented, detailed interrogation of single genetic requirements and combinatorial dependencies in cell lines as well as *in vivo* aiding in the process of identifying novel therapeutic targets. The corresponding technological innovations and advancements will be further discussed in the following chapters.

1.5 Phenotypic screening approaches for the identification of genes essential for cell proliferation and modulating cancer drug sensitivity

Following the concept of magic bullet treatment agents, the identification of novel cancer vulnerabilities and pharmacological protein targets requires the ability of high-throughput and -content-based screening systems. Hypothesis-driven “one-target-one-drug” at a time based approaches fail in keeping pace with the enormous complexity observed in the development and progression of human cancers. The field of chemical biology focuses on the phenotypic interrogation of cellular reactions upon chemical perturbations at large scale and has proven to be a powerful tool in the discovery of novel chemical agents with potential translation into clinical settings for various diseases (Stockwell, 2004). The obtainability of cellular material (e.g. cell lines, mouse model-derived tissue, primary patient cells) at large quantities is a prerequisite and allows for the application of diverse chemical libraries, spanning from small-scale FDA (US Food and Drug Administration) or EMA (European Medicines Agency)-approved molecule inhibitor groups to large-scale collections covering broad chemical scaffold space (Macarron *et al*, 2011; Moffat *et al*, 2014).

Large-scale compound screening campaigns focusing on cell line collections like the cancer cell line encyclopedia (CCLE) have identified well known associations but also revealed novel drug-cancer pairs and cell lineage-dependent vulnerabilities for potential clinical use (Barretina *et al*, 2012; Basu *et al*, 2013). The upcoming questions about cancer cell lines as representative surrogates has sparked the development of protocols to harness clinical samples for *ex vivo* screening therefore being closer to the clinical cancer patient (Tyner *et al*, 2008; Pemovska *et al*, 2013; van de Wetering *et al*, 2015). The resulting complexity in *ex vivo* specimens, containing both cancerous as well as non-cancerous, healthy cells created the necessity to phenotypically focus on selected cell populations as a screening read-out. This has been adapted for the identification of compounds with differential cancer stem cell (CSC) killing properties and newer high-content-based approaches are being developed at the moment (Gupta *et al*, 2009b; Hartwell *et al*, 2013).

Furthermore, the integration of drug treatment with gene expression-based profiles has been adapted in numerous projects (e.g. connectivity map) in order to stratify compound perturbations to cell type-specific signatures or patient samples in order to select subpopulations most likely to benefit from a specific treatment (Stegmaier *et al*, 2004; Ng *et al*, 2016) or attribute compound perturbation signatures to specific phenotypes as done in the connectivity map project (Lamb *et al*, 2006).

However, difficulties in the reproducibility of screening campaigns due to predominantly chemical agent discrepancies rather than cell line diversities (Haibe-Kains *et al*, 2013; Haverty

et al, 2016), annotation accuracy of chemical libraries (Arrowsmith *et al*, 2015), complexity of chemical structures representing both opportunity and challenge, and often limited reagent amounts represent obstacles to be considered. Moreover, chemical screening usually requires elaborate second-line experimental follow up strategies for target identification and validation using for example proteomic-based technologies (Rix & Superti-Furga, 2009) as well as *in vitro* purified target protein collections for enzymatic inhibition screening (Karaman *et al*, 2008). However, due to often observed polypharmacological compound action with concentration-dependent increase in off-target inhibition, narrowing down the molecular mode of action to one or even a view targets can be a challenging endeavor.

Hence, in parallel to the advancement of chemical biology, different genomic and genetic screening technology platforms have been developed in the last 10 to 15 years allowing the research community to delineate the wiring of physiological and pathological cell states on a gene level basis (Nijman, 2015) (Figure 8).

1.6 Loss-of-function genetic screening

Molecular tools for the manipulation of genomic DNA in model organisms like yeast have uncovered many fundamental cellular processes. However, the modulation of gene expression and introduction of changes into genomes of human cells has for a long time faced seemingly unsurmountable challenges. The introduction of transposon-based mutagenesis screens (Klinakis *et al*, 2000; Keng *et al*, 2009) as well as chemical mutagenesis approaches (e.g. N-ethyl-N-nitrosourea (ENU) mutagenesis (Forment *et al*, 2017)) has provided a first advancement into studying cellular pathways and phenotypes in an unbiased way. Their limitations in dynamic regulation, targeting specificity, saturation as well as recovery of mutant alleles at ease has nevertheless limited their broad application. The discovery of RNAi and subsequent adaptation as a technological tool for the regulated intervention in gene expression of mammalian cells has jump-started a new area in loss-of-function screening-based explorations of cell biology (Figure 8). This has also for the first time opened the possibility to study the requirement of individual genes for cell survival at a near genome-wide scale and enabled the first genomic interrogations for vital mediators of drug sensitivity (Mohr *et al*, 2014). The advent of zinc-finger nucleases (ZNF) (Kim *et al*, 1996; Hockemeyer *et al*, 2009) and transcription activator-like effector nucleases (TALEN) (Boch *et al*, 2009; Moscou & Bogdanove, 2009; Hockemeyer *et al*, 2011) has permitted researchers for the first time to manipulate genomic sequences at base-pair resolution in human cells. However, the complex assembly by protein-domain joining to achieve precise sequence recognition and thereby limited scalability with respect to generating complex libraries has similarly hindered their broad application in unbiased screening campaigns.

To this end, the usage of transposon and gene-trap-based targeting of genomic loci in human and murine cells has been restricted by the simple fact that the presence of each gene in two copies in a diploid genome makes the generation of complete loss-of-function alleles in complex mutagenized cell populations highly unlikely. The identification of rare subpopulations in a human hematopoietic cancer cell line displaying a near haploid karyotype (namely the CML cell line KBM-7 (Andersson *et al*, 1987)) has sparked the game-changing idea to reutilize viral gene-trap-based mutagenesis and created for the first time a fully functional genetic screening tool with unprecedented precision and reproducibility in human cells (Carette *et al*, 2009) (Figure 8). At the beginning being restricted to only two human haploid cancer cell lines, the derivation of murine (Leeb & Wutz, 2011; Elling *et al*, 2011) and later on human (Sagi *et al*, 2016) haploid embryonic stem cells has tremendously broadened the phenotypic space for gene-trap-based screening.

The limiting capabilities in cell line availability as well as single gene modification at will have been dramatically eased with the arrival of a novel revolutionary genetic precision tool kit: the clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR associated protein (Cas) technology (Doudna & Charpentier, 2014; Hsu *et al*, 2014). The discovery and adaptation of the bacterial RNA-guided nuclease system for the use in human cells has allowed to edit genomes with similar precision as ZFN or TALEN systems yet increased efficiency. The simplicity of directing the Cas nuclease protein by RNA guidance overcomes many limiting factors inherently linked to previous screening technologies and opened the door for even more sophisticated interrogation of gene functions in the culture dish as well as *in vivo* (Cox *et al*, 2015) (Figure 8).

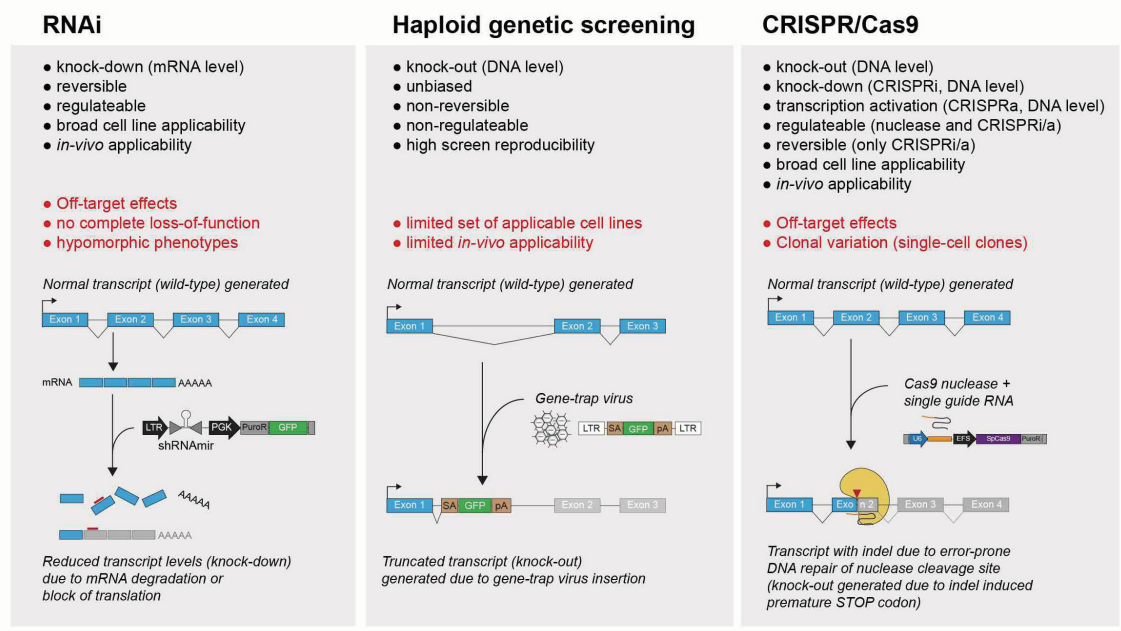

Figure 8: Overview and comparison of different genetic screening technologies. *(Adapted from Winter et al, 2009, Blomen et al, 2015 and Wang et al, 2015)*

1.6.1 RNAi-based functional genomic screening

The RNAi pathway has first been identified in *Caenorhabditis elegans* and has been awarded with the Nobel Prize to A. Z. Fire and C. C. Melo in 2006. Apart from its importance for the regulation of a multitude of biological processes, it has been adapted as a transformative technology allowing to interrogate gene function in a controllable way up to genome-wide scale in different cellular model systems *in vitro* and *in vivo*.

It is based on the mechanistic structural insight of double stranded RNA being able to regulate gene expression on a posttranscriptional level. The miRNA processing pathway forms an enzymatic cascade whereby the endonuclease DROSHA cleaves pri-miRNA into pre-miRNA, followed by XPO5-mediated nuclear export and a second DICER endonuclease-based cleavage step generating small RNA duplexes. These duplexes are in a final step bound by the AGO proteins, selecting one of the two duplex fragments, and taken up into the RNA-induced silencing complex (RISC). Subsequent posttranscriptional modulation of gene expression is achieved either by mRNA degradation or interference with protein translation (Fellmann & Lowe, 2013).

In its simplest form small interfering RNA (siRNA) or endoribonuclease-prepared siRNA (esiRNA) duplexes have been employed directly in single-well or array-format experiments whereby their direct loading into the RISC complex leads to gene suppression under transient transfection conditions (Kim *et al*, 2013).

In the following step retro- and lentiviral vectors have been adapted to stably express RNAi reagents allowing for the first time to perform complex library screens in pooled cell populations due to their traceability over time (Bernards *et al*, 2006). One strategy was the use of RNA polymerase III (Pol III) promoter-based expression of stem-loop short hairpin RNAs (shRNAs) whereby resulting shRNA transcripts would enter the miRNA pathway at the pre-miRNA stage (Brummelkamp *et al*, 2002; Paddison *et al*, 2004). In a second strategy, different endogenous miRNA scaffolds, like miR-30 or miR-155 (Winter *et al*, 2009; Fellmann & Lowe, 2013) have been adapted to express shRNA sequences within an endogenous miRNA backbone (shRNAmir) (Dickins *et al*, 2005). This arrangement allows shRNAmirs to enter the miRNA pathway similarly to its endogenous counterparts, leading to reduced off-target silencing, reduced toxicity to the endogenous miRNA metabolism and therefore unaltered endogenous miRNA regulation (Premsrirut *et al*, 2011). Recently, these reagents have been further optimized to increase silencing potency and the number of potent shRNA sequences. This has been achieved by performing reporter-based mRNA tiling assays to deduce ideal design rules to identify potent shRNA sequences (Fellmann *et al*, 2011) as well as optimized miRNA backbones like miR-E (Fellmann *et al*, 2013) or UltramiR (Knott *et al*, 2014) increasing shRNAmir processing and hence gene silencing. The additional advantage of using

shRNAmirs is their RNA polymerase II (Pol II) promoter-driven expression which allows for inducible expression *in vivo* (Dickins *et al*, 2007; Premsrirut *et al*, 2011).
Numerous pooled as well as arrayed screens have since then been performed providing novel insights into the pathway regulation and vulnerabilities of cancer. This has led for example to the identification of *Brd4* (Zuber *et al*, 2011c) and *Itgb3* (Miller *et al*, 2013) as targets in murine models of *MLL-AF9*-driven AML or the dependency of multiple myeloma cells on *IRF4* (Shaffer *et al*, 2008). Moreover, genome-wide shRNA screens have provided a draft of the gene-sets required for cancer cell proliferation and drug sensitivity (Schlabach *et al*, 2008; Luo *et al*, 2008). Additionally, the development of shRNAmir-based *in vivo* RNAi screening protocols has enabled to dissect tumor initiation and cancer cell dependencies as well as drug resistance in a native tissue context querying cell-autonomous and non-autonomous factors in models of lymphoma, hepatocellular and squamous cell carcinoma (Miething *et al*, 2014; Schramek *et al*, 2014; Rudalska *et al*, 2014).
However, doubts have remained due to several large-scale RNAi studies, as exemplified by two reports about the identification of gene vulnerabilities in KRAS mutant cancer cells, that failed to reproduce in follow-up experiments as well as similar RNAi screens displaying a substantial lack in overlapping results (Luo *et al*, 2009a; Barbie *et al*, 2009; Babij *et al*, 2011). These observations can be partially attributed to heterogeneous knock-down efficiencies, off-target silencing effects due to inaccurate shRNA processing, and overload toxic effects on the endogenous miRNA machinery (Jackson *et al*, 2003). The limitations in early generation RNAi reagents and heterogeneity in obtainable screening results has revealed the need for additional novel and precise genetic screening reagents.

1.6.2 Haploid genetic screening

Loss-of-function genetic screening capabilities offered by the haploid genome of yeast cells like *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae* have been difficult to translate into mammalian cells. Whereas in the context of a diploid genome the second allele provides a buffering fail safe mechanisms, single loss-of-function alleles in a haploid genome directly create a functional gene knock-out (KO). The identification of a nearly haploid human leukemia cell line, KBM-7 derived form a CML patient in the late ‘80s, has enabled the lab of Thijn Brummelkamp to develop a novel tool kit to perform genetic screens in human cells (Carette *et al*, 2009).
Haploid genetic screening employs a viral gene-trap cassette-containing vector in haploid human cells. Upon viral infection saturating mutagenesis is achieved by random integration of the pro-viral gene-trap sequence throughout the genome. Insertion of a gene-trap cassette, containing splice acceptor site, reporter gene (resistance marker or reporter fluorophore) and

an mRNA polyadenylation signal, within the genomic locus of a specific gene will lead to aberrant splicing and hence premature termination of the mRNA transcript leading to a functional knock-out allele. Furthermore, the gene-trap cassette is dependent on genomic integration directionality. Whereas sense orientation integrations lead to mRNA truncation, antisense orientations do not interfere with normal transcript generation providing an additional parameter for the interpretation of screening results. The integrated provirus sequence in the target cell genome provides a genetic handle to recover viral insertions. This is achieved by using inverse PCR or linear amplification-based (LAM)-PCR protocols allowing to recover mutagenic insertions on a large scale using deep sequencing, originally developed for mapping viral integrations and monitor clonal variation in gene therapy settings (Schmidt *et al*, 2007). Interestingly, analysis on unselected control cell populations have revealed and confirmed the tendency of retroviruses to integrate preferentially in the 5' prime region of the gene body and into actively transcribed genes (Wu *et al*, 2003; Carette *et al*, 2011a; Bürckstümmer *et al*, 2013). Many studies have until now employed haploid cell genetic technology primarily in positive selection screening scenarios. This has led to the identification of gene loss-of-functions important for drug sensitivity (Carette *et al*, 2009; 2011a; Birsoy *et al*, 2013). Gene-trap mutagenesis screens in HAP1 cells, an adherent haploid KBM-7 derivative cell line, have identified the intracellular cholesterol transporter Niemann-Pick C1 (*NPC1*) as the essential receptor for Ebola virus entry (Carette *et al*, 2011b). Fluorescent activated cell sorting (FACS)-assisted screens have identified novel pathway members important for the glycosylation of α-dystroglycan and thereby have helped in annotating the repertoire of genes mutated in Walker-Warburg syndrome as well as important for Lassa virus entry (Jae *et al*, 2013; 2014). The combination of FACS and gene-trap-based methods has furthermore enabled the identification of a novel protein complex involved in the epigenetic maintenance of transcriptional silencing, termed the human silencing hub (HUSH) complex consisting of transgene activation suppressor protein (*FAM208A*), M-phase phosphoprotein 8 (*MPHOSPH8*) and periphilin (*PPHLN1*) (Tchasovnikarova *et al*, 2015).

Interestingly, recent observations support the notation that positive selection screens using retroviral gene-trap mutagenesis can not only identify loss-of-function phenotypes but in selected cases also reveal gene expression activation-induced phenotypes. Dubey et al. uncovered in a doxorubicin resistance screen a significant enrichment of antisense insertions in the 5’ prime region of the gene *ABCB1*, encoding a drug efflux pump, leading to enhanced protein expression and increased cell survival upon drug treatment (Dubey *et al*, 2016).

The availability of only two different yet somehow similar human haploid cancer cell lines has limited the scope of biological questions to be addressed with this screening tool. Though, identification, isolation and characterization of murine (Leeb & Wutz, 2011; Elling *et al*, 2011) and human (Sagi *et al*, 2016) haploid embryonic stem cells has dramatically extended the

experimental space in performing genetic screens focusing for example on developmental biology or cellular differentiation phenotypes (Yilmaz *et al*, 2016). With these novel haploid cell lines and NGS analysis being available to the research community, also transposon and chemical mutagenesis reagents have regained attention in being used as genetic tool kits for genome-wide perturbation screens (Pettitt *et al*, 2013; Forment *et al*, 2017).

1.6.3 CRISPR/Cas9-based genetic screening

The initial discovery of CRISPR has been based on the observation of its role as a bacterial immune system, creating a memory of and cleaving intruding nucleic acids. This finding has been sparked by the initial sequence-based discovery of interspaced sequence repeats in the *Escherichia coli* genome (Ishino *et al*, 1987). First key insights were substantiated by the fact that these sequences are derived from exogenous phage sources or extrachromosomal stretches (Bolotin *et al*, 2005) and lie within close proximity to an expressed *cas* nuclease enzyme (Jansen *et al*, 2002). Functional experiments further established CRISPR being a genetically encoded bacterial immune system that maintains memory of previous infections by acquiring spacer sequences encoding fragments of previously faced threats (Barrangou *et al*, 2007). Different types of CRISPR/Cas systems (type I-VI) have since been identified with distinct protein components and modes of RNA-guided target DNA recognition and destruction (Wright *et al*, 2016). Key insights that sparked the development of CRISPR as a novel transformative gene editing tool in mammalian cells have come from functional characterizations of type II systems. CRISPR/Cas systems possess the ability to cut plasmid and phage DNA *in vivo* (Garneau *et al*, 2010) and the existence of a *trans*-encoded small RNA (tracrRNA) aids in the functional processing of CRISPR RNAs (crRNAs) for combating foreign nucleic acids (Deltcheva *et al*, 2011).

The discovery that the *Streptococcus pyogenes* encoded Cas9 enzyme, containing the two nuclease domains HNH and RuvC-like (Makarova *et al*, 2006), uses a tracrRNA and crRNA assembly to cleave DNA (Jinek *et al*, 2012) and furthermore requires a sequence feature, called protospacer adjacent motif (PAM) on the double-stranded target DNA for recognition-induced cleavage (Shah *et al*, 2013), has fueled its functional tool development. Collectively, these findings have furthermore opened the door for its translation to other species and subsequent work has adapted and applied CRISPR/Cas as a novel functional tool in genome engineering in eukaryotic cells (Cong *et al*, 2013; Mali *et al*, 2013; Cho *et al*, 2013). Additionally, fusion of tracrRNA and crRNA into one single guide RNA (sgRNA) allows for Watson-Crick base pair binding-enabled DNA site recognition and RNA double-strand scaffold-based Cas9 recruitment to DNA with one single RNA moiety (Jinek *et al*, 2012). Inspired by the adaptation of the type II CRISPR/Cas9 system found in *Streptococcus pyogenes,* additional CRISPR

effectors from *Staphylococcus aureus*, *Francisella novicida* and many others have been characterized in the meantime, displaying smaller protein size, altered sgRNA requirements as well as PAM sequence preferences, providing alternative tools for experimental and therapeutic genome editing approaches (Zetsche *et al*, 2015; Ran *et al*, 2015).

The development of genome engineering approaches offers the unique ability to correct preexisting nucleotide alterations causing disordered cellular and organism-wide physiology as well as construction of novel advantageous mutations in bioengineering and agricultural processes (Hsu *et al*, 2014; Cox *et al*, 2015). However, the prime application of these reagents in biomedical research is the generation of gene loss-of-functions in cell lines and *in vivo*. Endogenously-occurring, environmentally-induced or experimentally nuclease-induces DNA double strand breaks (DSB) are sensed and repaired by a complex interplay of genome-guarding repair pathways. DSBs induced by CRISPR/Cas9 cutting are predominantly substrate for the error-prone non-homologous end joining (NHEJ) repair process, leading to the insertion and deletion (indel) of small DNA stretches at a given cutting site. The introduction of these indels within the coding sequence of a given gene will lead to an alteration of the WT reading frame generating phenotypic loss-of-function alleles (Doudna & Charpentier, 2014). Providing DNA repair templates of variable length with homology to the endogenous targeted locus, which can encode distinct variants or reporter sequences, homology-directed repair (HDR) or micro-homology-mediated end-joining (MMEJ) pathways can mediate incorporation at CRISPR induced DSB sites (Yang *et al*, 2013; Suzuki *et al*, 2016).

Using CRISPR/Cas9 upon transient expression has allowed to establish different cancer and developmental models very rapidly and with unprecedented ease. The spectrum ranges from the generation of single cell knock-out or knock-in cell lines and primary cells (Gundry *et al*, 2016), generation of *in vivo* mouse models (Wang *et al*, 2013b; Xue *et al*, 2014), removal of chromosomal fragments as well as engineering of chromosomal alterations and translocations like *EML4-ALK* or *NPM1-ALK in vivo* (Maddalo *et al*, 2014). Using human stem cell-derived intestinal organoids in combination with CRISPR/Cas9 has allowed to reconstruct sequential mutation acquisition processes driving CRC development (Drost *et al*, 2015). Moreover, employment of repair template-aided modification to the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene locus in organoid cultures has model-wise provided an intriguing template for the use of CRISPR in hereditary diseases (Schwank *et al*, 2013).

The simplicity of RNA-guided recruitment of Cas nucleases and sgRNAs being the main determining factor of locus selection sparked the idea to harness vector reagents and screening systems, established in the course of RNAi technology developed. The placement of sgRNA and Cas9 expression cassettes within one or two viral vectors has enabled until now numerous targeted as well as genome-wide genetic screens displaying enhanced sensitivity and specificity in comparison to previous RNAi-based approaches (Shalem *et al*, 2014; Wang

et al, 2014b; Koike-Yusa *et al*, 2014). Along the same line, since CRISPR is an exogenous system being introduced into mammalian cells, it does not rely on endogenous RNA pathway processing in comparison to RNAi therefore providing the advantage to study the biology of many so far not amenable cellular processes (Doudna & Charpentier, 2014). Guided by exemplary studies performed to identify predictive factors for shRNAmir efficiency and to limit off-target binding mitigated events (Fellmann *et al*, 2011), several studies have identified and provided tools to incorporate important features (e.g. favoring or disfavoring of nucleotides (nt) at certain positions within the 20nt sgRNA sequence) influencing sgRNA targeting efficiency and off-target mutagenesis (Hsu *et al*, 2013; Xu *et al*, 2015; Chari *et al*, 2015; Doench *et al*, 2016). Collectively these screens have helped in the identification of novel genes being important for the vemurafenib sensitivity of BRAF V600E mutant melanoma (Shalem *et al*, 2014; Doench *et al*, 2016) or the etoposide sensitivity of human AML cells (Wang *et al*, 2014b). Furthermore, lentiviral CRISPR/Cas9 has allowed to model TSG combinations in murine models of AML (Heckl *et al*, 2014) and by harnessing the differential effect of indel formation to delineate the selective requirement of protein domains on cancer cell proliferation by CRISPR-based domain scanning approaches (Shi *et al*, 2015). In comparison to RNAi technology being dependent on post-transcriptional regulation, viral delivery of sgRNA pools targeting enhancer regions has enabled the functional annotation of these non-protein coding genomic regions for the first time (Canver *et al*, 2015).

In an effort to circumvent limitations associated with the protein size of *S. pyogenes* Cas9 (*SpCas9*), the development of Cas9 transgenic mice eases adaptation of *in vivo* screening applications, only requiring the delivery of sgRNAs to destined tissues for phenotypic interrogation (Platt *et al*, 2014).

The *S. pyogenes* Cas9 is a 1368 aa spanning multi-domain protein with the two nuclease domains, HNH and RuvC-like, being indispensable for DNA cutting (Nishimasu *et al*, 2014). Whereas the HNH domain cleaves the guide RNA complementary DNA strand, the RuvC-like domain mediates cleavage of the opposite one. Inactivating mutations in each of these two domains individually creates a nuclease derivative that mediates single strand breaks, also referred to as nickase approach (Cas9n) (Ran *et al*, 2013). Inactivation of both domains creates a nuclease-defective RNA-guided DNA binding protein (dCas9) that upon fusion with novel effector domains can dramatically expand the tool box of targeted genome modification (Jinek *et al*, 2012). Specifically, fusion of dCas9 with the Krüppel associated box (KRAB) domain for transcriptional interference (CRISPRi) or a VP16 tetrameric repeat (VP64) domain for activation (CRISPRa), respectively, has enabled to repurpose CRISPR as a time- and dosage-depend regulate able tool to control transcription in numerous cell types (Qi *et al*, 2013; Gilbert *et al*, 2014; Horlbeck *et al*, 2016). Therefore, CRISPR/Cas systems not only represent a revolutionary tool in DNA editing applications, but also for the first time allows as a genetic

programmable and unifying tool to bridge the gap between loss- and gain-of-function studies amenable to high-throughput pooled experimental setups.

1.7 Gain-of-function screening approaches enabling focused to genome-wide characterizations of cancer drug resistance

The identification of mediators and novel mechanisms leading to the development of drug resistance has for the longest time relied on culturing malignant cells with increasing inhibitor concentrations allowing for cellular drug adaptation and the subsequent collection of resistant sub clones. Comparison of these clones to the initial starting population using various different omics technologies (e.g. transcriptomics, expression proteomics) has allowed to identify proteins involved in enhanced neoplastic cell survival upon drug perturbation (Donato *et al*, 2003; Gioia *et al*, 2011; Wacker *et al*, 2012). However, the heterogeneity of hereby obtained cell pools has made the unequivocal mechanistic identification and causal relationship of individual gene candidates cumbersome.

Adaptation of viral vectors for the delivery of cDNA libraries containing different gene candidates or mutation-carrying variants into cell pools has enabled to interrogate and characterize inhibitor resistances in a more controlled fashion. A focused approach employing *in vitro* random mutagenesis of the *BCR-ABL1* cDNA and subsequent cell transduction, inhibitor treatment and sequencing-based recovery of aa variants from resistant single cell clones has revealed a broad set of mutations leading to reduced imatinib sensitivity. Convincingly, a great amount of the hereby identified BCR-ABL tyrosine kinase domain mutations have subsequently been recovered in resistant CML patients and provided the basis for mutation-informed clinical selection and stratification of different TKIs (Azam *et al*, 2003). Advancements in on-chip synthesis protocols has enabled to modify coding sequences in a more targeted manner and deep sequencing can aid substantially in the identification of enriched variants from complex mixtures of cells (Majithia *et al*, 2016).

The assembly of lentiviral open reading frame (ORF) libraries has expanded the applicability of overexpression-based screening approaches (Yang *et al*, 2011). Using these collections, important insights have been obtained about the auxiliary function of kinases like COT and TFs like microphthalmia-associated transcription factor (*MITF*) in enabling cell survival upon BRAF V600E inhibition in melanoma (Johannessen *et al*, 2010; 2013). Moreover, comprehensive sets of WT and mutant ORFs have collectively been identified in mediating resistance to targeted ALK (Wilson *et al*, 2015) or EGFR inhibition in lung cancer (Sharifnia *et al*, 2014) or pan-drug resistance in various malignant entities (Martz *et al*, 2014). Furthermore, this approach has been utilized to functionally screen vector-adapted cDNA libraries obtained from individual AML samples to identify protein variants with the potential to mediate cytokine-independent growth in murine hematopoietic cells (Yoda *et al*, 2015).

Nevertheless, sequence size constrains of viral vectors negatively impinging on packaging and transduction efficiencies, codon composition features or alterations, and cell type-specific

variabilities of employed (endogenous or exogenous) promoters, can decrease or even prevent sufficient cDNA expression levels which renders the interrogation of overexpression phenotypes challenging.

The development of dCas9-based transcription activation systems has provided a novel promising avenue implementing overexpression screens at a genome-wide level in reachable distance. Yet, preliminary results have demonstrated that, in contrast to dCas9-KRAB-mediated silencing, dCas9-VP64 induced transcription was lacking substantial dynamic strength (Konermann *et al*, 2015). Henceforth, various different technological improvements have been developed in increasing the magnitude of achievable transcriptional activation.

The first approach takes advantage of a new tag system, named SunTag, that allows the assembly of multiple antibody-effector fusion proteins along a protein scaffold tag leading to dramatic signal amplification in both transcriptional activation applications as well as imaging studies (Tanenbaum *et al*, 2014). A second approach harnessed the ability of minimal hairpin aptamers being bound by bacteriophage-derived coat protein dimers, named MS2. Incorporation of these aptamers into the 3' prime scaffold part of sgRNAs allows for the additional recruitment of MS2-fused activation domains derived from the NF-κB p65 subunit and heat-shock factor 1 (HSF1) to the dCas9-VP64 protein complex enabling strong synergistic activation (referred to as synergistic activation mediator (SAM)) (Konermann *et al*, 2015). The third approach utilizes a triple activator fusion conformation by attaching VP64, p65 and replication and transcription activator (RTA) as tandem to dCas9 (referred to as VPR) (Chavez *et al*, 2016). A following head-to-head comparison revealed increased functionality of all systems compared to dCas9-VP64 alone, with overall similar increased efficiency of the SAM, SunTag and VPR approach (Chavez *et al*, 2016). Interestingly, the ability to activate transcription of a specific gene is inversely correlated to its basal expression state (Konermann *et al*, 2015). However, whereas the combination of different dCas9-linked activation complexes does not add further benefit, placement of several sgRNAs within one gene promoter can elicit synergistic activation effects indicative that current tools are functioning in non-saturating conditions (Chavez *et al*, 2016). Additionally, a recent study has identified that incorporation of nucleosome DNA binding localization information as well as cap analysis gene expression (CAGE)-based precise transcription start site (TSS) annotations can dramatically improve sgRNA-induced gene expression activation propensity (Horlbeck *et al*, 2016).

Associated work to the establishment of the SAM and SunTag CRISPRa system have convincingly demonstrated that these tools can be used in genome-wide pooled screening campaigns to delineate phenotypic consequences of overexpression-induced genetic perturbations. Performed pilot screens have identified known and novel genes facilitating enhanced survival upon inhibition of the BRAF V600E oncogene in melanoma cells as well as

cholera-diphtheria fusion toxin induced cell killing of leukemic cells illustrating their novel unique experimental capabilities (Konermann *et al*, 2015; Gilbert *et al*, 2014).

The mechanistic understanding of gene candidates identified by above mentioned functional genetic screening approaches is greatly aided through the use of proteomic technologies that allow to detect and characterize the associated protein-protein interaction networks. Selected experimental approaches that are frequently employed by the biomedical research community will be described in more detail in the following sections.

1.8 Proteomic approaches to study protein complex regulation in cellular physiology

The attempt to systematically study the sum of all expressed proteins in a given cell, the proteome, in an unbiased way has been greatly empowered by the development of mass spectrometry (MS)-based protein identification. The advancements in mass spectrometer technologies including electrospray ionization and MS-based peptide analysis as well as further improvements in liquid chromatography (LC)-centered peptide fractionations have facilitated the identification of high, medium to even low abundant proteins in complex cell or tissue lysates (Aebersold & Mann, 2003; 2016).

Enhancements in acquisition and identification speed of MS machines has allowed to quantitatively compare the proteomes of different samples within reasonable timeframes enabling transcriptome-like proteomic studies. Importantly, it is now possible to directly study the dynamic state of signaling pathways in time and cellular space without the dependency on validated antibody reagents or using mRNA expression levels as a proxy read-out for protein dynamics (Choudhary & Mann, 2010; Kolch & Pitt, 2010). Furthermore, enrichment protocols for the identification of highly dynamic protein-linked PTMs has allowed to gain even deeper insights into the wiring and molecular underpinnings of interaction networks and pathway activation states (Altelaar *et al*, 2013; Gingras & Wong, 2016).

NGS-focused studies, "reading" the genetic code of cellular states as well as phenotypic screening approaches reveal novel, often uncharacterized gene alterations and compounds with poorly characterized mechanisms of action (MoA), creating the necessity of unequivocally defining their impact on protein function. Apart from global proteomics procedures, classical methods for protein complex identifications have been developed relying on candidate protein affinity purification coupled to MS analysis of obtained eluates. Improved protocols have been established to study and elucidate the dynamic nature of protein-protein interactions involving proximity labeling or cross-linking strategies as well as pulse-chase epitope labeling technologies (Mehta & Trinkle-Mulcahy, 2013) (Figure 9). Additionally, complementary approaches have been developed to confirm the structural basis of proteins and the related complexes they are embedded in mainly using X-ray crystallography and nuclear magnetic resonance (NMR) beyond others (Marsh & Teichmann, 2015).

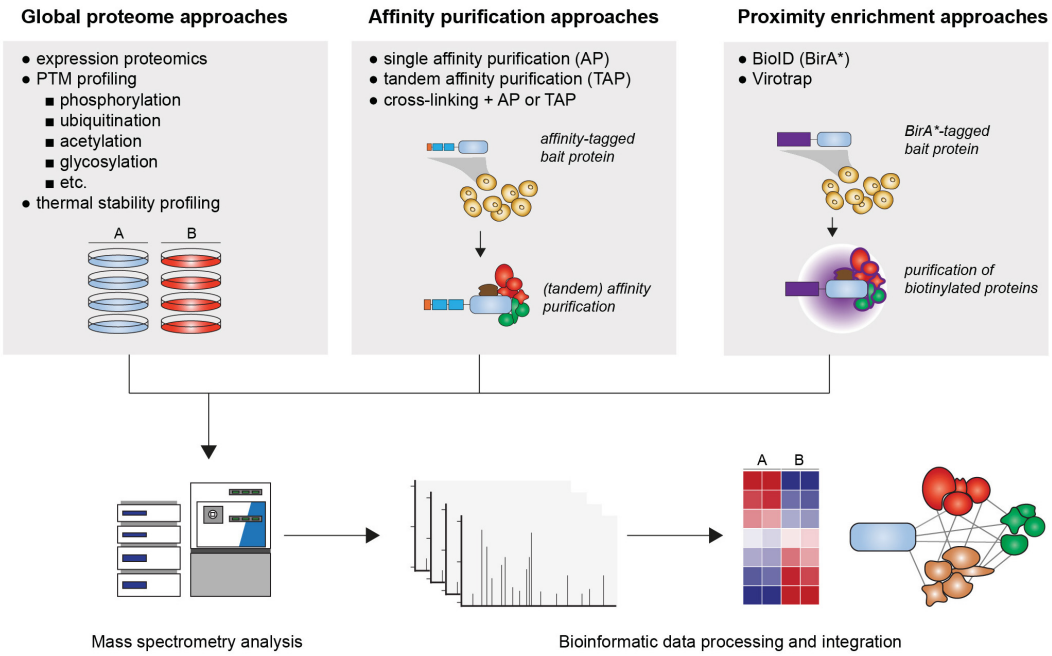

Figure 9: Overview and comparison of different proteomic work flows for the identification and quantitation of protein expression changes, PTMs and protein-protein interactions. *(Adapted from Mehta & Trinkle-Mulcahy, 2013, Rebsamen et al, 2013, and Bigenzahn et al, 2016)*

1.8.1 Global mass spectrometry-based approaches

Expression proteomics defines the MS-based undertaking of identifying (and eventually quantifying) all proteins expressed at a specific time point within a given cellular sample. Cell pools or tissue samples are disrupted in lysis buffers with application-dependent compositions and subsequently digested with one or more protease enzymes (e.g. trypsin) with preferential cleavage sites generating distinct identifiable peptide pools. LC-based peptide fractionation is directly linked to MS-based analysis, followed by bioinformatics processing of generated spectra for peptide and final protein identification (Aebersold & Mann, 2003; 2016). Whereas earlier studies were restricted by preferentially identifying highly abundant proteins, the technological improvements regarding resolution and speed of peptide identification as well as combination of different proteases for protein digestions have allowed to chart (nearly) complete proteome maps of human and murine cells providing isoform resolution (Kim *et al*, 2014; Wilhelm *et al*, 2014). This furthermore allows to identify differentially expressed proteins between different tissues and cell states (Munoz *et al*, 2011) or define their secreted protein fraction under steady state conditions or stimulation (Meissner *et al*, 2013).

Integrative attempts have established genomic- and proteomic-based characterization of cancer cell lines and primary patient material for bridging the genotype-phenotype border enabling identification of novel therapeutic targets and potential clinical biomarkers of distinct disease states (Gstaiger & Aebersold, 2009). In seminal studies this has provided the chance

to directly correlate the influence of nucleotide variants and epigenetic alterations on protein expression levels and led to the definition of distinct subtypes in comparison to transcriptome analysis in CRC (Zhang *et al*, 2014). Similarly, work on a collection of genetically characterized breast cancer samples has identified novel regulators of EGFR signaling and activation of GPCR signaling missed by transcriptome analysis (Mertins *et al*, 2016).

Essential to the comparative interrogation or cellular proteomes was the development of quantification strategies permitting assessment of protein abundance changes between samples. Different ways to evaluate changes in the proteome have been developed, of which three main types can be distinguished: label-free quantification uses spectral information to derive a measurement of quantitative abundance without the need to further introduce any artificial labeling (Asara *et al*, 2008; Li *et al*, 2012; Anand *et al*, 2017). In contrast, metabolic labeling approaches like stable isotope labeling with amino acids in cell culture (SILAC) utilize the introduction of specifically heavy labeled aa into the proteome in the culture dish for quantitation (Oda *et al*, 1999; Ong *et al*, 2002) and isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tags (TMT) labeling approaches attach balanced mass marks on digested peptides (Thompson *et al*, 2003; Ross *et al*, 2004). These quantitative methods have enabled more accurate in-between sample comparisons especially in the experimental settings of perturbation experiments using drug treatments (Cohen *et al*, 2008; Pan *et al*, 2009) or oxidative stress challenges (Vogel *et al*, 2011).

