MEDICAL UNIVERSITY OF VIENNA

Chemical modulation of $CUL3^{LZTR1}$-mediated proteostatic RAS regulation

Doctoral thesis at the Medical University of Vienna

for obtaining the academic degree

Doctor of Philosophy

Submitted by

Sophie Piech, MSc.

Supervisor

Univ.-Prof. Dr. Giulio Superti-Furga

CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences

Lazarettgasse 14, AKH BT25.3, 1090 Vienna, Austria

Center for Physiology and Pharmacology, Medical University of Vienna

Währinger Straße 13A/HP, 1090 Vienna, Austria

Vienna, 01/2025

Declaration

The work described in this thesis is presented in a cumulative form and contains one research publication. The experiments in this study were carried out by the author in the Superti-Furga laboratory at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, unless stated otherwise, under the supervision of Univ.-Prof. Dr. Giulio Superti-Furga and the co-supervision of Dr.med.univ. Johannes Bigenzahn, PhD. This thesis was solely written by Sophie Piech with input provided by Giulio Superti-Furga and Johannes Bigenzahn. The author of the thesis is also the first author of the research publication. The individual contributions of each co-author are outlined in the prologue preceding the research paper. All materials and results that are not original to this work have been cited and referenced.

The research article in the results section was published in *ACS Chemical Biology*: S. Piech, S. Brüschweiler, J. Westphalen, K.M. Siess, J. García Murias, R. Konrat, J.W. Bigenzahn, G. Superti-Furga, **Identification and Characterization of Novel Small-Molecule Enhancers of the CUL3^{LZTR1} E3 Ligase KRAS Complex**, *ACS Chemical Biology (2024)*, DOI: 10.1021/acschembio.4c00077

Table of Contents

List of Figures and Tables

Abstract

The prevalent role of oncogenically active RAS GTPases in the development and growth of human cancers makes them a significant therapeutic target. A detailed mechanistic understanding of this signaling pathway is expected to guide the development of novel therapeutics that would address a high clinical demand. The identification of the leucine zipper-like transcriptional regulator 1 (LZTR1) as a substrate adaptor of the Cullin 3 RING E3 ubiquitin ligase (CUL3), able to control the proteostasis of RAS GTPases, has added an important layer to the regulation of RAS.

LZTR1 can bind all four main RAS family members, including the frequently mutated oncoprotein KRAS. Structurally, LZTR1 bears a substrate recognition domain, the Kelch domain, that facilitates protein interaction with KRAS and two BTB/BACK domains that mediate interaction with the CUL3 ubiquitin-ligase complex. Recently, new pharmacological agents able to inhibit RAS specifically have heightened the enthusiasm for targeting this class of proteins. Pharmacological modulation of $CUL3^{LZTR1}$-based RAS proteostasis using a molecular glue degrader approach might provide a new therapeutic modality for targeting RAS-driven cancers. Molecular glue degraders are small molecules in the field of targeted protein degradation (TPD), that chemically enhance the interaction between a substrate protein and its E3 ubiquitin ligases.

The focus of this thesis is to understand the regulatory mechanisms of LZTR1-dependent KRAS degradation and to identify chemical fragments that, in a molecular glue-like manner, potentiate the protein-protein interaction (PPI) between KRAS and LZTR1, leading to enhanced KRAS degradation. I developed a split-luciferase assay to monitor the KRAS-LZTR1 PPI in a high throughput screening-compatible manner. Using this assay, I screened fragment libraries leading to the identification of two small molecule fragments, C53 and Z86, that potentiate this PPI in a dose-dependent manner. Further orthogonal validation methods, including thermal shift assay, proximity biotinylation (BioID) and NMR spectroscopy confirmed KRAS-ligand interaction and fragment-dependent KRAS-LZTR1 complex recruitment. This represents a proof-of-concept for molecular agents capable of inducing RAS-LZTR1 complex formation.

In summary, I anticipate that my findings will advance the biochemical understanding of the LZTR1-KRAS protein-protein interface and LZTR1-regulated KRAS degradation, providing a new avenue to develop RAS-focused therapeutic modalities based on the $CUL3^{LZTR1}$ E3 ligase. Moreover, the outlined chemical biology approach can easily be tailored

to other pharmacologically challenging oncogene-E3 ligase pairs to screen for small molecule PPI enhancers for the discovery of novel therapeutic entities.

Zusammenfassung

Onkogene Aktivierung von RAS GTPasen spielt bei der Entstehung und dem Wachstum von menschlichen Krebserkrankungen eine zentrale Rolle und macht sie zu einem bedeutenden therapeutischen Angriffsziel. Ein detailliertes mechanistisches Verständnis dieses Signalwegs ist sowohl von entscheidender klinischer Bedeutung, als auch wichtig für die erfolgreiche Entwicklung von neuen Therapeutika. Die Identifikation des Leucine Zipper-like Transcriptional Regulator 1 (LZTR1) Substratrezeptor enthaltenden Cullin-3 RING E3-Ubiquitin Ligase (CUL3) Komplexes, hat die proteostatische Regulation von RAS-GTPasen als eine zusätzliche wichtige regulatorische Ebene aufgezeigt. LZTR1 kann alle vier Hauptmitglieder der RAS GTPase Familie binden, einschließlich des häufig mutierten Onkoproteins, KRAS. Strukturell enthält LZTR1 ein Substratsbindungsdomäne, die Kelch-Domäne, die die Proteininteraktion mit KRAS ermöglicht, sowie zwei BTB/BACK-Domänen, die mit dem CUL3 Ubiquitin Ligase Komplex interagieren und somit den KRAS-Proteinabbau induzieren. Angesichts der Einschränkungen der derzeit verfügbaren RAS-zielgerichteten Wirkstoffe, die erst kürzlich erste vielversprechende klinische Erfolge gezeigt haben, könnte die pharmakologische Modulation der $CUL3^{LZTR1}$-abhängigen RAS-Degradierung mittels eines „Molecular Glue“ Ansatzes eine neue therapeutische Modalität zur Behandlung von RAS-getriebenen Krebserkrankungen bieten. Molecular Glue Degrader sind eine chemische Modalität im Bereich des pharmakologisch-induzierten Proteinabbaus („Targeted Protein Degradation“, TPD), und sind in der Lage die Interaktion zwischen einem Substratprotein und dessen E3 Ubiquitin Ligase chemisch im Sinne eines Klebstoffes zu verstärken.

Das Hauptziel dieser Arbeit ist es, die regulatorischen Mechanismen des LZTR1-abhängigen KRAS-Abbaus zu verstehen und chemisch auszunutzen sowie chemische Fragmente zu identifizieren, die auf molekulare Klebstoff-ähnliche Weise die Protein-Protein-Interaktion (PPI) zwischen KRAS und LZTR1 verstärken und so einen verbesserten KRAS-Abbau bewirken.

Zu diesem Zweck habe ich einen Split-Luciferase-Assay entwickelt, um die KRAS-LZTR1-PPI in einem für Hochdurchsatz-Screening kompatiblen Format zu monitieren. Mit diesem Assay habe ich Fragmentkollektionen gescreent und zwei kleine Molekülfragmente, C53 und Z86, identifiziert, die diese Proteininteraktion dosisabhängig verstärken können. Weitere orthogonale Validierungsmethoden, wie Thermal Shift Assays, Proximity Biotinylation (BioID) und NMR-Spektroskopie, bestätigten die KRAS Molekülfragment Interaktion und die fragmentabhängige Rekrutierung des KRAS-LZTR1-Komplexes.

Zusammenfassend erwarte ich, dass meine Ergebnisse das biochemische Verständnis des KRAS-LZTR1 Proteinkomplexes und des LZTR1-regulierten KRAS-Abbaus voranbringen und eine neue Grundlage für die Entwicklung RAS-fokussierter therapeutischer Modalitäten auf Basis der $CUL3^{LZTR1}$ E3 Ligase schaffen. Darüber hinaus kann der beschriebene chemisch-biologische Ansatz leicht auf andere pharmakologisch herausfordernde Onkogen-E3 Ligase Paare angepasst werden, um neue PPI-verstärkende Moleküle zu identifizieren, und so neuartige therapeutische Wirkstoffe zu entwickeln.

(translated with the help of Chat-GPT)

Abbreviations

^{1}H-^{15}N-HSQC	Heteronuclear Single Quantum Coherence
AMG-510	Amgen-510, Sotorasib
AUTACs	Autophagy-Targeting Chimeras
BioID	BirA*-based proximity biotinylation
cIAP1	Cell inhibitor of apoptosis protein 1
CML	Chronic myeloid leukemia
CRL	Cullin RING E3 ubiquitin ligases complex
CRBN	Cereblon
CUL3	Cullin 3 RING E3 ubiquitin ligase
CypA	Cyclophilin A
DUBTACs	Deubiquitinase-Targeting Chimeras
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor alpha
ERK	Extracellular signal-regulated kinase
FBDD	Fragment-based drug discovery
FRET	Fluorescence resonance energy transfer
FP	Fluorescence polarization
FTase	Farnesyltransferase
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GDP	Guanosine diphosphate
GGTase	Geranylgeranyltransferase
GTP	Guanosine triphosphate

HECT	‘Homologous to the E6-AP Carboxyl Terminus’ domain
HNSCC	Head and neck squamous cell carcinoma
HTRF	Homogeneous time-resolved fluorescence
HTS	High-throughput screen
HVR	Hypervariable region
IMiD	Immunomodulatory imide drug
ITC	Isothermal titration calorimetry
LE	Ligand efficiency
LYTACs	Lysosome-Targeting Chimeras
LZTR1	Leucine zipper-like transcriptional regulator 1
MDM2	Mouse double minute 2 human homolog
MEK	Mitogen-activated protein kinase kinase
NAE1	NEDD8-activating enzyme E1 subunit 1
Nedd8	Neural precursor cell expressed developmentally down regulated 8
NMR	Nuclear magnetic resonance
PDAC	Pancreatic ductal adenocarcinoma
PPI	Protein-protein interaction
POI	Protein of interest
RAS	Rat sarcoma virus
RBR	RING-between-RING
TKI	Tyrosine kinase inhibitor
TPD	Targeted protein degradation
SAR	Structure-activity relationship
SLC	Solute carrier protein
SH2	Sequence homology 2 domain
SOS	Son of Sevenless

SPR	Surface plasmon resonance
STD	Saturation transfer difference
UPS	Ubiquitin-proteasome system
VHL	Von Hippel-Lindau Tumor Suppressor

Acknowledgements

Foremost, I would like to thank my supervisor Prof. Giulio Superti-Furga for giving me the opportunity to work in his lab, and Dr. Johannes Bigenzahn for trusting in me to take on the drug-discovery work of the project that he started. Prof. Giulio Superti-Furga has continuously supported me, despite certain aspects of the projects appearing impossible to solve. Johannes was an incredible teacher and mentor and could address any questions or concerns I had regarding the project. I'm so thankful for their guidance. I would like to thank the members of the GSF laboratory, particularly Dr. Andras Boeszoermenyi, even though we didn't manage to purify LZTR1 he always supported me and provided valuable input. Additionally, I want to express my gratitude to Julio García Murias for conducting revision experiments during my maternity leave. Many thanks to Josepha Westphalen, she's a wonderful student, fun to have around and managed to complete crucial experiments towards the end of the project. To Robert Konrat and Sven Brüschweiler, thank you for the friendly and fruitful collaboration. The NMR part of the project was critical, and I couldn't have asked for better collaborators.