The characterization of selected PTMs has been hampered by the availability of suitable antibody reagents for modified target proteins. The aspiration for performing global, proteome-wide analysis of PTMs under normal culture conditions or upon perturbation using MS technology has stimulated research in numerous fields (Mann & Jensen, 2003). The concept of phosphoproteomics is based on the enrichment of serine and threonine as well as tyrosine phosphorylated peptides by ion metal affinity chromatography (IMAC) or antibodies respectively (Huang & White, 2008; Karisch *et al*, 2011). This has allowed for the first time to quantitatively monitor the global phosphorylation state of cells in culture and *in vivo* (Olsen *et al*, 2006; Huttlin *et al*, 2010). Unexpectedly, stimulation experiments using epidermal growth factor (EGF) or targeted T-cell receptor stimulation have revealed broad cellular changes in the phosphorylation states of proteins not only limited to receptor stimulation-proximal events (Pan *et al*, 2009; Mayya *et al*, 2009). Along the same line, drug-induced perturbations as exemplified by the treatment of BCR-ABL positive CML and ALL cells with dasatinib uncovered reduced phosphorylation events on numerous cellular processes yet not always directly connected to BCR-ABL signaling (Pan *et al*, 2009; Rubbi *et al*, 2011). Application of tyrosine phosphoproteomics in non-small-cell lung cancer (NSCLC) cell lines and primary samples allowed to characterize activation states and pinpoint important driver proteins like PDGFRα

or epithelial discoidin domain-containing receptor 1 (DDR1) involved in the oncogenic state (Rikova *et al*, 2007).

For the identification of ubiquitin based PTMs, tryptic digestion of lysates containing ubiquitinated proteins offers a unique opportunity for antibody-based enrichment due to formation of a characteristic glycine-glycine (diGly) peptide motif after tryptic digestion indicative of the covalent attachment of ubiquitin onto a given target protein (Kim *et al*, 2011; Udeshi *et al*, 2013). This approach has allowed to globally identify and quantitate the ubiquitinated state of the proteome under regular culture conditions and inhibition of the proteasome, cullin neddylation or deubiquitinating enzymes (Kim *et al*, 2011; Udeshi *et al*, 2013; Rose *et al*, 2016). Additionally, MS-based ubiquitination profiling allows for pinpointing specific lysine residues being subject of ubiquitination, providing further functional insights and entry points for biochemical validation experiments. Expectedly, similarly to phosphoproteomic analysis, inhibition of the cellular protein degradation machinery as well as EGF and B-cell receptor stimulations induce strong alterations in the ubiquitination of cellular signaling networks (Argenzio *et al*, 2011; Satpathy *et al*, 2015). Moreover, the combination of expression and ubiquitination proteomic approaches has been crucial in the elucidation of target proteins being licensed for degradation upon treatment with IMiDs like thalidomide or its analogues (Krönke *et al*, 2015; Winter *et al*, 2015).

A fairly recent addition to the armamentarium of global proteomic profiling has been the adaptation of thermal shift protein stability profiling. Based on the ability of chemical compounds or metabolites to increase the thermal stability of proteins upon binding, work from the lab of Pär Nordlund has demonstrated that this approach can be used to identify drug target proteins without the necessity for chemical modification of the examined agent (Martinez Molina *et al*, 2013). The availability of TMT quantification reagents and high-resolution global MS analysis enables the unbiased interrogation of target protein engagement within increasing temperature gradients applied to compound treated lysates, termed cellular thermal shift assay (CETSA)-MS (Savitski *et al*, 2014; Huber *et al*, 2015).

1.8.2 Interaction proteomic technologies

The conceptual appreciation that no single protein within a cell functions completely independent but rather is embedded into a web of protein-protein interactions has sparked interest early on to map these associations in an unbiased way. The identification of protein complexes purified out of their native cellular environment using affinity purification protocols followed by MS analysis has greatly enhanced our knowledge about function and modularity of interactions (Gingras *et al*, 2007; Köcher & Superti-Furga, 2007; Gingras & Wong, 2016). Interestingly, without further insight into the biology of a given protein using guilt-by-association analysis on identified interactors allows to predict potential biological functions based on the

derived embedding within the larger interactome and provides the basis for hypothesis-driven follow-up functional studies (Oliver, 2000; Köcher & Superti-Furga, 2007).

Affinity purification (AP) and tandem affinity purification (TAP) technology approaches allow the identification and characterization of protein-protein interactions in a broad variety of cellular model systems (Rigaut *et al*, 1999; Bürckstümmer *et al*, 2006; Glatter *et al*, 2009; Dunham *et al*, 2012). Whereas AP uses a single tag (e.g. FLAG, HA, V5), TAP utilizes various different tag combinations (e.g. STREP+HA (SH), FLAG+HA, Protein A+CBP) for sequential purification steps preceding MS analysis (Li, 2010; 2011). The procedure of combining different sequential affinity handles for protein purification allows to reduce the amount of identified background proteins, considered as non-specific binders. However, the TAP methodology comes at the disadvantage of reduced sensitivity for detecting transient, low-abundant and low-stoichiometric interactions (Li, 2010; 2011).

In general, both AP and TAP rely on the expression of affinity tagged bait-proteins within the cell system of relevance to the bait. Cell lysis is performed using non-denaturing conditions preserving protein-protein interactions during the purification procedure and lysates are subsequently loaded onto chromatography columns harboring a solid support matrix able to bind to the bait-attached affinity handle. Sequential wash steps are followed by a final elution in which the bait with its bound interacting prey-proteins is again released from the matrix. In the end, the purified fraction is processed by proteolytic digestion and analyzed by MS (Bürckstümmer *et al*, 2006).

Numerous studies have been performed using AP or TAP to map interactions of specific protein families like kinases, phosphatases, deubiquitinating enzymes, transcription factors, RNA binding proteins, virus-encoded proteins, fusion oncogenes, as well as proteins involved in cellular processes like autophagy, endoplasmic-reticulum-associated protein degradation (ERAD), chromatin-remodeling complexes, hippo pathway or innate immune signaling (Bouwmeester *et al*, 2004; Sowa *et al*, 2009; Brehme *et al*, 2009; Glatter *et al*, 2009; Behrends *et al*, 2010; Christianson *et al*, 2011; Li *et al*, 2011; Jäger *et al*, 2011; Pichlmair *et al*, 2012; Hauri *et al*, 2013; 2016).

Inspired by seminal work in identifying all protein complexes in yeast using TAP (Gavin *et al*, 2006; Krogan *et al*, 2006) efforts in recent years have focused on establishing a comprehensive map of the entire human protein-protein interactome mostly relying on AP methodology with the first part being completed in the year 2015 (Ewing *et al*, 2007; Hein *et al*, 2015; Huttlin *et al*, 2015).

Adapted forms of AP-based interaction proteomics have gained popularity in the field of drug discovery and compound mechanism of action studies, namely chemical proteomics (Rix & Superti-Furga, 2009; Rix *et al*, 2007) and kinobeads-based MS (Bantscheff *et al*, 2007; 2011). In the case of chemical proteomics, the bait protein is replaced with a small molecule drug,

that is chemically modified to be able to bind to a solid support matrix and therefore purify potential target proteins. The inclusion of conditions whereby excesses of unmodified compound can compete with the attached one allows to derive an estimate for binding specificity within these experimental datasets (Rix & Superti-Furga, 2009). Kinobeads-based MS makes use of solid support matrices coupled to a defined set of fairly unspecific inhibitors for a specific enzyme class (e.g. kinases, HDACs) providing close to full coverage. Incubation of cellular lysates with or without a selected kinase or HDAC inhibitor allows for the identification of specific drug binders by MS-guided competition analysis (Bantscheff *et al*, 2007; 2011).

The identification of highly complex and dense interaction data sets generates the need of statistically assessing each individual bait-prey interaction pair in their reproducibility and significance for being specific or unspecific within the entire dataset. Several bioinformatics platforms like significance analysis of interactome (SAINT) (Sardiu *et al*, 2008) or comparative proteomic analysis software suite (CompPASS) (Sowa *et al*, 2009) have been developed to aid in the analysis of proteomic experiments. An orthogonal filtering approach is offered by the recently established community-wide repository, CRAPome, which offers a collection of "negative" control purification datasets performed in various different cell lines using diverse purification technics (Mellacheruvu *et al*, 2013). Orthogonal approaches like yeast-two-hybrid screenings (Rolland *et al*, 2014) or luminescence-based mammalian interactome mapping (LUMIER) (Barrios-Rodiles *et al*, 2005; Taipale *et al*, 2012) can furthermore deliver an instrumental estimate about the binding potential of each individual human protein pair.

Proteins are highly dynamic entities, being embedded in modular complexes opening up the call to use AP/TAP-MS analysis to capture perturbation and nucleotide variation induced interactome changes (Przytycka *et al*, 2010; Bennett *et al*, 2010). Exemplary studies have started to address these questions by looking at the dynamic protein-protein interaction changes upon stimulation of the RTK adaptor protein GRB2 (Bisson *et al*, 2011) or by profiling of mutant versions of bait-proteins like CDK4 and the thereby altered interactions (Lambert *et al*, 2013; Sahni *et al*, 2015).

Limiting factors including cellular lysis, buffer conditions, salt concentrations, amount of detergents or pH can affect protein complex formation and preservation during the entire purification procedure (especially during wash steps) until tryptic digestion and MS analysis. Cross-linking approaches have been developed to "freeze" protein complexes in their native environment within the cell to enable recovery of even transient interactions using AP or TAP. These protocols can provide further insights into structural assembly features important for complex association, however can be challenging to analyze due to the difficult annotation of cross-linked peptides identified by MS (Holding, 2015).

1.8.3 Proximity-based protein interaction approaches

In order to extend beyond the interactome space covered by AP and TAP procedures novel methods have been developed on the basis of proximity labeling or trapping principles in order to capture transient interactions or associations within cellular subdomains being difficult to solubilize while preserving protein-protein interactions.

Proximity-dependent biotin identification (BioID) proteomics resembles a novel approach by adopting the bacterial enzyme BirA for labeling applications coupled to AP and MS analysis. BirA is a biotin ligase enzyme derived from *E. coli* that mediates biotinylation of target proteins involved in metabolic processes (Roux *et al*, 2012; Rees *et al*, 2015; Varnaitė & MacNeill, 2016). Whereas the original form displays restricted substrate specificity, an altered form of BirA (R118G), BirA*, enables promiscuous labeling of proteins in close proximity upon excessive supply of exogenous biotin in mammalian cells. Fusion of BirA* as an exogenous tag to a given bait protein will therefore induce biotinylation in a limited surrounding radius representative of close proximity to the enzyme-bait fusion protein (Roux *et al*, 2012). Coupling of BirA*-bait inducible expression with coordinate supply of exogenous biotin allows furthermore to fine tune the degree and magnitude of prey protein labeling in a time dependent manner (Roux *et al*, 2012; Couzens *et al*, 2013). Since the labeling is happening in cells, stringent lysis conditions can be applying allowing for enhanced protein recovery especially from disfavored subcellular compartments (e.g. membrane, nucleus). Marked bait and prey proteins are similar to AP protocols captured on streptavidin-coated beads, subjected to extensive washing and finally eluted and processed for MS analysis. Different laboratories have quickly adapted this complementary technology and recent works have illustrated especially the great value of combining in parallel AP and BirA*-mediated proximity biotinylation for the identification of novel components in the Hippo signaling pathway (Couzens *et al*, 2013), of chromatin associated protein complexes (Lambert *et al*, 2015), cullin E3 ligase substrates (Coyaud *et al*, 2015), the centrosome-cilium interface (Gupta *et al*, 2015) as well as novel phosphatase regulatory factors (St-Denis *et al*, 2016). Further vector adaptations will undoubtedly broaden the applicability of this tool kit and open up the possibility for *in vivo* applications.

In addition, a virotrap-based proteomic approach has recently been developed, expanding the scope by providing yet another complementary method for the identification of proteins in the close vicinity of specific target baits (Eyckerman *et al*, 2016). This technology is based on the usage of the HIV-1 GAG protein as a fusion tag thereby allowing trapping of protein interactors by viral particle sorting. Affinity tag-assisted recovery and purification of particle content coupled to MS analysis allows to identify protein complexes with high reproducibility. The current limitation to cytoplasmic proteins however confounds the broad applicability of this

technology (Eyckerman *et al*, 2016). Future work will be needed to address scalability of this tool towards other cellular compartments.

Enabled by numerous innovative developments described above, the interconnection of global and targeted interaction-proteomic as well as genetic screening approaches to dissect the functional wiring of protein-protein interactions and their impact on cellular physiology will be an important and prosperous playground for future research campaigns (Collins *et al*, 2007).

1.9 Aims of this thesis

Nowadays targeted therapeutic agents represent an important armamentarium in the treatment of many diseases including malignancies. In order to develop novel therapeutic molecules and increase the rate of success in subsequent clinical trials, a deep rooted mechanistic understanding of the underlying pathways and protein complexes intended to be targeted, including their involvement in pathological disease states, is of utmost importance.

Haploid genetic screens offer the opportunity to identify essential genes under standard culture conditions with unprecedented precision. In a collaborative project, the lab of Thijn Brummelkamp has mapped the repertoire of genes important for cell proliferation in different cancer cell lines. Interestingly, the cell biological function of a proportion of identified candidate essential genes was unexplainable by available data. Within this thesis, we aimed **(1)** to use TAP-MS to identify the binding partners of a preselected group of uncharacterized proteins, important for cell proliferation, in order to assigned molecular functions based on their engagement with known protein complexes.

The study of protein-protein interactions has been enabled by numerous technological innovations in the field of chromatography and mass spectrometry analysis. SH-based TAP approaches have demonstrated clear experimental benefits for the identification of protein complexes in mammalian cells, however face limitation with the current restricted subtypes of targetable cell lines available. Here within the scope of this thesis, we aimed to **(2)** further develop a new, versatile, inducible vector system to enable SH-based TAP-MS analysis in a broad range of cell lines as well as *in vivo* applications. Next **(3)** to demonstrate applicability of this toolbox in identifying interaction partners of oncogenic proteins, exemplified by the use of NRAS G12D in murine hematopoietic cells and toxic, cell-death inducing proteins, exemplified by the use of MLKL S358D in human CRC cells.

The development of targeted agents for treatment of CML patients has paved the way for a new area in cancer medicine. By using gene loss-of-function genetic screens in a haploid CML cell line we aimed **(4)** to identify the collection of genes mediating sensitivity towards small molecule-based BCR-ABL inhibition in this MPN subtype. We furthermore aimed to **(5)** characterize the cellular mode of action of identified candidate genes in their ability to modulate the cellular sensitivity to 1st, 2nd and 3rd generation BCR-ABL TKIs thereby providing a high-confidence data set for future clinical drug resistance follow-up studies.

2 Results

2.1 Prologue

The lab of Thijn Brummelkamp addressed the long standing question of identifying essential genes important for cancer cell proliferation in suspension and adherent cells under normal cell culture conditions. The adaptation of LAM-PCR-based library preparation protocols has drastically enhanced the recovery of independent gene-trap provirus integration sites from mutagenized cell populations. The high statistical power associated with the identification of 100 to 10 000 insertions per gene locus opened up the opportunity to perform negative selection screens in haploid cells. The ratio of sense and antisense gene-trap integrations becomes a direct measurement of the impact of mutagenic gene perturbation on cellular fitness. Surprisingly, within the set of recurrently identified gene candidates, resembling the “core essentialome”, a significant number remained uncharacterized with regard to their cell biological function.

Here, we inducibly expressed a preselected group of 18 essential genes in human HEK-293 cells and used TAP-MS analysis thereof to derive a first characterization of their biological function based on the identified interacting proteins. For instance, we were able to deduce a functional involvement of the transmembrane protein 258 (TMEM258/*C11orf10*) within the regulation of the oligosaccharyltransferase (OST) complex. For clarity, the bait proteins characterized by TAP-MS analysis with their identified prey proteins are listed in the following table preceding the manuscript (Table 2).

Gene name	Alternative name/protein name		Interactors identified (number, protein names)	
C1orf131	Chromosome 1 Open Reading Frame 131	NEPRO/ NET17	25	RARS, EIF5B, IARS, KARS, MYBBP1A, PPP2R1A, LRRC59, RPL23A, QARS, RPS15, DDX21, NAT10, AIMP2, PRPF40A, SNRNP200, RPL17, SMARCC1, DDX18, PPP2R2A, EPRS, EIF2S3, IQGAP1, PRPF8, EIF3L, SMARCB1
C3orf17	Chromosome 3 Open Reading Frame 17		19	POP1 , UBE2O, NPM1, RPP38, RPLP2, RPP25L, EMD, HIST1H1C, YBX1, POP7, PPM1G, NAP1L1, ATP1A1, LDHB, RPL27A, ATP2A2, RPL13, RPP30, RPL28
TMEM258	Transmembrane Protein 258	C11orf10	16	RPN1, RPN2, STT3A, DDOST, STT3B, MAGT1, MLEC, C4orf32, DAD1, SCCPDH, FKBP8, CCDC167, PTPLAD1, HS2ST1, STX12, FUT8
FAM210A	Family With Sequence Similarity 210 Member A	C18orf19	7	COPA, COPB2, RCN2, COPE, MYL6, DNAJA2, LDHB
C21orf59	Chromosome 21 Open Reading Frame 59	CILD26/ FBB18/ Kur	2	CTNNA1, CTNNB1
FAM204A	Family With Sequence Similarity 204 Member A	C10orf84	6	HAT1, KPNB1, NPEPPS, KPNA2, KPNA3, COPG1
SPATA5	Spermatogenesis Associated 5	AFG2/ SPAF/ EHLMRS	9	SPATA5L1, CINP, C1orf109, WBSCR16, PFDN5, ATAD3A, RCN2, AIFM1, PFDN2
TTC27	Tetratricopeptide Repeat Domain 27		13	EFTUD2, SNRNP200, PRPF8, CCT5, CCT6A, CCT4, CCT7, CCT2, AAR2, TCP1, CCT3, NCDN, ECD
LENG1	Leukocyte Receptor Cluster (LRC) Member 1		6	MARS, RARS, DARS, IARS, LARS, AIMP1
NHLRC2	NHL Repeat Containing 2		1	ERC1
ZNF207	Zinc Finger Protein 207	HBuGZ	8	BUB3, HSPH1, ZNF207, PUF60, SRSF11, DPY30, S100A9, SETD1A
DIEXF	Digestive Organ Expansion Factor Homolog (Zebrafish)	C1orf107/ DEF/ UTP25	7	AP2A1, PSME3, AP2B1, MPHOSPH10, AP2A2, AP2S1, IMP3
ARMC7	Armadillo Repeat Containing 7		5	CKAP4, RBM48, MPRIP, PHB, PHB2
C9orf78	Chromosome 9 Open Reading Frame 78	HCA59/ HSPC220	3	EFTUD2, SNRNP200, PRPF8
WBSCR22	Williams-Beuren Syndrome Chromosome Region 22	MERM1/ WBMT	2	WBSCR22, TRMT112
NLE1	Notchless Homolog 1 (Drosophila)		5	CCT2, CCT4, CCT6A, CCT5, CCT7
CEP85	Centrosomal Protein 85	CCDC21	4	PSMA7, PSMB5, PSMC2, PSMA6
METTL16	Methyltransferase Like 16	METT10D	2	MEPCE, KPNA6

Table 2: Protein interactions identified by TAP-MS analysis of essential proteins identified in the „core essentialome“ with unknown cellular function. *(Adapted from Blomen et al, 2015)*

2.2 Manuscript #1

Blomen VA*, Májek P*, Jae LT*, Bigenzahn JW, Nieuwenhuis J, Staring J, Sacco R, van Diemen FR, Olk N, Stukalov A, Marceau C, Janssen H, Carette JE, Bennett KL, Colinge J[#], Superti-Furga G[#] & Brummelkamp TR[#] (2015) Gene essentiality and synthetic lethality in haploid human cells. Science 350: 1092–1096
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A detailed description of the individual author contributions can be found within the declaration section of this doctoral thesis.

Gene essentiality and synthetic lethality in haploid human cells

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Abstract

Although the genes essential for life have been identified in less complex model organisms, their elucidation in human cells has been hindered by technical barriers. We use extensive mutagenesis in haploid human cells to identify approximately 2,000 genes required for optimal fitness under culture conditions. To study the principles of genetic interactions in human cells we created a synthetic lethality network focused on the secretory pathway based exclusively on mutations. This revealed a genetic crosstalk governing Golgi homeostasis, an additional subunit of the human oligosaccharyltransferase complex, and a Phosphatidylinositol 4-Kinase Beta adaptor hijacked by viruses. The synthetic lethality map parallels observations made in yeast and projects a route forward to reveal genetic networks in diverse aspects of human cell biology.

Main Text

Single cell organisms can tolerate inactivating mutations in the majority of genes (*1-3*), but it is unclear whether human cells require more essential genes due to increased complexity, or fewer, due to added redundancy. To study this we used mutagenesis in the near-haploid chronic myeloid leukemia (CML) cell line KBM7 (karyotype 25, XY, +8, Ph+), and its non-hematopoietic derivative HAP1 which is haploid for all chromosomes (fig. S1A) (*4*). More than 34.3 million and 65.9 million gene-trap integrations were identified in KBM7 and HAP1 cells, respectively. The employed gene-trap vector was unidirectional by design (fig. S1B) and for most genes the number of intronic integrations in the sense direction was similar to that in the antisense direction (e.g. pro-apoptotic factor *BBC3*, Fig. 1A and B) (*5-7*). For a fraction of genes, however, disruptive mutations were underrepresented, indicative of impaired fitness: some genes (e.g. *STAT5B*) appeared essential in one cell type (fig. S1C), whereas others in both (such as *RPL13A)* (Fig. 1A and B and tables S1-3).

2,054 genes in KBM7 cells (table S1, figs. S2-3) and 2,181 genes in HAP1 (table S2, figs. S2-3) appeared to be needed for viability or optimal fitness under the experimental growth conditions (referred to as 'essential' although the approach does not distinguish between the two). The 1,734 genes identified in both cell lines were designated as 'core essentialome' (table S3). Importantly in KBM7 cells, genes on chromosome 8 (present in two copies) tolerated disruptive mutations, underscoring the specificity of the approach (fig. S1D). Furthermore, nearly all subunits of the proteasome were identified as essential (fig. S4). In general, essential genes are overrepresented in categories such as translation or transcription but not signaling (Fig. 1C, fig. S5 and S6).

Many genes required for fitness in yeast were also essential in human cells. Exceptions were largely explained by paralogs in the human genome or by yeast-specific requirements (fig. S7A and table S4) (*1*). We estimated the evolutionary age of essential genes and found that 77% emerged in pre-metazoans ('*old*' essential genes) (fig. S7B). Essential genes had fewer paralogs and higher protein abundance and contained fewer single nucleotide polymorphisms (SNPs) predicted to impair function (Fig. 1D). Proteins encoded by essential genes displayed more protein-protein interactions (fig. S8A-D) and these occurred more frequently with other essential proteins (49.8%) (fig. S9A) and within the same functional category (fig. S5B). Remarkably, the products of *new* essential genes are more often connected with *old*

rather than other *new* essential gene products, suggesting that they largely function within ancient molecular machineries (fig. S9B and C).

To identify proteins interacting with products of 18 uncharacterized essential genes we used tandem affinity purification coupled to mass spectrometry (fig. S10). Interactors were frequently essential proteins (52.4%, P<2.5E-36, hypergeometric test) involved in processes like splicing, translation, and trafficking (fig. S11, and table S5). The small transmembrane protein TMEM258 associated with components of the conserved oligosacharyltransferase (OST) complex (Fig. 2A and fig. S12A) essential for protein N-glycosylation (*8*). TMEM258 localized to the endoplasmic reticulum (fig. S12B) and depletion (fig. S12C-D) impaired OST catalytic activity as monitored by hypoglycosylation of prosaposin (Fig. 2B) (*9*). This also rationalizes the observed clustering of TMEM258 with OST complex subunits in a recent genetic screen (*10*). Thus, TMEM258 constitutes a subunit of the human OST complex and although homology searches (fig. S12E) do not identify a yeast ortholog, TMEM258 may relate to the similarly sized yeast transmembrane protein OST5 (*11*).

Whereas most genes appear nonessential, their function may be buffered by other genes such that only simultaneous disruption is lethal (*12-15*). The frequency of such synthetic lethal interactions between human genes is debated and challenging to address experimentally (*16, 17*). We studied the small guanosine triphosphatases (GTPases) RAB1A and RAB1B, by creating individual knockout lines and assessing the genes needed for fitness in these backgrounds (Fig. 3A and fig. S13A). Whereas neither *RAB1A* nor *RAB1B* were essential in wild-type cells, *RAB1A* became indispensable in *RAB1B* knockout cells and vice versa (Fig. 3A and fig S13B). To explore the breadth of synthetic lethality we probed the secretory pathway using three independent knockout cell lines (fig. S14) for *RAB1A, RAB1B, GOSR1* (a subunit of the Golgi SNAP receptor), (*18*) and *TMEM165* (a Golgi-resident Ca^{2+}/H^{+} antiporter whose deficiency impairs glycosylation) (*19*) (Fig. 3B, figs. S15-16, and table S6). Most of their genetic interactions impinged on the secretory pathway (Fig. 3B and table S7) and many were found synthetic lethal with *PTAR1*. Synthetic lethality screens in PTAR1 deficient cells confirmed these genetic interactions and additionally identified the uncharacterized gene *C10orf76* (Fig. 3B and fig. S17A). Validation using *C10orf76* as query gene confirmed synthetic lethality with *PTAR1*, and (reciprocally) identified *TSSC1,* which was recently reported to interact with the Golgi associated retrograde protein complex (GARP) (*20*) (Fig. 3B). The human genes we studied display on

average ~20 synthetic lethal interactions, a number comparable to that in yeast (*12*), although this varies between genes, with *PTAR1* (causing a fitness defect when deleted alone) having close to 60 interactions (fig. S17B). This illustrates that synthetic lethal interactions can be identified and validated using reciprocal haploid screens and that, similar to yeast, interactions frequently occur between genes whose products act in related processes (fig. S17B) (*13, 16*). However, we acknowledge a caveat that this approach cannot readily distinguish between synthetic lethal or synthetic 'sick' interactions.

The impaired growth of *PTAR1*-deficient cells (table S2) was suppressed by loss of the Golgi factor GOLGA5 (*21*) (Fig. 4A, fig. S18). *PTAR1*-deficient cells had an abnormally dilated Golgi morphology (fig. S19A) which was partially corrected by co-deletion of *GOLGA5* (Fig. 4B and fig. S19B). Functionally, *PTAR1*-deficiency impaired glycosylation (*7*) (fig. S19C), possibly due to dysregulation of RAB proteins (*22*). Indeed, *PTAR1*-deficient cells showed attenuated geranylgeranylation of RAB1A and RAB1B (fig. S19D). Partial correction of the Golgi morphology in cells lacking both *PTAR1* and *GOLGA5* could relate to the effect of GOLGA5, itself a RAB effector, on Golgi fragmentation (*21, 23*). Thus, the interaction map reveals *PTAR1* and *GOLGA5* as opposing handles tuning Golgi morphology and homeostasis.

Genetic analysis suggested a link between the unstudied gene *C10orf76* and *PI4KB* which were both synthetic lethal with *PTAR1* (Fig. 3B). A host factor screen using coxsackievirus A10 also identified *C10orf76* as well as *PI4KB* (fig. S20A and B) and a proteomics survey (*24*) suggested association between *C10orf76* and *PI4KB*. We confirmed this interaction in immunoprecipitation experiments with cells expressing FLAG-tagged C10orf76 (Fig. 4C). PI4KB regulates abundance of phosphatidylinositol 4-phosphate [PI(4)P] (*25*) and has a role in genome replication of various RNA viruses including coxsackieviruses (*26*). Infection studies confirmed that *C10orf76* knockout cells were particularly resistant to coxsackievirus A10 (fig. S20C). Although virus entry occurred normally, replication of viral RNA was decreased in *C10orf76* knockout cells (Fig. 4D and fig. S20D). Enteroviruses hijack PI4KB activity to construct 'replication factories' which were abundant in wild-type cells but rare in *C10orf76*-deficient cells (fig. S20E). Amounts of PI(4)P were decreased in these cells and Golgi retention of PI4KB after chemical inhibition (*27*) was largely dependent on C10orf76, which also localized to this compartment under these conditions (fig. S21A-B). Thus, C10orf76 is a PI4KB-associated factor hijacked by specific picornaviruses for replication.

This study identifies approximately 2,000 genes required for optimal fitness of cultured haploid human cells. Despite technical limitations, the identification of gene essentiality shows high concordance with the gene-trap and CRISPR data reported in the accompanying manuscript of Wang *et al.* (supplemental discussion and fig. S22). This suggests that the increase in total number of genes in humans as compared to that in yeast yielded a system of higher complexity rather than more robustness through added redundancy. Nonessential human genes appear to frequently engage in synthetic lethal interactions. Our studies start to reveal an interconnected module of genetic interactions affecting the secretory pathway and link it to uncharacterized genes. The experimental strategy is applicable to various cellular processes and may help unravel the genetic network encoding a human cell.

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Figure legends

Figure 1. Identification of genes required for fitness in KBM7 and HAP1 cells through insertional mutagenesis. (**A**) Unique gene-trap insertions were mapped in KBM7 and HAP1 cells, and their orientation relative to the affected genes was counted. Per gene the percentage of sense orientation gene-trap insertions (Y-axis) and the total number of insertions in a particular gene (X-axis) are plotted. (**B**) Gene-trap insertions identified in the sense (S, yellow) or antisense orientation (AS, blue) in a non-essential gene (*BBC3*), a gene essential only in KBM7 cells (*STAT5B*), and a gene essential in both cell lines (*RPL13A*). (**C**) KEGG pathway enrichment analysis of essential genes shared between or unique to KBM7 or HAP1 cells. (**D**) Properties of 'new' and 'old' essential genes compared to the human genome. Averages for the sets are displayed, except for protein abundance where median emPAI values are shown.

Figure 2. The essential gene *TMEM258* encodes a component of the OST complex. (**A**) High-confidence protein-protein interactions associated with TMEM258. Green proteins indicate members of the oligosaccharyltransferase (OST) complex. Dashed lines indicate the OST complex subnetwork. (**B**) Effects of depletion of TMEM258 with siRNAs on the glycosylation of endogenous prosaposin. Cells were pulsed with ^{35}S-methionine/cysteine, lysed and subjected to immunoprecipitation using anti-prosaposin antibodies. Precipitated proteins were detected by phosphorimaging and hypoglycosylated prosaposin species are indicated. Tunicamycin treatment and depletion of the established OST subunit DDOST served as positive controls.

Figure 3. Synthetic lethality network generated based on mutations. (**A**) Essentiality of *RAB1A* and *RAB1B* in wild-type HAP1 cells and cells deficient for *RAB1A* or *RAB1B*. (**B**) Genetic interaction network indicating synthetic lethal/sick interactions that were identified by scoring genes for fitness reduction in three nuclease-generated knockout clones per genotype. This revealed an interconnected network with many genes that could be functionally assigned to the secretory pathway (labeled in green). Reciprocal interactions, scored in either query genotype, are

indicated by red edges. Edge thickness reflects the effect size of the interaction (compared to wild-type cells).

Figure 4. Roles of *PTAR1* and *C10orf76* in Golgi homeostasis and virus replication. (**A**) A bias for sense-orientation integrations in *GOLGA5* observed in *PTAR1*-deficient HAP1 cells but not wild-type cells. (**B**) Electron micrographs of the Golgi apparatus (orange highlight) in the indicated genotypes. (**C**) Interaction of Flag-tagged C10orf76 with PI4KB in HAP1 cells detected by immunoprecipitation using anti-Flag antibodies. (**D**) Coxsackievirus A10 amplification in wild-type and *C10orf76*-deficient cells measured by single molecule fluorescent in situ hybridization (smFISH) to localize individual viral genomes (red). Intracellular viral RNA was first detected after 30 minutes. Increased RNA signal after 300 minutes indicates RNA replication.

Supplementary Materials

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Materials and Methods

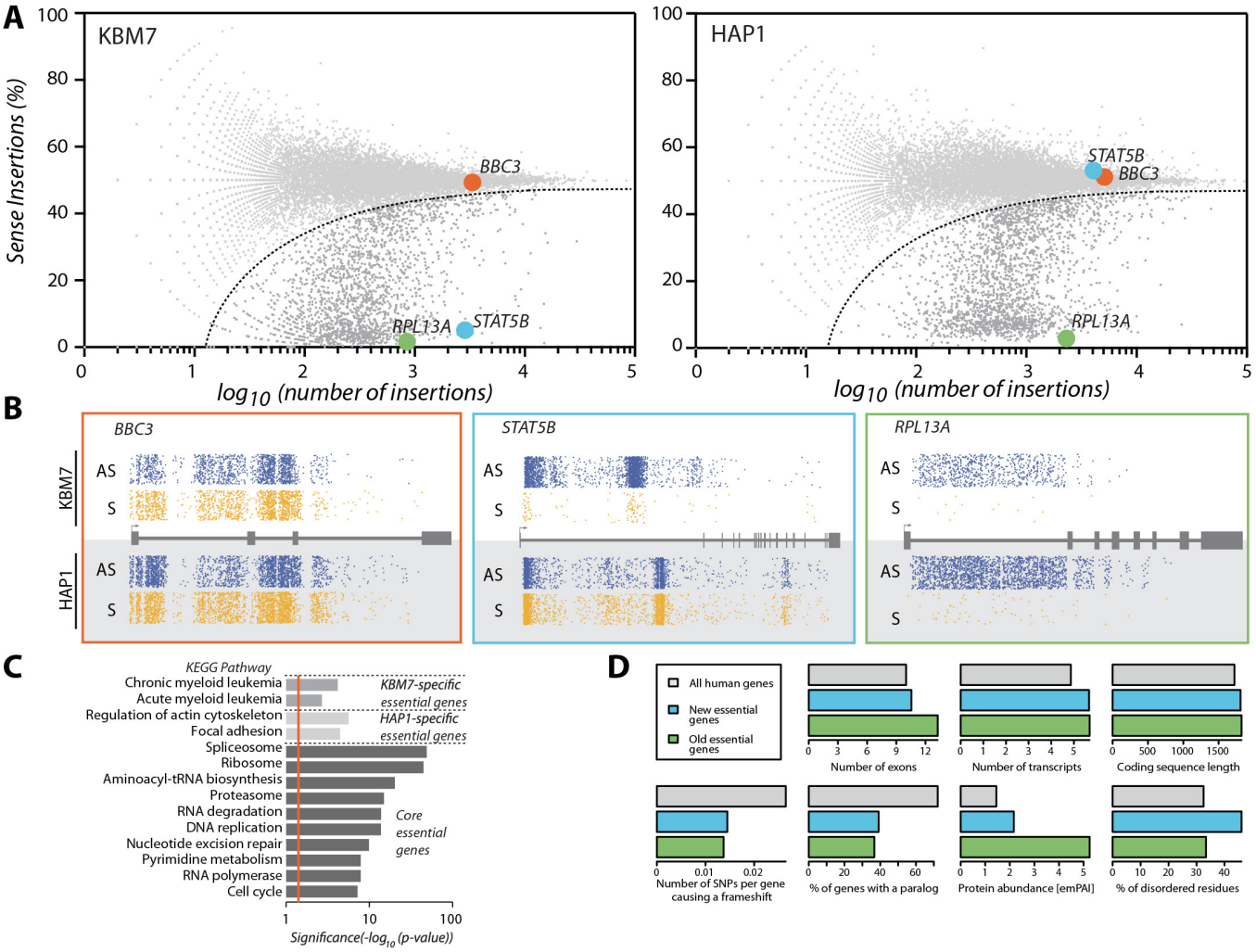
Supplemental Discussion

Figs. S1 to S23

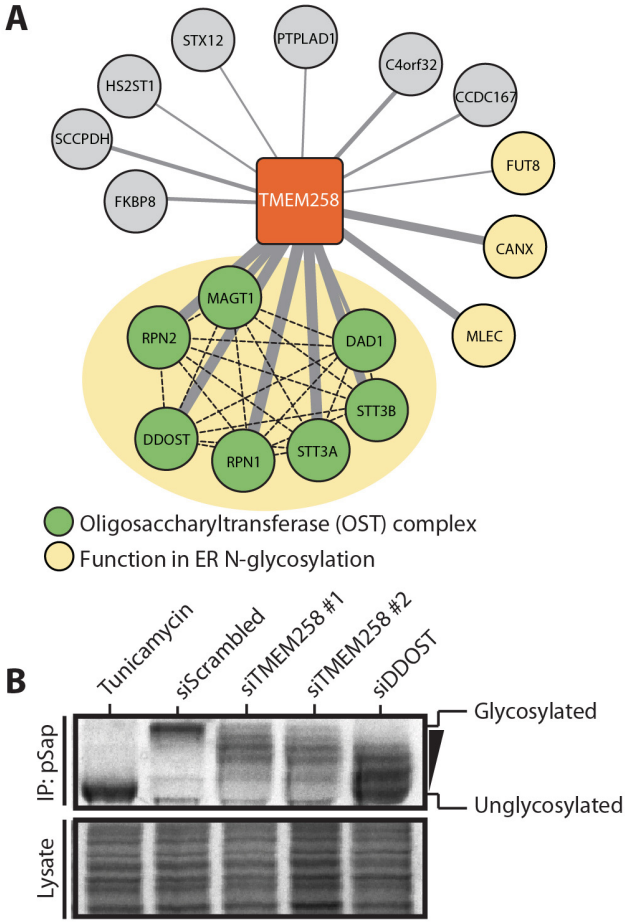
Tables S1 to S8

References (*28-61*)

Blomen et al., Figure 1



Blomen et al., Figure 2



Blomen et al., Figure 3

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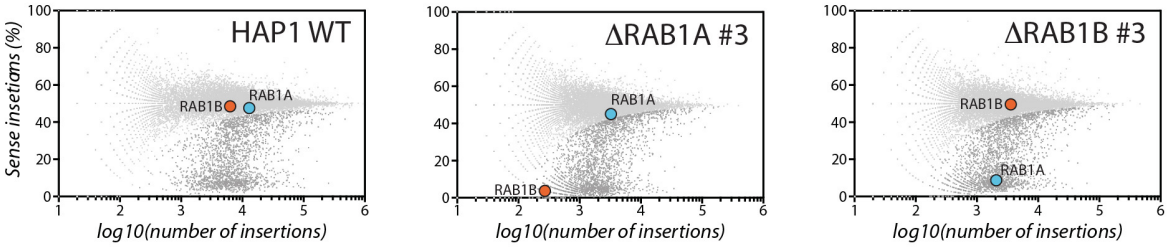