I'm so fortunate to have so many people that were cheering me on along the way. Thank you to my husband, Arthur, he is my biggest supporter, and everything is better with him. I'm thankful for you every day. I am deeply grateful for the arrival of our precious son, Joseph Mendel, whose presence has filled our lives with boundless love and joy. To my parents and sister, thank you for always being proud of me. Thank you to my friends and the PhD cohort of 2021 for always being there for me and helping me along the way. Writing this dissertation has brough about a lot of life changes and the experience has been amazing.

"To defend a country you need an army, but to defend a civilization you need education."

– Jonathan Sacks

1. Introduction

1.1 RAS GTPases

Rat sarcoma virus (RAS) proteins belong to the class of small GTP binding and hydrolyzing proteins (GTPases) that regulate critical cellular processes, such as proliferation, migration, survival and differentiation (Longo and Carroll 2022). Their switch mechanism allows them to cycle between the active guanosine triphosphate (GTP)-bound and the inactive guanosine diphosphate (GDP)-bound states. This binary switch mechanism allows them to mediate the “ON” and “OFF” states of the signal transduction pathways they are embedded in (Simanshu, Nissley et al. 2017). The transition between these states is regulated by guanine nucleotide exchange factors (GEFs) that enhance GDP to GTP exchange and GTPase-activating proteins (GAPs) that accelerate GTP hydrolysis (Longo and Carroll 2022).

The most intensely studied RAS-mediated signaling event is regulation of proliferation signaling via the RAS-RAF-MEK-ERK mitogen activated protein kinase (MAPK) signaling pathway. This pathway is tightly controlled by transmembrane tyrosine kinase receptors, like the epidermal growth factor receptor (EGFR), that is activated in response to the extracellular binding of growth factors such as epidermal growth factors (EGF) (Zheng and Chang 2014). Nonetheless, other upstream receptors such as integrins, serpentine receptors, and cytokine receptors can also activate RAS signaling (Molina and Adjei 2006). Once EGF binds, EGFR oligomerizes which subsequently leads to kinase activation on the cytoplasmic domain, resulting in transphosphorylation of the receptor (Schlessinger 2000). Successively, the transphosphorylation facilitates the binding of adapter protein GRB2 that bind sequence homology 2 (SH2) domains, thereby, leading to the recruitment of the GEF, Son of Sevenless (SOS) (Molina and Adjei 2006). SOS enables the critical switch between the inactive (GDP)-bound state and the active (GTP)-bound and, consequently, activated RAS in turn recruits the serine/threonine RAF kinases (Marais, Light et al. 1995, Martinez Molina, Jafari et al. 2013). Upon RAF engagement, MAPK signaling is activated via phosphorylation, which results in the activation of the mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) (Bahar, Kim et al. 2023). Thereafter, ERK translocates to the nucleus, where it regulate various transcription factors thereby altering gene expression (Roberts and Der 2007). This well characterized cascade, depicted in Figure 1, is essential for transducing proliferative signal to the nucleus, prompting cell growth (Bahar, Kim et al. 2023). Importantly, one of the main GAP proteins responsible for turning off RAS GTPases is called neurofibromin, encoded by the *NF1* gene (Longo and Carroll 2022). Neurofibromin stimulates the intrinsic GTPase activity of RAS, leading to the hydrolysis of GTP to GDP, which switches RAS from

its active to its inactive state, thus downregulating the MAPK signaling pathway activity (Khrenova, Grigorenko et al. 2015, Longo and Carroll 2022).

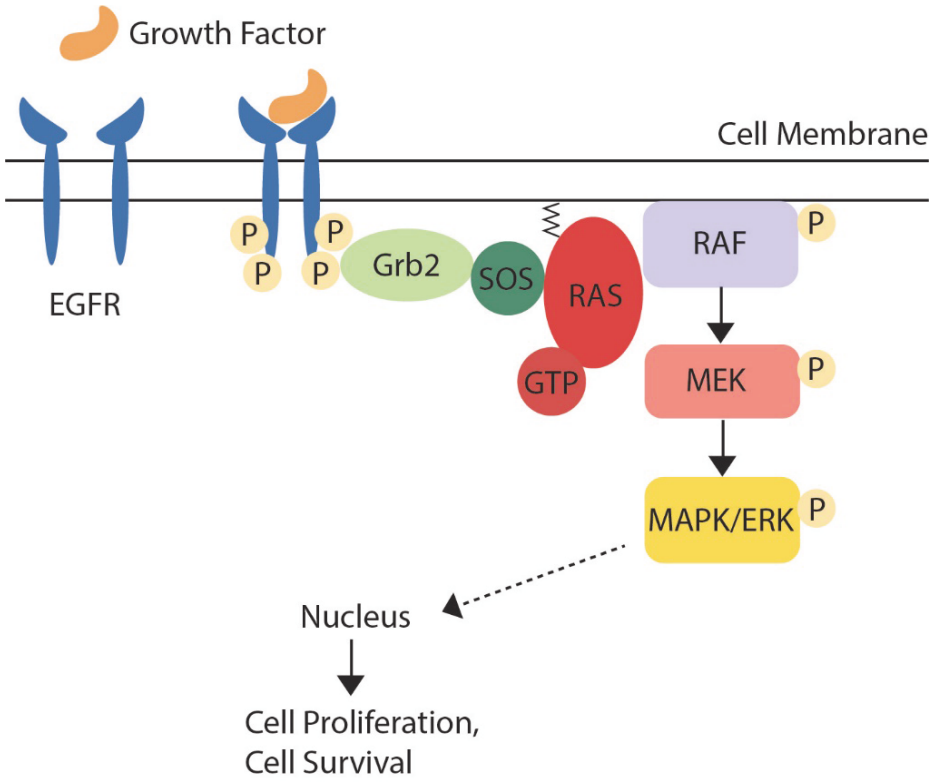


Figure 1: Schematic representation of the RAS-RAF-MEK-ERK MAPK signaling cascade. The activation of the signaling pathway via the binding of a growth factor like EGF to EGFR leads to autophosphorylation. Upon SOS-mediated GDP/GTP exchange, RAS becomes active (GTP-bound state), initiating the RAF-MEK-ERK cascade leading to ERK-mediated gene expression changes in the nucleus.

Mutational alterations leading to oncogenic activation of RAS proteins, KRAS4A, KRAS4B, NRAS, and HRAS, are found in a significant percentage of human cancers, with KRAS being the most commonly mutated form (85%), followed by NRAS (11%) and HRAS (4%) (Hobbs, Der et al. 2016). Conjointly, they are considered one of the most common oncogenes detected in cancers, where KRAS is the frequently mutated oncogenic driver in colorectal, pancreatic and non-small cell lung cancers (Azmi and Philip 2017, Hofmann, Gmachl et al. 2021). Normally, there is a balanced cycle of RAS in the active and inactive state (Azmi and Philip 2017). Indeed, when RAS family GTPases contain activating mutations, the equilibrium shifts towards the active, GTP-bound state, and GAP-assisted GTP hydrolysis is suppressed, promoting cancer growth and developmental defects (Ladygina, Martin et al.

2011, Azmi and Philip 2017). Thereby, aberrant hyperactivation of RAS signaling prompts a persistent activation of the RAS-RAF-MEK-ERK signaling pathway which directly impacts cell division and growth (Roberts and Der 2007, Azmi and Philip 2017). Furthermore, ERK-induced transcriptional changes can create an autocrine growth feedback loop where hyperactive RAS and the overexpression of EGFR ligands further promote tumor growth (Roberts and Der 2007). Collectively, this continuous cell division and proliferation leads to malignant cell transformation and tumorigenesis (Weinmann and Ottow 2007).

Even though RAS proteins are ubiquitously expressed, mutant codons and frequency of mutations vary by cell type, though RAS mutations are predominantly located at codon 12, 13 and 61 (Mukhopadhyay, Vander Heiden et al. 2021). These codons are collectively referred to as mutational “hot spots” (Mukhopadhyay, Vander Heiden et al. 2021). RAS proteins display a distinctive isoform codon mutation signature with 80% of KRAS mutations occurring at codon 12, and rarely at 61. Conversely, almost 60% of NRAS mutations are observed at codon 61 versus 35% at codon 12 (Prior, Lewis et al. 2012). HRAS mutations are split up with 50% occurring at codon 12 and 40% at codon 61 (Prior, Lewis et al. 2012). Similarly, although mutated RAS isoforms vary by cancer type, again, there seems to be a trend when looking at large-scale tumor profiling. For instance, 90% of pancreatic tumors harbor a KRAS mutation while NRAS mutations are largely observed in hematopoietic malignancies or melanoma (Prior, Lewis et al. 2012). Notably, the *KRAS* gene locus encodes two splice variants, giving rise to two KRAS isoforms, namely KRAS4A and KRAS4B. These two isoforms differ in their hypervariable region and the expression of the KRAS4B isoform in cancer has shown to be higher compared to KRAS4A (Nussinov, Tsai et al. 2016).