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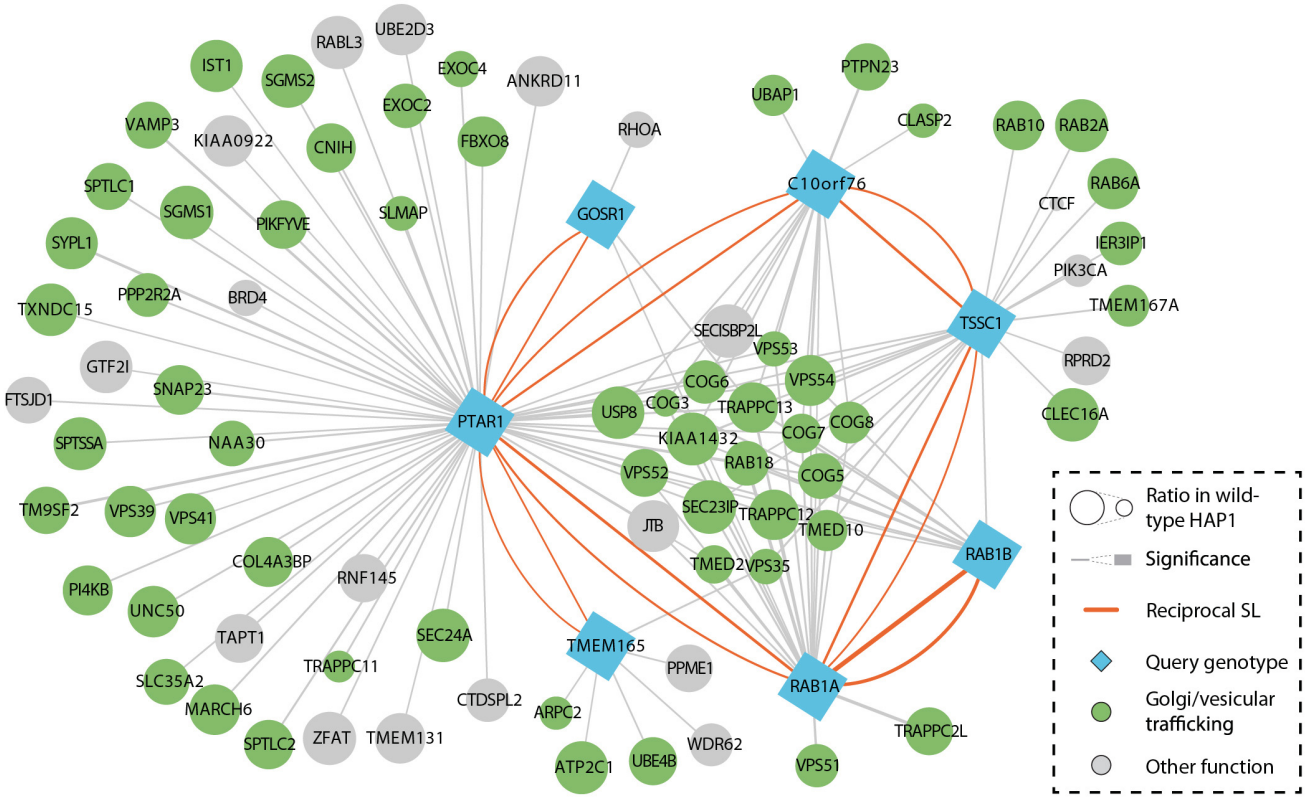

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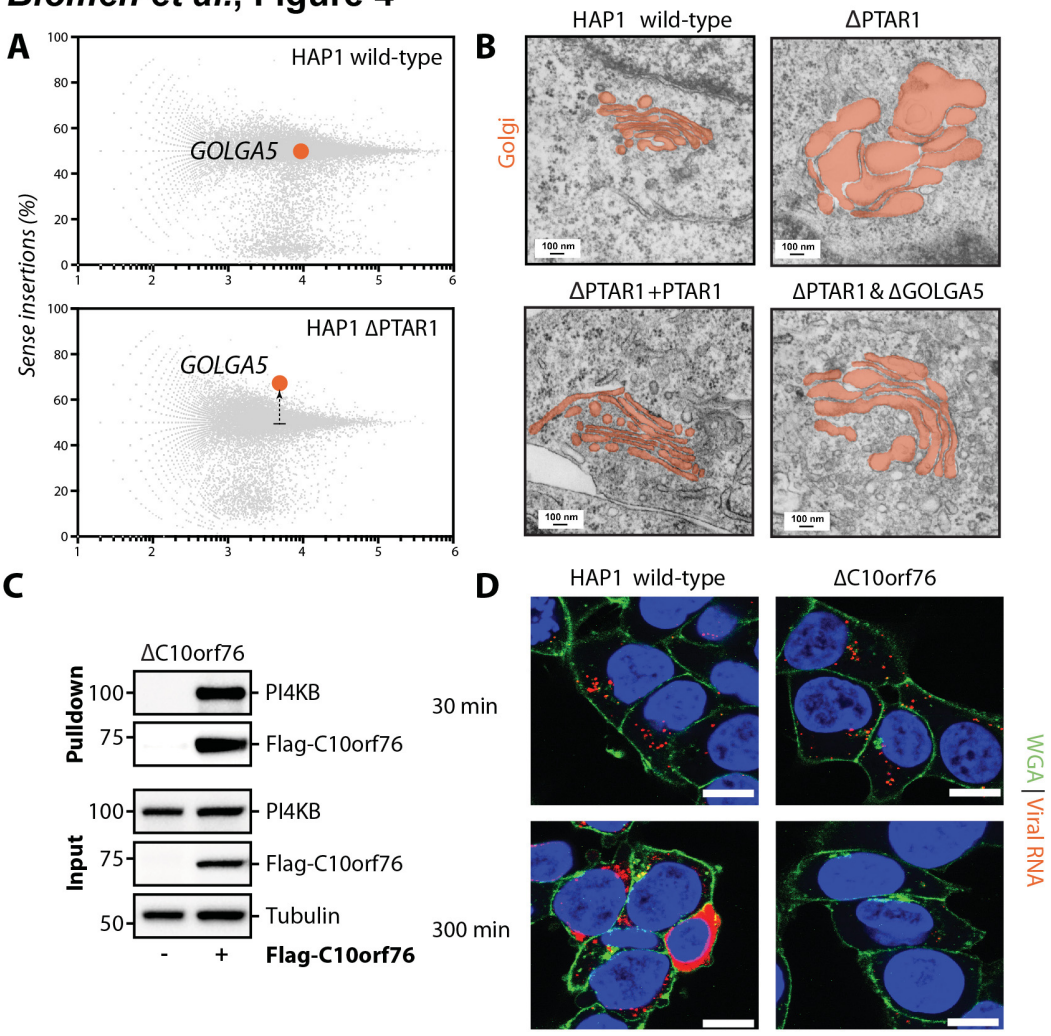

2.3 Interlude

The elucidation of protein complexes within their most physiological or disease-representative tissue and cellular model system is of great importance to appropriately capture and annotate the human interactome. The growing number of identified cancer-associated gene alterations as well as signaling hubs involved in the development of drug resistance requires a scalable system to identify mutant-specific binding partners and capture dynamic protein-protein interactions.

Here we have developed a versatile retroviral vector system pRSHIC (retroviral expression of SH-tagged proteins for interaction proteomics and color-tracing) that enables TAP procedures utilizing the SH tandem tag combination in a wide-ranging set of cell lines. Additional linked expression of the mCherry reporter fluorophore allows tracking of SH-tagged bait protein expressing cell pools by flow cytometry and microscopy. We have utilized pRSHIC in two biological settings to chart the interactors of the tumorigenic NRAS G12D protein in murine hematopoietic cells and the regulated cell death inducing mutant MLKL S358D protein in a human CRC cell line. The integration of proteomic as well as biochemical and functional validation data uncovered an essential role of HSP90 in the proteostatic regulation of MLKL-induced necroptosis that can be targeted by using different HSP90 inhibitors.

2.4 Manuscript #2

Bigenzahn JW*, Fauster A*, Rebsamen M, Kandasamy RK, Scorzoni S, Vladimer GI, Müller AC, Gstaiger M, Zuber J, Bennett KL & Superti-Furga G (2016) An Inducible Retroviral Expression System for Tandem Affinity Purification Mass-Spectrometry-Based Proteomics Identifies Mixed Lineage Kinase Domain-like Protein (MLKL) as an Heat Shock Protein 90 (HSP90) Client. Mol. Cell Proteomics 15: 1139–1150

**: equal contribution*

A detailed description of the individual author contributions can be found within the declaration section of this doctoral thesis.

An Inducible Retroviral Expression System for Tandem Affinity Purification Mass-Spectrometry-Based Proteomics Identifies Mixed Lineage Kinase Domain-like Protein (MLKL) as an Heat Shock Protein 90 (HSP90) Client*[S]

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Tandem affinity purification–mass spectrometry (TAP-MS) is a popular strategy for the identification of protein–protein interactions, characterization of protein complexes, and entire networks. Its employment in cellular settings best fitting the relevant physiology is limited by convenient expression vector systems. We developed an easy-to-handle, inducible, dually selectable retroviral expression vector allowing dose- and time-dependent control of bait proteins bearing the efficient streptavidin-hemagglutinin (SH)-tag at their N- or C termini. Concomitant expression of a reporter fluorophore allows to monitor bait-expressing cells by flow cytometry or microscopy and enables high-throughput phenotypic assays. We used the system to successfully characterize the interactome of the neuroblastoma RAS viral oncogene homolog (NRAS) Gly12Asp (G12D) mutant and exploited the advantage of reporter fluorophore expression by tracking cytokine-independent cell growth using flow cytometry. Moreover, we tested the feasibility of studying cytotoxicity-mediating proteins with the vector system on the cell death-inducing mixed lineage kinase domain-like protein (MLKL) Ser358Asp (S358D) mutant. Interaction proteomics analysis of MLKL Ser358Asp (S358D) identified heat shock protein 90 (HSP90) as a high-confidence interacting protein. Further phenotypic characterization established MLKL as a novel HSP90 client. In summary, this novel inducible expression system enables SH-tag-based interaction studies in the cell line proficient for the respective phenotypic or signaling context and constitutes a valuable tool for experimental approaches requiring inducible or traceable protein expression. ***Molecular & Cellular Proteomics 15: 10.1074/mcp.O115.055350, 1139–1150, 2016.***

Protein–protein interactions are the basis of most cellular processes and characterizing the complexes associated with a given protein greatly increases understanding of the biological function (1). Tandem affinity purification (TAP)[1] (2, 3) coupled to mass spectrometry (MS) constitutes a powerful technique for identifying high-confidence interaction partners of

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[1] The abbreviations used are: TAP, tandem affinity purification; CDC37, cell division cycle 37; Flp, Flippase; Flp-*FRT*, flippase-flippase recognition target; GFP, green fluorescent protein; GTP, guanosine triphosphate; HSP90, heat shock protein 90; IRES, internal ribosome entry site; MEK, mitogen-activated protein kinase kinase; MLK3, mixed lineage kinase 3; MLKL, mixed lineage kinase domain-like protein; Nec-1, necrostatin-1; NRAS, neuroblastoma RAS viral oncogene homolog; NSA, necrosulfonamide; PK3CG, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform; RAF1, RAF proto-oncogene serine/threonine-protein kinase; RAS, rat sarcoma; RIN1, Ras and Rab interactor 1; RIPK, receptor-interacting serine/threonine-protein kinase; rtTA3, reverse tet transactivator protein 3; RIEP, rtTA3-IRES-ecotropic receptor-PGK-PuroR; S6K1, ribosomal protein S6 kinase; SH, streptavidin-hemagglutinin-tag; STAT5, signal transducer and activator of transcription 5; TRE, tetracycline-responsive element; pRSHIC, retroviral expression of SH-tagged proteins for interaction proteomics and color-tracing.

tagged bait proteins (4–6). The reduction of nonspecific background binding due to dual-affinity purification has made TAP-MS the method of choice for protein interaction mapping (7–9), and more than 30 different tandem tags have been established so far by alternative combination of affinity handles (10, 11). Specifically, the purification procedure for the recently developed SH-tag (12) shows particularly high bait protein recovery (10). In combination with the flippase–flippase recognition target (Flp-*FRT*) recombination system, SH-based TAP-MS has been successfully applied to the in-depth analysis of human signaling networks (12–15) and virus–host interactions (16). A detailed interlaboratory comparative analysis of highly standardized procedure using HEK293 cells revealed a reproducibility within an individual laboratory of 98% and a reproducibility between two laboratories of more than 80%, suggesting robustness of the method using workhorse cell lines (15).

Charting the interactome of a specific protein in the relevant physiological setting, in context of its functional signaling pathway, requires performing interaction proteomics in different cellular backgrounds. Highly efficient gene delivery to a variety of cell lines, including cell types that are difficult to transfect, can be achieved by viral-vector-mediated gene transfer (17). Temporal and reversible control of bait protein expression can be achieved by using inducible expression systems, further enabling the analysis of proteins with toxic ectopic expression. Tetracycline (Tet)-On systems (18) have proven to be valuable tools for inducible expression of cDNAs or short hairpin RNAs in cell lines and animal models (19, 20).

To date, TAP-MS experiments are based on Flp-In technology or viral-based transgene delivery of bait proteins fused to different affinity tags with a diverse range of expression and bait recovery efficiency (10, 11, 21). While the SH-tag has comparably high bait recovery (10) and strong interlaboratory reproducibility (15), its application has so far been restricted to the limited number of Flp-In system-competent cell lines. To overcome this limitation and widen the reach of SH-based TAP-MS studies, we established and characterized retroviral expression of SH-tagged proteins for interaction proteomics and color tracing (pRSHIC). This novel retroviral, doxycycline-inducible Tet-On vector system is suitable for expression of SH-tagged target proteins in a wide range of cell systems. In addition to enlarging the existing toolbox for TAP-MS-based interaction proteomics, the features and versatility of pRSHIC make it a valuable tool for a broad set of phenotypic analyses. To illustrate the features of pRSHIC, we charted the interactome of the oncogenic NRAS G12D mutant protein (22, 23), as delineating the network properties of such cancer-associated gene variants is crucial to understand their impact on the disease (24). Furthermore, we demonstrated the applicability of pRSHIC to study cytotoxicity-inducing proteins using the MLKL mutant S358D (25). MLKL is the key molecule required for executing necroptosis, a form of programmed necrotic cell death (26–28). Our study identified MLKL to associate with HSP90 and functionally validated MLKL as a novel client protein of HSP90.

MATERIALS AND METHODS

Cell Lines and Reagents—HEK293T was obtained from ATCC (Manassas, VA) and K-562 and KCL-22 from DSMZ (Braunschweig, Germany). HT-29 was kindly provided by P. Schneider (Lausanne). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) or RPMI medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Gibco, Grand Island, NY) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) (Sigma-Aldrich). Ba/F3 was obtained from DMSZ and grown in RPMI supplemented with 10% (v/v) FBS (Gibco) and 1–3 ng/ml recombinant murine IL-3 (213–13, PeproTech, Rocky Hill, NJ). The reagents used were as follows: doxycycline (D9891, Sigma-Aldrich), necrostatin-1 (N9037, Sigma-Aldrich), necrosulfonamide (480073, Merck Millipore, Billerica, MA), geldanamycin (G-1047, AG Scientific, San Diego, CA), MG132 (C2211, Sigma Aldrich), chloroquine (C6628, Sigma Aldrich), selumetinib (S1008, Selleck Chemicals, Houston, TX), trametinib (S2673, Selleck Chemicals), and ponatinib (S1490, Selleck Chemicals).

Antibodies—Antibodies used were HA (SC-805, Santa Cruz, Dallas, TX), HA-7-HRP (H6533, Sigma-Aldrich), MEK1/2 (#9126, Cell Signaling, Danvers, MA), phospho-MEK1/2 (#2338, Cell Signaling), ERK1/2 (M5670, Sigma-Aldrich), phospho- ERK1/2 (#4370, Cell Signaling), STAT5 (610191, BD Biosciences, Franklin Lakes, NJ), phospho-STAT5A/B (05–886R, Merck Millipore), phospho-p70 S6 kinase (#9234, Cell Signaling), p70 S6 kinase (SC-230, Santa Cruz), RIPK3 (#12107, Cell Signaling), HSP90 (610418, BD Transduction Laboratories), actin (AAN01-A, Cytoskeleton, Denver, CO), and tubulin (ab7291, Abcam, Cambridge, UK). The secondary antibodies used were goat anti-mouse HRP (115–035-003, Jackson ImmunoResearch, West Grove, PA), goat anti-rabbit HRP (111–035-003, Jackson ImmunoResearch), and Alexa Fluor 680 goat anti-mouse (A-21057, Molecular probes, Grand Island, NY).

Plasmids and Cloning—Inducible retroviral expression vectors are derived from the pQCXIX self-inactivating retroviral vector backbone (pSIN, Clontech). pRSHIC vectors were assembled using standard cloning techniques and final expression constructs contain the following elements: pSIN-TREtight or TRE3G-HA-StrepII-Gateway cassette-IRES-mCherry-PGK-BlastR for N-terminal StrepHA tagging and pSIN-TREtight or TRE3G-Gateway cassette-StrepII-HA-IRES-mCherry-PGK-BlastR for C-terminal StrepHA tagging. Detailed cloning strategies, primers, and vector information are available upon request. NRAS coding sequence was PCR-amplified from K562 cDNA and cloned into the Gateway-compatible pDONR221 entry vector using BP recombination (Invitrogen, Grand Island, NY). The G12D mutant version of NRAS was generated by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using the following primers 5′-GTGGTGGTTGGAGCAGATGGTGTTGGGAAAAGC-3′ and 5′-GCTTTTCCCAACACCATCTGCTCCAACCACCAC-3′. Cloning of RIPK3, MLKL, and MLKL S358D has been described elsewhere (48). Following sequence verification, the cDNAs were transferred by Gateway cloning using LR recombination (Invitrogen) into pRSHIC vectors. All vectors are available upon request.

Generation of Inducible Cell Lines—Human cell lines were retrovirally transduced using vector pMSCV-rtTA3-IRES-EcoR-PGK-PuroR (pMSCV-RIEP) (29), and murine cell lines were transduced with pMSCV-rtTA3-PGK-PuroR (pMSCV-RP) (29) to generate rtTA3 and ecotropic receptor-coexpressing (RIEP) or rtTA3-expressing (rtTA3) Tet-on competent cell lines, respectively. Briefly, HEK293T cells were transiently transfected with the retroviral packaging plasmids pGAG-POL, pVSV-G, pADVANTAGE, and pMSCV-RIEP or pMSCV-RP. The medium was exchanged 24 h later and replaced with the medium for

the respective target cell line. After 48 h the virus-containing supernatant was harvested, filtered (0.45 μm), supplemented with 8 μg/ml protamine sulfate (Sigma-Aldrich) and added to 40–60% confluent target cell lines. Suspension cells were subjected to spinfection (2000 *rpm*, 45 min, room temperature). 24 h after infection the medium was exchanged and replaced with fresh medium. Another 24 h later, the medium was supplemented with 1–2 μg/ml puromycin (Sigma-Aldrich) to select for infected cells. Following puromycin selection, RIEP- or rtTA3-expressing cell lines were similarly transduced with retrovirus produced in HEK293T cells using the respective target gene-encoding pRSHIC vector, and pGAG-POL, pADVANTAGE, and pEcoEnv. Infected cells were selected by addition of 15–25 μg/ml blasticidin (InvivoGen). Target gene expression was induced by addition of 1–2 μg/ml doxycycline.

Immunoblotting—Cells were lysed using Nonidet-40 lysis buffer (50 mM HEPES (pH 7.4), 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, one tablet of EDTA-free protease inhibitor (Roche, Indianapolis, IN, USA) per 50 ml) or IP lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml TPCK and protease inhibitor mixture) for 10 min on ice. Lysates were cleared by centrifugation (13000 *rpm*, 10 min, 4 °C). The proteins were quantified with BCA (Pierce, Grand Island, NY) or Bradford assay using γ-globin as a standard (Bio-Rad, Hercules, CA). Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes Protran BA 85 (GE Healthcare, Little Chalfont, UK). The membranes were immunoblotted with the indicated antibodies. Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western blotting system (Thermo Scientific, Waltham, MA) or Odyssey Infrared Imager (LI-COR, Lincoln, NE).

Immunoprecipitation—Cells were washed in PBS and lysed in ice-cold HENG buffer (50 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 20 mM Na_2MoO_4, 2 mM EDTA, 5% glycerol, 0.5% Triton X-100, one tablet of EDTA-free protease inhibitor (Roche) per 50 ml, 20 mM NaF, and 0.4 mM Na_3VO_4) for 10 min on ice. Lysates were cleared by centrifugation (13000 *rpm*, 10 min, 4 °C), quantified with BCA (Pierce), and precleared (30 min, 4 °C) on Sepharose6 beads (Sigma-Aldrich). Subsequently, lysates were incubated (3 h, 4 °C) with monoclonal anti-HA agarose antibody (Sigma-Aldrich). Beads were recovered by centrifugation and washed three times with lysis buffer before analysis by SDS-PAGE and immunoblotting.

Affinity Purifications and Sample Preparation for Liquid Chromatography Mass Spectrometry—Tandem affinity purifications were performed as previously described (15, 61). Affinity purifications were performed as biological replicates and cell lines expressing SH-tagged GFP were used as negative controls. In brief, cell lines were incubated with 1–2 μg/ml doxycycline for 7–24 h to induce expression of SH-tagged bait proteins. Whole cell extracts were prepared in 50 mM HEPES (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor mixture. Cell lysates were cleared by centrifugation (13000 *rpm*, 20 min, 4 °C). Proteins were quantitated by Bradford assay using γ-globin as standard (Bio-Rad). 50 mg total lysate were incubated with StrepTactin Sepharose beads (IBA, Göttingen, Germany). Tagged proteins were eluted with D-biotin (Alfa-Aesar, Ward Hill, MA) followed by a second purification step using HA-agarose beads (Sigma-Aldrich). Protein complexes were eluted with 100 mM formic acid and immediately neutralized with triethylammonium bicarbonate buffer (Sigma-Aldrich). Samples were digested with trypsin (Promega, Fitchburg, WI), and the resultant peptides desalted and concentrated with customized reversed-phase tips (62). The volume of the eluted samples was reduced to ~2 μl in a vacuum centrifuge and reconstituted with 5% formic acid.

Reversed-Phase Liquid Chromatography Mass Spectrometry—Mass spectrometry was performed on a hybrid linear trap quadrupole Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Waltham, MA) using the Xcalibur version 2.1.0 coupled to an Agilent 1200 HPLC nanoflow system (dual pump system with one precolumn and one analytical column) (Agilent) via a nanoelectrospray ion source using liquid junction (Proxeon, Odense, Denmark). Solvents for liquid chromatography mass spectrometry separation of the digested samples were as follows: solvent A consisted of 0.4% formic acid in water and solvent B consisted of 0.4% formic acid in 70% methanol and 20% isopropanol. From a thermostatic microautosampler, 8 μl of the tryptic peptide mixture were automatically loaded onto a trap column (Zorbax 300SB-C18 5 μm, 5 $\times$ 0.3 mm, Agilent) with a binary pump at a flow rate of 45 μl/min. 0.1% TFA was used for loading and washing the precolumn. After washing, the peptides were eluted by back-flushing onto a 16 cm fused silica analytical column with an inner diameter of 50 μm packed with C18 reversed phase material (ReproSil-Pur 120 C18-AQ, 3 μm, Dr. Maisch, Ammerbuch-Entringen, Germany). The peptides were eluted from the analytical column with a 27 min gradient ranging from 3 to 30% solvent B, followed by a 25 min gradient from 30 to 70% solvent B, and, finally, a 7 min gradient from 70 to 100% solvent B at a constant flow rate of 100 nl/min. The analyses were performed in a data-dependent acquisition mode using a top 15 collision-induced dissociation method. Dynamic exclusion for selected ions was 60 s. A single lock mass at *m/z* 445.120024 was employed (63). The maximal ion accumulation time for MS in the Orbitrap and MS^2 in the linear trap was 500 and 50 ms, respectively. Automatic gain control was used to prevent overfilling of the ion traps. For MS and MS^2, automatic gain control was set to 10^6 and 5,000 ions, respectively. Peptides were detected in MS mode at a resolution of 60,000 (at *m/z* 400). The threshold for switching from MS to MS^2 was 2,000 counts. All samples were analyzed as technical, back-to-back replicates.

Data Analysis—The acquired raw MS data files were processed with msconvert (ProteoWizard Library v2.1.2708) and converted into Mascot generic format (mgf) files. The resultant peak lists were searched against either the human or mouse SwissProt database v2014.03_20140331 (40,055 and 24,830 sequences, respectively, including isoforms obtained from varsplic.pl (64) and appended with known contaminants) with the search engines Mascot (v2.3.02, MatrixScience, London, UK) and Phenyx (v2.5.14, GeneBio, Geneva, Switzerland) (65). Submission to the search engines was via a Perl script that performs an initial search with relatively broad mass tolerances (Mascot only) on both the precursor and fragment ions ($\pm$10 ppm and $\pm$0.6 Da, respectively). High-confidence peptide identifications were used to recalibrate all precursor and fragment ion masses prior to a second search with narrower mass tolerances ($\pm$ 4 ppm and $\pm$0.3 Da, respectively). One missed tryptic cleavage site was allowed. Carbamidomethyl cysteine and oxidized methionine were set as fixed and variable modifications, respectively. To validate the proteins, Mascot and Phenyx output files were processed by internally developed parsers. Proteins with $\leq$2 unique peptides above a score T1 or with a single peptide above a score T2 were selected as unambiguous identifications. Additional peptides for these validated proteins with score $>$T3 were also accepted. For Mascot and Phenyx, T1, T2, and T3 peptide scores were equal to 16, 40, 10 and 5.5, 9.5, 3.5, respectively (p value $<10^{-3}$). The validated proteins retrieved by the two algorithms were merged and any spectral conflicts discarded and grouped according to shared peptides. By applying the same procedure against a reversed database, a false-positive detection rate of $<$1 and $<$0.1% (including the peptides exported with lower scores) was determined for proteins and peptides, respectively. The significance of the interactions from affinity purification-mass spectrometry (AP-MS) experiments was assessed using the SAINT software (51)

and the CRAPome database (53). GFP pulldowns were used as the negative control. Commonly known contaminants including trypsin and keratin were removed. Visualization of interaction data was performed using R statistical environment (66). All prey proteins with a SAINT score of >0.95 were identified as high-confidence interactors. Supplemental Tables S1 and S2 give the TAP-LC-MSMS analysis results for NRAS G12D and MLKL S358D, respectively. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (67) via the PRIDE partner repository with the dataset identifier PXD002855.

Cell Viability Assays—Cells were seeded in 96-well plates at the appropriate cell density. For drug sensitivity experiments, cells were incubated with increasing drug concentrations for 72 h. For cell death assays, cells were incubated with the indicated compounds as stated or overnight (14 h). Cell viability was determined using CellTiter Glo Luminescent Cell Viability Assay (Promega) according to the instructions provided by the manufacturer. Luminescence was recorded with a SpectraMax M5Multimode plate reader (Molecular Devices, Sunnyvale, CA). Data were normalized to values of untreated controls.

Flow Cytometry—Samples were analyzed on an LSR Fortessa (BD Biosciences), and data analysis was performed using FlowJo software version 7.6.3 (Tree Star Inc., Ashland, OR).

Proliferation Competition Assay—To analyze the influence of inducible SH-tagged bait protein expression on cell proliferation and survival, pRSHIC-NRAS G12D (mCherry+) and pRSHIC-GFP (mCherry+/GFP+) transduced Ba/F3 rtTA3 cells were induced with 1 μg/ml doxycycline. After 24 h, cells were mixed in a 1:1 ratio and cultured in the presence of doxycycline with or without IL-3. The percentage of mCherry+ and mCherry+/GFP+ populations was monitored daily by flow cytometry, gating only viable cells (FSC/SCC).

Microscopy—Microscopy images were taken at 10× with a Leica DFC310 FX on a Leica DM IL LED microscope (Leica Microsystems, Wetzlar, Germany) or at 20× on an Operetta automated confocal microscope (PerkinElmer, Waltham, MA) and analyzed with ImageJ 1.44p (NIH, open source). The fluorophores used contained no overlapping spectrums and channels were imaged sequentially.

Experimental Design and Statistical Rational—Tandem affinity purifications were performed as biological replicates ($n = 2$) and analyzed by LC-MSMS as technical duplicates. Cell viability assay data were normalized to untreated control and are shown as mean value ± s.d. of at least two independent experiments ($n \geq 2$) performed in triplicates. Flow-cytometry-based proliferation competition assay data are shown as mean value ± s.d. of at least two independent experiments ($n \geq 2$). Flow cytometry and immunoblot results shown are representative of at least two independent experiments ($n \geq 2$).

RESULTS AND DISCUSSION

Generation of a Retroviral Expression System for Inducible, Dose-Dependent, and Reversible Expression of SH-Tagged Bait Proteins—We assembled an inducible expression system in a self-inactivating retroviral vector containing a tetracycline response element tight (TREtight) promoter (29). For expression of N- or C-terminally TAP-tagged cDNAs, we inserted a gateway-cloning cassette preceded or followed by two streptavidin and one hemagglutinin epitope(s) (12) (Fig. 1*A*). The recombination efficiency of the gateway system allows high-throughput cloning, and thus, the vector is suitable for use with gateway-compatible cDNA and ORF libraries. Furthermore, we linked a fluorescent mCherry marker to the cDNA expression cassette via an internal ribosome entry site (IRES) sequence to enable tracing of bait protein-expressing cell populations by flow cytometry or microscopy. The doxycycline-controlled reverse tet transactivator protein 3 (rtTA3) (30) in combination with different TRE promoters has proven to be effective in inducing transgene expression in a broad range of cell lines and tissues *in vivo* (31). To generate Tet-On proficient cell lines, the respective target cells are first stably transduced with rtTA3 or a combination of rtTA3 and the ecotropic receptor (RIEP), the latter also providing enhanced biosafety (32). Cell lines with inducible bait protein expression are then established by retroviral transduction of rtTA3 transgene-harboring target cells with the respective pRSHIC constructs (Fig. 1*B*). Transduced cells are selected using blasticidin, and transgene expression in the target cell lines can be assessed by flow cytometry or immunoblotting prior to TAP-MS and follow-up experiments.

To characterize the properties of this novel expression system, we transduced human leukemia K-562 RIEP, KCL-22 RIEP and colorectal adenocarcinoma HT-29 RIEP cells with a vector construct encoding SH-tagged green fluorescent protein (GFP). Following selection using blasticidin, the cells were cultured in the presence of doxycycline for 24 h to induce GFP expression. In all three cell lines, >85% of the cell population efficiently induced gene expression as determined by the detection of the mCherry reporter using flow cytometry (Figs. 2*A*–2*C*). Target protein expression was confirmed by immunoblotting for SH-tagged GFP (Figs. 2*D*–2*F*). Additionally, we observed strong correlation between GFP and mCherry fluorescence (Fig. 2*G* and Supplemental Figs. 1*A*–1*C*), indicating that flow cytometry-based detection of the mCherry marker provides a reliable surrogate measure for efficient induction of transgene expression. The TREtight promoter exhibits low basal expression while promoting high-level transcription upon induction. Depending on the promoter used, the efficiency of inducible expression by Tet-regulated systems and the basal expression levels can vary between different cell types (31). For bait proteins with elevated basal expression levels in the context of the TREtight promoter, we additionally created a set of vectors harboring a TRE3G promoter (Supplemental Fig. 2*A*), which provides strongly reduced basal expression compared with earlier versions of the TRE promoter (33) (Supplemental Fig. 2*B*). As demonstrated in K-562 RIEP GFP cells, expression of bait proteins can be modulated by the addition of increasing concentrations of doxycycline (Fig. 2*H*). Furthermore, we monitored induction kinetics, indicating that GFP was induced within hours after doxycycline addition and continued to accumulate over 24 h (Fig. 2*I*). Removal of doxycycline led to a decline in GFP levels, illustrating the reversibility of bait expression (Fig. 2*I*). Altogether, these data establish pRSHIC as a versatile inducible vector system that enables scaling and reversible expression of SH-tagged bait proteins in multiple mammalian cell types.

Phenotypic Characterization and Interaction-Proteomic Analysis of NRAS G12D in the Murine Pro B Cell Line Ba/F3—Cancer genome sequencing projects continue to reveal novel

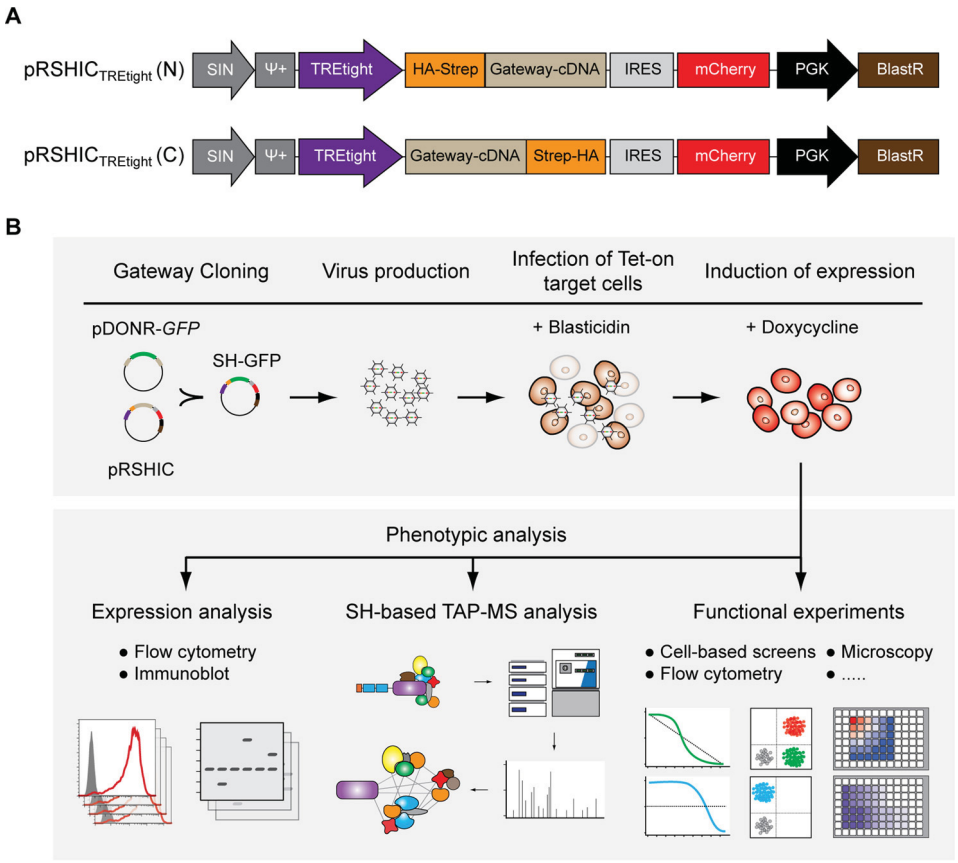

FIG. 1. **Main features of pRSHIC and workflow for generation of inducible cell lines.** (*A*) Schematic illustration of inducible TREtight-driven expression vectors with Gateway-cloning cassette fused to N- (upper) or C-terminal (lower) SH-tag. (*B*) Workflow for generation of inducible cell lines amenable to TAP-MS and follow-up experiments.

gene mutations and fusions (23). Understanding the molecular function of these genetic alterations requires characterization of their phenotypic impact on transformation and specific influence on protein–protein interactions (34, 35). We therefore chose to exemplify utility of pRSHIC through phenotypic analysis of the oncogenic G12D mutant of NRAS, a member of the rat sarcoma (RAS) family (H-, K-, and NRAS) of guanosine triphosphate (GTP)-binding proteins and frequently mutated in hematological malignancies (22). We demonstrated the growth-promoting effects and delineated the interactome of NRAS G12D in the murine bone-marrow-derived pro-B cell line Ba/F3. This cell line requires interleukin (IL)-3 for survival and proliferation and thus constitutes a convenient tool for studying oncogene-induced growth factor independence (36). We generated Tet-On competent Ba/F3 cells inducibly expressing N-terminal SH-tagged NRAS G12D or a GFP control (Supplemental Figs. 3*A* and 3*B*). To examine NRAS G12D-mediated growth factor independence, we performed flow cytometry-based proliferation-competition assays. While both cell populations showed equal growth in the presence of IL-3, NRAS G12D-expressing cells rapidly out-competed GFP-expressing control cells upon IL-3 withdrawal (Fig. 3*A*). Cytokine removal led to loss of signal transducer and activator of transcription 5 (STAT5) phosphorylation in both cell lines; however, NRAS G12D cells maintained elevated mitogen-activated protein kinase (MEK) 1/2 phosphorylation and hence activation of the mitogen-activated protein kinase pathway (Fig. 3*B*). Consequently, NRAS G12D-expressing cells showed marked sensitivity to the MEK 1/2 inhibitors trametinib (GSK1120212) (Fig. 3*C*) and selumetinib (AZD6244) (Fig. 3*D*) in the absence of IL-3, as increasing drug concentrations reduced mitogen-activated protein kinase pathway activation and ribosomal protein S6 kinase 1 (S6K1) phosphorylation (Supplemental Fig. 3*C*). In order to map the interactome of NRAS G12D, we induced bait protein expression for 24 h with doxycycline in the presence of IL-3 and performed TAP coupled to one-dimensional gel-free liquid chromatography tandem mass spectrometry (TAP-LC-MSMS). Significance analysis of interactome (SAINT) analysis using GFP purifications as a control for nonspecific protein interactions identified Ras and Rab interactor 1 (RIN1) among the high-confidence interacting proteins of NRAS G12D (Fig. 3*E* and Supplemental Table 1). Indeed, RIN1 has been described as associating with harvey rat sarcoma viral oncogene homolog (HRAS) and to preferentially bind active, GTP-loaded RAS (37). RIN1 competes with the RAF proto-oncogene serine/

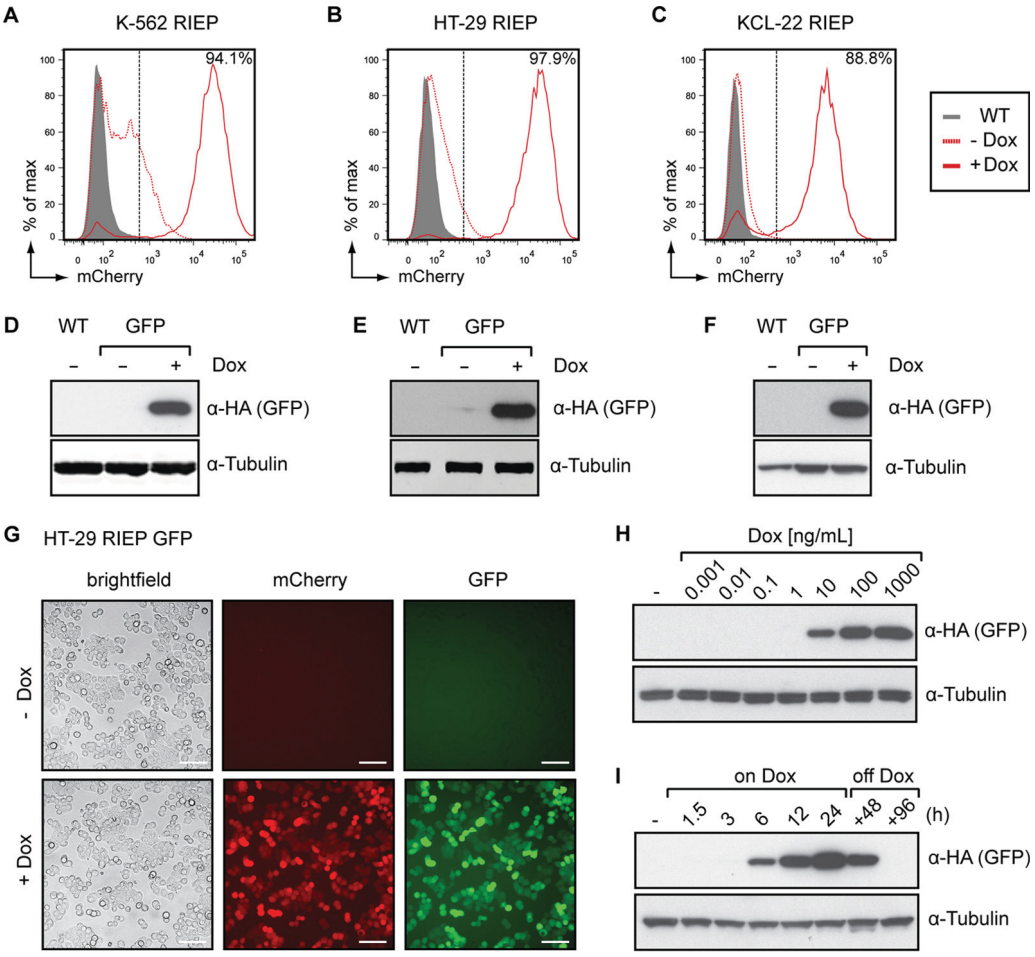

FIG. 2. **pRSHIC allows inducible, dose-dependent, and reversible expression of SH-tagged bait proteins.** (*A–F*) Flow cytometry and immunoblot analysis of K-562 RIEP (*A, D*), HT-29 RIEP (*B, E*) and KCL-22 RIEP (*C, F*) GFP cells, untreated or treated with 1–2 μg/ml doxycycline for 24 h. Immunoblots were probed with the indicated antibodies. Wild-type (WT) cells act as a baseline control. (*G*) Microscopy (20×; brightfield, fluorescence) of HT-29 RIEP GFP cells induced or not for 24 h with 2 μg/ml doxycycline (scale bar: 100 μm). (*H*) K-562 RIEP GFP cells were treated with increasing concentrations of doxycycline for 24 h. Cells were lysed and immunoblotted as indicated. (*I*) K-562 RIEP GFP cells were induced with 1 μg/ml and doxycycline subsequently withdrawn for the indicated time span. Cells were lysed and immunoblotted with the indicated antibodies. Results are representative of two independent experiments ($n = 2$).

threonine-protein kinase (RAF1) for RAS binding (38). Furthermore, we identified phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform (p110γ; PK3CG) of the phosphoinositide-3-kinase (PI3K) complex as a significant interactor. Binding of active RAS isoforms to p110γ leads to activation of the PI3K-pathway (39, 40) and the interaction with p110α (PK3CA) is important for mutant RAS-induced cancer formation and maintenance *in vivo* (41, 42). In summary, by recapitulating the interaction partners and phenotypic features of the oncogenic NRAS G12D protein, we showed that pRSHIC is an efficient tool to functionally annotate and mechanistically characterize proteins bearing cancer-relevant mutations.

Phenotypic Analysis of a Cell Death-Inducing MLKL S358D Mutant Protein—The possibility of tightly controlling the timing and extent of protein expression is necessary when investigating proteins that trigger cell death. The pseudokinase MLKL plays a key role in the execution of necroptosis, a form of nonapoptotic programmed cell death relying on the receptor-interacting serine/threonine kinase 1 (RIPK1) and RIPK3 that in recent years has been the subject of very intense research efforts (26–28). Upon activation by RIPK3-mediated phosphorylation, MLKL triggers destabilization and rupture of membranes, resulting in rapid cell death (43–47). We expressed and analyzed a constitutively active MLKL mutant, known to trigger necroptosis (25, 46). We chose to study the RIPK3-phosphorylation mimicking MLKL S358D mutant (48) in the human colorectal adenocarcinoma cell line HT-29, proficient to undergo necroptosis. We observed robust expression of the MLKL S358D mutant in HT-29 RIEP cells within 6 h of doxycycline addition (Fig. 4*A* and Supplemental Fig. 4*A*). As we have shown previously (48), exogenous expression of constitutively active mutant versions of MLKL induces toxicity in these cells. Indeed, MLKL S358D triggered cell death within

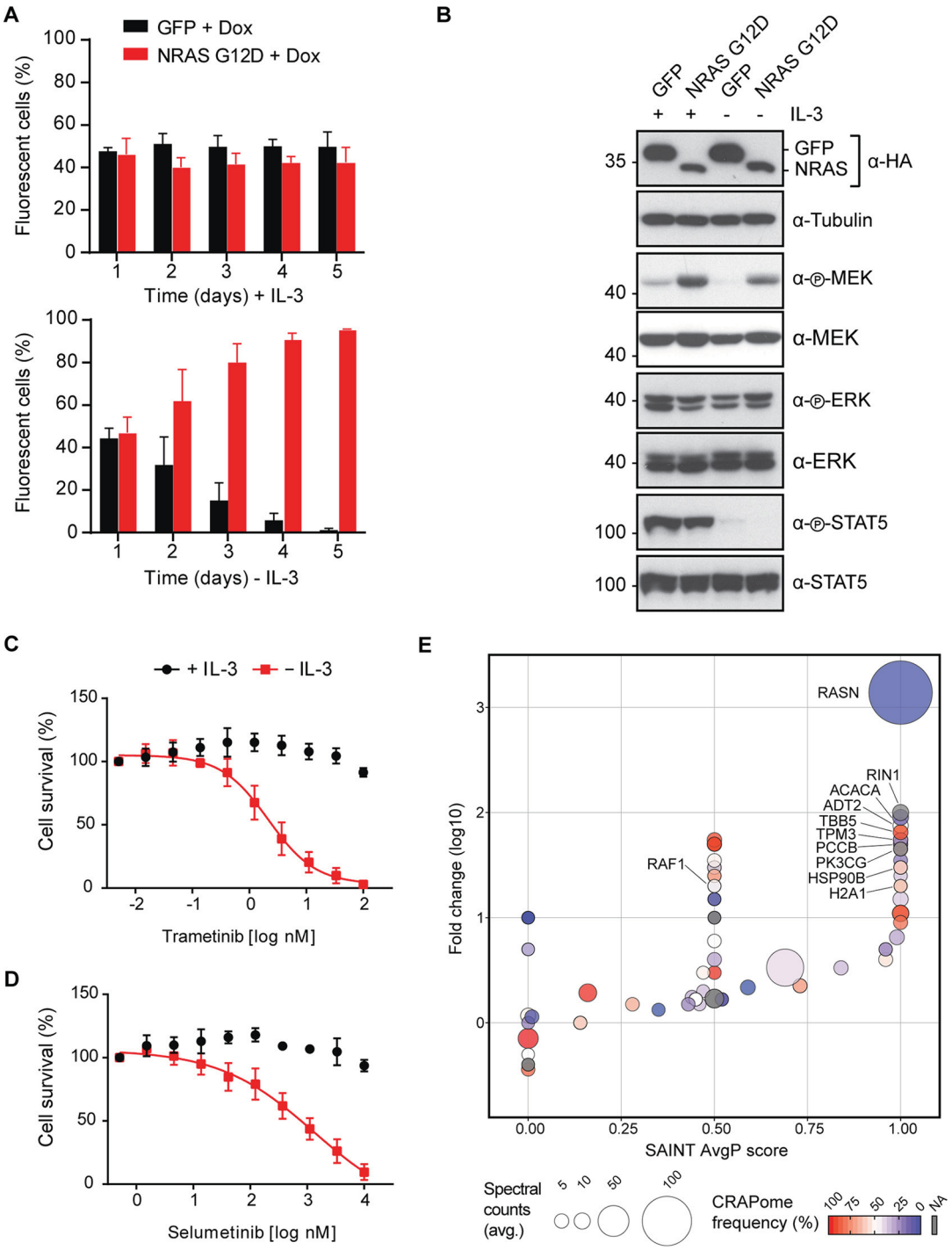

FIG. 3. **Phenotypic characterization and interaction-proteomic analysis of NRAS G12D in Ba/F3 cells.** (*A*) Flow cytometry-based proliferation competition assay for Ba/F3 rtTA3 cells expressing NRAS G12D (mCherry+) or GFP (mCherry+/GFP+). After 24 h doxycycline induction cells were mixed at a 1:1 ratio and grown in the presence of 1 μg/ml doxycycline with or without IL-3. The distribution of cell populations was monitored at the indicated time points using flow cytometry. Data represent mean value ± s.d. of at least two independent experiments. (*B*) Ba/F3 rtTA3 GFP and NRAS G12D cells were induced with 1 μg/ml doxycycline in the presence of IL-3 for 48 h. Cells were then washed once, cultured in the presence of 1 μg/ml doxycycline with or without IL-3 for 12h, lysed, and immunoblotted with the indicated antibodies. (*C–D*) Cell viability of Ba/F3 rtTA3 NRAS G12D-expressing cells in the presence or absence of IL-3 upon treatment with trametinib (C) or selumetinib (*D*) as indicated. Data represent mean value ± s.d. of at least two independent experiments performed in triplicates and normalized to untreated control. (*E*) Scatter plot summarizing the SAINT-based significance and CRAPome frequency analysis of NRAS G12D TAP-LC-MSMS experiments. Ba/F3 rtTA3 NRAS cells were grown in presence of IL-3 and induced for 24 h with 1 μg/ml doxycycline. Data shown are based on two independent experiments (n = 2), each analyzed as technical duplicates and using Ba/F3 rtTA3 GFP-expressing cells as negative control.

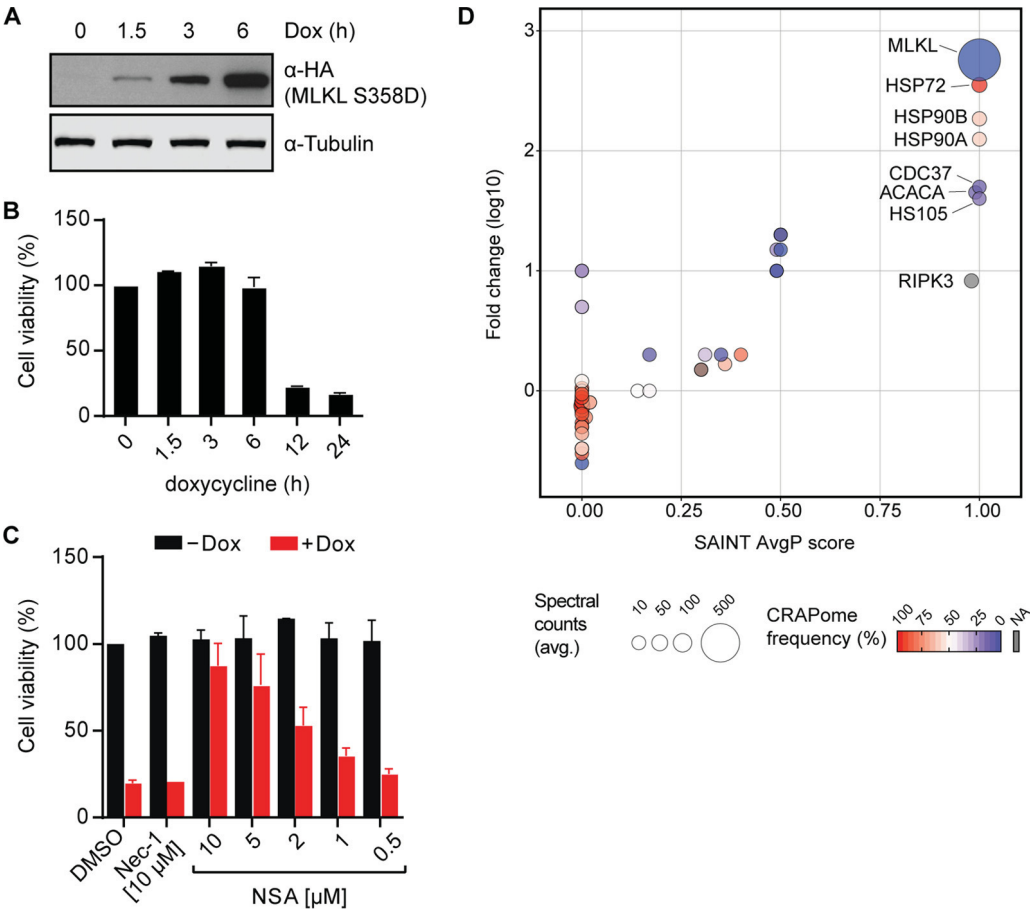

FIG. 4. **Phenotypic and TAP-LC-MSMS analysis of the cell death-inducing MLKL S358D mutant.** (*A*) HT-29 RIEP MLKL S358D cells were treated with 2 μg/ml doxycycline for the indicated time. Cells were lysed and immunoblotted with the indicated antibodies. (*B*) Cell viability of HT-29 RIEP MLKL S358D cells induced with 2 μg/ml doxycycline for the indicated time. Data represent mean value ± s.d. of two independent experiments performed as triplicates and normalized to the untreated control. (*C*) Cell viability was examined in HT-29 RIEP MLKL S358D cells untreated or treated overnight with 2 μg/ml doxycycline and the compounds Nec-1 (10 μM) or NSA, as indicated. Data represent the mean value ± s.d. of two independent experiments performed as triplicates and normalized to the untreated control. (*D*) Scatter plot summarizing the SAINT-based significance and CRAPome frequency analysis of MLKL S358D TAP-LC-MSMS experiments. HT-29 RIEP MLKL S358D cells were induced for 7 h with 2 μg/ml doxycycline. Data shown are based on two independent experiments ($n = 2$), each analyzed as technical duplicates with HT-29 RIEP GFP-expressing cells used as the negative control.

12 h after induction as demonstrated by cell viability measurement (Fig. 4*B*) and microscopy (Supplemental Fig. 4*B*). The MLKL inhibitor necrosulfonamide (NSA) (46) inhibited MLKL S358D-induced cell death (48) in a dose-dependent manner (Fig. 4*C*). Conversely, the RIPK1 inhibitor necrostatin-1 (Nec-1) (49) that blocks necroptosis signaling upstream of MLKL, did not confer protection. These data demonstrate that pRSHIC enables expression and, consequently, phenotypic analysis of proteins that promote cell death.

TAP-LC-MSMS Analysis Identifies MLKL S358D as an HSP90 Client Protein—To identify novel protein interaction partners of MLKL S358D, the cells were induced for 7 h with doxycycline before harvest and TAP-LC-MSMS analysis. The known interactor RIPK3 (47) was significantly enriched in MLKL S358D pulldowns (Fig. 4*D*). Furthermore, heat-shock-related 70 kDa protein 2 (HSP72), HSP90A/B, and the kinase-adaptor cochaperone cell division cycle 37 (CDC37) (50) were identified as high-confidence interactors based on SAINT analysis (51). These heat shock proteins act as molecular chaperones, assisting other proteins to attain and maintain proper folding (52). The comparably high contaminant repository for affinity purification (CRAPome) frequencies (53) assigned to HSP90 and HSP72 likely reflect the large number of client proteins they functionally interact with. Chemical inhibition of HSP90 function leads to client protein destabilization and degradation. Importantly, the HSP90 inhibitor geldanamycin (54) has been shown to block necroptotic cell death (55). This inhibitory effect has been attributed to the destabilizing effect on the two main kinases involved in necroptosis signaling, RIPK1 and RIPK3. Both have been demonstrated to depend on HSP90 (56–58). Our TAP-MS analysis would, however, suggest that the interaction of MLKL with HSP90 may also contribute to this inhibitory effect (Fig. 4*D*). In order to investigate the functional relevance of HSP90 for MLKL

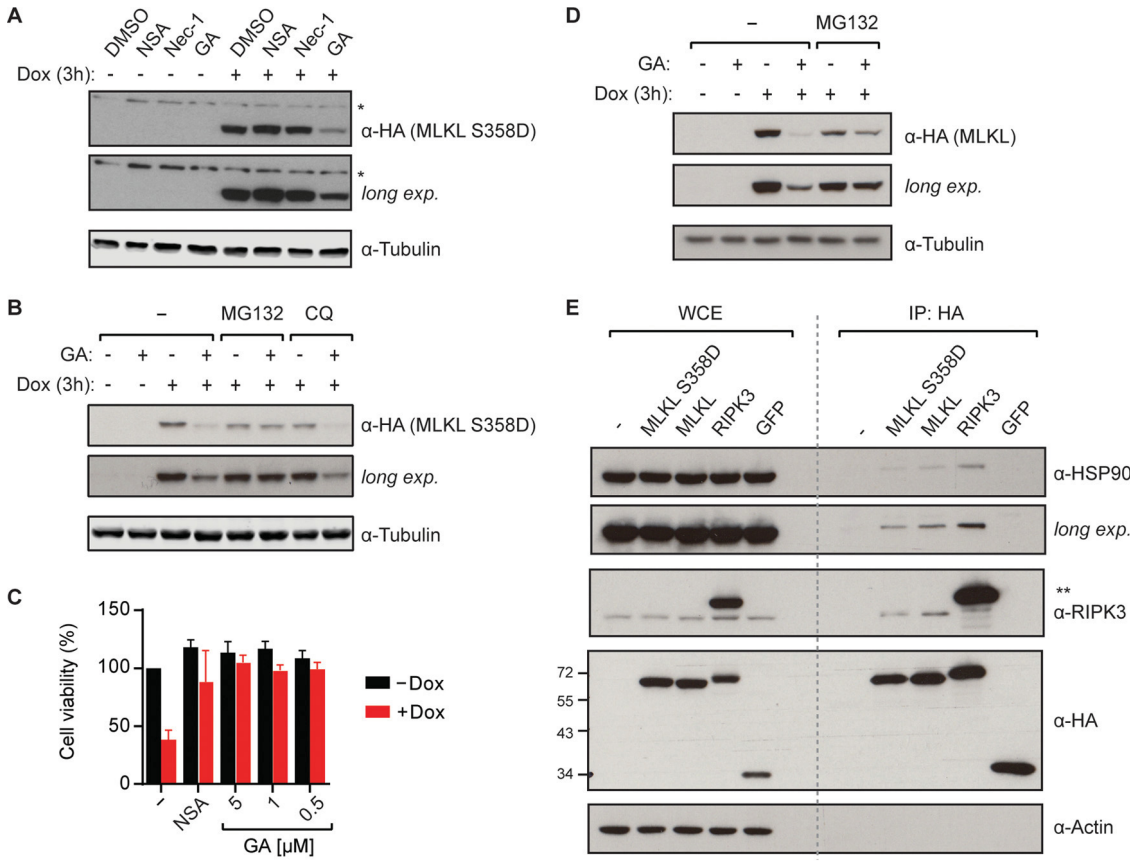

FIG. 5. **MLKL is a novel HSP90 client protein.** (*A*) HT-29 RIEP MLKL S358D cells were treated with 2 μg/ml doxycycline and NSA (10 μM), Nec-1 (10 μM) or geldanamycin (GA, 1 μM) for 3 h. Cells were lysed and immunoblotted with the indicated antibodies. Asterisk (*) denotes nonspecific band. Data shown are representative of three independent experiments. (*B*) HT-29 RIEP MLKL S358D cells were pretreated for 1 h with 10 μM MG132 or 10 μM chloroquine (CQ) before induction with 2 μg/ml doxycycline and the addition of 1 μM GA or DMSO. After 3 h of incubation, cells were harvested, lysed, and immunoblotted with the indicated antibodies. Data shown are representative of two independent experiments. (*C*) Cell viability was assessed in HT-29 RIEP MLKL S358D cells induced with 2 μg/ml doxycycline and treated with 10 μM NSA or GA as indicated for 14 h. Data represent mean value ± s.d. of three independent experiments performed as triplicates and normalized to the untreated control. (*D*) HT-29 RIEP MLKL cells were pretreated for 1 h with 10 μM MG132 before induction with 2 μg/ml doxycycline and addition of 1 μM GA or DMSO. After 3 h of incubation, cells were harvested, lysed, and immunoblotted with the indicated antibodies. Data shown are representative of two independent experiments. (*E*) Expression of the indicated bait proteins was induced in HT-29 cells with 2 μg/ml doxycycline for 6 h. Cell lysates were immunoprecipitated and whole cell extracts (WCE) and immunoprecipitates (IP) were analyzed by immunoblotting with the indicated antibodies. Asterisks (**) denote SH-tagged RIPK3. Data shown are representative of two independent experiments.

S358D, we induced expression in HT-29 RIEP MLKL S358D cells by doxycycline addition for 3 h in the presence of geldanamycin, Nec-1, or NSA. Geldanamycin led to a strong decrease in MLKL S358D protein levels, whereas the other inhibitors had no effect (Fig. 5*A*). To exclude the possibility that geldanamycin interfered with the inducible expression system *per se*, we verified that the mCherry reporter was equally expressed in both control and geldanamycin-treated samples by flow cytometry (Supplemental Fig. 4*C*). The rapid degradation of MLKL S358D upon HSP90 inhibition suggested that this protein constitutes a novel HSP90/CDC37 client. Indeed, the closely related mixed lineage kinase 3 (MLK3) has previously been shown to be stabilized by association with HSP90 and the cochaperone CDC37 (59). The geldanamycin-induced loss of MLKL S358D protein could be prevented by simultaneous treatment with the proteasome inhibitor MG132 (Fig. 5*B*), whereas blocking lysosomal protein degradation using chloroquine had no effect. This data suggested that MLKL S358D was subjected to proteasomal degradation in the absence of HSP90-mediated stabilization, similar to previously described HSP90 client proteins (57). Neither Nec-1 nor ponatinib, recently described to inhibit both RIPK1 and RIPK3 (48, 60), blocked MLKL S358D-induced cell death, indicating that it proceeded independently of these kinases. Yet, the HSP90 inhibitor geldanamycin efficiently blocked MLKL S358D-dependent necroptotic cell death in HT-29 cells (Fig. 5*C*), further corroborating the requirement of HSP90 for MLKL S358D.

Finally, we investigated the requirement of HSP90 function for the MLKL wild-type protein. Similar to the S358D mutant,

geldanamycin induced destabilization of the wild-type MLKL protein and this degradation could be blocked by concomitant MG132 treatment (Fig. 5*D*). To confirm the interaction between HSP90 and wild-type MLKL as well as the MLKL S358D mutant, we performed coimmunoprecipitation experiments. MLKL copurified HSP90, similar to the previously described HSP90 client protein RIPK3 (58) (Fig. 5*E*). As demonstrated by the identification and characterization of MLKL as a novel HSP90 client, pRSHIC is an efficient tool to perform phenotypic and TAP-MS analysis of toxicity-promoting proteins.

CONCLUSIONS

We have established a retroviral-based expression system that expands the repertoire of cell lines amenable to SH-based TAP-MS experiments and thus enables interaction proteomic experiments in the physiologically relevant cellular background. The IRES-linked fluorescent reporter protein allows quick evaluation of bait protein induction by flow cytometry, fluorescence-activated cell sorting of specific cell populations and live tracing of bait-expressing cells to assess phenotypic changes (*i.e.* morphology, surface marker expression, drug resistance). Intracellular localization of the bait proteins can be assessed by probing for the N- or C-terminally fused SH-tag. Moreover, the inducibility of bait expression allows proteins that promote cell death to be studied and opens the opportunity to perform targeted chemical screens in the cell system of choice.

Here, we demonstrated efficiency and applicability of pRSHIC for TAP-MS-based interaction proteomics studies on the oncogenic NRAS G12D mutant protein (22) in murine Ba/F3 cells. Furthermore, we performed interaction proteomics and detailed phenotypic analysis of the cell death-inducing MLKL S358D mutant protein (25) in HT-29 cells, leading to the identification of MLKL as a novel HSP90 client protein.

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[S] This article contains supplemental material Supplemental Tables S1–S4 and Supplemental Figs. S1–S4.

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Conflict of interest: The authors declare no competing financial interest.

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2.5 Interlude

The development of targeted therapeutic agents has substantially been influenced by the identification and clinical advancement of BCR-ABL TKIs in CML. The development of resistance due to the acquisition of TyrK domain mutations has been recognized as a pressing constraint in the broad and durable application of kinase inhibitor-based therapy in CML and beyond. In order to design potential novel combination treatments as alternative therapeutic options and to derive novel candidate biomarkers of suboptimal response in resistant patients, we rationalized the necessity to identify the repertoire of genes important for kinase inhibitor-mediated blockade of cancer cell proliferation.

Here we have performed gene-trap based mutagenesis screens in a human haploid CML cell line upon treatment with the six 1st, 2nd and 3rd generation BCR-ABL inhibitors. Integrative analysis has allowed us to identify a common set of 6 gene candidates which upon loss-of-function lead to cellular resistance to pharmacological BCR-ABL inhibition. More specifically, we could identify the LZTR1 protein as an adaptor protein for the CUL3 E3 ligase complex and being critically important for the regulation of RAS and MAPK pathway activation state in a broad range of cell lines. Furthermore, these observations enabled us to experimentally demonstrate that *LZTR1* mutations identified in Noonan syndrome (NS), Schwannomatosis (SWNMT) and glioblastoma (GBM) are loss-of-function mutations affecting RAS/MAPK signaling.

2.6 Manuscript #3

Johannes W. Bigenzahn, Giovanna M. Collu, Gregory I. Vladimer, Leonhard X. Heinz, Melanie Pieraks, Fiorella Schischlik, Astrid Fauster, Manuele Rebsamen, Vincent A. Blomen, Georg E. Winter, Robert Kralovics, Thijn R. Brummelkamp, Marek Mlodzik, Giulio Superti-Furga. *"Genetic drug resistance screen identifies the cullin adaptor LZTR1 as RAS regulator mutated in human disease"* ‡

‡ submitted for publication

A detailed description of the individual author contributions can be found within the declaration section of this doctoral thesis.

Genetic drug resistance screen identifies the cullin adaptor LZTR1 as RAS regulator mutated in human disease

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Introductory paragraph

Small molecule tyrosine kinase inhibitor (TKI)-based treatment of chronic myeloid leukemia (CML), directed by the presence of the Philadelphia chromosome (Ph^+) encoded BCR-ABL tyrosine kinase, is a paradigm of targeted cancer therapy[1,2]. However, the development of TKI resistance limits the long-term success of these therapeutics[3]. We used a genetic screening approach in the near-haploid CML cell line KBM-7[4] to identify six genes whose individual loss-of-function led to TKI drug resistance. Among these, we here investigated the role of the leucine zipper like transcription regulator 1 (*LZTR1*) gene, as it was mechanistically enigmatic despite its conspicuous genetic involvement in a variety of human developmental and oncological diseases. LZTR1 protein is localized to the endosomal compartment of human cells and interacted with all three RAS GTPase proteins (K-, N- and HRAS). By acting as an adaptor protein for the cullin (CUL)-3 E3 ligase protein complex, LZTR1 protein mediated KRAS ubiquitination. Investigation of LZTR1 function in *Drosophila,* a classical model system to study the RAS pathway, showed that loss of the *LZTR1* orthologue *CG3711 in vivo* increased ectopic wing vein formation and rescued $Ras^{V12\,C40}$-mediated R7 photoreceptor loss, consistent with a role for LZTR1 in RAS pathway regulation. In CML cells, loss of LZTR1 activity led to enhanced mitogen-activated protein kinase (MAPK) pathway activation and reduced TKI sensitivity in a KRAS-dependent manner. To genetically validate the interaction between LZTR1 and RAS, we tested whether loss of *LZTR1* was functionally rescued through simultaneous ablation of the *KRAS* gene. Indeed, genetic inactivation of *KRAS* reverted MAPK pathway hyperactivation. *LZTR1* missense mutations identified in Noonan syndrome (NS)[5] and glioblastoma (GBM)[6] on the other hand, failed to revert the loss-of-function phenotype, providing a mechanistic rationale for the involvement of LZTR1 in RASopathies and cancers. Our genetic survey for TKI drug resistance genes thus identified LZTR1 as a CUL3 adaptor for RAS family members whose role in modulating stimulus-dependent MAPK signaling appears to be critical for specific developmental and growth regulatory processes.

Main text

CML is characterized by the expression of the constitutively active oncogenic tyrosine kinase fusion BCR-ABL originating from the Philadelphia chromosomal (Ph^+) translocation t(9;22)(q34;q11)[2]. The proliferation and survival of Ph^+ CML cells depends on the activation state of key cellular signaling cascade networks including the MAPK, PI3K/AKT and JAK/STAT pathways[2]. The development of the TKI imatinib has provided a successful targeted therapeutic example, although development of resistance mainly due to kinase domain mutations proved to be a persistent challenge[1,3]. Different 2nd and 3rd generation TKIs have been developed to combat drug resistance induced by kinase domain mutations[3].

We have previously used haploid genetic screens in the near-haploid CML cell line KBM-7 to show that the experimental set up could be successfully employed for the unbiased identification of candidate genes affecting BCR-ABL inhibitor resistance by alternative mechanisms[4]. Encouraged, we decided to here mount a full haploid genetic screening and validation campaign with the six TKIs that were in clinical use or evaluation with the hope that results could lead to a more comprehensive understanding of drug resistance mechanisms (Extended Data Fig. 1a). Cells were mutagenized using a retroviral gene-trap and subsequently exposed to each of the small molecule inhibitors at concentrations corresponding to IC^{50}-IC^{70} dosage (Extended Data Fig. 1b). Resistant cell populations were collected after 14-21 days of selection and genomic gene-trap insertions identified by deep sequencing. Each screen resulted in significant enrichment of disruptive insertions in 5 to 18 different genes (Fig. 1a-f and Extended Data Fig. 1c). We identified a recurrent set (≥4 screens) of six genes (*NF1*, *WT1*, *PTPN1*, *PTPN12*, *LZTR1*, *BAP1* – "TOP6" set) (Fig. 1g) with significant overrepresentation of disruptive genomic gene-trap integrations strongly indicating a selective advantage upon drug treatment (Extended Data Fig. 2a,b).

We employed a lentiviral CRISPR/Cas9 multi-color competition assay (MCA)-based co-culture system to evaluate gene loss-of-function-mediated drug resistance effects upon small molecule inhibitor treatment. Here, *SpCas9* expressing KBM-7 ($KBM\text{-}7^{Cas9}$) cells were infected with lentiviral guide RNA (sgRNA) vectors co-expressing reporter fluorophores enabling color

tracing of mixed knock-out and control cell populations by flow cytometry in the same well (Extended Data Fig. 3a). Cells infected with individual sgRNAs targeting the TOP6 genes exhibited a strong decrease in cognate protein levels (Extended Data Fig 3b-g) and efficient indel formation (Extended Data Fig. 3h). As expected, mixed sg*Ren* (targeting *Renilla luciferase*) control cell populations (GFP^+ vs. $mCherry^+$) did not show any preferential out-growth of resistant cells upon 14 days of TKI treatment. In contrast, KBM-7^{Cas9} GFP^+ cells harboring sgRNAs targeting TOP6 genes demonstrated enhanced cell survival and out-growth upon increasing concentrations of imatinib (Fig. 1h) and rebastinib (Extended Data Fig. 3i). Thus, we could establish by individual functional validation the TOP 6 genes important for drug action in *BCR-ABL*$^+$ CML cells.

The RAS GTPase activating protein neurofibromin (*NF1*) and the tyrosine-protein phosphatase non-receptor type 1 (*PTPN1*) have been identified previously in genome-wide shRNA screens, reducing sensitivity to BCR-ABL inhibitors via loss of negative RAS activity regulation and enhanced BCR-ABL - GRB2 recruitment, respectively [7]. Additionally, tyrosine-protein phosphatase non-receptor type 12 (*PTPN12*) has been shown to negatively impinge on MAPK pathway activation in mammosphere formation [8]. All three genes share the ability to modulate MAPK pathway activation (Fig 2a). In contrast, transcription factor Wilms tumor protein (*WT1*) and deubiquitinating enzyme BAP1 exert both oncogenic as well as tumor suppressor functions due to their involvement in transcriptional regulation[9,10] (Fig 2a).

In contrast to these five genes, we could not deduce any mechanistic explanation for the role of leucine zipper like transcription regulator 1 (*LZTR1*) in enhanced CML cell survival upon BCR-ABL inhibition from the existing literature. To exclude cell line specific effects, we first confirmed that loss of *LZTR1* expression induced resistance to imatinib and rebastinib in different CML cell lines (Extended Data Fig. 4b, d-e and data not shown). While we identified significant *LZTR1* enrichment only in four of the genetic screens, K-562^{Cas9} sg*LZTR1* cells exhibited various degrees of resistance against all tested BCR-ABL TKIs (Fig. 2a,b). In contrast to other BTB domain-containing proteins LZTR1 displays a reverse domain orientation with the N-terminal kelch domain preceding the two BTB and partial BACK domains[11-13]. We

tested whether both N- and C-terminal domains are essential for the drug resistance phenotype using a CRISPR/Cas9 based domain scanning strategy[14] to separately target kelch, BTB-1 and BTB-2 domains with individual sgRNAs. All protein domain-targeting sgRNAs showed efficient indel formation (Extended Data Fig. 4a) and convincingly induced resistant out-growth of domain-targeted cell populations with increasing concentrations of rebastinib, suggesting that the entire protein is required for inhibitor sensitivity (Fig. 2c). In order to determine whether LZTR1 exerts its function only in a CML specific context, we tested its drug sensitivity modulating effect in acute myeloid leukemia (AML) cells driven by the oncogenic FLT3-ITD tyrosine kinase[15]. We infected FLT3 inhibitor sensitive MV4-11^{Cas9} AML cells with *LZTR1* targeting sgRNAs (Extended Data Fig. 4c) and observed out-growth of resistant cells upon tandutinib (Fig 2d), ponatinib (Fig 2e) or quizartinib treatment (Extended Data Fig. 4f) underlining a role for LZTR1 in the drug response of hematopoietic cancers driven by different tyrosine kinases.

KBM-7^{Cas9} CML cells infected with different sgRNAs targeting *LZTR1* displayed enhanced phosphorylation of MEK and ERK, indicative of augmented MAPK pathway activation (Fig. 2f). In contrast, global tyrosine phosphorylation, as well as phosphorylation of S6K1, S6 and the direct BCR-ABL substrate STAT5 remained unchanged (Extended Data Fig. 5a). Additionally, *BCR-ABL*$^{+}$ CML (K-562) and *FLT3-ITD*$^{+}$ AML (MV4-11) cell lines demonstrated similar enhanced MAPK pathway activation under normal growth conditions (Fig. 2f). Deregulation of the MAPK pathway was detectable also by sustained MEK and ERK phosphorylation levels in the presence of increasing imatinib concentrations (Extended Data Fig. 5b). *LZTR1* full-length cDNA complementation in K-562^{Cas9} sg*LZTR1* cells reverted both enhanced MEK/ERK phosphorylation as well as TKI resistance (Fig. 2g-h). Interestingly, in comparison to CML cells, neither HeLa^{Cas9} nor HEK293T^{Cas9} sg*LZTR1* cells presented enhanced MAPK pathway activation under normal culture conditions (Extended Data Fig. 5c). However, upon serum stimulation after starvation, HEK293T^{Cas9} sg*LZTR1* cells displayed a more pronounced MEK and ERK activation than control cells (Extended Data Fig. 5d). As the BCR-ABL activity may be considered functionally equivalent to constitutive growth factor stimulation this would

suggest the interesting notion that LZTR1 function may only become manifest upon pathway stimulation[2].

Recently, several genetic studies have identified *LZTR1* mutations in glioblastoma (GBM)[6], schwannomatosis (SWNTS)[13] and Noonan syndrome (NS)[5], a developmental syndrome which is part of the larger group of RASopathies characterized by mutations in members of the RAS/MAPK pathway [16]. Identification of *NF1* and *LZTR1* loss-of-function induced MAPK pathway activation in our haploid resistance screens combined with human *LZTR1* mutations in NS raised to us the hypothesis that LZTR1 could be directly involved in regulation of RAS or RAS like GTPases.

The genetic involvement of different pathway components to the RAS/MAPK pathway has historically been best discovered using fly development as a model. *Drosophila* wing vein formation and eye development serve as excellent read-outs to dissect RAS signaling[17,18,19]. *CG3711* encodes the *Drosophila* orthologue of mammalian *LZTR1*, which contains a unique N-terminal domain (amino acids 1-184), found in *Drosophila* species only. This is followed by the highly conserved remaining part of the protein (53.69% sequence identity) (Extended Data Fig. 6a-c). We first used a systemic knock-down RNAi approach of *CG3711* to identify loss-of-function phenotype(s) related to RAS signaling. Although knock-down of *CG3711* using *act5C-Gal4* was viable, the majority of wings of these flies in three independent RNAi lines displayed wing vein defects characterized by extra veins and vein tissue (Fig. 3a-c). This phenotype closely resembles a gain-of-function increase of RAS/MAPK signaling[19,20]. For this reason, we next asked whether *CG3711* could genetically interact with *Ras*. *Drosophila* R7 photoreceptor induction serves as paradigm and was instrumental in the dissection of the RAS pathway[17,18]. As there are no viable hypomorphic loss-of-function alleles of *dRas* (*Drosophila* Ras), we employed a mild dominant negative version, $Ras^{V12\ C40}$, which although locked in the GTP-bound state does not activate MAPK signaling[21]. $Ras^{V12C\ 40}$ expression in the developing eyes (via the *sevenless*/*sev-Gal4* expression system) led to a frequent loss of the R7 photoreceptor (~30% of ommatidia display R7 loss and some also lose other R-cells; Fig. 3d,g). As $Ras^{V12\ C40}$ is constitutively active it also causes ommatidial rotation defects, besides its R7 loss

effect[21,22], serving as internal control. Strikingly, upon *sev-Gal4* driven *CG3711* RNAi and $Ras^{V12\ C40}$ co-expression, the loss of R7 phenotype was almost completely suppressed (Fig. 3e,g ; note that all three *CG3711* RNAi lines display similar suppression of the R7 loss). Importantly, as a control, the ommatidial rotation defects are rather enhanced than suppressed as these involve MAPK independent RAS signaling[21], which is not blocked in $Ras^{V12\ C40}$ (Fig. 3e), consistent with an increase in RAS activity. Noteworthy, *CG3711* RNAi in the eye alone did not induce phenotypic changes, in contrast to ectopic vein formation in the wing (Fig 3f). Altogether, the data obtained using *Drosophila* genetics corroborate the hypothesis that LZTR1 is a RAS/MAPK pathway component with a negative regulatory role.

To obtain insight in the LZTR1 mode of action we co-expressed LZTR1 and different RAS isoforms in in HEK293T cells. LZTR1 co-immunoprecipitated with KRAS4A (Fig. 3h), NRAS, HRAS (Extended Data Fig. 7a,b), and, to a lesser extent, KRAS4B (Fig. 3i) suggesting direct or indirect physical association of LZTR1 with members of the RAS family of proteins. To investigate the subcellular localization of LZTR1 we switched to HeLa cells with a large cell body convenient for immunofluorescence. Immunostaining of inducibly expressed tagged LZTR1 revealed proximity and co-localization with the endosomal markers EEA1, RAB5 and RAB7, respectively, but not with golgi (Golgin97) or lysosomal (LAMP1) markers (Extended Data Fig. 8a-g). This is compatible with the notion that all three RAS GTPases can signal from endosomal compartments[23]. Interestingly, HRAS endosomal localization is regulated by ubiquitination resulting in modulation of MEK/ERK activity[24].