Isoforms favoring specific codon mutations and certain isoforms linked to specific cancer types are important when understanding structural and functional effects of those point mutations. KRAS mutations on G12, G13 and Q61 clearly impair the intrinsic hydrolysis of GTP in the Switch I/II pocket that is catalyzed by GAPs (Prior, Lewis et al. 2012). When GAPs hydrolyze GTP to GDP there is a cloud of negative charges that form within the KRAS active site, that is then neutralized by a the positively charged GAP arginine finger, forming the transition state. The resulting structural changes of G12 mutants, and likely G13, mutants fail to establish this transition state complex with GAP. This is attributable to side chains on these residues, that sterically disturb the interaction with the arginine finger and thus, reducing the hydrolysis rate (Scheffzek, Ahmadian et al. 1997). Mutations on codon 61, in contrast, stabilize the transition state established during GTP hydrolysis, thereby favoring the active state. In both instances, the equilibrium is shifted, leaving RAS in its active state and leading to the upregulation of the RAS signaling pathway (Azmi and Philip 2017).

Structurally, the first 165 amino acids of the RAS protein make up the G-domain, that is identical in all family members, and functions as the binding site for GEFs and GAPs (McCormick 2019). Similarly, the Switch I and Switch II regions and P-loop, which undergo confirmational change upon GTP and GDP binding and are also highly conserved (McCormick 2019). Noteworthy, isoform-specific codon mutations at codon 12 and 61 share identical amino acid sequences in all four main RAS GTPases (Prior, Lewis et al. 2012). The final 23-24 amino acids at the C-terminal region of RAS form the hypervariable region (HVR) and differs greatly between the family members, where less than 10-15% of the amino acid sequence is identical between the individual RAS GTPases (Jaumot, Yan et al. 2002).

All four RAS proteins contain a C-terminal CAAX motif (C meaning cysteine, A aliphatic amino acids and X is serine, leucine, glutamine or alanine), that is recognized by farnesyltransferase (FTase) and post-translationally farnesylated (Figure 2) (Ahearn, Zhou et al. 2018). Moreover, RCE1 (Ras-converting enzyme 1), ICMT (isoprenylcysteine carboxyl methyltransferase), and palmitoylation are vital post-translational modifications that regulate RAS protein localization, activation, and signaling. RCE1 cleaves the C-terminal CAAX motif of RAS, exposing a prenylated cysteine essential for membrane anchoring (Takahashi, Nakagawa et al. 2005). ICMT methylates this cysteine, enhancing RAS's affinity for membranes and ensuring proper localization in specific microdomains for effective signaling. Palmitoylation further modulates RAS membrane association and trafficking between cellular compartments, enabling spatial and temporal control of signaling. Together, these modifications ensure RAS interacts with its regulators and effectors precisely, maintaining signaling fidelity (Takahashi, Nakagawa et al. 2005).

As an alternative prenylation modification, both NRAS and KRAS can also be geranylgeranylated by geranylgeranyltransferase (GGTase) (Chenette and Der 2011). Essentially, prenylation modifications provides a hydrophobic moiety, required for anchoring RAS in the phospholipid bilayer of cellular membranes (Haluska, Dy et al. 2002). Interestingly, the divergent HVR sequences have also been shown to significantly impact trafficking and localization of RAS to different cellular compartments (Prior and Hancock 2012). Moreover, biophysical studies have shown the flexibility of the HVR, that alters between a proximal and distal confirmation, enables residues K180 to C185 to anchor in the plasma membrane (Van, López et al. 2020). Overall, the prenylation modification and the dynamic HVR is essential for RAS function, allowing for the intracellular trafficking, subcellular localization of RAS proteins to the cell membrane and engagement with effector and regulatory proteins such as RAF, to activate signaling clusters (Van, López et al. 2020).

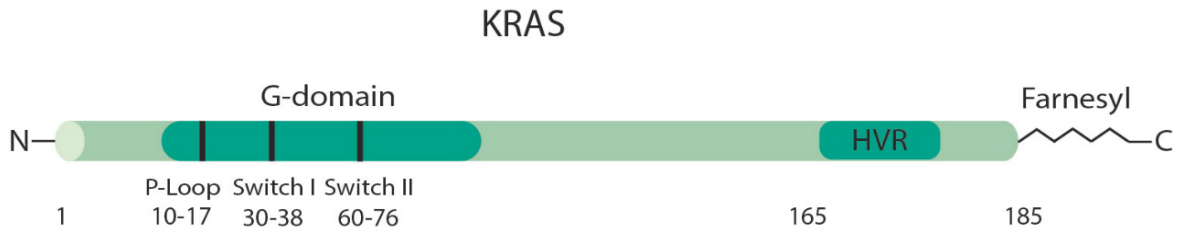


Figure 2: Illustration of the KRAS protein structure. Domains and their boundaries are depicted, including the G-domain with the P-loop, Switch I and Switch II regions, HVR and C-terminal farnesylation modification. Domain sizes are not drawn to scale.

1.1.1 Therapeutics Targeting RAS

Given its mutational prevalence, RAS is a major clinical cancer target that has been deemed undruggable for a long time, as it lacks any suitably accessible active site and possesses few well-defined hydrophobic pockets, that could be targeted by conventional small molecule inhibitors. Various chemical intervention efforts and drug campaigns have been launched to successfully target KRAS, its posttranslational modifications as well as up and downstream signaling flux, with only minor success until recently. Selected examples illustrating the broad spectrum of efforts will be discussed in more detail in the following paragraphs.

Farnesyl transferase inhibitors have been extensively investigated as a potential therapeutic approach, to interfere with the posttranslational processing of RAS proteins, essential for proper signaling activity, but have only shown modest success (Gilardi, Wang et al. 2020). Bearing in mind that farnesylation on the CAAX motif of RAS is a vital post-translational modification, for both the proliferative activity and proper subcellular localization of RAS, blocking FTases by small molecules appeared to be an attractive therapeutic intervention point. In the beginning, tetrapeptides mimicking the CAAX motif were developed to compete with the substrate. However, these struggled to be taken up by the cell or were intracellularly degraded (Basso, Kirschmeier et al. 2006). Subsequently, more stable peptides were tested, that replaced the aliphatic amino acids of the CAAX sequence with either benzodiazepine or aminomethylbenzoic acid (Basso, Kirschmeier et al. 2006). Ultimately, small molecules were identified through high-throughput screening (HTS), namely lonafarnib and tipifarnib, and advanced into clinical trials (Basso, Kirschmeier et al. 2006). Notably, clinical trials have been conducted in HRAS-mutant head and neck squamous cell carcinomas (HNSCC) utilizing tipifarnib (Moore, Rosenberg et al. 2020). Nevertheless, a multitude of proteins bear a CAAX motif sequence, that can be recognized by FTases for prenylation

(Haluska, Dy et al. 2002). Consequently, it calls into question whether FTase inhibitors provide the necessary specificity and balance between cancer cell-specific antiproliferative effects and bystander cell toxicity (Haluska, Dy et al. 2002). Hence, the mechanism of FTase inhibitors and their cytotoxic effect could be attributable to off-target mechanisms (Haluska, Dy et al. 2002). More importantly, following FTase inhibition, KRAS4A, KRAS4B and NRAS proteins can undergo alternative prenylation via GGTases, thus, maintaining their ability to localize to the cell membrane leading to FTase inhibitor treatment resistance (Basso, Kirschmeier et al. 2006).

On the premise that SOS is a crucial RAS activator, small molecules have been identified by means of NMR-based fragment screening, yielding promising candidates. In particular, low-affinity compounds that bind to the SOS-binding site of RAS, the Switch I/II pocket, are able to block the GDP/ GTP exchange reaction (McCormick 2019). As a result, these molecules disrupt the interaction between SOS and RAS, reducing the levels of active GTP-bound RAS (Hillig, Sautier et al. 2019). For example, SCH54292 was one of the early identified RAS binders, blocking nucleotide exchange, but has not been developed further (Taveras, Remiszewski et al. 1997). An additional example of an NMR-based fragment would be DCAI, a small molecule that can inhibit SOS-mediated exchange activity (Maurer, Garrenton et al. 2012). More recently, the development of BI-3406 created a significantly more potent and selective pan-RAS inhibitor, also binding to the SOS catalytic domain and preventing RAS activation (Hofmann, Gmachl et al. 2021). However, some studies of SOS inhibitors indicated complete inhibition of the MAPK signaling cascade in cells expressing wild type (WT) KRAS, yet only 50% reduction in phospho-ERK in mutant KRAS cells (Hillig, Sautier et al. 2019). Consequently, these molecules don’t solely target RAS mutants, but also prevent the normal function of RAS, potentially resulting in greater toxicities within healthy tissues (McCormick 2019).

Ultimately, targeting distinct mutant alleles of RAS can lead to higher specificity and avoids targeting all RAS isoforms, thereby, reducing drug-related toxicity (Moore, Rosenberg et al. 2020). The development of covalent RAS inhibitors, with the ability to target allele specific mutants have yielded for the first time promising clinical results, igniting a new wave of RAS-focused drug discovery campaigns. The development of Sotorasib (AMG-510) and Adagrasib (MRTX849), cysteine-reactive covalent ligands, that bind to the KRAS G12C mutant allele, has shown first encouraging results in clinical trials of KRAS-mutant cancers (Skoulidis, Li et al. 2021). The G12C mutation, frequently observed in lung adenocarcinoma (34%), is a transversion believed to be specifically caused by carcinogens found in tabaco smoke products (McCormick 2019). Given the high nucleophilicity and reactivity of cysteine, G12C makes a very appealing target. Irreversible binding of the covalent warhead disrupts the

Switch I/ Switch II active site and prevents GDP/GTP nucleotide exchange, hampering KRAS to interact with RAF (Ostrem, Peters et al. 2013). Considering that the active site of KRAS WT lacks additional cysteines, covalent KRAS G12C inhibitors allow for specific inhibition of codon 12 via covalent warheads (Ostrem, Peters et al. 2013, Moore, Rosenberg et al. 2020). However, these inhibitors only bind to KRAS G12C in the inactive-GDP bound state, preventing nucleotide exchange, yet fail to target already active GTP-bound KRAS G12C (Ostrem, Peters et al. 2013, Moore, Rosenberg et al. 2020). In this regard, early clinical trial data has shown that even direct targeting of the KRAS G12C mutant only provides an intermittent control of tumor growth, with patients likely requiring additional combinatorial treatments for sufficient long term disease control (Skoulidis, Li et al. 2021).