We sought to investigate whether LZTR1 affected ubiquitination of RAS family members. The ubiquitination of RAS proteins underlies dynamic stimulus dependent regulation[25,26] and can result in diverse outcomes such as βTrCP poly-ubiquitination-induced HRAS degradation[27], mono- and di-ubiquitination-mediated activity and effector protein binding regulation[28,29] or alteration of intracellular trafficking and stabilization of endosomal HRAS localization modulating ERK activation[24]. BTB domain-containing proteins serve as adaptor proteins for the CUL3 E3 ligase complex enabling specific substrate recognition and ubiquitination[30]. Therefore, we tested whether LZTR1 and CUL3 would bind. Upon co-expression in HEK293T

cells, CUL3 indeed co-immunoprecipitated with LZTR1 (Extended Data Fig. 7c) in agreement with previous reports [6,31]. We tested the possibility that the CUL3-LZTR1 E3 ligase complex ubiquitinated KRAS, thereby regulating KRAS signaling properties and MAPK pathway activation state in the presence of the proteasome inhibitor MG132 known to stabilize substrate interactions[32]. While co-expression of HA-ubiquitin with KRAS4A alone only demonstrated a basal state of ubiquitination, the addition of tagged CUL3 and LZTR1 led to a substantial increase in ubiquitination (Fig. 3j). In contrast, the two LZTR1 mutants R198G and G248R identified in GBM and NS, failed to induce a similar increase in ubiquitination on KRAS4A (Fig. 3k), suggesting a possible biochemical mechanism for these genetic mutations.

If indeed the action of RAS is epistatic to LZTR1, then loss of RAS function should compensate loss of LZTR1 activity. In this case, it should be possible to assess the disease-associated *LZTR1* mutations by their dependency on RAS activity. To validate the experimental system, we first confirmed the prediction that loss of LZTR1 function would enhance RAS activity in K-562 cells (Extended Data Fig. 9a). We then performed CRISPR/Cas9-based double knock-out MCA experiments (Extended Data Fig. 9b), infecting sg*LZTR1* cells with an additional set of sgRNAs targeting the three main *RAS* isoforms (*K*-, *N*- and *HRAS*), *RIT1* or sg*Ren* as negative control and treated these cells with imatinib (Extended Data Fig. 9c-e). As expected, single sg*LZTR1* and sg*LZTR1*/sg*Ren* double-infected cells displayed the expected resistance phenotype compared to control sg*Ren* (Fig. 4a). Intriguingly, sgRNAs targeting *KRAS* abolished cellular out-growth, whereas *NRAS*, *HRAS* and *RIT1* targeting sgRNAs failed to do so (Fig. 4a and Extended Data Fig. 9f). In line, sg*LZTR1*/sg*KRAS* cells displayed reduced MEK phosphorylation comparable to sg*Ren* cells whereas sg*NRAS* and sg*HRAS* cells still maintained enhanced MAPK pathway activation (Fig. 4b). Altogether this genetic interaction experiment provided formal proof for a functional involvement of RAS in the phenotypes induced by loss of LZTR1.

Collectively there are more than 50 different mutations that have been mapped to the human *LZTR1* gene in diseases as diverse as developmental RASopathies[5] and various hereditable predispositions or acquired forms of cancer[6,13]. As of now there is no molecular insight into the

mechanism involved. We chose to focus on missense mutations in NS and GBM that can be expressed in our experimental system (Fig. 4c). Different mutation-bearing *LZTR1* cDNAs were tested for their ability to complement K-562 CML cells made deficient for endogenous *LZTR1* (Extended Data Fig. 10a). In contrast to WT LZTR1, NS-associated Y199C, S247N, G248R, R284C, H287Y and GBM-associated W105R, R198G, G248R, T288I, R810W mutations failed to reduced MEK and ERK phosphorylation, despite being expressed at comparable or higher levels (Fig. 4d,e). GBM mutations W105R, R198G and the GBM/NS mutation G248R additionally failed to restore sensitivity to imatinib treatment (Extended Data Fig. 10b). These findings provide the first functional evidence that the human LZTR1 missense mutations identified in NS and GBM are loss-of-function mutations depending on RAS function and thus contribute to the understanding of the disease pathologies by providing a mechanistic rationale.

Through haploid genetic drug resistance screening we revealed that the so far unknown cellular function of LZTR1 lies in its ability as adaptor protein for the CUL3 E3 ligase complex to ubiquitinate and regulate RAS signaling and MAPK activation (Fig. 4f). The study provokes the important questions on the role of LZTR1-induced RAS ubiquitination. What type of ubiquitination is induced, where on the RAS protein and in the cell is the ubiquitination occurring and what is the functional consequence of the ubiquitination ? RAS has been previously reported to be ubiquitinated and other proteins have been suggested to regulate RAS signaling through ubiquitination before. However, there has been no consensus as to the type of ubiquitination or the functional consequences thereof and the previously characterized Rabex-5 E3 ligase has not yet been involved in any human condition. Clearly more work will be required to dissect the intricacies of RAS isoform ubiquitination. Our preliminary investigation using co-transfection systems led to the detection of mono- and di- as well as poly-ubiquitination on K48 and K63, possibly arguing for LZTR1 promoting a mixed linkage ubiquitination. While RAS ubiquitination has been observed in large scale proteomic studies we failed so far to detect dependency on LZTR1 for endogenous RAS ubiquitination. However,

an accompanying study points to LZTR1 promoting mono-ubiquitination of NRAS and subsequent impairment of p120GAP binding (Steklov, Baietti et al.).

In summary our study provides functional characterization of six genes involved in TKI sensitivity of CML cells as well as an unequivocal involvement of an LZTR1-RAS axis in MAPK pathway regulation leading to TKI therapy resistance. Future studies will be focused on assessing the clinical utility of all these observations. In general, the findings highlight the importance of RAS ubiquitination as a second layer of MAPK pathway signaling regulation in physiology, disease pathology and cancer biology.

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Author contributions

J.W.B. and G.S-F. conceived the study based on discussions with T.R.B.. J.W.B., G.I.V, M.P. performed research, G.M.C. and M.M. designed and performed *Drosophila* experiments. L.X.H., A.F. M.R. and G.W. generated reagents and provided scientific insight. F. S. analyzed haploid genetic screening data, created circos plots and the graphical display of insertion sites. V.A.B. and T.R.B. provided reagents and gave experimental advice. R.K. supervised sequencing data analysis and gave experimental advice. J.W.B., G.M.C., G.I.V., L.X.H., F.S., M.M. and G.S-F. analyzed and interpreted the data; J.W.B., M.M. and G.S-F. wrote the paper.

Competing financial interests

The authors declare no competing financial interest.

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MATERIALS AND METHODS

Cell lines and reagents.

HEK293T were obtained from ATCC (Manassas, VA, USA), K-562, KCL-22 and MV4-11 from DSMZ (Braunschweig, Germany). KBM-7 were obtained from T. Brummelkamp. HeLa were provided by M. Hentze. Cells were cultured in DMEM (Gibco, Grand Island, NY, USA), RPMI (Gibco) or IMDM medium (Gibco) supplemented with 10% (v/v) FBS (Gibco) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) (Gibco). Cell lines were checked for mycoplasma by PCR or ELISA. The reagents used were as follows: imatinib (S1026, Selleckchem, Houston, TX, USA), nilotinib (S1033, Selleckchem), dasatinib (S1021, Selleckchem), bosutinib (S1014, Selleckchem), rebastinib (DCC-2036, S2634, Selleckchem), ponatinib (S1490, Selleckchem), quizartinib (S1526, Selleckchem), tandutinib (S1043, Selleckchem), MG132 (S2619, Selleckchem) and doxycycline (D9891, Sigma-Aldrich, St. Louis, MO, USA).

Antibodies.

Antibodies used were HA (sc-805, Santa Cruz, Dallas, TX, USA), HA (901501, BioLegend, San Diego, CA, USA), FLAG (F1804, Sigma-Aldrich), FLAG (#14793, Cell Signaling, Danvers, MA, USA), V5 (R960-25, Thermo Fisher Scientific, Waltham, MA, USA), V5 (ab9116, Abcam, Cambridge, UK), NF1 (A300-140A, Bethyl Laboratories, Montgomery, TX, USA), PTPN1 (sc-1718, Santa Cruz), PTPN12 (A301-302A, Bethyl Laboratories), BAP1 (A302-243A, Bethyl Laboratories), WT1 (sc-192, Santa Cruz), LZTR1 (HPA071248, Sigma-Aldrich), phospho-MEK1/2 (#2338, Cell Signaling), MEK1/2 (#9126, Cell Signaling), phospho-ERK1/2 (#4370, Cell Signaling), ERK1/2 (M5670, Sigma-Aldrich), phospho-STAT5A/B (05-886R, Merck Millipore, Billerica, MA, USA), STAT5 (610191, BD Biosciences, Franklin Lakes, NJ, USA), phospho-S6 (#5364, Cell Signaling), p70 S6 kinase (sc-230, Santa Cruz), Golgin97 (ab84340, Abcam), EEA1 (610457, BD Biosciences), LAMP1 (ab25630, Abcam), RAB7 (#9367, Cell Signaling), HSP90 (610418, BD Biosciences), RIT1 (ab127041, Abcam), GAPDH (sc-365062, Santa Cruz) and tubulin (ab7291, Abcam). The secondary antibodies used were goat anti-

mouse HRP (115-035-003, Jackson ImmunoResearch, West Grove, PA, USA), goat anti-rabbit HRP (111-035-003, Jackson ImmunoResearch), donkey anti-goat (705-035-003, Jackson ImmunoResearch), Alexa Fluor 488 goat anti-mouse (A11001, Thermo Fisher Scientific), Alexa Fluor 488 goat anti-rabbit (A11008, Thermo Fisher Scientific), Alexa Fluor 680 goat anti-mouse (A21057, Thermo Fisher Scientific) and Alexa Fluor 680 goat anti-rabbit (A-21076, Thermo Fisher Scientific).

Plasmids and cloning.

CRISPR/Cas9-based knock-out cell line generation was performed using pLentiCRISPRv2 (Addgene plasmid #52961) or pLentiCas9-BlastR (Addgene plasmid #52962) and pLentiGuide-PuroR (Addgene plasmid #52963)[33]. An IRES-GFP or IRES-mCherry fragment was added to pLentiGuide-PuroR creating LGPIG (pLentiGuide-PuroR-IRES-GFP) and LGPIC (pLentiGuide-PuroR-IRES-mCherry) using standard cloning techniques to enable color tracing of targeted cells in multi-color competition assays (MCA). CRISPR cloning was performed as described elsewhere[34]. In brief, sgRNAs were designed using crispr.mit.edu, CHOPCHOP[35] and sgRNA Designer[36]. Oligonucleotides containing *BsmBI* restriction site-compatible overhangs were annealed, phosphorylated and ligated into pLentiCRISPRv2, LGPIG or LGPIC using standard cloning techniques and sequence verified using sanger sequencing. sg*Ren*.208 sgRNA (sg*Ren*) targeting *Renilla luciferase* coding sequence was used as a negative control. sgRNAs are labeled throughout the manuscript by gene name followed by the genomic targeting sequence position numbered according to the sequence position on the corresponding mRNA.

LZTR1 coding sequence was obtained as pENTR223 vector HsCD00351142 from DNASU plasmid repository (Arizona State University, Tempe, AZ, USA) and following sequence verification an existing single amino acid deletion was reverted to wild type by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). An sg*LZTR1*.466 sgRNA resistant cDNA version was generated and single HA or V5 tags were added to *LZTR1* cDNA using NEB Q5 site-directed

mutagenesis kit (E0554S, NEB, Ipswich, MA, USA). *KRAS4A* and *KRAS4B* coding sequences were designed as described elsewhere[37], ordered as gBlocks (IDT, Coralville, IO, USA) containing gateway cloning adaptor sites and inserted into pDONR221 entry vector using BP recombination (11789020, Thermo Fisher Scientific). Cloning of *NRAS* cDNA has been described elsewhere[38]. *HRAS* cDNA was obtained from Addgene (Plasmid #18662), PCR-amplified using KOD DNA polymerase (71085, Merck Millipore) and cloned into pDONR221 using BP recombination. Point mutations were introduced as stated previously or using *Pfu* DNA polymerase (M7741, Promega, Fitchburg, WI, USA) and *DpnI* restriction enzyme digestion (R0176S, NEB). Following sequence verification cDNAs in gateway-compatible pENTR223 or pDONR221 vectors were transferred by LR recombination (11791100, Thermo Fisher Scientific) into respective expression vectors.

For cDNA rescue experiments cells were either infected with empty vector MPBIG (pMSCV-PGK-BlastR-IRES-GFP) and MPBIC (pMSCV-PGK-BlastR-IRES-mCherry) or with cDNA-containing gateway vector MgwSHPBIC (pMSCV-gateway-StrepHA-PGK-BlastR-IRES-mCherry) all derived from pMSCV-PuroR (Clontech, Mountain View, CA, USA). For transient expression experiments HA- or V5-tagged *LZTR1* cDNAs were subcloned into pcDNA3.1-gw-6xMYC and RAS isoforms into pTO-3xFLAG-gw-FRT-HygroR. pcDNA3-MYC-CUL3 (Plasmid #19893, Y201C mutation was corrected by site-directed mutagenesis) and pRK5-HA-Ubiquitin-WT (Plasmid #17608) were obtained from Addgene. For immunofluorescence experiments HA- and V5-tagged *LZTR1* cDNAs were subcloned into doxycycline inducible gateway-compatible lentiviral vector pCW57.1 (Addgene plasmid #41393). pGAG-POL and pVSV-G retroviral packaging plasmids were obtained from T. Brummelkamp and pADVANTAGE from Promega (E1711). Lentiviral packaging plasmids psPAX2 (Plasmid #12260) and pMD2.G (Plasmid #12259) were obtained from Addgene.

Retroviral and lentiviral cell line generation.

For retroviral infections HEK293T cells were transiently transfected with pGAG-POL, pVSV-G, pADVANTAGE and retroviral expression vectors using Polyfect (301105, Qiagen). Similarly,

for lentiviral infections HEK293T cells were transiently transfected with psPAX2, pMD2.G and lentiviral expression vectors. In both cases the medium was exchanged 24h after transfection and replaced with the respective target cell line specific medium. After 48h the virus-containing supernatant was harvested, filtered (0.45 µm), supplemented with 8µg/mL protamine sulfate (Sigma-Aldrich) and added to 40-60% confluent target cell lines. Suspension cells were furthermore subjected to spinfection (2000 *rpm*, 45 min, room temperature). 24h after infection the medium was exchanged and replaced with fresh medium. Another 24h later, the medium was supplemented with the respective selection antibiotic for 5-7 days to select for infected target cells.

Haploid genetic screens and deep sequencing analysis.

Haploid genetic screening was implemented as described previously [4 39]. In brief, gene-trap retrovirus containing supernatant was produced by transient transfection of HEK293T cells with the gene-trap plasmid along with packaging plasmids pGAG-POL, pVSV-G and pADVANTAGE using Lipofectamine 2000 (Thermo Fisher Scientific). Virus-containing supernatant was collected three times every 24h followed by ultracentrifugation and concentrated virus was used to mutagenize 1×10^8 KBM-7 cells via spinfection. The mutagenized pool was expanded further, 1×10^8 gene-trapped cells were harvested as unselected control population and equal cell amounts were selected with one of the six BCR-ABL inhibitors each in 96-well plates (1×10^5 cells seeded in 100µL per well). Three days after drug treatment 200µL dilution media was added to each well. Plates were monitored for out-growth of drug-resistant clones for 2-3 weeks. Resistant cells were pooled thereafter, collected and expanded to a total cell number of 3×10^7 cells. Genomic DNA (gDNA) was isolated and retroviral insertion sites in resistance screens were recovered via an inverse PCR protocol and control cell populations via a linear amplification mediated (LAM)-PCR followed by deep sequencing analysis. Reads were aligned to human genome version hg19 and insertions 2 base pairs away from each other were removed. The significance of enrichment of insertions in a given gene was calculated by comparing the number of insertions of the BCR-ABL inhibitor

selected populations with the unselected control data set by applying a one-sided Fisher's exact test. *P*-values were adjusted for false discovery rate (FDR) using Benjamini-Hochberg procedure. Screen result plots were visualized using circos plot software[40], summary bubble plots and gene-trap insertion plots were generated via custom scripts using R statistical environment.

TIDE sequencing for analysis of CRISPR/Cas9 induced indel formation.

gDNA was isolated from control and knock-out cells using Qiagen DNeasy Blood & Tissue kit (69506, Qiagen, Hilden, Germany), 400-800bp fragments were PCR amplified using GoTaq DNA Polymerase (M3001, Promega, Madison, WI, USA), subsequently purified using Qiagen QIAquick PCR purification kit (28106, Qiagen) and sent for sanger sequencing. Sequencing tracks were analyzed using TIDE analysis[41] via the online web tool (tide.nki.nl) and results visualized using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Immunoblotting.

Cells were lysed using Nonidet-40 lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 1% NP-40, 1 mM PMSF and one tablet of Roche EDTA-free protease inhibitor cocktail (Sigma-Aldrich) per 50 mL) supplemented with Halt phosphatase inhibitor cocktail (78420, Thermo Fisher Scientific) for 10 min on ice. Lysates were cleared by centrifugation (13000 *rpm*, 10 min, 4°C). The proteins were quantified and normalized with Bradford assay using γ-globin as a standard (Bio-Rad, Hercules, CA, USA). Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes Protran BA 85 (GE Healthcare, Little Chalfont, UK). The membranes were immunoblotted with indicated antibodies and bound antibodies were visualized with horseradish peroxidase–conjugated secondary antibodies using the ECL Western blotting system (Thermo Fisher Scientific).

Immunoprecipitation.

HEK293T cells were transiently transfected with indicated constructs using Polyfect (Qiagen). 36-48h after transfection cells were washed once in PBS and lysed in IP lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.5% NP-40, 1 mM PMSF and protease inhibitor cocktail containing 1μg/mL leupeptin, 1μg/mL aprotinin, 10μg/mL soybean trypsin inhibitor) for 10 min on ice. Lysates were cleared by centrifugation (13000 *rpm*, 10 min, 4°C), quantified and normalized with Bradford assay using γ-globin as a standard (Bio-Rad). Subsequently, lysates were incubated (3h, 4°C) with anti-HA or anti-FLAG coupled beads (Sigma-Aldrich). Beads were recovered by centrifugation and washed three times with IP lysis buffer, bound proteins were eluted by addition of 4x Laemmli buffer and boiling for 5min before analysis by SDS-PAGE and immunoblotting.

***In vivo* ubiquitination assay.**

HEK293T cells were transiently transfected with indicated constructs using Polyfect. 24-30h after transient transfection cells were treated overnight with 5μM MG132, washed once in PBS and lysed in RIPA buffer (25mM Tris-HCl pH 7.5, 50mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and protease inhibitor cocktail containing 1μg/mL leupeptin, 1μg/mL aprotinin, 10μg/mL soybean trypsin inhibitor) supplemented with 25U/mL of Benzonase (71205, EMD Millipore Billerica, MA, USA) for 10 min on ice. Lysates were cleared by centrifugation, quantified and normalized with Bradford assay and incubated (3h, 4°C) with anti-HA coupled beads (Sigma-Aldrich). Beads were recovered by centrifugation, washed three times with RIPA lysis buffer without Benzonase, bound proteins were eluted by addition of 4x Laemmli buffer, boiled for 5min and analyzed by SDS-PAGE and immunoblotting.

Cell viability assay.

Cells were seeded in 96-well plates at the appropriate cell density. For drug sensitivity experiments, cells were incubated with increasing drug concentrations for 72h. Cell viability was determined using CellTiterGlo Luminescent Cell Viability Assay (Promega) according to

the instructions provided by the manufacturer. Luminescence was recorded with a SpectraMax M5 Multimode plate reader (Molecular Devices, Sunnyvale, CA, USA). Data were normalized to values of untreated controls.

Flow cytometry.

Samples were analyzed on an LSR Fortessa (BD Biosciences) and data analysis was performed using FlowJo software version 7.6.3 (Tree Star Inc., Ashland, OR, USA).

Flow cytometry based multi-color competition assay (MCA).

To analyze the long-term cellular response upon drug treatment, cell populations were marked with GFP or mCherry fluorescent reporters coupled to individual sgRNAs or cDNAs. In the case of CRISPR/Cas9-based knock-out cell competition experiments sg*Ren*.208-mCherry$^+$ reporter cells (LGPIC lentiviral sgRNA vector) were mixed with sg*Ren*.208 or target gene sgRNA-GFP$^+$ cells (LGPIG lentiviral vector) in a 1:1 ration. In the case of cDNA rescue competition experiments empty vector-GFP$^+$ (MPBIG retroviral vector) control cells were mixed with empty vector or cDNA-expressing vector-mCherry$^+$ (MPBIC or MgwSHPBIC retroviral vector) cells and mixed in a 1:1 ratio. Mixed cell populations were treated with increasing drug concentrations or DMSO as negative control. After ~14d the percentage of GFP$^+$ and mCherry$^+$ cell populations was monitored by flow cytometry, gating on viable cells only (FSC/SCC). The fold change of GFP$^+$ and mCherry$^+$ fluorescent cells from each treatment condition was calculated and normalized to DMSO control.

Confocal microscopy.

HeLa cells inducibly expressing HA- or V5-tagged LZTR1were seeded on glass coverslips and cDNA expression was induced by addition of 1-2 µg/mL doxycycline. After 24h of induction coverslips were fixed and permeabilized with 4% formalin/0.1% TritonX-100 in PBS. Subsequently, coverslips were incubated with 5% BSA in PBS for 1h, incubated with indicated primary antibodies in 5% BSA/PBS overnight and stained with isotype specific fluorescently

labeled secondary antibodies. After DAPI staining coverslips were mounted on glass slides using ProLong Gold (Thermo Fisher Scientific). Cells were visualized using a Zeiss Laser Scanning Microscope (LSM) 780 utilizing sequential laser line interrogation into two PMTs and a GaAsP spectral detector. Images were taken at 40× and analyzed with ImageJ (NIH, open source).

***Drosophila* crosses and stocks.**

Crosses were set up on standard cornmeal-agar food at 31°C for wing experiments and 29°C for eye experiments. Embedding and sectioning of eyes were performed as described [42]. Wings were removed from adult flies, equilibrated in PBS with 0.1%Triton X-100 and mounted in 80% glycerol in PBS.

Stocks used were: *sev-Gal4* (on III), *UAS-Ras*$^{V12\ C40}$ [21], *act5C-Gal4* (line FBst0003954/Bloomington stock center); *UAS-CG3711*^{IR} stocks were #1 – VDRC line 11164/GD, #2 – VDRC line 13008/GD, and #3 - TRiP/Bloomington line FBst0033422; control was *UAS-white*^{IR} line FBst0031088 (TRiP/Bloomington)

RAS activation quantification.

RAS activation state was measured by assaying binding of active RAS to the RAS binding domain (RBD) of RAF1 using the RAS Activation ELISA Assay Kit (17-497, Merck Millipore). The assay was performed according to manufacturer instructions and chemiluminescent signals were recorded with a SpectraMax M5 Multimode plate reader (Molecular Devices).

Experimental design, data plotting and statistical rational.

Cell viability assay data are normalized to untreated control and shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in triplicates. Flow cytometry-based multi-color competition assay (MCA) data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in duplicates if not otherwise stated. Immunoblot results shown are representative of at least two independent biological experiments (n ≥ 2).

Data calculations were performed using Microsoft Excel (Microsoft, Redmond, WA, USA), data plotting and statistical analysis was done using GraphPad Prism 6 (GraphPad Software) if not otherwise stated. A normal distribution of data was assumed and appropriate tests were applied. In *Drosophila* experiments, eyes from multiple individual adults were analyzed per genotype and over 200 ommatidia were categorized in total, wings from at least ten adults were analyzed per genotype and categorized as wild type, partial extra vein or extra vein depending on the amount of ectopic vein tissue present. Eye data were analyzed using the Fisher's exact test function of GraphPad Prism and wing data were analyzed using the χ^2 test.

Data availability.

Deep sequencing data of haploid genetic screens will be made publicly available upon manuscript acceptance prior to publication.

FIGURE LEGENDS

Figure 1 Haploid genetic screens identify gene knock-outs promoting BCR-ABL inhibitor resistance. a-f, Circos plots of haploid genetic screens in the CML cell line KBM-7 with the six clinically relevant 1st (blue), 2nd (orange) and 3rd (red) generation BCR-ABL inhibitors. Imatinib (**a**), ponatinib (**b**), nilotinib (**c**), dasatinib (**d**), bosutinib (**e**) and rebastinib (**f**). Each dot represents a mutagenized gene identified in the resistant cell population, the dot size corresponds to the number of independent insertions identified per gene and the distance from the circos plot center indicates the significance of enrichment compared to an unselected control data set. Hits with an FDR adjusted *P*-value lower than 10^{-4} are labeled by gene name. **g**, Bubble plot depicting the "TOP6" set of genes identified in four or more of the six haploid screens. The bubble size of each bubble corresponds to the number of independent insertions per gene and the color gradient depicts the FDR adjusted *P*-value of enrichment significance. **h**, Multi-color competition assay (MCA)-derived fold change of KBM-7 *SpCas9* (KBM-7^{Cas9}) CML cells after imatinib treatment transduced with sgRNAs targeting the "TOP6" genes or sg*Ren*.208 (targeting *Renilla luciferase*) as negative control. sgRNA-infected cell populations were mixed in a 1:1 ratio, treated with increasing drug concentrations and analyzed by flow cytometry after 14 days. Data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in duplicates. sgRNAs are labeled by gene name followed by the genomic targeting sequence position numbered according to the sequence position on the corresponding mRNA.

Figure 2 Loss of *LZTR1* promotes resistance to BCR-ABL and FLT3-ITD inhibition and enhances MAPK pathway activation. a, Bubble plot depicting the six top hits identified in four or more screens. Coloring indicates functional pathway association of identified genes and bubble size corresponds to the number of independent insertions per gene. **b**, MCA-derived fold change of sg*LZTR1*.466-transduced KBM-7^{Cas9} after 14 days of treatment with each of the six BCR-ABL inhibitors. 1st, 2nd and 3rd generation BCR-ABL inhibitors are colored

in blue, orange and red respectively. **c**, MCA-derived fold change of KBM-7^{Cas9} cells transduced with sgRNAs targeting different domains of the LZTR1 protein after treatment with increasing concentrations of rebastinib for 14 days. **d**-**e**, MCA-derived fold change of sgRNA-transduced MV4-11 *SpCas9* (MV4-11^{Cas9}) AML cells after treatment with increasing concentrations of tandutinib (**d**) and ponatinib (**e**) for 14 days. **f**, KBM-7^{Cas9} and K-562 *SpCas9* (K-562^{Cas9}) CML cells as well as MV4-11^{cas9} AML cells were transduced with the indicated sgRNAs and immunoblotted with the indicated antibodies. **g**, K-562^{Cas9} CML cells expressing sg*Ren*.208 were transduced with empty vector, sg*LZTR1*.466-expressing cells with empty vector or an *LZTR1*-cDNA-containing MSCV retrovirus, and immunoblotted with the indicated antibodies. **h**, MCA-derived fold change of K-562^{Cas9}*9* sg*Ren*.208 cells transduced with empty vector and sg*LZTR1*.466-transduced cells with empty vector or *LZTR1* cDNA after after treatment with increasing concentrations of imatinib for 14 days. In **b,** data of a representative experiment are shown as mean value ± s.d. performed in duplicates, **c**-**e** and **h** data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2). In **f**-**g** immunoblot results are representative of at least two independent biological experiments (n ≥ 2).

Figure 3 LZTR1 regulates RAS/MAPK pathway activation *in vivo* and mediates KRAS ubiquitination. a-b, Panels show representative adult wings from *act5C-Gal4, UAS-w^{IR}* (*act>w^{IR}* for short) and *act>CG3711^{IR}* #1 RNAi lines (**a**) and higher magnifications of representative adult wings from *act>CG3711^{IR}* RNAi lines (**b**). **c**, Quantification as percentage of wings with ectopic wing vein formation. *P*-value for RNAi #1 and #3 in the wing is <0.0001 (****) and for #2, based on the criteria of expected values, and tolerance of values of 0, the χ^2 test is not valid (although the *P*-value can be calculated to 0.0255). **d-f**, Representative images of tangential eye sections (upper panels) and schematics of ommatidial orientations (lower panels) as observed in *sev-Gal4, UAS-RasV12C40* flies (or *sev>Ras$^{V12\ C40}$* for short) (**d**), *sev>Ras$^{V12\ C40}$, >CG3711^{IR}* #2 (**e**) and *sev>CG3711^{IR}* #2 (**f**) genotypes. **g**, Quantification as percentage of photoreceptor number defects in the indicated RNAi lines/genotypes. *P*-value for Ras* suppression in each of the RNAi experiments in the eye is <0.0001(****). **h-i**,

HEK293T cells were transiently transfected with empty vector, 3xFLAG-tagged KRAS4A (**h**) or KRAS4B (**i**) and V5-tagged LZTR1 constructs for 36-48h. **j**, HEK293T cells were transiently transfected with 3xFLAG tagged GFP or KRAS4A, HA-ubiquitin, MYC-CUL3 and V5-LZTR1. **k**, HEK293T cells were transiently transfected with 3xFLAG-KRAS4A, HA-ubiquitin, MYC-CUL3 and V5-LZTR1 WT, R198G or G248R. After 24-30h cells were treated overnight with 5μM MG132 (**j**-**k**). Following immunoprecipitation of cell lysates, immunoprecipitates (IP) and whole cell extracts (WCE) were analyzed by immunoblotting with the indicated antibodies (**h**-**k**). WT, wild type. Long exp., long exposure. Results are representative of at least two independent biological experiments (n≥2).

Figure 4 Loss of *LZTR1* protein function leads to enhanced RAS/MAPK pathway activation in CML cells, in a KRAS-dependent manner, and in human disease. a, MCA-derived fold change of K-562 ^{Cas9} CML sg*Ren*.208 cells and sg*LZTR1*.466 cells additionally transduced with sg*Ren*.208 or sgRNAs targeting *KRAS*, *NRAS* or *HRAS,* treated with increasing concentrations of imatinib for 14 days. Data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2). **b**, Single and double sgRNA-expressing K-562^{Cas9} CML cells were immunoblotted with the indicated antibodies at the starting time of the MCA in panel (**a**). **c**, Domain organization of LZTR1 and arrows indicating amino acid position of missense mutations identified in Noonan syndrome (NS, brown) and glioblastoma (GBM, blue). **d-e**, K-562^{Cas9} and sg*Ren*.208-expressing cells were retrovirally transduced with empty vector, sg*LZTR1*.466-expressing cells were transduced with either empty vector, LZTR1 WT, or LZTR1 mutations identified in Noonan syndrome (brown) (**d**) or glioblastoma (blue) (**e**) and thereafter immunoblotted with the indicated antibodies. The LZTR1 G248R mutation has been identified in both diseases. Immunoblot results are representative of at least two independent biological experiments (n ≥ 2). **f**, Mechanistic model of CUL3-LZTR1-mediated RAS ubiquitination and loss of *LZTR1* function-mediated enhanced MAPK pathway activation and BCR-ABL inhibitor drug resistance.

ASSOCIATED CONTENT

Extended Data Figure 1 Haploid genetic screening workflow, BCR-ABL inhibitor sensitivity of KBM-7 cells and summary view of genes identified in BCR-ABL inhibitor-focused haploid screens. a, Experimental outline of haploid genetic screens. Near-haploid KBM-7 CML cells are infected with a retroviral gene-trap, transduced cell populations are treated individually with one of the six 1st (blue), 2nd (orange) or 3rd (red) generation BCR-ABL TKIs individually for 14-21 days, colonies of resistant cells are pooled, followed by gDNA isolation, recovery of genomic gene-trap insertions using an inverse-PCR based library preparation protocols and deep sequencing analysis. The enrichment of identified insertions per gene is calculated by comparison to an unselected gene-trap mutagenized control cell population harvested at the time before TKI addition. **b**, Cell viability of KBM-7 cells after 72h of treatment with increasing concentrations of the six BCR-ABL inhibitors. 1st, 2nd and 3rd generation BCR-ABL inhibitors are colored in blue, orange and red respectively. Results are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in triplicates. **c**, Bubble plot summarizing significantly enriched genes (FDR adj. *P*-value ≤10^{-4}) identified in the six BCR-ABL TKI-focused haploid screens. The bubble size corresponds to the number of independent insertions per gene and the color gradient depicts the FDR adjusted *P*-value of enrichment significance.

Extended Data Figure 2 Retroviral gene-trap insertions identified in the ponatinib screen and LZTR1 insertions identified in four BCR-ABL inhibitor screens. a-b, Genomic location of gene-trap insertions identified in the ponatinib screen depicting the top six genes (identified in ≥4 screens) (**a**) and insertions affecting the *LZTR1* gene identified in the Imatinib, bosutinib, rebastinib and ponatinib screens (**b**). Insertions within exons function via gene disruption and generate a gene knock-out independent of insertional orientation. Intronic gene-trap insertions disrupt transcript expression in an orientation-dependent manner. Sense

integrations (same transcriptional orientation) lead to a gene knock-out (red), whereas antisense integrations (blue) have no effect on gene expression in most of the cases.

Extended Data Figure 3 CRISPR/Cas9-based validation of gene loss-of-function-induced BCR-ABL inhibitor drug resistance. a, Experimental validation strategy of CRISPR/Cas9 based validation of gene loss-of-function-induced BCR-ABL inhibitor drug resistance using a multi-color competition assay. KBM-7^{Cas9} cells were lentivirally infected with sgRNA-expressing LentiGuide-PuroR vectors additionally harboring a GFP (LGPIG) or mCherry (LGPIC) fluorescent marker enabling to monitor respective knock-out cell populations by flow cytometry. sg*Ren*.208-mCherry control sgRNA infected cells were mixed with sg*Ren*.208-GFP control or gene targeting sgRNAs in a 1:1 ratio and treated with the indicated inhibitor for 14 days. Cells were analyzed by flow cytometry gating on the remaining viable population and the fold change of control or gene targeting GFP$^+$ vs mCherry$^+$ control cells, indicative of enhanced survival and/or proliferation upon drug treatment, was calculated normalized to untreated control. **b**-**g**, KBM-7^{Cas9} cells were transduced with the indicated sgRNAs targeting *NF1* (**b**), *WT1* (**c**), *PTPN1* (**d**), *PTPN12* (**e**), *BAP1* (**f**) or *LZTR1* (**g**) and immunoblotted with the indicated antibodies. **h**, Analysis of editing efficiency as well as scatter plots showing size and frequency of insertions and deletions (indels) induced by sg*LZTR1*.620 and 466 in KBM-7^{Cas9} cells using sanger sequencing and TIDE analysis. **i**, MCA-derived fold change of KBM-7^{Cas9} CML cells after rebastinib treatment transduced with sgRNAs targeting the top six genes or sg*Ren*.208 as negative control. sgRNA-infected cell populations were mixed in a 1:1 ratio, treated with increasing drug concentrations and analyzed by flow cytometry after 14 days. Data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in duplicates.

Extended Data Figure 4 Analysis of indel formation by *LZTR1*-targeting sgRNAs leading to TKI resistance in CML and AML cell lines. a-c, Analysis of editing efficiency as well as scatter plots showing size and frequency of indels by LZTR1 protein domain targeting

sgRNAs in K-562^{Cas9} cells (**a**), by sg*LZTR1*.620 and 466 in KCL-22^{Cas9} CML cells (**b**) and MV4-11^{Cas9} AML cells (**c**) using sanger sequencing and TIDE analysis. **d-e**, MCA-derived fold change of KCL-22^{Cas9} CML cells transduced with indicated sgRNAs and treated with increasing concentrations of imatinib (**d**) or rebastinib (**e**) for 14 days. **f**, MCA-derived fold change of MV4-11^{Cas9} *FLT3-ITD*$^{+}$ AML cells transduced with indicated sgRNAs and treated with increasing concentrations of quizartinib for 14 days. MCA data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in duplicates.

Extended Data Figure 5 Loss of *LZTR1* expression induced MAPK pathway activation. a, KBM-7^{Cas9} cells were transduced with sg*Ren*.208, sg*LZTR1*.620 or 466 sgRNAs and cell lysates were analyzed by immunoblot using the indicated antibodies. **b**, K-562^{Cas9} cells transduced with sg*Ren*.208 or sg*LZTR1*.466 were treated with increasing concentrations of imatinib for 3h. Cell lysates were immunoblotted with the indicated antibodies. **c**, K-562^{Cas9}, HeLa^{Cas9} and HEK293T^{Cas9} cells were transduced with the indicated sgRNAs and immunoblotted with the indicated antibodies. **d**, HEK293T^{Cas9} cells transduced with sgRNAs as in panel (**b**) were serum starved overnight and then serum stimulated for the indicated time duration. Cell lysates were immunoblotted with the indicated antibodies. In **a**-**d** immunoblot results are representative of at least two independent biological experiments (n ≥ 2).

Extended Data Figure 6 Human and *Drosophila* LZTR1 protein domain organization and sequence alignment of LZTR1 in different species. a, Domain organization of human LZTR1 and *Drosophila* CG3711 protein. **b-c**, Tree view plotting average distance using % sequence identity (**b**) and multiple sequence alignment colored according to % sequence identity (**c)** of LZTR1 in different species. Sequence comparisons were generated using Clustal Omega and visualized using JalView.

Extended Data Figure 7 LZTR1 is an adaptor protein for the CUL3 E3 ligase complex, and binds to RAS GTPase proteins. a-c, HEK293T cells were transiently transfected with

empty vector control, 3xFLAG-NRAS (**a**) or 3xFLAG-HRAS (**b**) and V5-LZTR1, LZTR1-STREP-HA (SH) or SH-GFP and MYC-CUL3 (**c**) for 36-48h. Following immunoprecipitation of cell lysates (**a**-**c**), immunoprecipitates (IP) and whole cell extracts (WCE) were analyzed by immunoblotting with the indicated antibodies. Immunoprecipitation and immunoblot results are representative of at least two independent biological experiments (n ≥ 2).

Extended Data Figure 8 LZTR1 localizes to endosomes. a-b, HeLa cells were transduced with an inducible lentiviral vector constructs harboring HA- (**a**) or V5- (**b**) tagged LZTR1. Cells were induced with 1-2 µg/mL doxycycline for 24h and analyzed by immunoblotting using the indicated antibodies. Long exp., long exposure. **c**-**g**, Confocal microscopy images of doxycycline-induced HeLa cells as described in panel (**a**-**b**) and immunostained with anti-HA or -V5 and RAB5 (**c**), RAB7 (**d**), EEA1 (**e**), LAMP1 (**f**) or Golgin97 (**g**). Stars are indicating direct overlap and arrows are neighboring vesicles. Representative cells are shown. Scale bar indicates 20 µm on full-sized images and 2 µm on magnifications.

Extended Data Figure 9 CRISPR/Cas9-based double knock-out MCA assay identifies KRAS GTPase as LZTR1 substrate mediating drug resistance in CML cells. a, RAS activation was quantified in K562^{Cas9} cells expressing control or *LZTR1*-targeting sgRNAs using an RAS activation ELISA-based assay. Data of a representative experiment are shown as mean value ± s.d. of detected relative light units (RLU) and mean values of fold change of sg*LZTR1* compared to sg*Ren* cells were calculated. Corresponding input sample lysates were analyzed by immunoblotting using the indicated antibodies. **b**, Scheme of the CRISPR/Cas9-based double knock-out MCA assay in K-562^{Cas9} CML cells to genetically identify LZTR1 substrates mediating BCR-ABL inhibitor drug resistance. sg*LZTR1*.466-GFP^{+} K-562^{Cas9} cells were infected with sg*Ren*.208-, sg*KRAS*-, sg*NRAS*-, sg*HRAS*- or sg*RIT1*-mCherry^{+} vectors generating GFP^{+}/mCherry^{+} double knock-out cells, mixed in a 1:1 with single positive sg*Ren*.208-mCherry^{+} K-562^{Cas9} cells and treated with imatinib for 14 days. Cells were analyzed

by flow cytometry and the fold change of double positive cells vs. single positive cells was calculated normalized to untreated control. **c-d**, Analysis of editing efficiency (**c**) as well as scatter plots showing size and frequency of indels (**d**) by *K*-, *N*- and *HRAS* targeting sgRNAs in K-562^{Cas9} sg*LZTR1*.466 cells using sanger sequencing and TIDE analysis. **e**, K-562^{Cas9} sg*LZTR1*.466 cells were transduced with sg*Ren*.208 or sg*RIT1*.250 and immunoblotted with the indicated antibodies. **f**, MCA-derived fold change of K-562^{Cas9} sg*Ren*.208 cells and sg*LZTR1*.466 cells additionally transduced with sg*Ren*.208 or sg*RIT1*.250 treated with increasing concentrations of rebastinib for 14 days. Data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in duplicates.

Extended Data Figure 10 *LZTR1* missense mutations identified in NS and GBM are loss-of-function mutations. a, Scheme of CRISPR knock-out and cDNA reconstitution MCA to characterize *LZTR1* missense mutations. K-562^{Cas9} cells are either infected with sg*Ren*.208 control sgRNA and MSCV-GFP empty vector control or with sg*LZTR1*.466 and MSCV-mCherry empty vector, LZTR1 WT and missense mutations identified in Noonan syndrome and glioblastoma. Cells were mixed in a 1:1 ratio, treated with BCR-ABL inhibitor for 14 days and analyzed by flow cytometry. **b**, MCA-derived fold change of K-562 CRISPR/Cas9 knock-out and cDNA reconstituted cells as described in panel (**a**). Cells were treated with increasing concentrations of imatinib and the fold change of GFP$^+$ vs. mCherry$^+$ cells was calculated normalized to untreated control. Data are shown as mean value ± s.d. of at least two independent experiments (n ≥ 2) performed in duplicates.

Supplementary Table 1 Haploid genetic screen results listing disruptive gene-trap insertions by genes identified in KBM-7 cells after imatinib, nilotinib, dasatinib, bosutinib, rebastinib and ponatinib selection. Tables listing for each inhibitor screen the identified disruptive gene-trap insertions per gene, total inactivating insertions in other genes identified in the screens, disruptive insertions and total insertions in other genes in the control

population, *P*-value and FDR adjusted *P*-value of enrichment. Identified genes with a FDR adjusted *P*-value lower than 10^{-4} were categorized as significantly enriched and considered for further analysis.

Supplementary Table 2 sgRNA and primer sequences used in the study.

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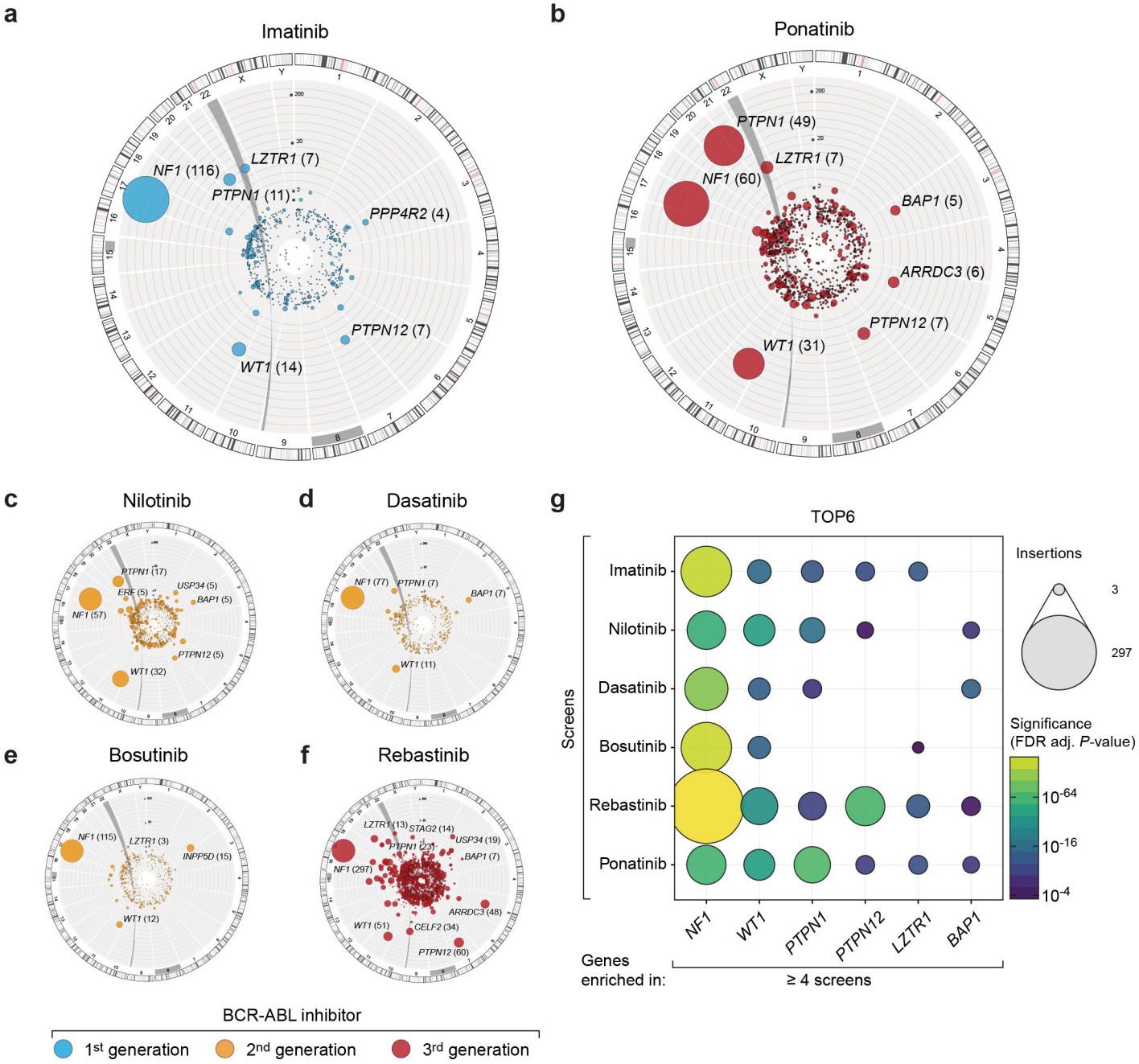
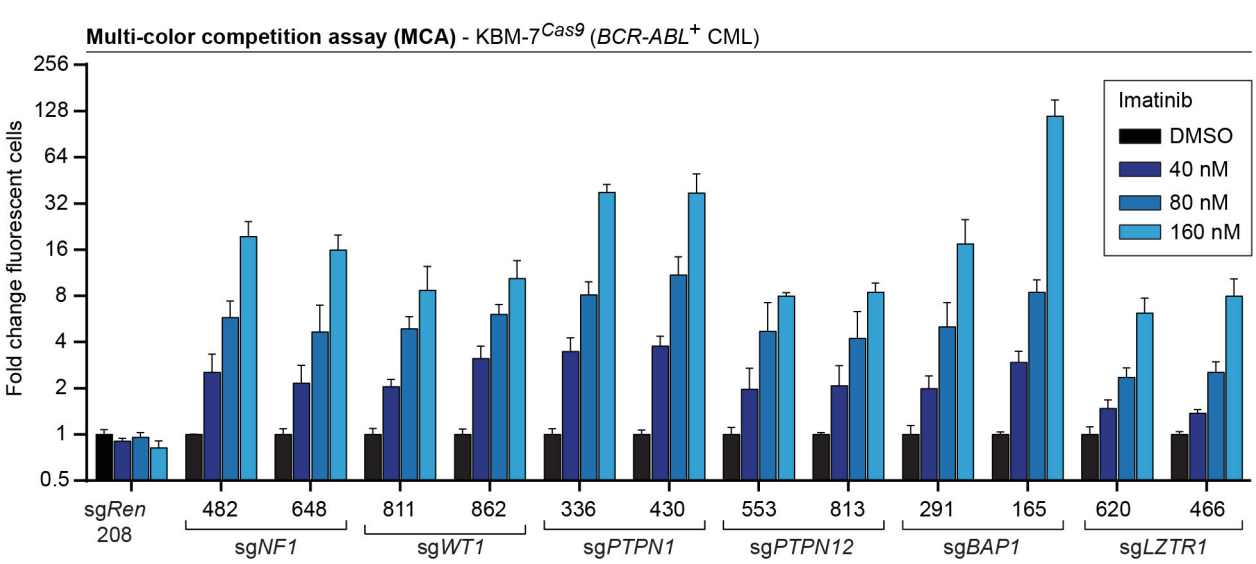
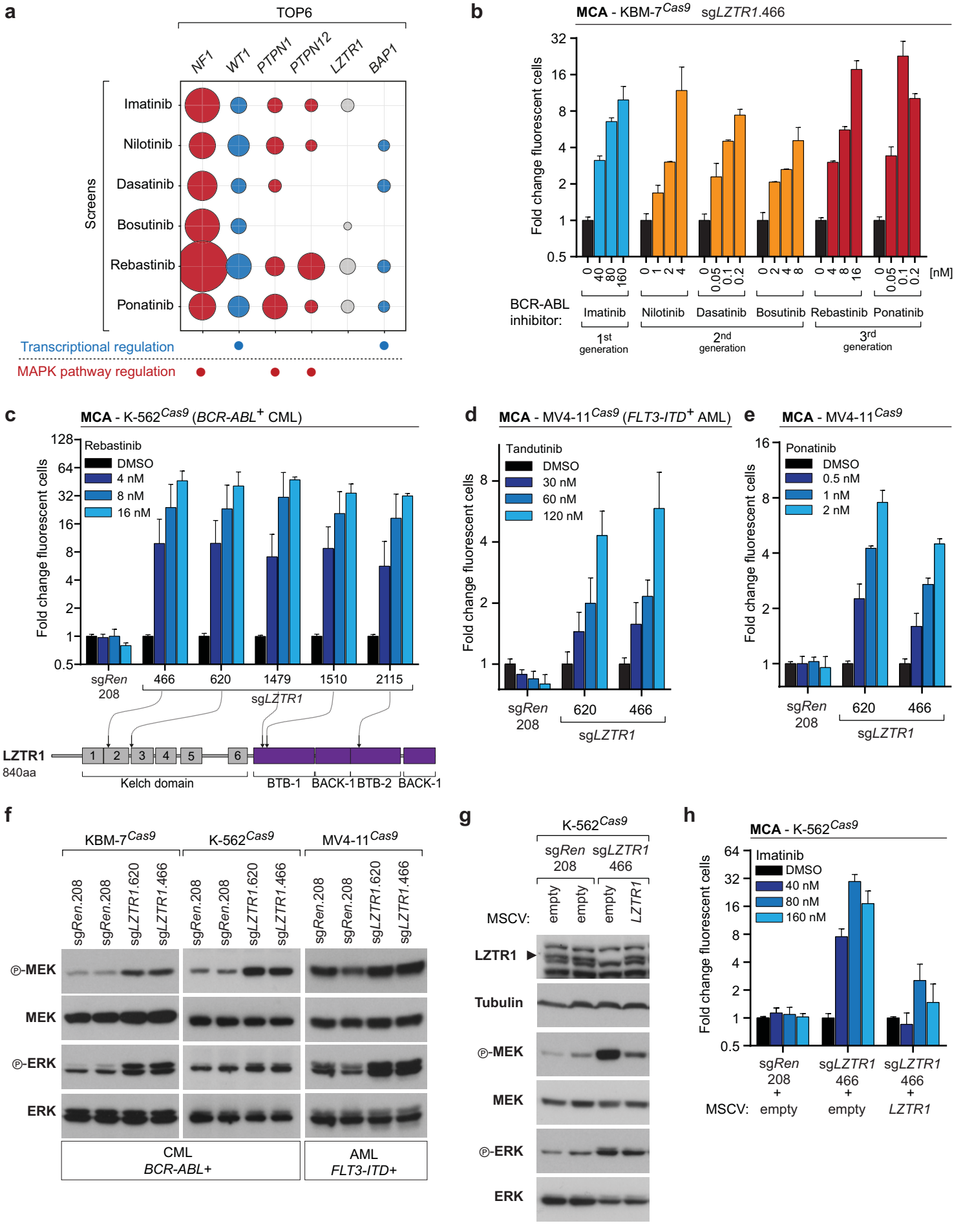
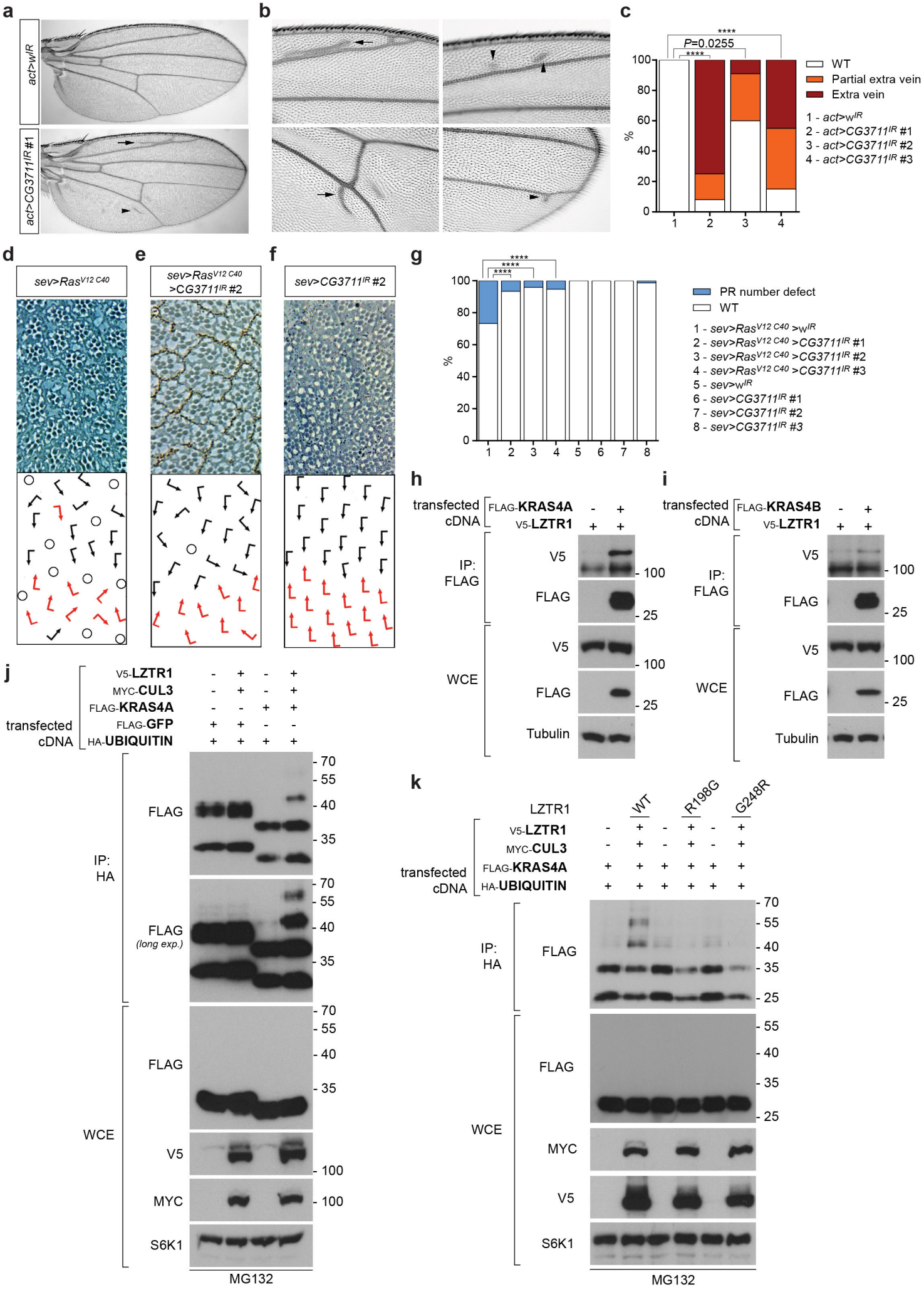
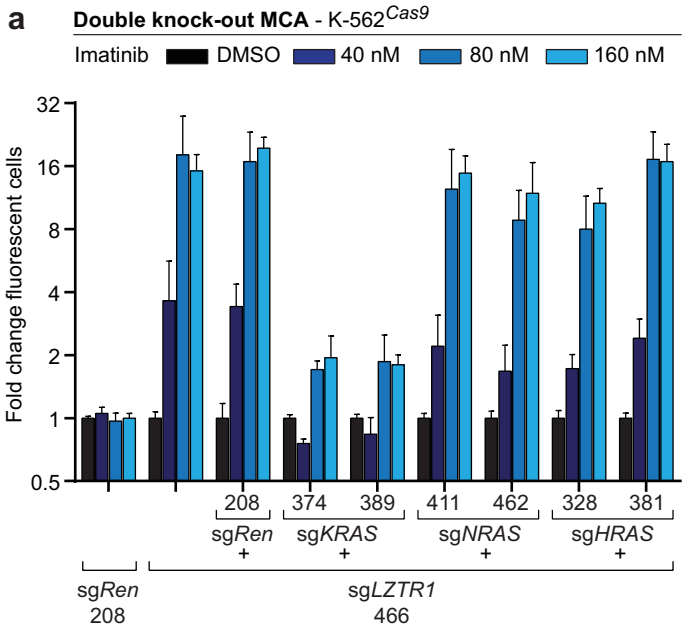
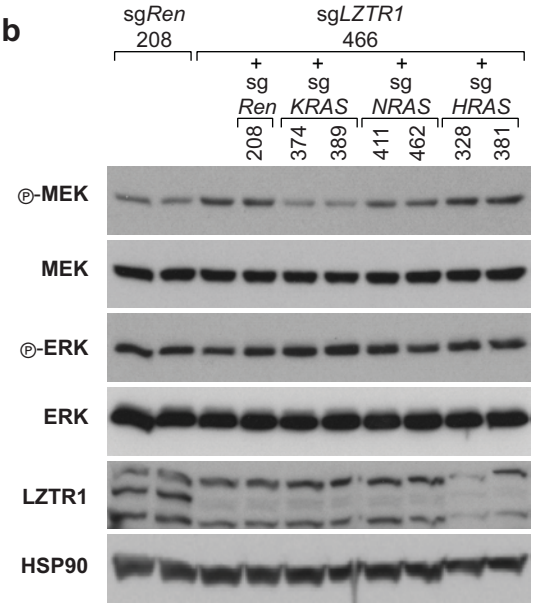

a
Imatinib
NF1 (116)
LZTR1 (7)
PTPN1 (11)
PPP4R2 (4)
PTPN12 (7)
WT1 (14)
b
Ponatinib
PTPN1 (49)
LZTR1 (7)
NF1 (60)
BAP1 (5)
ARRDC3 (6)
PTPN12 (7)
WT1 (31)
c
Nilotinib
PTPN1 (17)
USP34 (5)
ERF (5)
BAP1 (5)
NF1 (57)
PTPN12 (5)
WT1 (32)
d
Dasatinib
NF1 (77)
PTPN1 (7)
BAP1 (7)
WT1 (11)
e
Bosutinib
NF1 (115)
LZTR1 (3)
INPP5D (15)
WT1 (12)
f
Rebastinib
LZTR1 (13)
STAG2 (14)
PTPN1 (23)
USP34 (19)
BAP1 (7)
NF1 (297)
ARRDC3 (48)
WT1 (51)
CELF2 (34)
PTPN12 (60)
BCR-ABL inhibitor
1st generation
2nd generation
3rd generation
g
TOP6
Imatinib
Nilotinib
Dasatinib
Bosutinib
Rebastinib
Ponatinib
Screens
NF1
WT1
PTPN1
PTPN12
LZTR1
BAP1
Genes enriched in:
≥ 4 screens
Insertions
3
297
Significance (FDR adj. P-value)
10-64
10-16
10-4

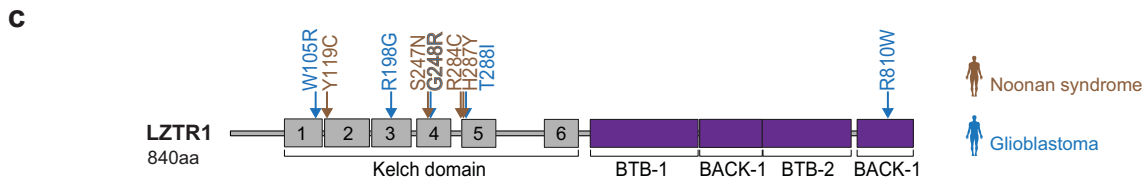

h
Multi-color competition assay (MCA) - KBM-7Cas9 (BCR-ABL+ CML)
Fold change fluorescent cells
256
128
64
32
16
8
4
2
1
0.5
Imatinib
DMSO
40 nM
80 nM
160 nM
sgRen 208
482
648
sgNF1
811
862
sgWT1
336
430
sgPTPN1
553
813
sgPTPN12
291
165
sgBAP1
620
466
sgLZTR1

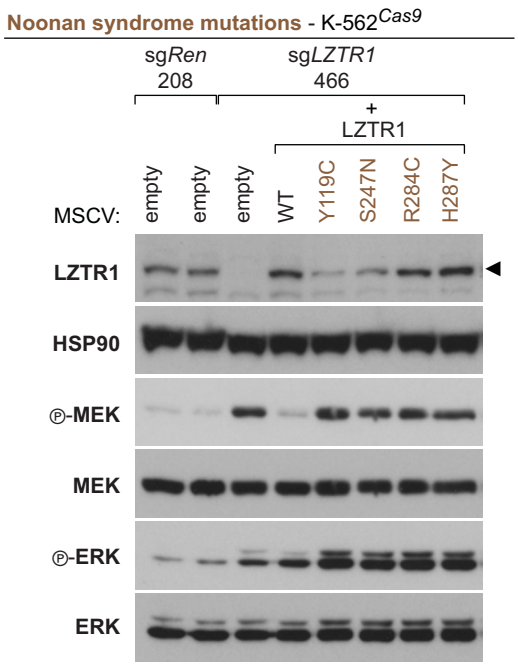

a
TOP6
NF1
WT1
PTPN1
PTPN12
LZTR1
BAP1
Screens
Imatinib
Nilotinib
Dasatinib
Bosutinib
Rebastinib
Ponatinib
Transcriptional regulation
MAPK pathway regulation
b
MCA - KBM-7Cas9 sgLZTR1.466
Fold change fluorescent cells
[nM]
BCR-ABL inhibitor:
Imatinib
Nilotinib
Dasatinib
Bosutinib
Rebastinib
Ponatinib
1st generation
2nd generation
3rd generation
c
MCA - K-562Cas9 (BCR-ABL+ CML)
Rebastinib
DMSO
4 nM
8 nM
16 nM
Fold change fluorescent cells
sgRen 208
466
620
1479
1510
2115
sgLZTR1
LZTR1
840aa
Kelch domain
BTB-1
BACK-1
BTB-2
BACK-1
d
MCA - MV4-11Cas9 (FLT3-ITD+ AML)
Tandutinib
DMSO
30 nM
60 nM
120 nM
Fold change fluorescent cells
sgRen 208
620
466
sgLZTR1
e
MCA - MV4-11Cas9
Ponatinib
DMSO
0.5 nM
1 nM
2 nM
Fold change fluorescent cells
sgRen 208
620
466
sgLZTR1
f
KBM-7Cas9
K-562Cas9
MV4-11Cas9
sgRen.208
sgRen.208
sgLZTR1.620
sgLZTR1.466
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK
CML BCR-ABL+
AML FLT3-ITD+
g
K-562Cas9
sgRen 208
sgLZTR1 466
MSCV:
empty
empty
empty
LZTR1
LZTR1
Tubulin
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK
h
MCA - K-562Cas9
Imatinib
DMSO
40 nM
80 nM
160 nM
Fold change fluorescent cells
sgRen 208 + MSCV: empty
sgLZTR1 466 + empty
sgLZTR1 466 + LZTR1

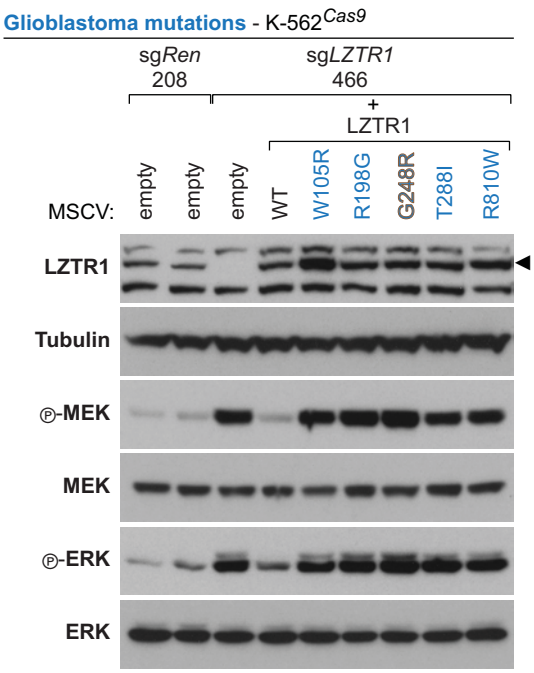

a
act>w^IR
act>CG3711^IR #1
b
c
P=0.0255
****
****
%
WT
Partial extra vein
Extra vein
1 - act>w^IR
2 - act>CG3711^IR #1
3 - act>CG3711^IR #2
4 - act>CG3711^IR #3
d
sev>Ras^V12 C40
e
sev>Ras^V12 C40 >CG3711^IR #2
f
sev>CG3711^IR #2
g
PR number defect
WT
1 - sev>Ras^V12 C40 >w^IR
2 - sev>Ras^V12 C40 >CG3711^IR #1
3 - sev>Ras^V12 C40 >CG3711^IR #2
4 - sev>Ras^V12 C40 >CG3711^IR #3
5 - sev>w^IR
6 - sev>CG3711^IR #1
7 - sev>CG3711^IR #2
8 - sev>CG3711^IR #3
h
transfected cDNA
FLAG-KRAS4A
V5-LZTR1
IP: FLAG
V5
FLAG
WCE
Tubulin
i
FLAG-KRAS4B
j
V5-LZTR1
MYC-CUL3
FLAG-KRAS4A
FLAG-GFP
HA-UBIQUITIN
IP: HA
FLAG
FLAG (long exp.)
WCE
V5
MYC
S6K1
MG132
k
LZTR1
WT
R198G
G248R
V5-LZTR1
MYC-CUL3
FLAG-KRAS4A
HA-UBIQUITIN
IP: HA
FLAG
WCE
MYC
V5
S6K1
MG132

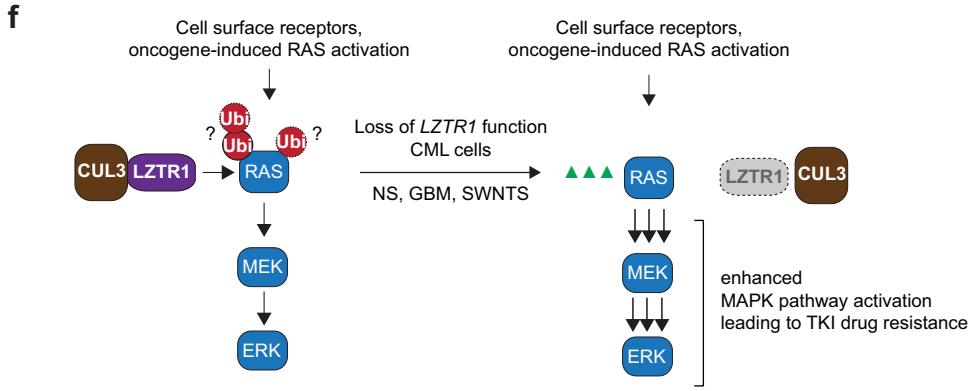

a
Double knock-out MCA - K-562Cas9
Imatinib
DMSO
40 nM
80 nM
160 nM
Fold change fluorescent cells
32
16
8
4
2
1
0.5
208
374
389
411
462
328
381
sgRen +
sgKRAS +
sgNRAS +
sgHRAS +
sgRen 208
sgLZTR1 466


b
sgRen 208
sgLZTR1 466
+ sg Ren
+ sg KRAS
+ sg NRAS
+ sg HRAS
208
374
389
411
462
328
381
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK
LZTR1
HSP90

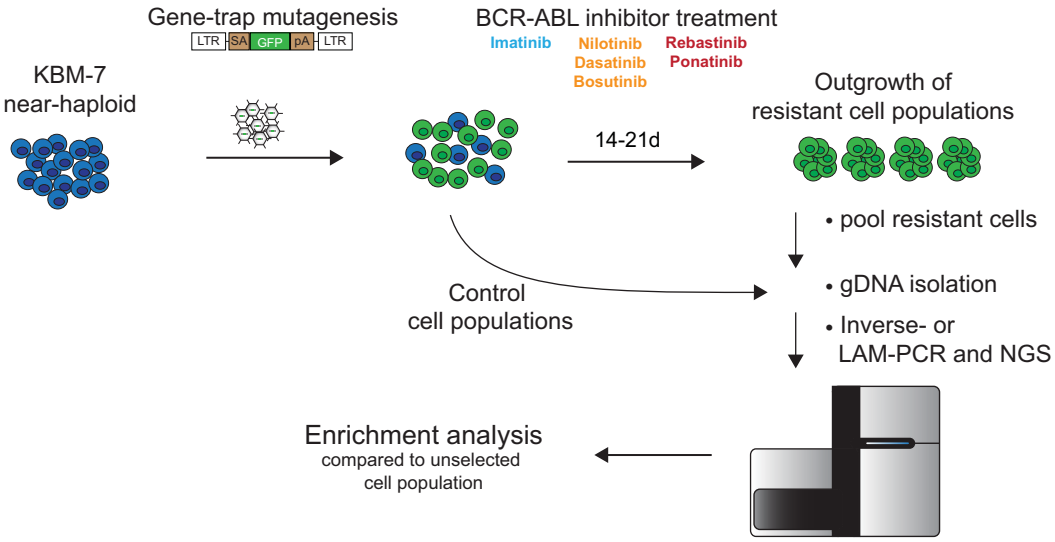

c
W105R
Y119C
R198G
S247N
G248R
R284C
H287Y
T288I
R810W
LZTR1
840aa
1
2
3
4
5
6
Kelch domain
BTB-1
BACK-1
BTB-2
BACK-1
Noonan syndrome
Glioblastoma


d
Noonan syndrome mutations - K-562Cas9
sgRen 208
sgLZTR1 466
+ LZTR1
MSCV:
empty
empty
empty
WT
Y119C
S247N
R284C
H287Y
LZTR1
HSP90
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK

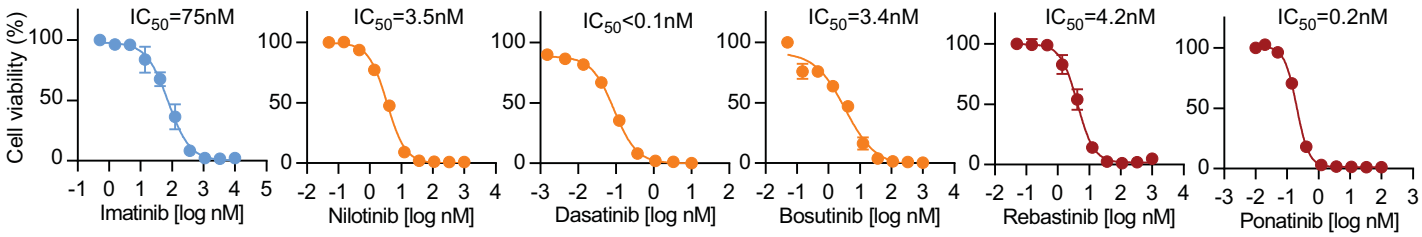

e
Glioblastoma mutations - K-562Cas9
sgRen 208
sgLZTR1 466
+ LZTR1
MSCV:
empty
empty
empty
WT
W105R
R198G
G248R
T288I
R810W
LZTR1
Tubulin
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK


f
Cell surface receptors, oncogene-induced RAS activation
Cell surface receptors, oncogene-induced RAS activation
Ubi
CUL3
LZTR1
RAS
MEK
ERK
Loss of LZTR1 function
CML cells
NS, GBM, SWNTS
enhanced
MAPK pathway activation
leading to TKI drug resistance

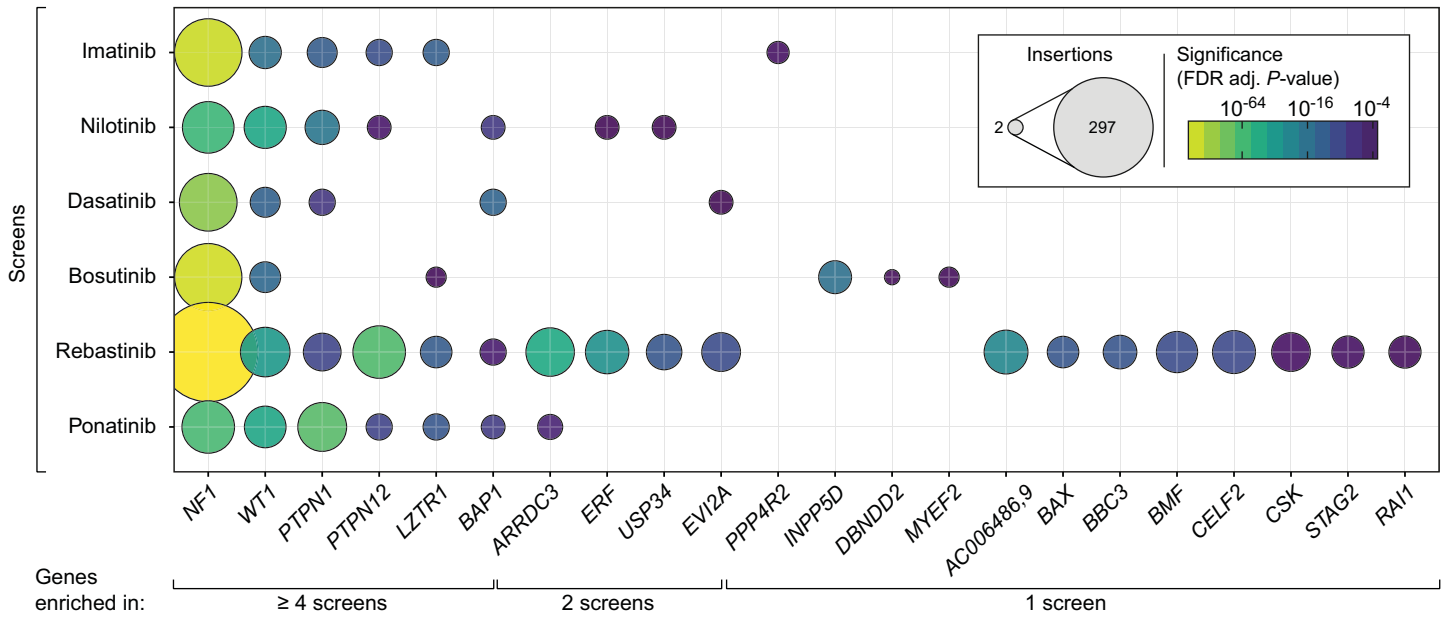
a



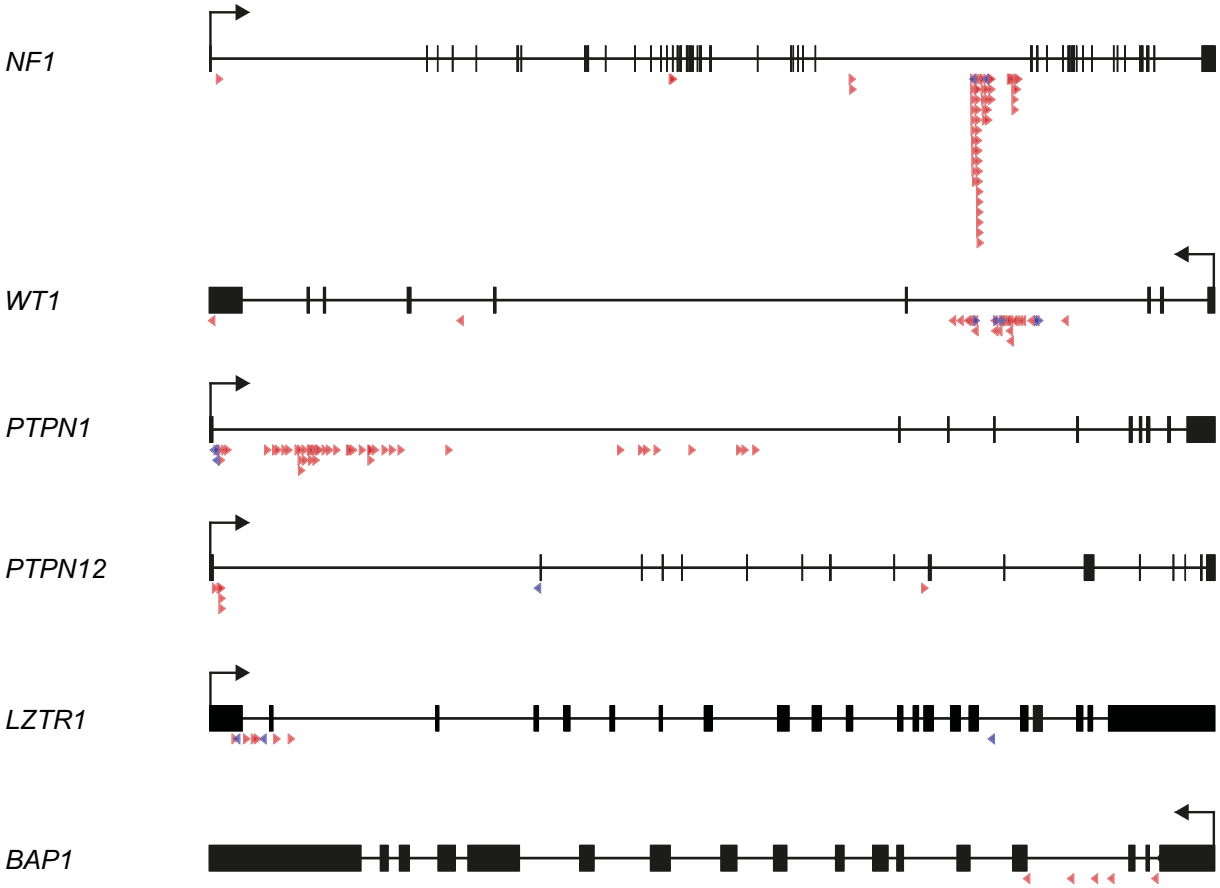
b **KBM-7** (*BCR-ABL*$^+$ CML)