Currently, there are just a small number of covalent warheads that can target cysteines, most of them being acrylamides (Spradlin, Zhang et al. 2021). Emerging research focuses on expanding the chemical scope of electrophiles and nucleophiles employed in covalent protein modulation, addressing many challenges when it comes to targeting less reactive amino acids such as aspartic acid found in the KRAS G12D mutation (McGregor, Jenkins et al. 2017). Commonly found in pancreatic ductal adenocarcinoma (PDAC) and in nearly 30% of human cancers, a novel attempt to target KRAS G12D has been launched and produced encouraging pre-clinical data (Mao, Xiao et al. 2022, Wang, Allen et al. 2022). Compound MRTX1133, a KRAS G12D reversible inhibitor for treating PDAC, has been identified with the ability to bind the active and inactive state of KRAS, to suppress KRAS G12D signaling and possesses a thousand-fold higher affinity for KRAS G12D compared to KRAS WT (Wang, Allen et al. 2022). Through a hydrogen bonding and ion pair interaction in the Switch I/II pocket, the inhibitor prevents SOS-mediated nucleotide exchange and/or blocks the PPI interaction with RAF (Wang, Allen et al. 2022). Moreover, compounds like MRTX133 could potentially pave the way to develop a covalent ligand that specifically targets the aspartic acid on KRAS G12D (Tang and Kang 2023). Nevertheless, targeting noncatalytic amino acids remains a significant bottleneck and additional efforts are needed to identify electrophiles with the ability to label a wider range of nucleophiles on KRAS (McGregor, Jenkins et al. 2017).

Small interfering RNAs (siRNAs) targeting RAS have been developed in the hope of achieving mutant specific therapies. These antisense oligonucleotides targeting RAS isoform-specific mRNA are delivered to cells via nanoparticles and could downregulate RAS-driven proliferative pathways by depleting RAS mRNA (Ross, Revenko et al. 2017). The first siRNA, AZD4785, showed inhibition of KRAS expression in preclinical xenograft models, but was not effective in reducing KRAS protein levels in subsequent clinical trials (Ross, Revenko et al. 2017, Moore, Rosenberg et al. 2020). Nevertheless, a subsequent candidate, siG12D-LODER, in combination with chemotherapy did achieve minor success in clinical phase II trials

for treating PDAC (Golan, Khvalevsky et al. 2015). Ultimately, siRNA RAS-targeting therapies require more efficient cellular uptake and should be refined to make them into a more effective treatment. An overview of RAS-targeted therapies and other drugs are summarized in Table 1 below.

Table 1: List of RAS-targeting therapies and clinically developed inhibitors. *Table adapted from Moore, Rosenberg, et al. 2020. Additional data compiled from ClinicalTrials.gov.*

RAS-targeted Therapies	Drugs/ Compounds
Inhibitors of RAS processing	• Tipifarnib • Cysmethynil • UCM-1336 • Deltarasin • NHTD
SOS inhibitors	• BI-1701963 • BAY-293 • BI-3406
Allele-specific RAS inhibitors	• AMG510 • MRTX849 • LY3499446 • ARS-3248 • ARS-1620 • ARS-853 • RM-007 • RM-008 • MRTX1133
RAS antisense oligonucleotides	• AZD4785 • siG12D LODER

1.2 Protein Degradation

Proteins undergo degradation through a mechanism known as proteolysis, wherein proteins are broken down into small peptides and ultimately into individual amino acids. Protein degradation is essential for maintaining cellular homeostasis and regulating protein quality by removing damaged, misfolded, or excess proteins. It plays a critical role in cellular processes such as signal transduction, stress responses and cell cycle progression. Additionally, the controlled degradation of proteins ensures proper turnover, enabling adaptation to changing environmental and metabolic conditions while preventing the

accumulation of toxic aggregates that could disrupt cellular function (Flick and Kaiser 2012). Two primary cellular pathways govern protein degradation: the ubiquitin-proteasome system (UPS) and autophagy-lysosome system (Nandi, Tahiliani et al. 2006). In the context of this dissertation, my emphasis will be on the UPS, which will be outlined in this section.

The ATP-dependent 26S proteasome is a large complex responsible for protein degradation in eukaryotic cells, in both the cytosol and the nucleus (Nandi, Tahiliani et al. 2006). It consists of a barrel shaped 20S subunit core and two 19S regulatory subunits at both ends. The cellular roles of the 26S proteasome encompass maintaining overall protein balance, stress response, and regulating cell division and signal transduction (Bard, Goodall et al. 2018). The proteasome recognizes the covalently bound ubiquitin modification that occurs through an isopeptide linkage between the C-terminal glycine of ubiquitin and the ε-amino lysine of the target protein (Nandi, Tahiliani et al. 2006). Cellular proteins targeted for degradation undergo linked poly-ubiquitination, where a polymer of ubiquitin conjugated onto an protein serves as the substrate of the 26S proteasome, which in turn facilitates processive proteolysis of the target protein (Pickart 2001).

Ubiquitination conjugation onto a target protein occurs through three enzymatic steps. First an activating enzyme (E1) catalyzes the formation of a thiol ester with the carboxyl group and activates the C-terminus of ubiquitin for nucleophilic attack. E1 enzymes carry two ubiquitin molecules where the thiol ester formation of one ubiquitin involves E1-mediated ATP-AMP exchange (Pickart 2001). Subsequently, a conjugating enzyme (E2), that holds the activated ubiquitin molecule in form of the thiol ester, is then carried from the E2 by a ligase (E3) to covalently ligate the C terminus of ubiquitin to a lysine residue on the substrate or facilitates ubiquitin-ubiquitin ligation (Pickart 2001) (Figure 3). Polyubiquitination catalysis requires K48 isopeptide bonds between sequential ubiquitin molecules for substrates that will be targeted for proteasomal degradation (Pickart 2001). This crucial step involves three key components: a substrate protein, an E3 ubiquitin ligase and an E2 ubiquitin-conjugating enzyme. This is also known as ternary complex formation and facilitates the transfer of ubiquitin molecules onto the substrate (Wurz, Rui et al. 2023). In the human genome, there are two members of the E1 enzymes, about 40 E2 enzymes, and over 600 E3 ubiquitin ligases (Kleiger and Mayor 2014) (Figure 3). E3 ligases have garnered the greatest focus in the field of UPS, since they are crucial for substrate recognition. Ubiquitination is a reversible process and modifications on substrate proteins can be removed by deubiquitinating enzymes (Lu, Wang et al. 2021).

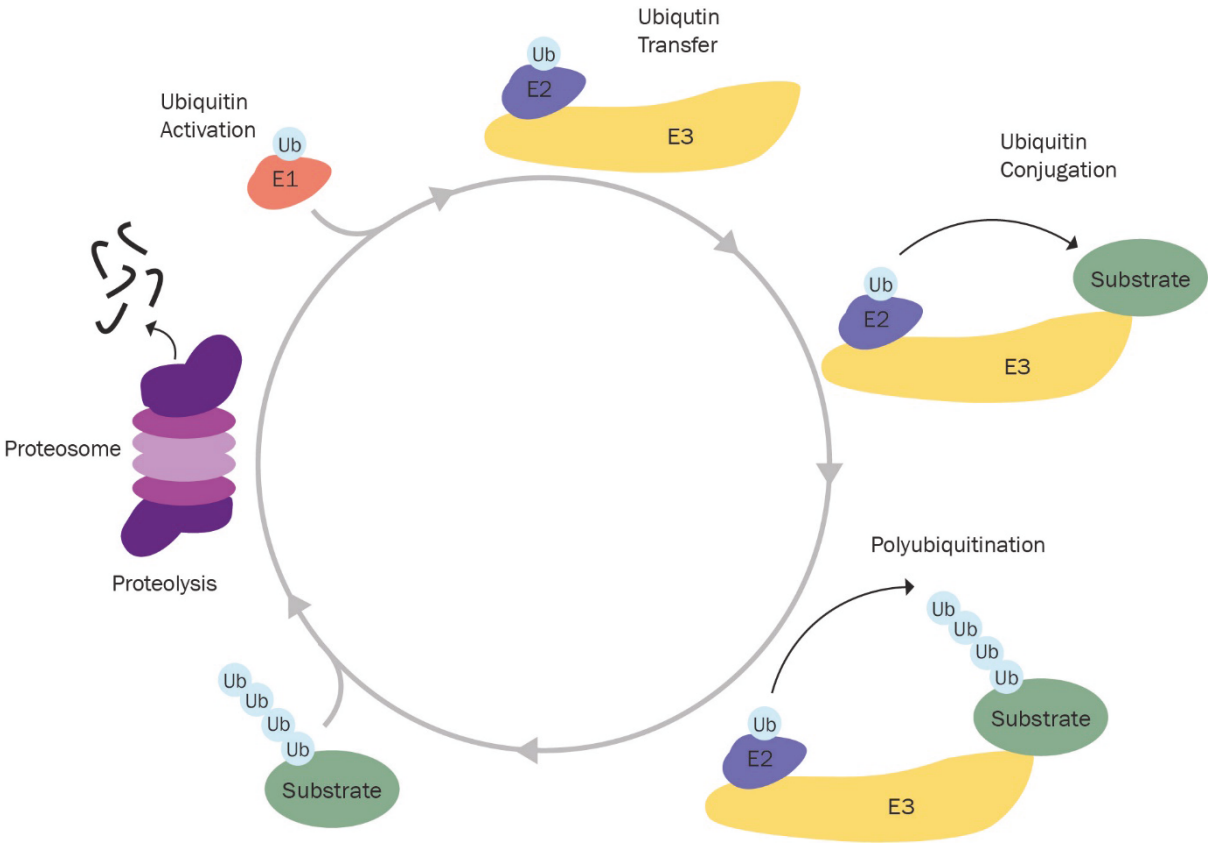


Figure 3: Cullin-based Ubiquitin-Proteasome System. Ubiquitination of protein substrates is carried out by the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Zhao, Zhao et al. 2022). Next, polyubiquitinated substrates are recognized by the proteasome and subsequently undergo proteolysis. *Adapted from Zhao et al. 2022.*

E3 enzymes play a pivotal role in determining the specificity of ubiquitination by recognizing so called ubiquitination signals which is a primary sequence or structural motifs in the substrate (Pickart 2001). E3 ligases are categorized into four main families: HECT, RING-finger, U-box, and PHD-finger (Nakayama and Nakayama 2006). Among these, two prominent families are the homologous to E6-AP C terminus (HECT) E3 ligases and the RING-between-RING (RBR) E3 ligases (Cowan and Ciulli 2022). HECT E3 ligases form the thioester intermediate with ubiquitin that are then transferred from the E2 to the E3 for subsequent ubiquitin ligation to the substrate (Cowan and Ciulli 2022). Cullin-RING ligases (CRL), the largest family of RING E3 ligases, carry the ubiquitin-E2 enzymes and the substrate into proximity for ubiquitin conjugation directly from the E2 onto the substrate. The Really Interesting New Gene (RING) finger domain coordinates two Zn^{2+} ions that form a recognition

platform for the binding of E2 enzymes, suggesting that the RING domain serves as a molecular scaffold that brings other proteins into proximity (Metzger, Pruneda et al. 2014). Domains of the RING finger then recognize the ubiquitination modification on the substrate.

1.2.1 Cullin-RING Ligases

In the interest of the dissertation, I will concentrate on CRL ligases, as they also represent the most abundant subfamilies of RING E3 ligases, compromising over 300 complexes and are responsible for mediating the degradation of approximately 20% of all cellular proteins (Lu, Wang et al. 2021). Due to the extensive diversity of CRL substrate-receptor subunits, multiple substrate receptors can be recruited to the CRL core (Petroski and Deshaies 2005). Specifically, CRLs can combine with numerous substrate receptors via the N-terminal domain, creating ubiquitin ligases with a conserved catalytic core, but diversifying the range of substrates that they can recognize and thus ubiquitinate (Petroski and Deshaies 2005). There are four intermediate adaptor proteins associated with CRLs: SKP1 corresponds to CUL1 and CUL7, ELONGIN B/C to CUL2 and CUL5, and DDB1 to CUL4A and CUL4B, while CUL3 lacking an adaptor protein. Furthermore, distinct substrate receptor families assemble to different CRLs: F-box proteins to CUL1, VHL-box proteins to CUL2, BTB-Kelch proteins to CUL3, DCAF/WD40 repeat-containing proteins to CUL4A/B, SOCS-box proteins to CUL5, and FBXW8 to CUL7 (Lu, Wang et al. 2021) (Table 2).

Table 2: Overview of Cullin-RING ligase family members. *Table reproduced from Lu et al. 2021. Additional data compiled from Petroski, Deshaies et al. 2005.*

Cullin	Adaptor	Substrate Receptor
CUL1	SKP1	F-box protein
CUL2	Elongin B/C	VHL-box protein
CUL3	-	BTB protein
CUL4A/B	DDB1	DCAF
CUL5	Elongin B/C	SOCS
CUL7	SKP1/CUL7	FBXW8
CUL9	Undefined	Undefined

Small RING proteins, RBX1 (RING box 1) or in the case of CUL5 RBX2, assemble with the Cullin scaffold via its C-terminal domain and interact with ubiquitin-carrying enzymes that transfer ubiquitin onto substrate receptor bound proteins (Henneberg, Singh et al. 2023). Moreover, a crucial activation step of CRLs is mediated via neddylation, attachment of a ubiquitin-like NEDD8 modification (neural precursor cell expressed developmentally down regulated 8) of the C-terminal Cullin WHB subdomain, through NAE1 (NEDD8-activating enzyme E1 subunit 1) and UBA3, which in turn allosterically activates ubiquitin-carrying enzymes (Schmaler and Dubiel 2010, Henneberg, Singh et al. 2023). Recently, MLN4924 was identified as a potent molecule that selectively inhibits NAE1, preventing CRL activation and thereby disrupting CRL-mediated substrate degradation (Soucy, Smith et al. 2009). Eventually, after proteasomal degradation of a substrate, CRLs are then deneddylated by the COP9 signalosome, a metalloprotease complex, and disassemble from their substrate receptor (Schmaler and Dubiel 2010, Henneberg, Singh et al. 2023). CRLs activation and deactivation, through neddylation and deneddylation, are tightly regulated, determining when CRLs are in ON or OFF state (Henneberg, Singh et al. 2023) (Figure 4).

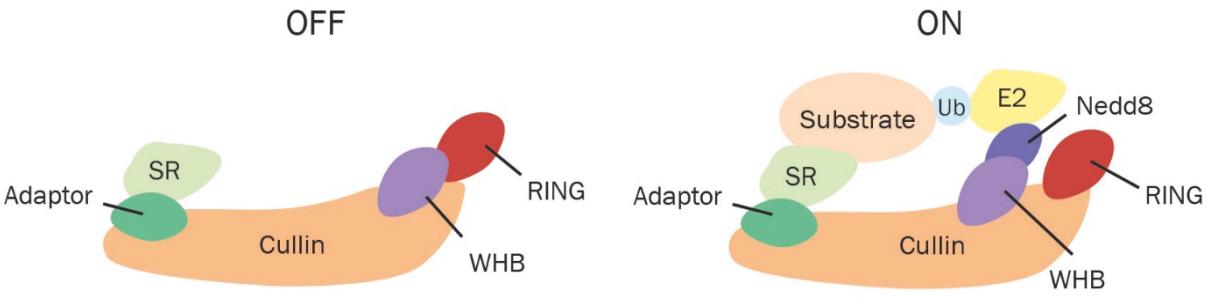


Figure 4: Activation and assembly of CRLs. Neddylation of WHB domain pushes CRLs into the active conformation (ON state). Neddylated CRL recruits and activates the E2 enzyme, facilitating ubiquitin transfer on a substrate that is recruited to the CRL via a substrate receptor (SR).

1.3 LZTR1

The Leucine Zipper Like Transcription Regulator 1 (*LZTR1*) gene, is located on human chromosome 22q11.21 and encodes the LZTR1 protein (Evans, Messiaen et al. 2021). Genetic studies have identified LZTR1 mutations in different diseases ranging from the

RASopathy developmental disorder, Noonan syndrome (Yamamoto, Aguena et al. 2015), to various cancer entities (Tidyman and Rauen 2009). While the molecular mechanism of LZTR1 has remained enigmatic for a long time, it has now become clear that the LZTR1 protein is involved in various cellular processes predominantly through its role in the regulation of the RAS-MAPK signaling pathway (Bigenzahn, Collu et al. 2018) (Steklov, Pandolfi et al. 2018) (Castel, Cheng et al. 2019).

Specifically, LZTR1 has been identified as a substrate recognition element of Cullin 3 RING E3 ubiquitin ligase (CUL3) complex, mediating the proteostatic regulation and hence degradation of the main RAS GTPase proteins KRAS, NRAS, and HRAS, as well as the closely related members MRAS and RIT1 (Ras Like Without CAAX 1) (Bigenzahn, Collu et al. 2018) (Bigenzahn, Collu et al. 2018, Castel, Cheng et al. 2019). Pathological mutations in *LZTR1*, associated with different human diseases, inhibit the formation of the $CUL3^{LZTR1}$ complex and/or the interact with RAS proteins itself (Bigenzahn, Collu et al. 2018, Steklov, Pandolfi et al. 2018). Hence, LZTR1 mutations associated with disease appear to hinder RAS ubiquitination by preventing assembly of the RAS-LZTR1-CUL3 complex (Steklov, Pandolfi et al. 2018). Consequently, a loss of LZTR1 function leads to an increase in RAS abundance and subsequently activation, leading to enhanced MAPK pathway activation. Considering that LZTR1-mediated ubiquitination of KRAS attenuates the activation and downstream signaling of the MAPK pathway, LZTR1 acts a negative regulator of the RAS-MAPK signaling pathway by moderating RAS levels (Figure 5).

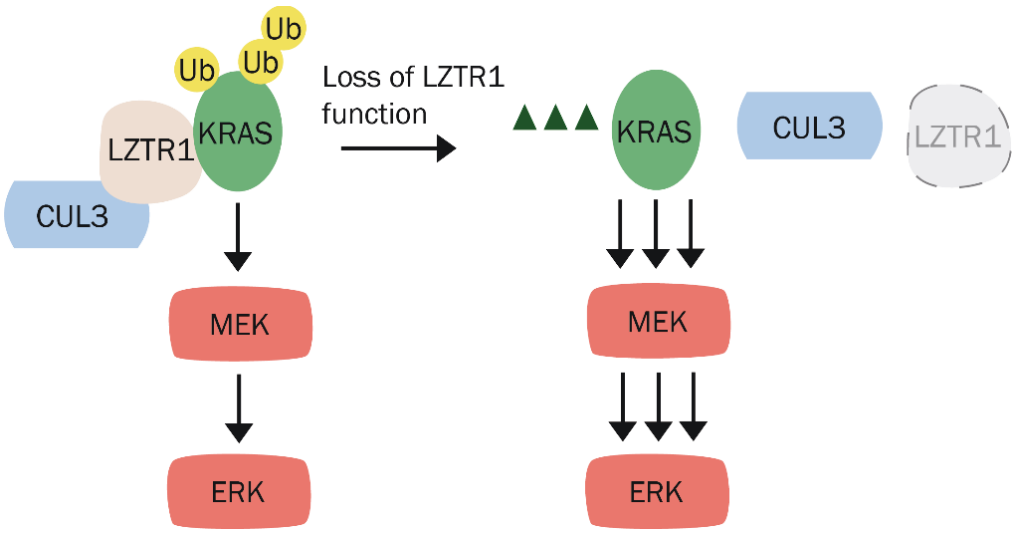


Figure 5: Schematic representation of the KRAS-$CUL3^{LZTR1}$ complex. $CUL3^{LZTR1}$ mediates (poly-)ubiquitination and degradation of KRAS. The absence of LZTR1 leads to enhanced

KRAS protein abundance and subsequent upregulation of MAPK pathway activity. *Adapted from Bigenzahn et al.* (Bigenzahn, Collu et al. 2018).