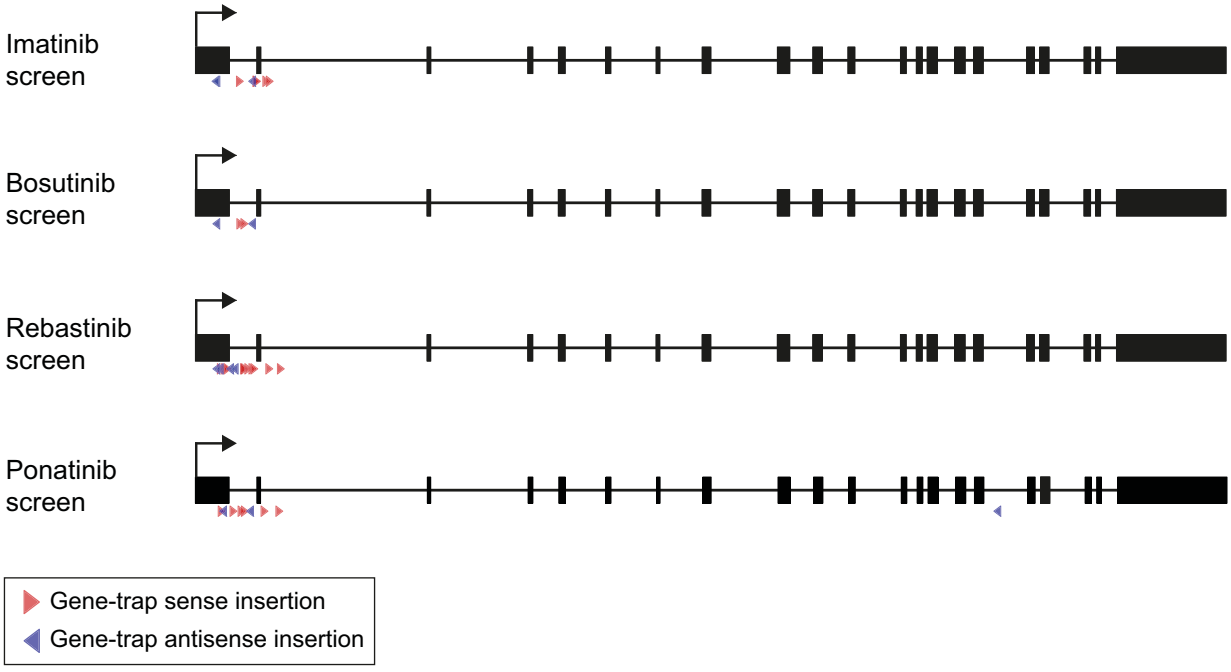
c



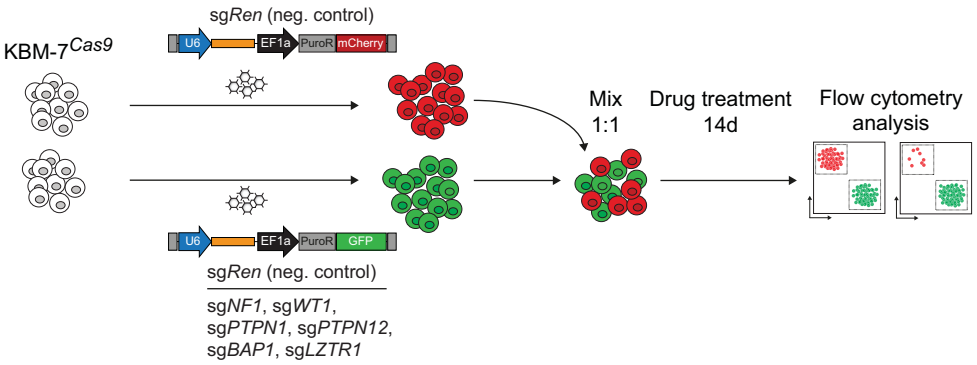
a Identified insertions in “TOP6” set genes in the ponatinib screen



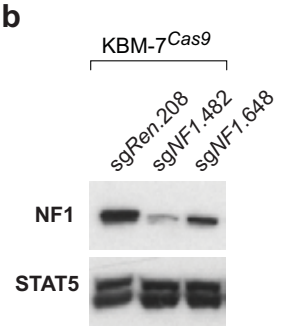
b Identified *LZTR1* insertions



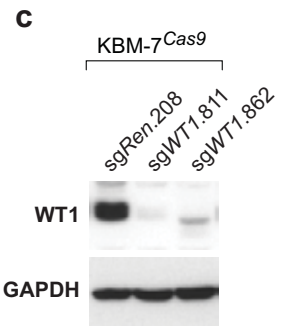
a **CRISPR based multi-color competition assay (MCA) for the validation of cellular drug resistance**



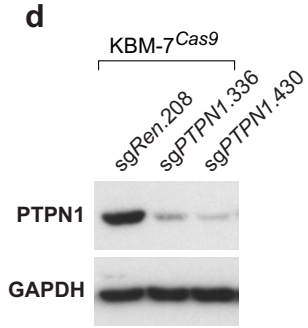
b



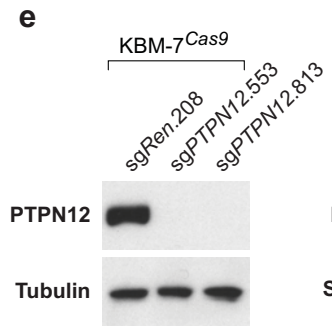
c



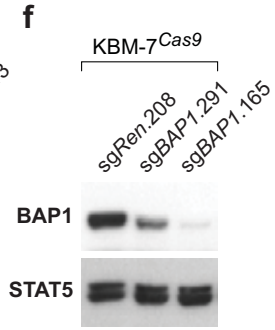
d



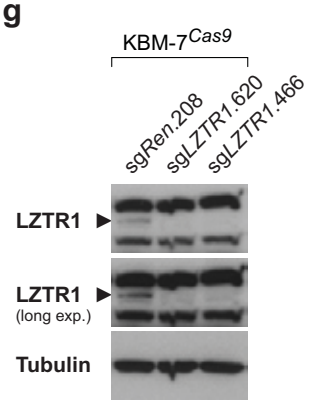
e



f

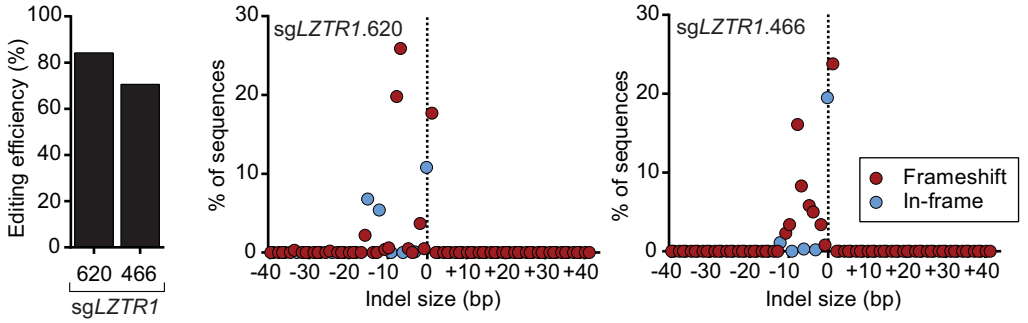


g



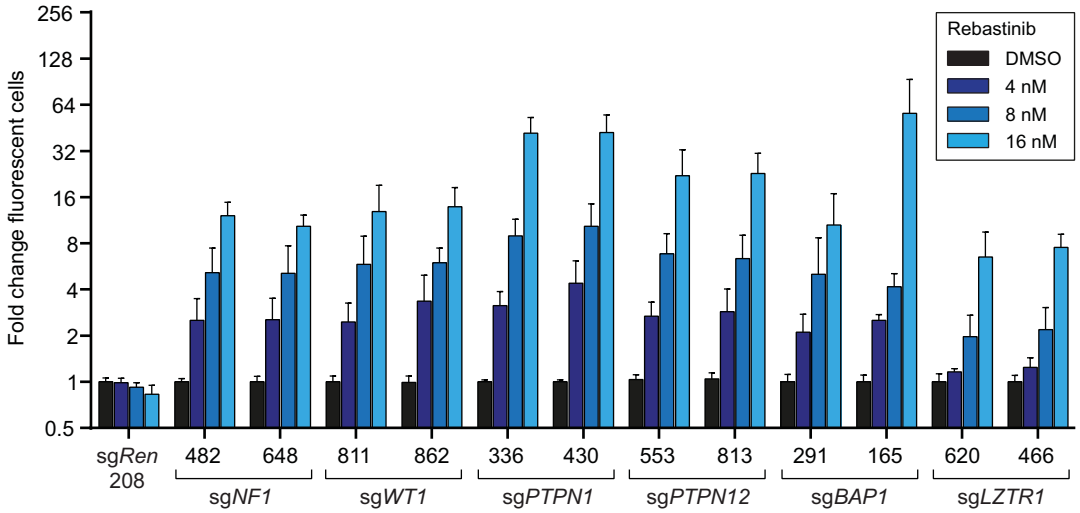
h

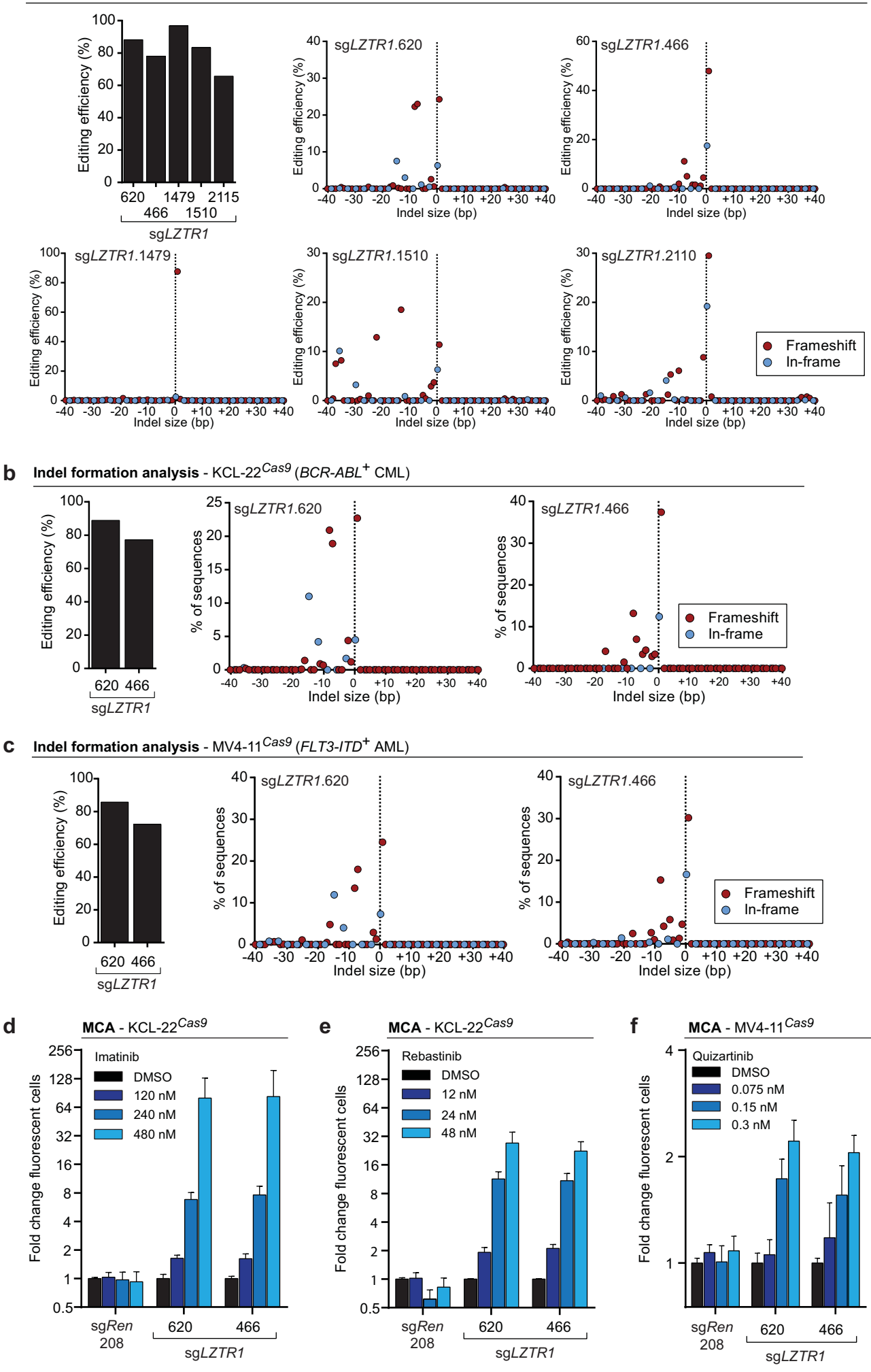
Indel formation analysis - KBM-7^{Cas9}

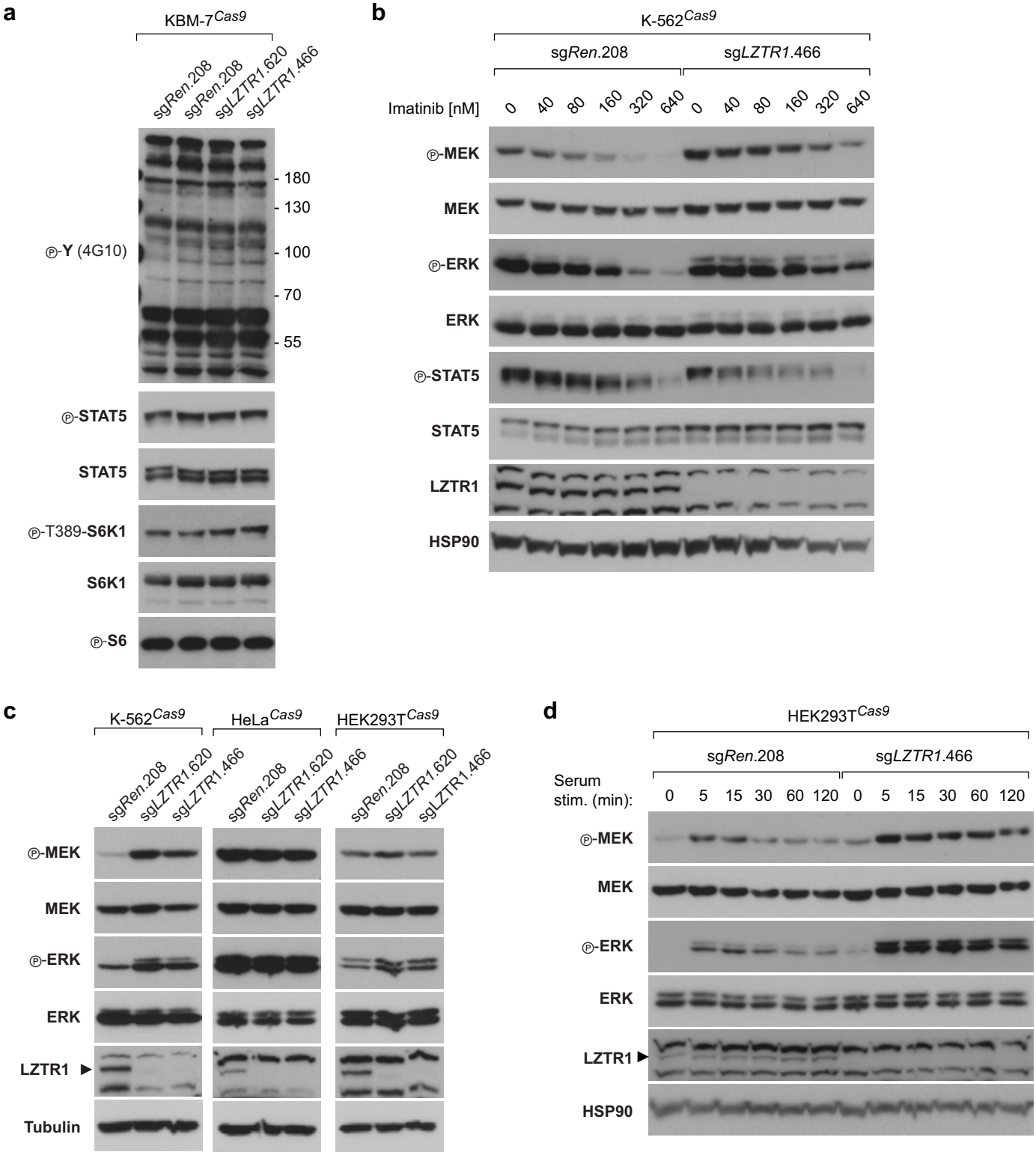


i

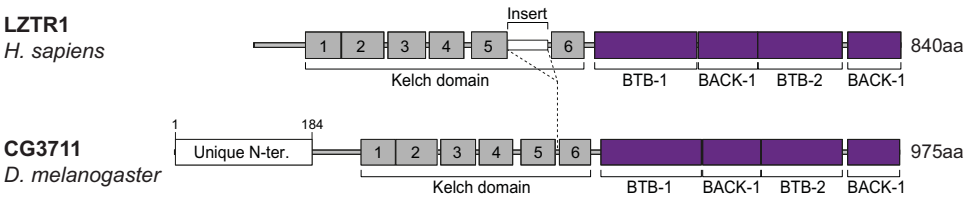
MCA - KBM-7^{Cas9} (*BCR-ABL*$^{+}$ CML)



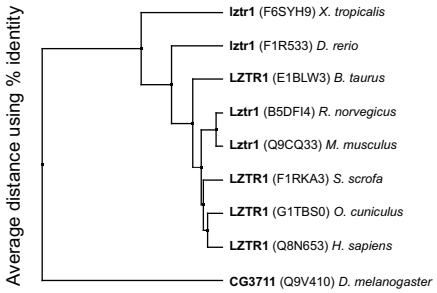

a
Indel formation analysis - K-562Cas9 (BCR-ABL+ CML)
Editing efficiency (%)
620 1479 2115
466 1510
sgLZTR1
sgLZTR1.620
sgLZTR1.466
sgLZTR1.1479
sgLZTR1.1510
sgLZTR1.2110
Indel size (bp)
Frameshift
In-frame
b
Indel formation analysis - KCL-22Cas9 (BCR-ABL+ CML)
% of sequences
620 466
c
Indel formation analysis - MV4-11Cas9 (FLT3-ITD+ AML)
d
MCA - KCL-22Cas9
Imatinib
DMSO
120 nM
240 nM
480 nM
Fold change fluorescent cells
sgRen 208
e
MCA - KCL-22Cas9
Rebastinib
12 nM
24 nM
48 nM
f
MCA - MV4-11Cas9
Quizartinib
0.075 nM
0.15 nM
0.3 nM


a
KBM-7Cas9
sgRen.208
sgRen.208
sgLZTR1.620
sgLZTR1.466
180
130
100
70
55
Ⓟ-Y (4G10)
Ⓟ-STAT5
STAT5
Ⓟ-T389-S6K1
S6K1
Ⓟ-S6
b
K-562Cas9
sgRen.208
sgLZTR1.466
Imatinib [nM]
0 40 80 160 320 640 0 40 80 160 320 640
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK
Ⓟ-STAT5
STAT5
LZTR1
HSP90
c
K-562Cas9
HeLaCas9
HEK293TCas9
sgRen.208
sgLZTR1.620
sgLZTR1.466
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK
LZTR1 ▶
Tubulin
d
HEK293TCas9
sgRen.208
sgLZTR1.466
Serum stim. (min):
0 5 15 30 60 120 0 5 15 30 60 120
Ⓟ-MEK
MEK
Ⓟ-ERK
ERK
LZTR1 ▶
HSP90

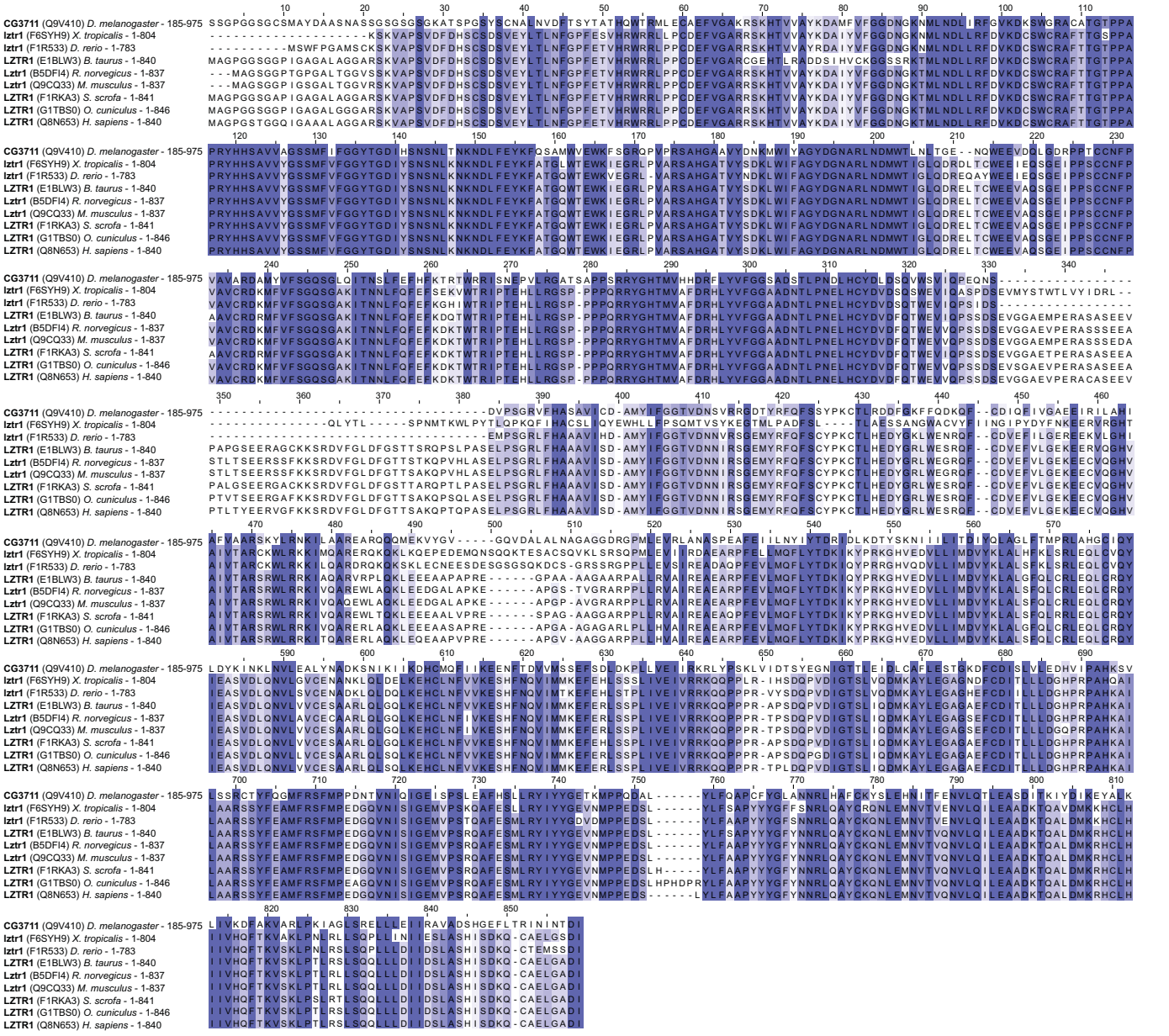
a

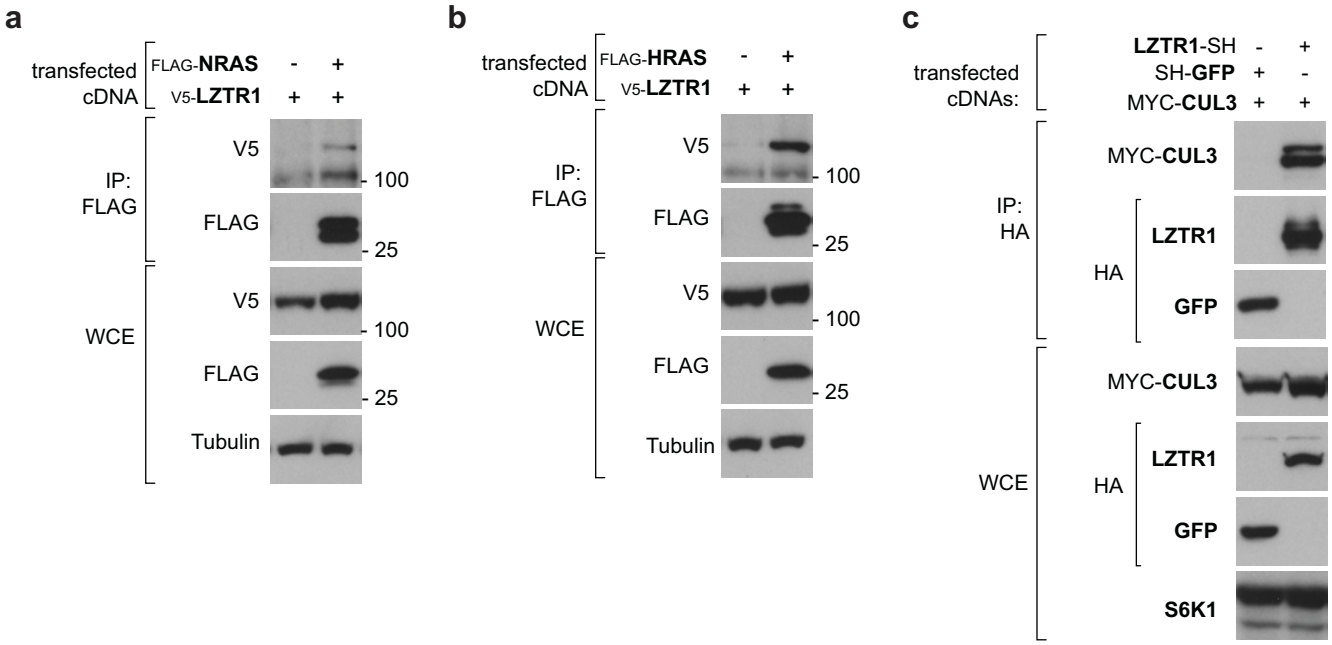

LZTR1
H. sapiens
Insert
1
2
3
4
5
6
840aa
Kelch domain
BTB-1
BACK-1
BTB-2
BACK-1
1
184
CG3711
D. melanogaster
Unique N-ter.
975aa

b


Average distance using % identity
Iztr1 (F6SYH9) X. tropicalis
Iztr1 (F1R533) D. rerio
LZTR1 (E1BLW3) B. taurus
Lztr1 (B5DFI4) R. norvegicus
Lztr1 (Q9CQ33) M. musculus
LZTR1 (F1RKA3) S. scrofa
LZTR1 (G1TBS0) O. cuniculus
LZTR1 (Q8N653) H. sapiens
CG3711 (Q9V410) D. melanogaster

c


CG3711 (Q9V410) D. melanogaster - 185-975
Iztr1 (F6SYH9) X. tropicalis - 1-804
Iztr1 (F1R533) D. rerio - 1-783
LZTR1 (E1BLW3) B. taurus - 1-840
Lztr1 (B5DFI4) R. norvegicus - 1-837
Lztr1 (Q9CQ33) M. musculus - 1-837
LZTR1 (F1RKA3) S. scrofa - 1-841
LZTR1 (G1TBS0) O. cuniculus - 1-846
LZTR1 (Q8N653) H. sapiens - 1-840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transfected cDNA
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V5-LZTR1 + +
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V5
FLAG
100
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WCE
V5
FLAG
Tubulin
100
25
b
transfected cDNA
FLAG-HRAS - +
V5-LZTR1 + +
IP: FLAG
V5
FLAG
100
25
WCE
V5
FLAG
Tubulin
100
25
c
transfected cDNAs:
LZTR1-SH - +
SH-GFP + -
MYC-CUL3 + +
IP: HA
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HA
LZTR1
GFP
WCE
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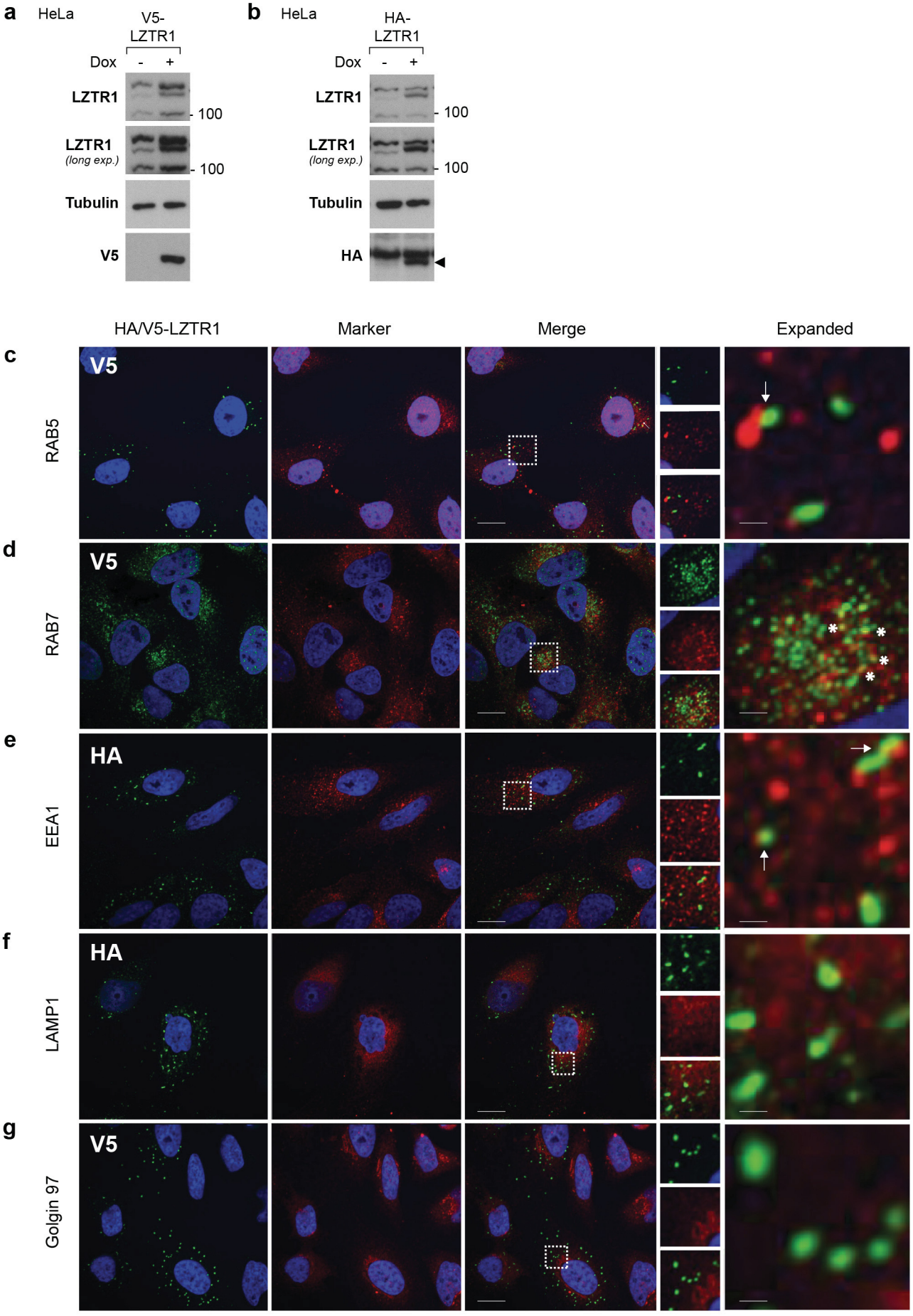
GFP
S6K1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HeLa
V5-LZTR1
Dox - +
LZTR1
LZTR1 (long exp.)
Tubulin
V5
100
100
b
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HA-LZTR1
Dox - +
LZTR1
LZTR1 (long exp.)
Tubulin
HA
100
100
HA/V5-LZTR1
Marker
Merge
Expanded
c
RAB5
V5
d
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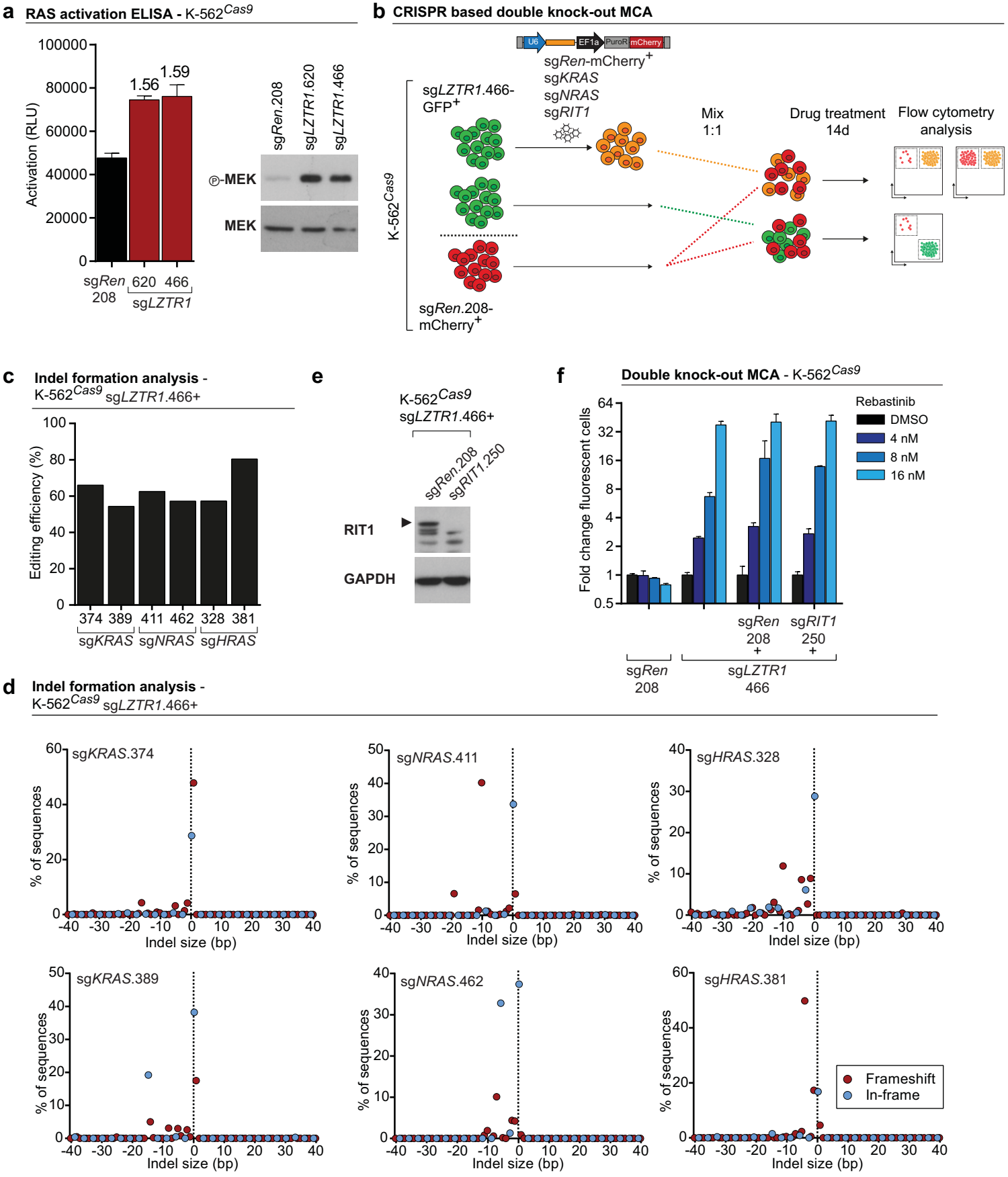
HA
g
Golgin 97
V5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RAS activation ELISA - K-562Cas9
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1.59
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sgLZTR1.466
P-MEK
MEK
sgRen 208
sgLZTR1 620 466
b
CRISPR based double knock-out MCA
U6
EF1a
PuroR
mCherry
sgRen-mCherry+
sgKRAS
sgNRAS
sgRIT1
sgLZTR1.466-GFP+
K-562Cas9
sgRen.208-mCherry+
Mix 1:1
Drug treatment 14d
Flow cytometry analysis
c
Indel formation analysis - K-562Cas9 sgLZTR1.466+
Editing efficiency (%)
374 389 411 462 328 381
sgKRAS sgNRAS sgHRAS
e
K-562Cas9 sgLZTR1.466+
sgRen.208
sgRIT1.250
RIT1
GAPDH
f
Double knock-out MCA - K-562Cas9
Fold change fluorescent cells
Rebastinib
DMSO
4 nM
8 nM
16 nM
sgRen 208 +
sgRIT1 250 +
sgRen 208
sgLZTR1 466
d
Indel formation analysis - K-562Cas9 sgLZTR1.466+
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sgNRAS.411
sgHRAS.328
sgKRAS.389
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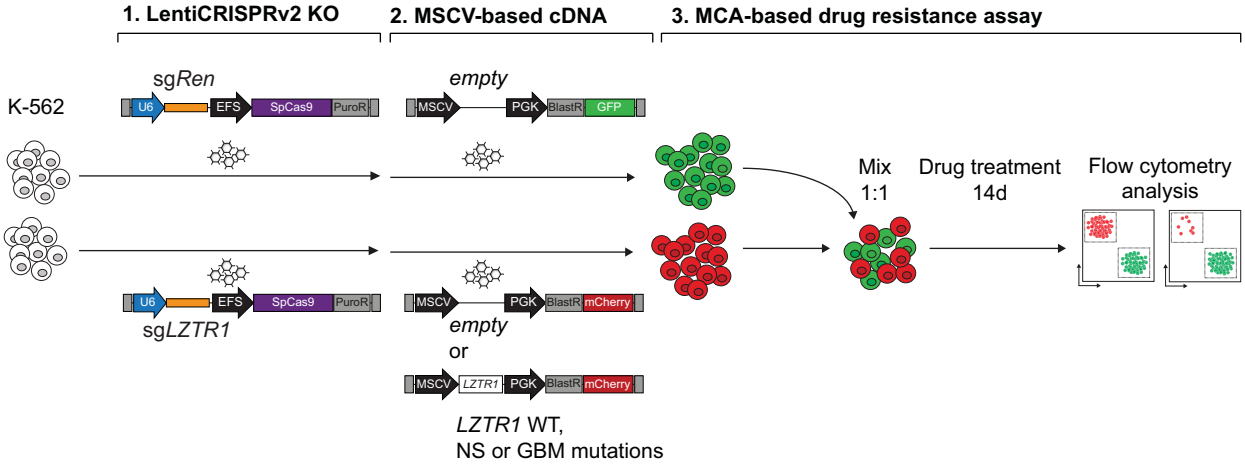
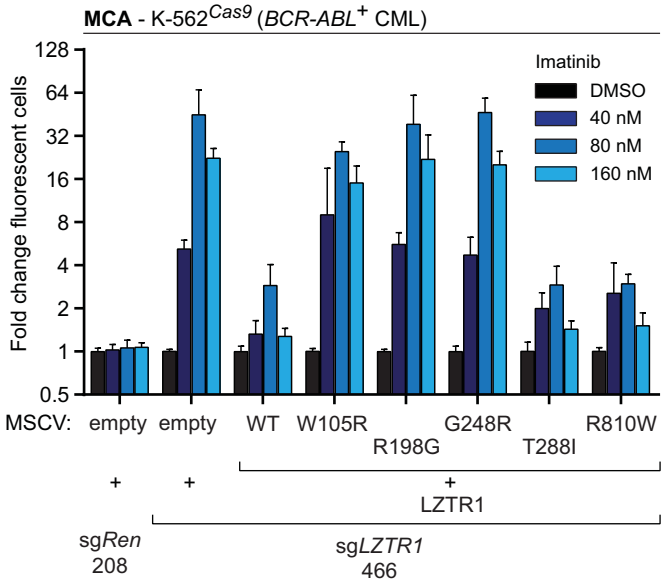
% of sequences
Indel size (bp)
Frameshift
In-frame

a

CRISPR knock-out and cDNA reconstitution MCA to phenotypically characterize LZTR1 missense mutations



b



3 Discussion

3.1 General discussion

Genetic screening approaches coupled with MS-based interactome analysis are a powerful and transformative technology duo for the delineation of cell biological processes and the functional characterization of disease-causing pathway alterations at large scale (Bouwmeester *et al*, 2004; Behrends *et al*, 2010; Grebien *et al*, 2015; Blomen *et al*, 2015). The development of novel functional genetic reagents broadening the tool box from RNAi to haploid cell genetics and CRISPR/Cas9 allows nowadays the research community to identify genes important for maintaining the cellular transformed state and governing the response to therapeutic interventions with unprecedented precision and resolution (Fellmann *et al*, 2016). On the other hand, technological innovations in the field of liquid chromatography and mass spectrometry combined with novel purification and enrichment strategies allow researchers to study the functional organization and dynamicity of the proteome in an unbiased way (Aebersold & Mann, 2016).