Structurally, LZTR1 is part of the BTB/BACK domain-containing protein family, harboring an N-terminal Kelch domain, followed by two BTB/BACK domains (Nacak, Leptien et al. 2006) (Frattini, Trifonov et al. 2013). The Kelch domain consists of a beta-propeller structure and acts as the substrate binding element, whereas, the BTB/BACK domains act as interaction site with the CUL3 ligase complex and appear to contribute to the assembly of the LZTR1-CUL3 complex. To date there is no experimentally solved structure of LZTR1 in a soluble form. Nevertheless, structural insights have been obtained through alternative means, such as sequence-based homology modeling and domain predictions, suggesting that the BTB proteins are capable of forming homo or heterodimers with themselves or other BTB proteins (Motta, Fidan et al. 2019). Predictably, LZTR1 forms a homodimer, interacting with RAS proteins via its Kelch domain (Figure 6) (Motta, Fidan et al. 2019).

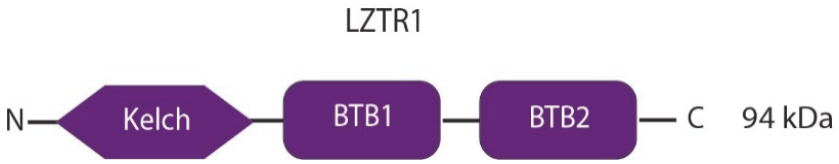


Figure 6: Diagram illustrating the LZTR1 domain organization. Schematic depiction of the LZTR1 domain organization, displaying the N-terminal Kelch domain followed by two BTB/BACK domains (Abe, Umeki et al. 2020). Protein domains not drawn to scale.

In the context of glioblastoma, a very highly aggressive type of brain cancer, *LZTR1* has been identified as a potential tumor suppressor gene, where loss of function mutations appear to contribute to disease development and progression (Frattini, Trifonov et al. 2013, Piotrowski, Xie et al. 2014). In contrast, schwannomatosis is a rare genetic disorder characterized by the development of numerous noncancerous tumors, called “schwannomas”, affecting the corresponding cells that surround peripheral nerves. In terms of schwannomatosis, monoallelic *LZTR1* mutations have been identified in a subset of patients, where mutations after acquisition of a secondary genetic alteration are associated with enhanced schwannoma growth (Piotrowski, Xie et al. 2014).

Moreover, *LZTR1* mutations have been associated with Noonan syndrome and predispose to distinct pediatric neoplasms (Yamamoto, Aguena et al. 2015). Noonan

syndrome is a genetic disorder within the larger group of RASopathies. Patients are characterized by facial dysmorphism, congenital heart abnormalities, developmental delays, predisposition to certain cancers and reduced postnatal growth (Motta, Fidan et al. 2019). Germline mutations occur in *PTPN11*, *SOS1*, *RAF1*, *RIT1* and *KRAS* (Ko, Kim et al. 2008) thereby leading to aberrant regulation of the RAS-MAPK pathway (Motta, Fidan et al. 2019). Noonan syndrome-associated *LZTR1* mutations cause enhanced RAS-MAPK signaling, attributable to a higher abundance of RAS proteins (Bigenzahn, Collu et al. 2018, Steklov, Pandolfi et al. 2018, Castel, Cheng et al. 2019). Specifically, mutations in LZTR1 affect the Kelch domain binding to RAS and thereby hinder ubiquitination and degradation of RAS. This, in turn, leads to the upregulation of MAPK signaling (Motta, Fidan et al. 2019). Taken together, these LZTR1 mutations underscore its functional connection to the RAS-MAPK pathway, where it plays a negative regulatory role in controlling RAS protein levels.

Pertaining to cancer therapy, enhanced MAPK pathway activation can prompt reduced sensitivity to tyrosine kinase inhibitors (TKIs), such as imatinib, a BCR-ABL-targeting tyrosine kinase inhibitor (TKI) for the treatment of chronic myeloid leukemia (CML) (Bigenzahn, Collu et al. 2018), or quizartinib, a FLT3-targeting TKI, used in the treatment of FLT3 mutant acute myeloid leukemia (AML) (Chen, Vedula et al. 2022). These findings underscored these alterations in *LZTR1*, leading to a dysregulation of RAS degradation and thereby hyperactivation of the MAPK pathway can pose a significant challenge for effective, targeted treatment underscoring the importance of controlling RAS protein levels in cancers driven by different driver oncogenic alterations.

Interestingly, the $CUL3^{LZTR1}$ complex is not only able to proteostatically regulate the main group of RAS GTPase proteins but is also responsible for the abundance regulation of the closely related GTPases MRAS (Muscle RAS Oncogene Homolog) and RIT1 (Ras-like without CAAX 1) (Castel, Cheng et al. 2019). This has been substantiated by Castel and colleagues, showing that mutations in either RIT1 or LZTR1 led to impaired degradation of RIT1, resulting in accumulation of RIT1 protein levels and hyperactivation of MAPK signaling (Castel, Cheng et al. 2019). RIT1 shares a 44% sequence homology with K-, N-, and HRAS (Fang, Marshall et al. 2016) and akin to RAS, RIT1 acts as a molecular switch, facilitating the transmission of signals from the plasma membrane to the nucleus. Furthermore, mutations in RIT1 have been identified in Noonan syndrome patients, while somatic mutations in RIT1 drive the proliferation of lung adenocarcinomas (Fang, Marshall et al. 2016). Crucially, this parallels the role of RAS mutations in these diseases, where genetic mutations in the *RIT1* gene can disrupt the RAS-MAPK pathway, owing to uncomplete protein degradation of RIT1 (Castel, Cheng et al. 2019).

1.4 Targeted Protein Degradation

Currently, the majority of drugs in the research and development are comprised of small organic molecules (Santos, Ursu et al. 2017). These molecules typically target protein classes such as GPCRs, ion channels, kinases, proteases, and nuclear receptors (Santos, Ursu et al. 2017). They usually interfere with the catalytic functionality of these proteins either by inhibiting hydrophobic active sites or large binding pocket space (Santos, Ursu et al. 2017). Considering that proteins do not act as single entities but rather interact with other proteins to form larger and dynamic complexes, targeting protein-protein interactions (PPI) is an emerging field in drug discovery. The protein-protein interaction interfaces of these complexes tend to be flat and usually do not readily contain binding pockets amenable to classical drug development-based approaches. A novel therapeutic strategy, termed targeted protein degradation (TPD), involves the development of chemical molecules that are able to recruit active E3 ligases and by relying on the UPS degrade a specific protein of interest (Zhao, Zhao et al. 2022). There are two main embodiments of TPD drugs, namely proteolysis targeting chimeras (PROTACs) and molecular glue degraders. The majority of TPD drug development strategies like PROTACs and molecular glues depend on the UPS and predominantly target intracellular proteins (Figure 7) (Jaeger and Winter 2020, Zhao, Zhao et al. 2022). Emerging approaches such LYTACs (Lysosome-Targeting Chimeras), DUBTACs (Deubiquitinase-Targeting Chimeras) and AUTACs (Autophagy-Targeting Chimeras) are novel targeted protein degradation or stabilization technologies designed to manipulate cellular protein levels by leveraging specific pathways like lysosomal, deubiquitinase and autophagy degradation activity (Alabi and Crews 2021). PROTACs and molecular glues are the main approaches of proximity inducing compounds and will be explained in the following sections.

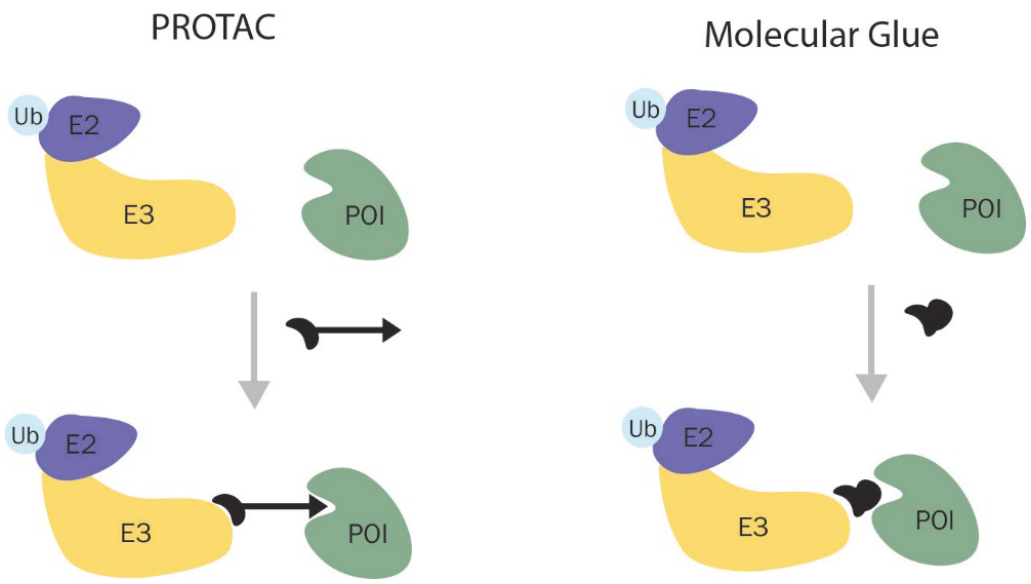


Figure 7: Schematic depiction of PROTACs and molecular glues. PROTACs are heterobifunctional compounds composed of an E3 ligase binding ligand attached to a protein of interest (POI)-binding ligand via a linker. Molecular glues are smaller in size and act as chemical stabilizers by binding to either the E3 ligase or POI.

1.4.1 PROTACs

PROTACs are heterobifunctional molecules compromised of two chemical domains, namely an E3-ligase-spefic ligand, acting as an “anchor”, and a POI-binding ligand, commonly referred to as “warhead”. These two molecules are connected via a chemically functionalized polyethylene glycol (PEG) or alkyl chain linker (Zhao, Zhao et al. 2022). Consequently, the E3-specific ligand binds to the ligase, and thereby, recruits the POI to the E3 ligase, resulting in ternary complex formation (Zhao, Zhao et al. 2022). Subsequently, ubiquitination and degradation of the protein of interest is prompted. Even if the ligase is not the native ligase of the POI, meaning that it wouldn’t endogenously bind and lead to the degradation of the POI, E3s can be chemically “hijacked” through PRTOACs and recruit a wide range of proteins and new substrates. These novel substrates are referred to as neo-substrates (Zhao, Zhao et al. 2022). Thus, PROTACs repurpose existing binders of target proteins by incorporating a degradation mechanism into the mode of action of existing drugs, enhancing potency and selectivity, particularly for challenging targets like non-receptor or non-enzymatic proteins, including transcription factors and oncoproteins (Fuchs 2023). Several E3 ligases have been harnessed for the use in PROTAC technology, including Cereblon (CRBN, CUL4 E3 ligase

complex), cell inhibitor of apoptosis protein (cIAP1), Von-Hippel-Lindau (VHL, CUL2 E3 ligase complex) and murine double minute 2 (MDM2) (Zhao, Zhao et al. 2022, Cieślak and Słowianek 2023). Recently, additional E3 ligases have been identified to be deployable for PROTAC applications, nevertheless, the majority of recently reported and clinically developed PROTAC candidates still rely on either CRBN or VHL (Bricelj, Steinebach et al. 2021) (Figure 8).

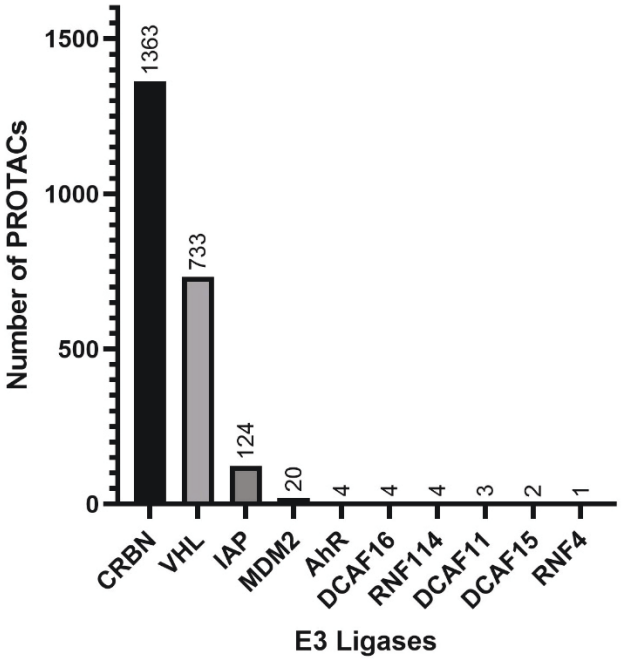


Figure 8: Number of PROTACs utilizing individual E3 ligase proteins or complexes.
Histogram reproduced from Guenette, Yang et al. 2022.

The $CUL4^{CRBN}$ E3 ubiquitin ligase complex consists of the substrate binding receptor CRBN, the Cullin 4 (CUL4A/4B) scaffold as well as the damaged DNA-binding protein 1 (DDB1) adaptor that links CUL4 to CRBN, and regulator of cullins 1 (RBX1) as the catalytic E3 part (Cieślak and Słowianek 2023).

Thalidomide and its analogs, lenalidomide and pomalidomide, are known as immunomodulatory imide drugs (IMiDs) and based on their CRBN binding propensity have led to the development of CRBN-recruiting PROTACs, with the ability to target nuclear receptors, kinases, transcription factors, regulatory proteins, neurodegeneration-related proteins, or metabolic enzymes for degradation (Ito, Ando et al. 2010, Cieślak and Słowianek 2023, Fuchs 2023). Originally, thalidomide gained notoriety due to its teratogenic effects on unborn children during the late 1950s and early 1960s. Initially it was marketed under the trademark name “Contergan” and was given to pregnant women for treating morning sickness and nausea,

given its sedative and anti-emetic effects (Melchert and List 2007, Ito, Ando et al. 2010, Rehman, Arfons et al. 2011). Its devastating teratogenic effects caused thousands of infants to be born with limb malformations, such as phocomelia, where the limbs were severely shortened or absent (Rehman, Arfons et al. 2011). It was therefore withdrawn from the market in 1961 and led to significant changes in pharmaceutical approval and regulation worldwide (Melchert and List 2007, Vargesson 2015). Despite its tragic history, thalidomide and its analogs resurfaced decades later for their anti-tumor activity and are used as drugs for the treatment for hematological malignancies, including myelofibrosis and multiple myeloma (Latif, Chauhan et al. 2012). In the following section on molecular glues, I will provide a detailed explanation of the mechanism of thalidomide, focusing on its molecular interactions and therapeutic applications.

Since the discovery of high-resolution structures and well-characterized ligands of VHL, specifically hydroxyproline-based molecules, VHL PROTACs have gained interest in the field of drug discovery due to their potential applications in treating various diseases, including cancer (Bhela, Ranza et al. 2022, Wang, Zhang et al. 2022). This includes targets like ALK, a tyrosine kinase that participates in several fusion oncoproteins, making it a significant cancer driver gene, and α-synuclein, which regulates synaptic vesicle release and is a powerful target for treating Parkinson's disease (Wang, Zhang et al. 2022). An additional powerful target for VHL-based PROTACs is BRD4, a member of the bromo- and extra-terminal (BET) family of proteins, which play critical roles in transcriptional regulation (Gadd, Testa et al. 2017). BRD4 overexpression in cancer contributes to oncogenesis an also plays a role in inflammatory disease (Wang, Zhang et al. 2022).

Since the initial small-molecule PROTAC was documented in the literature, the TPD technology has transitioned from academia to industry. Due to the typically large size of PROTACs, there was considerable skepticism regarding their ability to be transported across cell membranes, their safety profile, and their capacity to effectively induce therapeutic effects *in vivo*. Several biotechnology and pharmaceutical companies unveiled programs in preclinical and early clinical development, with the entry of two PROTACs into first-in-human trials: ARV-110 (NCT03888612) and ARV-471 (NCT04072952) (Békés, Langley et al. 2022). Both ARV-110 and ARV-471 have successfully entered phase 2 clinical trials with a manageable tolerability profile and clinical efficacy in the field of oncology, for the treatment of prostate and metastatic breast cancer respectively (Békés, Langley et al. 2022). ARV-110 exhibited protein degradation within metastatic castration resistant prostate cancer, showing reductions in prostate-specific antigen levels and preliminary signs of anti-tumor activity (Békés, Langley et al. 2022). ARV-471 is an ER degrader for ER+/HER2− metastatic breast cancer and is being tested as single agent and in combinational therapy with CDK4/CDK6 inhibitors (Békés,

Langley et al. 2022). Although at present, the majority of PROTACs in clinical development are being developed for cancer treatments, the field is diversifying to explore PROTACs for addressing conditions such as autoimmunity, inflammation, and neurodegeneration as well as antiviral defense (Békés, Langley et al. 2022).

1.4.2 Molecular Glues

Molecular glues make up another subgroup of TPD, that facilitates a therapeutic intervention on the pathological state of altered protein pathways. Given that interacting surfaces of PPIs tend to be flat and usually do not contain binding pockets amenable to classical drug discovery-based approaches, molecular glues can exploit and harness these properties, enhancing pre-existing PPIs or inducing neo-PPIs, resulting in desired biological activity and therapeutic effects (Hughes and Ciulli 2017, Kozicka and Thomä 2021). Furthermore, they are compounds that can bind to multiple proteins simultaneously, acting as chemical stabilizers for complex formation by modifying the contact surface, supplementing more extensive PPIs and thus, increasing the affinity for two proteins (Stevers, Sijbesma et al. 2018). This can lead to a range of biochemical effects, such as activating or inhibiting protein function, regulating protein degradation, promoting PPIs in cellular pathways and regulating transcription or protein folding (Zhao, Zhao et al. 2022). Contrasting PROTACs, molecular glue degraders form an interaction between a POI and an E3 ligase by binding to either the E3 ubiquitin ligase or the POI itself, thus changing the interaction propensities of a given protein surface area, thereby facilitating a ternary complex formation. Therefore, molecular glues do not contain two separate warheads, they lack a linker, typically possess a lower molecular weight (below 400 kDa), and usually only have weak or moderate affinity to either protein in isolation (Geiger, Schäfer et al. 2022, Zhao, Zhao et al. 2022). There are instances of both natural products and synthetic compounds in molecular glue discovery. Auxins serve as an example of natural molecular glues by promoting protein-protein interactions between the TIR1 receptor and specific substrates, leading to targeted protein degradation in plants (Tan, Calderon-Villalobos et al. 2007). Furthermore, it is important to highlight that while molecular glue degraders are indeed PPI stabilizers, they may exhibit distinctions from other PPI modulators as they facilitate targeted protein degradation.

Initial examples of molecular glue action were natural product discovered in the nineties, which include cyclosporin A, rapamycin and FK506 (Geiger, Schäfer et al. 2022). Cyclosporin A’s mode of action is to allosterically inhibit the phosphatase calcineurin, a protein critical for T-cell activation, by forming a complex with cyclophilin (Gaali, Gopalakrishnan et al. 2011).

Consequently, by blocking calcineurin, cyclosporin inhibits transcription of genes involved in the immune response, including IL-2 and IL-4, thereby, facilitating immunosuppression (Gaali, Gopalakrishnan et al. 2011). Rapamycin's effect is not attributable to calcineurin inhibition, but through the induction of ternary complex formation of FKBPs, or FK506 binding proteins, and mTOR, a protein involved in cell growth and proliferation. Rapamycin binds to FKBP12 with very high affinity and leads to the formation of this FKBP12-Rapamycin-mTOR ternary complex, which in turn blocks mTORC1 to interact with its substrate S6K (Hausch, Kozany et al. 2013). Each of these compounds are widely used for organ transplantation and in treatment of various autoimmune diseases, such as rheumatoid arthritis (Fanigliulo, Lazzerini et al. 2015).