This thesis has aimed to make use of the afore mention technologies as well as create novel tools to shed light on how different genes enable cancer cells to maintain cellular homeostasis, integrate and execute growth-stimulatory signals, and adapt to pharmacological perturbations in order to uphold cellular viability. Specific aspects and implications related to obtained experimental findings will be discussed in the following sections in more detail linked to the corresponding manuscripts respectively.

3.2 Functional annotation of uncharacterized essential genes using TAP-MS analysis

There is the long-standing interest in identifying the minimal crucial repertoire of genes important for life. The ease of genetically manipulating the yeast genome has already more than a decade ago provided the first insights into the sets of genes encoding core cellular processes necessary to maintain cellular physiology (Giaever *et al*, 2002; Tong *et al*, 2001). The development of selective gene-targeting and RNAi reagents has for the first time allowed to address similar questions in multicellular organisms like *C. elegans* or *D. melanogaster* or murine models in a focused manner (Green *et al*, 2011; Dietzl *et al*, 2007; Skarnes *et al*, 2011; White *et al*, 2013). Additionally, this led to the first initiatives in mapping gene essentiality in human cancer cells covering numerous tissues with near genome-wide resolution (Luo *et al*, 2008; Cowley *et al*, 2014). However, especially the attempts in human cells have suffered from the limitations of available RNAi reagents as mentioned previously, namely inconsistencies in

genome coverage, phenotypic heterogeneity related to incomplete gene knock-down and low reproducibility in between different studies closely linked to RNAi mediated off-target silencing effects (Hart *et al*, 2015).

Two technological innovations have reinvigorated the venture to interrogate and chart the human set of essential genes - the "essentialome". Haploid genetic screens have demonstrated their superiority in identifying important factors for viral entry, post translational modifications and drug sensitivity with unprecedented exactness. Inspiring pilot experiments in KBM-7 cells, which are dependent on the expression of *BCR-ABL1* for proliferation, have revealed that gene-trap sense insertions get depleted (negatively selected) from the cell population over time indicative of their transcript-abrogating function demonstrating that this technology using saturating mutagenesis on a large scale can be used to call gene knock-out related fitness defects with high statistical significance (Carette *et al*, 2011a). By applying stringent thresholds this has led to the identification of 2054 essential genes in the suspension cell line KBM-7 and 2181 genes in the adherent counterpart HAP1 with a core set of 1734 genes being required in both cell lines (Blomen *et al*, 2015).

The second technological innovation for large scale, pooled growth phenotypic interrogations came from the identification and utilization of CRISPR/Cas9 for genome-wide screening (Shalem *et al*, 2014; Wang *et al*, 2014b). Whereas work from the Sabatini lab has exploited both gene-trap mutagenesis and CRISPR/Cas9 screening in a comparative setting defining a set of 1878 genes (Wang *et al*, 2015), the Moffat lab has used CRISPR technology alone in different cell lines and primary cells uncovering a common set of 1580 genes required for cellular fitness (Hart *et al*, 2015).

All three studies have validated and recapitulated the requirement of core cellular processes like transcription, splicing, translation, ribosome biogenesis, cellular metabolism, proteasome complex organization beyond many others for cell growth. In addition, in between cell line comparisons allowed for the identification of specific liabilities outside the "core essentialome". For example, *SHC1*, *GRB2*, *GAB2*, *KRAS* and *STAT5B* showed preferential depletion in KBM-7 compared to HAP1 cells, constituting key downstream signaling components of the *BCR-ABL1* oncogene (Blomen *et al*, 2015). Similarly, *EGFR*, *SHC1*, *GRB2*, *SOS1* were selectively required in a subset of KRAS mutant CRC cell lines whereas BRAF mutant melanoma cells failed to demonstrate analogous dependencies (Hart *et al*, 2015). These findings highlight the effectiveness of these approaches for the identification of cancer subtype-specific therapeutic targets when expanded to a larger panel of different tumor entities.

Unifying to all three essentialome studies was the finding that a significant proportion of identified essential genes remains functionally non-annotated. The work of the Sabatini lab has identified 330 essential genes with unknown molecular function, encoding proteins that showed enrichment for domains involved in RNA processing and nucleolar localization (Wang

et al, 2015). The work of the Moffat lab has also identified essential genes with unknown MoA providing for the first time functional annotations to selected examples which are involved in mRNA splicing (*ZNF830*, *CCDC84*) and protein folding (*ANKRD49*) (Hart *et al*, 2015).

We have, in a collaborative effort with the Brummelkamp lab, characterized 18 essential genes using TAP-MS, which revealed that these proteins interact again commonly with essential proteins (Blomen *et al*, 2015). Most interestingly, we identified a new integral member of the oligosaccharyltransferase (OST) complex, a protein network important for protein N-linked glycosylation (Kelleher & Gilmore, 2006; Shrimal *et al*, 2015; Cherepanova *et al*, 2016). Proteomic analysis of TMEM258 identified the OST complex members STT3A, STT3B, DDOST, DAD1, RPN2 and MAGT1 within the group of high-confidence interacting proteins. A very recent study has independently confirmed the interaction of TMEM258 with the OST complex and deletion of *TMEM258* in colon tissue organoids leads to ER stress. Additionally, heterozygous knock-out mice display exacerbated intestinal inflammation in a colitis model (Graham *et al*, 2016). Furthermore, small molecule-based therapeutic targeting of the OST complex in RTK-driven cancers has been demonstrated by the induction of cellular senescence and growth arrest due to interference with EGFR or FGFR glycosylation (Lopez-Sambrooks *et al*, 2016). To this end, these cases provide paradigmatic examples of the scientific value of the growing number of haploid cell genetic and CRISPR-based essentialome datasets for future research.

However, several aspects need consideration for the comprehensive understanding of obtained results and the adaptation of future screens aiming to identify potential therapeutic targets. Whereas essentiality has been categorized in a qualitative way, yes or no, it actually covers a long gradient from clear essentiality to sickness and weak essentiality due to reduced cell proliferation. Future work will need to incorporate a way to quantify the severity of cell depletion in order to rank gene candidates based on their phenotypic impact. The integration of different read-outs for cell death initiation, cell cycle arrest or induction of differentiation might further provide a second layer of information, valuable for the interpretation of depletion phenotypes. It is noteworthy that most of the screening experiments so far have been done under normal cell culture condition using exogenous serum and media supply which needs consideration in the interpretation of gene candidates involved in metabolic processes. Targeted or small library *in vivo* validation experiments might provide an instructive alternative for the interrogation of such findings. Moreover, gene essentiality due to incompatibility with developmental processes cannot be identified in the currently utilized cellular model systems. However, the identification, characterization and adaptation of haploid embryonic stem cells combined with gene-trap or CRISPR technologies opens up new exciting territories to study the genetic requirement of tissue differentiation processes on a genome-wide level (Leeb & Wutz, 2011; Elling *et al*, 2011).

Our initial TAP-MS approach has shown that essential proteins tend to interact again with essential ones, providing a rational for potential genetic associations and interactions by forming functionally wired pathways (Blomen *et al*, 2015). Indeed, genetic interaction studies performed in yeast have identified dense wiring of cellular core processes assembling into functional protein complexes with high contextual dependencies (Collins *et al*, 2007; Costanzo *et al*, 2016). Pilot synthetic lethality experiments performed in HAP1 cells using gene-trap mutagenesis have started to uncover the genetic wiring of the Golgi organelle homeostasis and provide a primer for the genome-wide mapping of such interactions in human cells (Blomen *et al*, 2015). Furthermore, the proteomic interrogation of functionally uncharacterized essential genes will provide complementary insights into the pathway wiring of mammalian cells and their potential involvement in human diseases. This will undoubtedly involve the use of different affinity-based, proximity labeling-based or other alternative proteomic enrichment strategies depending on the biochemical and interaction properties of query proteins of interest. In the end, the knowledge about essentiality in culture system per se presents a unique, so far unavailable and experimentally important resource for many cell biological studies.

3.3 Establishment of an inducible retroviral expression system for TAP-MS-based protein complex identification

The proteomic characterization of proteins in their respective cell line or tissue background is of particular importance when studying genes with tissue-specific expression patterns or biological functions, and for the identification of cell type-specific interaction partners. Various studies focusing on transcription factors, deubiquitinating enzymes and other protein families have demonstrated the enhanced value of performing AP-MS analysis in selected tissue contexts (Wang *et al*, 2006; Dey *et al*, 2012). Furthermore, the identification of distinct cancer-associated mutations in a tissue-selective manner, as exemplified by association of different RAS isoforms with different cancer entities, further emphasizes the utility of targeted TAP-MS analysis and requires the availability of scalable tool kits (Cox *et al*, 2014).

The reliable usability of the rather small size SH combination tag compared to other larger tandem tags for proteomic approaches has been demonstrated in several important studies focusing on the characterization of kinase substrate interactions, phosphatase interaction networks and chromatin remodeling complexes (Glatter *et al*, 2009; Varjosalo *et al*, 2013; Hauri *et al*, 2016). We have developed the viral pRSHIC (retroviral expression of SH-tagged proteins for interaction proteomics and color-tracing) vectors that broaden the scope of amenable cell lines and primary cells for SH-based TAP-MS experimental work flows currently limited to a small set of flippase-flippase recognition target (Flp-FRT) recombination system-compatible

cell lines. The joint features of inducible expression synchronized to reporter fluorophore appearance further widens the space for potential mechanistic validation experiments based on flow cytometry or high-content fluorescence microscopy. Fluorescent tracking of cells provides a useful and easily accessible experimental handle to monitor cell population changes in inducible RNAi and cDNA (e.g. TSG) overexpression or rescue experiments as well as in the context of chemical screening (Zuber *et al*, 2011a; Hartwell *et al*, 2013).

We first focused on the oncogenic NRAS G12D variant, frequently found mutated in hematopoietic malignancies and melanoma (Cox *et al*, 2014) and inducibly expressed it in the murine pro-B cell line Ba/F3. This convincingly recapitulated the growth-promoting ability upon cytokine withdrawal. TAP-MS analysis of NRAS G12D revealed some of the known interactors most prominently Ras and Rab interactor 1 (RIN1) and one of the catalytic subunits of the PI3K protein complex, p110γ (PK3CG). These data demonstrated the functional validity of the newly established vector tool and opens up the possibility to map phenotypic changes and protein-protein interactions of cancer-associated nucleotide variants, truncations and oncogenic fusions (Yatim *et al*, 2012; Grebien *et al*, 2015; Klampfl *et al*, 2013).

We secondly focused on the ability to inducibly express SH-tagged bait proteins in a time controlled manner followed by TAP-MS analysis, that would otherwise upon stable cellular expression trigger cell death. We chose to profile a mutant form of the necroptosis-executing MLKL protein. Necroptosis is a form of regulated cell death contrasting apoptotic cell demise and is characterized by granulation, loss of cell membrane stability and release of intracellular material (Linkermann & Green, 2014; Vanden Berghe *et al*, 2014). Cell killing by regulated necrosis is a genetically encoded safety mechanism against virus infections and orchestrated by protein complexes containing RIPK1, RIPK3 and MLKL (Vanden Berghe *et al*, 2014). Recent work has identified necroptotic cell death as contributing factor to inflammation and organ failure (Linkermann *et al*, 2014), hence warranting the development of potential therapeutic agents to interfere with cell kill-induced tissue damage. The MLKL protein is the final executing protein of regulated necrosis leading to cellular membrane disruption, reminiscent to the mode of action of pro-apoptotic BCL2 family members by a yet not fully resolved molecular mechanism potentially involving membrane pore formation and/or ion influx (Sun *et al*, 2012). Activation of the pseudokinase MLKL is induced by RIPK3-mediated phosphorylation at T357 and S358 releasing its inactive conformation and N-terminal four-helix bundle domain (4HB) (Wang *et al*, 2014a; Hildebrand *et al*, 2014). Interestingly, MLKL activating mutations have been demonstrated to be toxic when expressed in human and murine cells making them an interesting tool to study the molecular mode of cell killing (Hildebrand *et al*, 2014; Murphy *et al*, 2013). We have used pRSHIC to inducibly express the phosphomimetic MLKL S358D mutant protein in human CRC cells. Expression led to induction of necroptotic cell death within 12 hours. Proteomic analysis after 6 hours of induction enabled

us to recover the known interaction with RIPK3 and further revealed a strong association with the protein chaperone complex members HSP90A, HSP90B and cell division cycle 37 (CDC37). Further purification experiments convincingly also identified an association of the MLKL WT protein with HSP90 excluding potential artefactual interactions due to the inducible expression of the mutant protein. The ability to block cell death induction upon pharmacological inhibition of HSP90 function using geldanamycin demonstrated the cell physiological importance of the detected interaction. Interestingly, the requirement of HSP90 and its kinase-specific co-chaperone CDC37 for the proper execution of necroptosis has previously been demonstrated by its importance for RIPK1 and RIPK3 protein function (Lewis *et al*, 2000; Li *et al*, 2015). Our data additionally suggests that also the final step in the necroptosis signaling cascade depends on proper HSP90-CDC37 chaperone function that allows therapeutic interference using small molecule inhibitors. The HSP90 protein complex is an important cellular proteostatic regulator and different chemical inhibitors have been developed for potential application in cancer therapy (Taipale *et al*, 2012; Wu *et al*, 2012; Trepel *et al*, 2010). Our findings in synopsis with previous study results warrant the further consideration and exploration of HSP90 inhibition as a potential candidate to therapeutically interfere with tissue damage induce due to pathological necroptosis as seen for example in ischemia reperfusion injury, pancreatitis, systemic inflammatory response syndrome or upon virus infection (Linkermann *et al*, 2013; Mocarski *et al*, 2015; Duprez *et al*, 2011).

3.4 Genetic screening for the identification of gene loss-of-functions mediating targeted cancer drug resistance

The successful development of oncogene-directed inhibitors in CML has conceptually transformed the field of cancer therapy triggering numerous research efforts for the therapeutic exploration of OA and NOA in a personalized manner. Similarly, the identification and subsequent characterization of resistance mechanisms to targeted cancer therapy has strongly been influenced by insights obtained from studying TKI-induced BCR-ABL inhibition. The identification of mutations in the drug target itself provide compelling evidence for the efficiency and strong evolutionary pressure these agents elicit on cancer cells to maintain proliferation and cell survival (Smith *et al*, 2012). This has not only been observed in numerous cases of kinase inhibitor resistance but also in cellular model systems resistant to the protein neddylation inhibitor MLN4924 whereby a single mutation in the NEDD8-activating enzyme subunit UBA3 abrogates drug binding showing full cellular adaptation to drug treatment (Soucy *et al*, 2009; Toth *et al*, 2012).

The development of resistance creates the necessity to identify patients at risk early on and provide alternative single agent or combination treatment regimens that interfere with the state of reduced drug sensitivity enabling again sufficient disease control. Positive and negative regulatory feedback loops can contribute to drug resistance and this raises the important question of which of these act in a non-redundant manner centering around important cellular signaling hubs. These hubs in many cases represent critical nodes with regard to OA and NOA and therefore can instruct the development, selection or repurposing of small molecules for novel therapeutic strategies. Genetic screens have been extremely instructive in identifying genes critically important for modulating cellular sensitivity towards targeted therapeutic interventions in different cancer entities (Bernards, 2014). Table 3 provides a summary of a selected set of phenotypically validated RNAi, haploid genetic as well CRISPR-based positive selection screens. It illustrates commonalities shared between different cancer entities and their respective pharmacological treatment as well as tissue subtype-specific genetic factors.

Gene	Breast cancer HER2i resistance	CML BCR-ABLi resistance	ALKi & EGFRi (lung), BRAFi (melanoma), MEKi (colon), RAFi (liver) resistance	Melanoma BRAFi resistance	Melanoma BRAFi resistance	Lung cancer EGFRi resistance	EGFRi (colon, lung), BRAFi (melanoma), MEKi (colon) resistance	Melanoma BRAFi resistance	NSCLC METi and ALKi resistance	Melanoma BRAFi resistance	CML BCR-ABLi resistance
	Berns et al. 2007	*Luo et al. 2008*	*Huang et al. 2012*	*Whittaker et al. 2013*	*Shalem et al. 2013*	*de Bruin et al. 2014*	*Bajpe et al. 2014*	*Sun et al. 2014*	*Papadakis et al. 2015*	*Doench et al. 2016*	*This thesis (2016)*
NF1		●		●	●	●				●	●
MED12			●		●					●	
PTEN	●									●	
PTPN1		●									●
SMARCE1		●							●		
CUL3					●					●	
NF2					●					●	
SMARCB1		●									
TADA1					●						
TADA2B					●						
SIRT2							●				
SOX10								●			
ARIH2										●	
KIRREL										●	
MED23										●	
PDCD10										●	
SUPT20H										●	
TAF6L										●	
TP53										●	
UBE2F										●	
WT1											●
PTPN12											●
LZTR1											●
BAP1											●

Table 3: Comparison of identified gene loss-of-functions in genetic drug resistance screens performed in different cancer types using pharmacological inhibition (i) of OA targets. *(Studies included in the comparison: Berns et al, 2007, Luo et al, 2008, Huang et al, 2012, Whittaker et al, 2013, Shalem et al, 2014, de Bruin et al, 2014, Bajpe et al, 2015, Sun et al, 2014, Papadakis et al, 2015, Doench et al, 2016 and screening results obtained within this doctoral thesis)*

3.5 Gene loss-of-functions contribute to tyrosine kinase inhibitor resistance in chronic myeloid leukemia

In this work we have taken advantage of the availability of a haploid human CML cell line that is exquisitely sensitive to 1st, 2nd and 3rd generation BCR-ABL TKIs. Performing gene-trap mutagenesis positive selection screens upon pharmacological BCR-ABL inhibition identified several gene knock-out alleles with an overlapping set of 6 gene candidates that repeatedly enriched upon TKI treatment. Whereas the BCR-ABL oncogene activates several pathways governing cellular survival, proliferation and the regulation of differentiation and cell cycle progression, the identification of *NF1* and tyrosine-protein phosphatase non-receptor type 1 (*PTPN1*) emphasized the importance of RAS/MAPK pathway reactivation in the initial recovery of CML cells upon incomplete inhibition or post-drug treatment reactivation of BCR-ABL. Convincingly, previous drug resistance RNAi screens focusing on imatinib have similarly identified these two genes in mediating resistance upon reduced expression in CML cell lines (Luo *et al*, 2008).

NF1 belongs to the family of GAP proteins and represents one of the most prominent negative regulators of RAS activation state by enhancing its intrinsic GTPase activity (Ballester *et al*, 1990; Scheffzek *et al*, 1998). Germline inactivating variants have been identified that lead to the development of neurofibromatosis type 1, which is phenotypically defined by the formation of benign peripheral cutaneous nerve tumors so called neurofibromas as well as benign plexiform neurofibromas carrying the possibility of malignant transformation (Ratner & Miller, 2015). These inherited mutations further present a risk for the development of a diverse set of other malignancies like JMML, gastrointestinal stromal tumor (GIST) or gliomas and somatic alterations in the *NF1* locus are frequently found in AML, melanoma, glioblastoma beyond many others (McGillicuddy *et al*, 2009; Parkin *et al*, 2010; Maertens *et al*, 2013; Lawrence *et al*, 2013). Reduced or complete absence of expression has furthermore been recognized in the process of resistance development to targeted inhibition of EGFR in lung cancer and RAF inhibition in BRAF V600E positive melanoma (Whittaker *et al*, 2013; de Bruin *et al*, 2014).

PTPN1 (also in the literature referred to as PTP1B) belongs to the group of protein tyrosine phosphatases which act as erasers in the phosphotyrosine signaling circuitry (Tonks, 2006). PTPN1 has been implicated as a key negative regulator of RTK signaling and more specifically is able to antagonize the transformation propensity of BCR-ABL by negatively regulating the BCR Y177 residue thereby interfering with GRB2 recruitment (Haj *et al*, 2003; LaMontagne *et al*, 1998). Moreover, recent genetic studies have identified somatic inactivating mutations in lymphoid malignancies, namely B cell and Hodgkin lymphoma (Gunawardana *et al*, 2014).

There are ongoing discussions about the degree of specificity and the spatial regulation of phosphatases in cellular signaling cascades (Tonks, 2013). Therefore, the identification of a

second protein tyrosine phosphatase, PTPN12 (also referred to as PTP-PEST), in mediating resistance to BCR-ABL TKIs was intriguing to us. In fact, *PTPN12* has been identified as a TSG in triple-negative breast cancer whereby loss-of-function mutations or reduced expression enhances transformation by increased activity of RTK driven MAPK signaling (Sun *et al*, 2011). Detailed time-resolved MS analysis has furthermore demonstrated that PTPN12 controls an important switch between pro-mitogenic and pro-survival signaling to cytoskeleton rearrangement and cellular invasion via the regulation of the adaptor protein SHC1 and its association with GRB2 and other signaling adaptor proteins (Zheng *et al*, 2013). In fact, TAP-MS analysis on the BCR-ABL core signaling complex has identified an association of PTPN12 and SHC1 in CML cells (Brehme *et al*, 2009). In conjunction, these findings suggest a model in which both tyrosine phosphatases, PTPN1 and PTPN12, govern sensitivity towards BCR-ABL-directed TKIs via the negative regulatory modulation of fusion oncogene-induced MAPK pathway activation.

Recent studies have started to accumulate evidence which emphasizes the importance of transcriptional regulation and chromatin remodeling in the induction of drug tolerant states (Sharma *et al*, 2010; Huang *et al*, 2012; Rathert *et al*, 2015). Using haploid genetic screens, we have identified that knock-out cells of the transcription factor Wilms tumor 1 (*WT1*) and the deubiquitinating enzyme BRCA1 associated protein 1 (*BAP1*) show reduced sensitivity towards BCR-ABL inhibition.

WT1 has been considered a double-faced gene by its capability to act as a driving OG as well as to fulfill TSG function (Yang *et al*, 2007; Huff, 2011). Within the hematopoietic system mutations in *WT1* have frequently been found in AML with prognosis predictive relevance, contributing to disease pathogenesis and potentially influencing therapeutic responses (Cancer Genome Atlas Research Network, 2013; Pemovska *et al*, 2013; Krauth *et al*, 2015). Interestingly, recent studies have identified mutual exclusivity between alterations in *WT1* and *IDH1*, *IDH2* or *TET2* suggestive of a shared pathway involvement regulating DNA methylation and hence altered gene transcription (Rampal *et al*, 2014).

In contrast *BAP1* is frequently mutated in pleural mesothelioma, renal cell carcinoma and uveal melanoma (Harbour *et al*, 2010; Bott *et al*, 2011; Peña-Llopis *et al*, 2012; Carbone *et al*, 2013). It has deubiquitinating enzyme function and was originally shown to interact with BRCA1 (Jensen *et al*, 1998). However, more recent work has identified its essential role in regulating cell proliferation via the modulation of HCF-1 (*HCFC1*) protein levels (Misaghi *et al*, 2009). Additionally, BAP1-associated protein complexes have been shown to further contain the polycomb group proteins ASXL1 and ASXL2 as well as the O-GlcNAc transferase OGT (Dey *et al*, 2012). Deletion of *Bap1* in the murine hematopoietic compartment leads to myeloid transformation mostly resembling human myelodysplastic syndrome supporting a potential collaborative function with ASXL1 (Dey *et al*, 2012).

To this end, both *WT1* and *BAP1* knock-out KBM-7 cells displayed reduced sensitivity towards BCR-ABL TKI treatment, however other related CML cell lines failed to demonstrate similar behavior. It is well established that the genetic and epigenetic plasticity in between cancer entities but also within on specific tumor can drastically influence the response to therapeutic agents (Sharma *et al*, 2010; Ding *et al*, 2012). A previous RNAi screen for imatinib resistance in a different CML cell line, K562, has failed to recover shRNAs targeting *WT1* or *BAP1* as candidate drug sensitivity modulators. However, the same experimental setup has identified reduced expression of the *SMARCB1* and *SMARCE1* genes encoding members of the SWItch/Sucrose Non-Fermentable (SWI/SNF) nucleosome remodeling complex in being able to mediate resistance to imatinib treatment (Luo *et al*, 2008) (Table 3). Clearly more work will be required to disentangle the complex wiring of epigenetic regulation and their ability to modulate the sensitivity towards pharmacological inhibition of the BCR-ABL oncogene in CML and Ph+ ALL cells.

In contrast, the mechanistic involvement of leucine zipper like transcription regulator 1 (*LZTR1*) in the regulation of BCR-ABL TKI sensitivity of CML cells remained elusive based on currently available literature. Initial cell biological and MS-based studies have shown localization of LZTR1 to the Golgi apparatus and binding to the CUL3 subunit of the cullin-RING E3 ligase (CRL3) complex (Nacak *et al*, 2006; Bennett *et al*, 2010; Emanuele *et al*, 2011). Association with CRL3 can further be rationalized by the presence of two C-terminal BTB domains in LZTR1 which bind CUL3 as well as six N-terminal kelch domains which act as substrate recognition domains (Xu *et al*, 2003; Lydeard *et al*, 2013). CRL complexes constitute a subclass of the large family of RING domain E3 ligases and there are six cullin proteins (CUL1-3, CUL4A, CUL4B and CUL5) encoded in the human genome (Zimmerman *et al*, 2010). These cullins form assembly platforms for multi protein complexes, containing an E3 enzyme (RBX1 or RBX2) and one or more sequential substrate adaptor protein assemblies that lead to the attachment of ubiquitin PTMs onto specific target proteins (Petroski & Deshaies, 2005). Interestingly, genetic studies have identified *LZTR1* mutations as potential pathogenic alterations in GBM, SWNMT and NS (Frattini *et al*, 2013; Piotrowski *et al*, 2014; Yamamoto *et al*, 2015). Particularly the association with Noonan syndrome, part of the group of RASopathies, which are characterized by increased activation of the RAS/MAPK pathway offered a compelling mechanistic explanation for the observed resistance phenotype. Indeed, several examined CML as well as FLT3-ITD mutant AML cells already under normal culture conditions displayed elevated MAPK activation. Further genetic and biochemical experiments revealed that the increased pathway activation in CML cells is dependent on the presence of KRAS and that the CRL3-LZTR1 complex is able to ubiquitinate RAS proteins. The ubiquitination of RAS proteins itself has been reported in different studies, yet contradictory in their postulated mechanistic outcome. Ubiquitination of HRAS however not KRAS can lead to altered

endosomal trafficking and mutation-induced blockade of ubiquitination enhances ERK activation (Jura *et al*, 2006). On the contrary, ubiquitination of KRAS can lead to enhanced activation and modulates the interaction with downstream substrates (Sasaki *et al*, 2011). Similarly, selective mono-ubiquitination can interfere with GAP functionality leading to enhanced signaling output (Baker *et al*, 2013). Ultimately, ubiquitination has been described to likewise trigger RAS degradation (Zeng *et al*, 2014). To this end, the biochemical consequences of CRL3-LZTR1-induced RAS ubiquitination leading to enhanced MAPK pathway activation still require further experimental work for clarification especially in light of discrepancies present in the current literature. Most notably, RABGEF1 (also commonly referred to as Rabex-5) has been shown to ubiquitinate RAS proteins and by using *Drosophila* as model system *in vivo* RNAi experiments have revealed wing vein phenotypes reminiscent of findings obtained with other NS candidate genes like *PTPN11* (Xu *et al*, 2010; Yan *et al*, 2010). However, no inactivating mutations in *RABGEF1* have been identified in human genetic studies focusing on patients with RASopathy-like clinical presentation. In contrast, reconstitution experiments done in CML cells showed that the identified *LZTR1* missense mutations are phenotypically loss-of-function alterations in a KRAS-dependent MAPK pathway activation state. In addition, this provides for the first time a potential mechanistic explanation for the so far unknown molecular link between *LZTR1* variants and their identification in GBM, SWNMT and NS. It however remains to be clarified whether RAS GTPases represent the only substrate proteins for LZTR1 or whether there are further candidates to be discovered that would contribute to the pathogenesis of the afore mentioned diseases.

Besides our findings on LZTR1 the CRL3 complex itself has previously been linked to the regulation of RTK-RAS/MAPK or PI3K/AKT pathway activation as well as the response to targeted cancer agents. CRISPR/Cas9 genetic knock-out screens in BRAF mutant melanoma have identified an enrichment of sgRNAs targeting *CUL3* upon RAF or MEK inhibition using vemurafenib or selumetinib treatment, respectively (Shalem *et al*, 2014; Doench *et al*, 2016). No further mechanistic validation experiments have been performed building on the initial screen observations. Nevertheless, reduced expression of CUL3 leads to alterations in late endosome maturation interfering with EGFR degradation (Huotari *et al*, 2012). Correspondingly, increased EGFR protein levels leading to enhanced PI3K/AKT activation have been recognized to induce acquired resistance to vemurafenib treatment in BRAF mutant melanoma as well as intrinsic resistance to RAF inhibition in BRAF mutant CRC (Prahallad *et al*, 2012; Sun *et al*, 2014). Additionally, the regulation of NF1 protein stability has been linked to the CRL3 activity, whereby NF1 is bound by the CUL3 BTB-Kelch-domain containing adaptor protein KBTBD7 mediating its proteasomal degradation (Hollstein & Cichowski, 2013). These findings further support the observed persistent proteostatic deregulation of NF1 contributing to glioblastoma development and growth (McGillicuddy *et al*, 2009).

In summary, our genetic screen findings provide another piece of evidence for the crucial importance of the RAS/MAPK pathway in maintaining cell growth of BCR-ABL-positive leukemias and in their response to targeted therapy. Preclinical studies including *in vivo* models of RAS-driven leukemias as well as case reports support the idea to therapeutically target MAPK signaling in CML (Nguyen *et al*, 2007; Pellicano *et al*, 2011; Burgess *et al*, 2014). A drug combination screen performed in BCR-ABL T315I mutant cells has identified a strong dependency on maintaining active MAPK signaling for cell survival. The uncovered synergistic anti-proliferative action of bosutinib and danusertib co-administration was dependent on efficient compound off-target-mediated inhibition of MEK and ERK activity (Winter *et al*, 2012). Similarly, selected TKIs like nilotinib in the presence of BCR-ABL T315I can in fact lead to enhanced ERK signaling due to paradoxical RAF activation which can be rescued by MEK inhibitor co-treatment (Packer *et al*, 2011). Moreover, treatment observations in a patient diagnosed with metastatic melanoma in the presence of CP CML revealed a complete HR upon combinatorial treatment with BRAF and MEK1/2 inhibitors demonstrating the potential clinical utility of combining BCR-ABL and MAPK pathway targeting agents (Andrews *et al*, 2015). Additionally, exemplary studies from BRAF mutant melanoma have further demonstrated that the combinatorial blockade within the MAPK cascade or in parallel the MAPK and PI3K pathway can significantly delay the onset of resistance and target different mechanism leading to treatment failure (Whittaker *et al*, 2015; Deuker *et al*, 2015).

In the end, it will be important to clarify which of the so far additionally identified and characterized resistance situations excluding direct drug target effects can be identified in clinically resistant patients (Kim *et al*, 2017). Identifying a way to estimate their phenotypic impact either by representative biomarkers or *ex vivo* profiling approaches will be crucial to make informed treatment decisions for the selection of 2nd line therapeutic agents or combinatorial regiments (Murtaza *et al*, 2013).

3.6 Conclusion and future prospects

In summary within the scope of this thesis I have employed TAP procedures aided by MS technology for the identification of protein complexes as well as gene-trap-based haploid genetic screens and CRISPR/Cas9-induced targeted genome engineering for the discovery and functional characterization of genes important for cancer cell growth and targeted pharmacological perturbations. The developed vector toolkit will be beneficial in the future for the mechanistic, MS-aided description of uncharacterized and tissue-selective essential gene candidates identified by genetic screening campaigns. The technological integration of proximity labeling strategies and advancements in affinity purification strategies for *in vivo* settings will offer alternative ways to especially capture transient and highly context-dependent or dynamic interactions. The adaptation of suitable CRISPR-based targeted integration strategies into endogenous genomic loci for subsequent TAP- or AP-MS analysis will be crucial for the exploitation of proteins currently not amenable to vector-based transductions due to size limitations.

Focused genetic screens on the requirements of CML cells for oncogene-directed TKI sensitivity has identified known and novel negative regulators of the BCR-ABL signaling network. The evaluation of their relevance and usability as clinical biomarkers for the identification of patient subpopulations at higher risk for either disease relapse after therapy cessation or progression to advance disease states will be critical (Bertotti *et al*, 2015). The findings obtained by studying the mechanistic basis of *LZTR1* loss-of-function-induced drug resistance have highlighted again the importance of ubiquitination and its interplay with other PTMs like phosphorylation in the orchestration and fine tuning of cellular signaling processes. The development and exploitation of novel therapeutic approaches by targeting alterations within the ubiquitin system as well as harnessing its proteostatic regulatory capabilities will dramatically expand the breadth of the currently available kinase and epigenetic-focused small molecule therapeutic armamentarium (Skaar *et al*, 2014; Huang & Dixit, 2016).

Improvements in genetic screening systems, the addition of inducibility and traceability features into existing CRISPR-based techniques as well as the adaptation of novel innovative viral vectors will broaden the scope of amenable cellular and phenotypic interrogations (Khan *et al*, 2011; Ran *et al*, 2015; Yin *et al*, 2016). Furthermore, the development of combinatorial genetic screening tool kits will provide a powerful orthogonal approach to well-established chemical biology platforms for the identification of synergistic pairs of druggable targets (Wong *et al*, 2016). The development of organoid and alternative *ex vivo* cell (co-)culture systems will be crucial to study gene essentiality and drug resistance development more closely to the actual clinical setting providing more accurate predictions and means to experimentally tackle phenotypic heterogeneities brought about by patient to patient variability (van de Wetering *et al*, 2015; Clevers, 2016).

In the end, the application of above listed technological innovations will increase our understanding of the genetic and phenotypic wiring of cancer cells as well as the complex interplay of targeted pharmacological agents with neoplastic cells. Extending the lessons learned in cancer drug discovery to the growing field of immuno oncology will empower the rise of innovative novel combinatorial treatment modalities for cancer with curative intent achieving durable therapeutic responses.

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5 Appendix

5.1 Curriculum vitae

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Education:

2010 - present Medical University of Vienna, Austria
PhD Program N094

2004 - 2010 Medical University of Vienna, Austria
Program of Medicine N202 (Completed July 2010)
Clinical rotations completed in Austria, USA, Australia and Japan

Research:

2011 - present Giulio Superti-Furga laboratory, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria
PhD thesis within the thematic program: *"Molecular signal transduction"*

2007 - 2010 Experimental Oncology Research Laboratory, Department of Otorhinolaryngology, Head and Neck Surgery, Medical University of Vienna, Austria (PI: ao. Univ.-Prof. Dr. Dietmar Thurnher)
Diploma thesis: *"Resveratrol and etoposide in head and neck cancer cell lines – an in vitro study"* (completed 2010)

Conferences, poster and oral presentations:

2016 EMBO Conference Series – Cellular Signaling & Cancer Therapy
Cavtat-Dubrovnik, Croatia
Poster presentation: *"Haploid genetic screens in CML cells identify mediators of BCR-ABL inhibitor resistance"*

2015 17th Annual John Goldman Conference on Chronic Myeloid Leukemia: Biology and Therapy
Congress Center Estoril, Portugal
Oral presentation: *"Haploid genetic screens in CML cells identify mediators of BCR-ABL inhibitor resistance"*
Invited oral presentation: Workshop for non-clinical scientists #2 - Proteomics
"Mass spectrometry based proteomics – a powerful tool from protein-protein interaction studies to drug mechanism of action discovery"

2014 10th YSA PhD Symposium, Vienna, Austria
Poster presentation: *"Haploid genetic screens using CML cells identifies profile of BCR-ABL inhibitor resistance"*

2014 EU-LIFE scientific workshop – Biology of Cancer: bridging basic and translational research
CRG, Barcelona, Spain
Poster presentation: *"Haploid genetic screens using CML cells identifies profile of BCR-ABL inhibitor resistance"*

2013 15th International Conference on Chronic Myeloid Leukemia: Biology and Therapy
Congress Center Estoril, Portugal
Oral presentation: *"Haploid genetic screens using CML cells identifies profile of BCR-ABL inhibitor resistance"*

2013 9th YSA PhD Symposium, Vienna, Austria
Poster presentation: *"Haploid genetic screens uncovering BCR-ABL inhibitor resistance"*

2012 3rd European Chemical Biology Symposium
Vienna, Austria
Poster presentation: *"Molecular BCR-ABL network modulations upon perturbation"*

2011 13th International Conference on Chronic Myeloid Leukemia: Biology and Therapy
Congress Center Estoril, Portugal
Poster presentation: *"Molecular BCR-ABL network modulations upon perturbation"*

2011 EACR-FEBS advanced lecture course: Molecular Mechanisms in Signal Transduction and Cancer
Spetses, Greece
Poster presentation: *"Molecular BCR-ABL network modulations upon perturbation"*

2011 7th YSA PhD Symposium, Vienna, Austria
Poster presentation: *"Molecular BCR-ABL network modulations upon perturbation"*

2011 FEBS Practical Course – In Silico Systems Biology: Network Reconstruction, Analysis and Network based Modelling
EMBL-EBI, Hinxton, UK

2011 Keystone Meeting – Omics Meets Cell Biology
Alpbach Congress Center, Alpbach, Austria

2010 54th Annual Meeting of the Austrian Society of Oto-Rhino-Laryngology, Head and Neck Surgery, Salzburg, Austria
Oral presentation: *"Targeting Polo-like kinase 1 in head and neck squamous cell carcinoma by a small molecule inhibitor in vitro"*

2008 3rd European conference on head and neck oncology, Zagreb, Croatia
Poster presentation: „*Resveratrol and etoposide in head and neck cancer cell lines"*

Peer review assistance:

Cancer Research, Biochimica et Biophysica Acta, Leukemia,
Nature Review Cancer, Cancer Cell, Nature Chemical Biology, eLife,
Oncotarget, Blood

Teaching:

2013/2014 Supervision of lab rotation and internship students

Awards and scholarships:

2014 10th YSA PhD Symposium, Vienna, Austria
Best poster presentation award

2009/2010 Scholarship JASSO-NUPACE (Nagoya University Program of Academic Exchange), Japan

2009 Research scholarship of the Medical University of Vienna, Austria

2008 Research scholarship of the Medical University of Vienna, Austria

References: available upon request

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