Another significant illustration of the molecular glues reintroduces the subject of thalidomide. Thalidomide and other IMiDs have been utilized in the treatment of hematologic malignancies, such as multiple myeloma, myelodysplastic syndromes, non-Hodgkin lymphoma and acute myeloid leukemia (AML) (Chamberlain and Hamann 2019). IMiDs, including thalidomide, lenalidomide, and pomalidomide, are a class of small molecules that act as molecular glue degraders, repurposing the E3 ubiquitin ligase CRBN to degrade specific protein substrates (Chamberlain and Hamann 2019). Originally developed without a clear understanding of their mechanism, it was later discovered that these drugs bind to CRBN and promote the recruitment and degradation of transcription factors such as Ikaros (IKZF1) and Aiolos (IKZF3), which are critical targets in the treatment of multiple myeloma and other diseases (Lu, Middleton et al. 2014, Chamberlain and Hamann 2019). In 2010 chemical proteomic approaches identified CRBN and DDB1 as the primary binding partners of thalidomide (Ito, Ando et al. 2010). The initial rational of thalidomide acting as a CRBN inhibitor didn't fully explain the mode of action of thalidomide or its analogs. Studies later illustrated a molecular-glue mechanism, revealing that the binding of IMiDs to CRBN prompted subsequent degradation of neo-substrates (Lu, Middleton et al. 2014). The identified neo-substrates include as mentioned above IKZF1 and IKZF3, as well as the zinc finger transcription factor ZFP91, GSPT1 and casein kinase 1 alpha (CSNK1A1) (An, Ponthier et al. 2017, Geiger, Schäfer et al. 2022). They do not share a so called "degron motif", or a primary sequence of amino acids within a protein that allows for an E3 ligase to recognize it, however they do share a glycine in a hairpin motif and hydrogen bonding of three backbone carbonyls, both crucial structural motifs for binding (An, Ponthier et al. 2017).

Large efforts have been launched to develop molecular glues that induce neo-interactions of otherwise non-interacting proteins. Nevertheless, another field of molecular glue development focuses on stabilizing existing native ligase-substrate pairs, where the innate ligase and substrate interface can be potentiated. Previous attempts to enhance the

degradation of a substrate with its E3 ligase through chemical means have been made, notably with the discovery of the small molecule NRX-252114 (Simonetta, Taygerly et al. 2019). This compound was found to strengthen the natural interaction between the oncogenic transcription factor β-Catenin and its associated E3 ligase, SCFβ-TrCP, leading to increased ubiquitination successive degradation of mutant β-Catenin (Simonetta, Taygerly et al. 2019). Mutations at S37 constitute approximately 10% of all identified β-catenin mutations, diminishing its binding to β-TrCP significantly. NRX-252114 is able to restore the interaction between pS/S37 β-catenin and β-TrCP(Simonetta, Taygerly et al. 2019).

Similarly, recent endeavors aimed at identifying novel molecules with "glue-like" properties and antiproliferative effects have uncovered several candidates that function, at least in part, by augmenting the existing weak affinity between substrate proteins and E3 ligase complexes (Kozicka, Suchyta et al. 2023). Noteworthy among these discoveries are the identified molecular glue degraders capable of enhancing the minimal affinity between CDK12 and the DDB1-CUL4-RBX1 E3 ligase complex (Kozicka, Suchyta et al. 2023).

Another intriguing category of molecular glues concentrates on stabilizing existing protein-protein interfaces yet, does not involve degradation of a substrate. Rather it aims at stabilizing a complex to modulate downstream signaling. Another remarkable example of a PPI stabilizer is the ‘trametiglue’, a molecular glue that originated from the MEK inhibitor trametinib (Mekinist®) (Khan, Real et al. 2020). Unlike regular inhibitors this cancer drug doesn’t only bind to MEK but engages in the MEK and the scaffold kinase repressor RAS (KSR) interface, stabilizing the KSR-MEK complex. Moreover, trametinib was slightly altered by exchanging an acetamide with a sulfamide bioisostere, transforming trametinib into trametiglue (Geiger, Schäfer et al. 2022). As a result, interfacial binding between these two proteins is enhanced, trapping MEK in an inactive KSR-MEK complex (Khan, Real et al. 2020).

An additional example of this would be the small molecule KI-MS2-008. Here, the MYC-associated factor X (MAX) functions as a transcription factor capable of forming homodimers or heterodimers with other transcription factors, notably MYC, a key regulator of cell growth and proliferation. Considering that MAX/MYC heterodimers are oncogenic drivers, drug discovery efforts were made to develop the small molecule KI-MS2-008, which stabilizes MAX homodimers instead of MAX/MYC heterodimers (Struntz, Chen et al. 2019). This in turn attenuates MYC-driven transcription and even reduces MYC proteins levels (Struntz, Chen et al. 2019).

One peculiar way of molecular glue-mediated degradation is degradation of a target protein via oligomerization. BCL6 is a transcription factor, linked to lymphoid malignancies, specifically diffuse large B-cell lymphoma. Inhibitors, targeting the interaction between the

BCL6-BTB domains, are being explored as potential cancer therapeutics (Słabicki, Yoon et al. 2020). In the optimization of screening hits for BCL6-BTB domain binders a molecule, BI-3802, was identified. Unexpectedly, cells treated with this molecule underwent proteasome-dependent degradation of BCL6 (Słabicki, Yoon et al. 2020). Cyro-EM studies revealed that the solvent-exposed dimethylpiperidine moiety of BI-3802 interacts with a neighboring BTB-domain homodimer, leading to BCL-6 polymerization. Subsequently, the SIAH1 E3 ubiquitin ligase has been identified being able to recognize this supramolecular structure, resulting in the rapid degradation of BCL6 (Słabicki, Yoon et al. 2020).

More molecular glues are being serendipitously uncovered given that numerous approved drugs and molecules have elusive mechanisms. It's plausible that many more of these compounds could act as molecular glues. Therefore, extensive efforts are being made, particularly in the field of proteomics, to identify alterations at the protein level and to capture instances of molecular glue action. Figure 9 shows the chemical structure of the molecule glue examples outlined above. Nevertheless, given the diversity of molecular weight and structure, it will be challenging to derive a rational design for molecular glues.

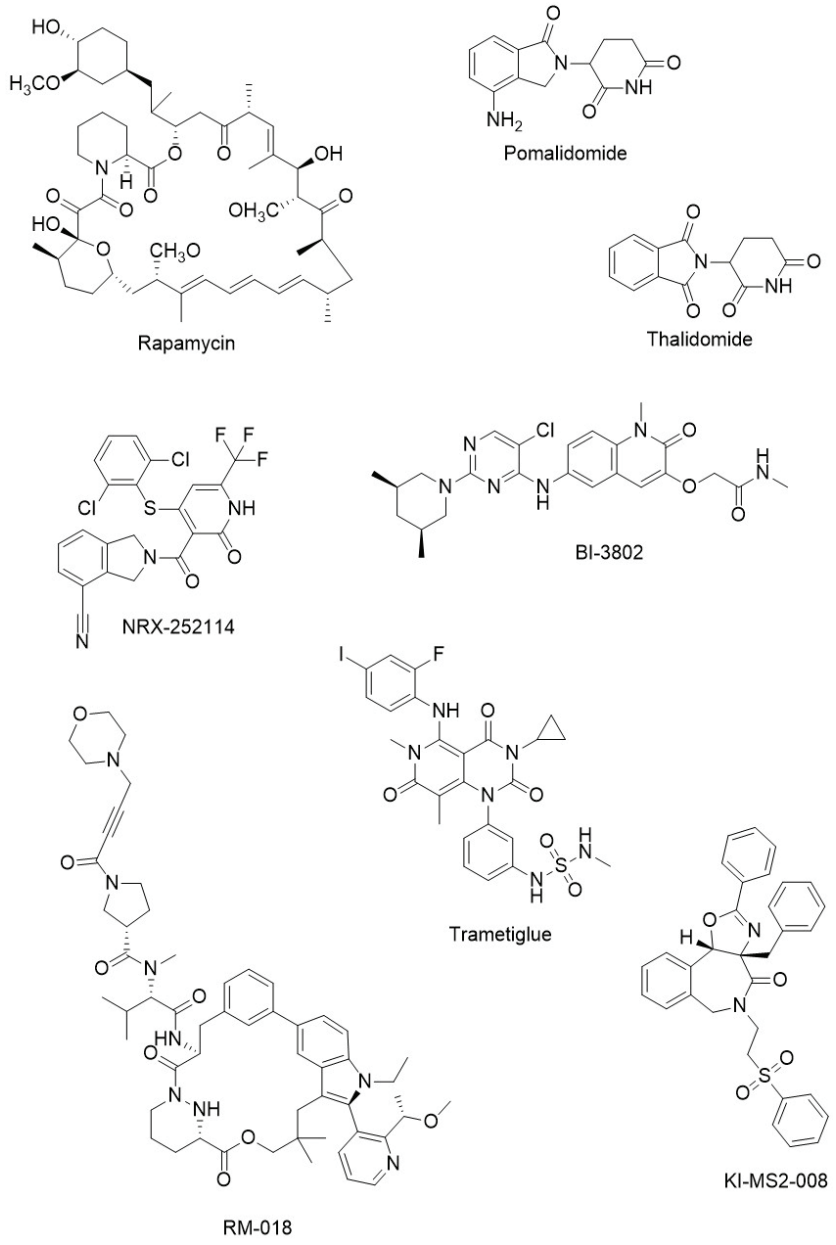


Figure 9: Examples of molecular glues. Showing the chemical structure and diversity of molecular glues described above, including rapamycin, thalidomide, pomalidomide, NRX-252114, BI-3802, trametiglue, RM-018 and KI-MS2-008.

1.4.3 TPD Drugs Targeting KRAS

In recent years, there has been an increasing amount of literature on PROTACs targeting KRAS. Several attempts have been made including a PROTAC targeting KRAS G12C, known as LC-2 (Bond, Chu et al. 2020). It is composed of the known KRAS inhibitor MRTX849 and utilizes the E3 ligase CRL2-VHL. The covalent binding of KRAS G12C to MRTX849, followed

by VHL recruitment, effectively degraded KRAS and thereby, inhibited MAPK signaling. (Hyun and Shin 2021). Interestingly, the degradation efficiency of LC-2 varied in cells with different KRAS G12C genotypes. In heterozygous NCI-H358 cells, approximately 50% degradation of KRAS G12C was observed, while in homozygous NCI-H2030 and MIA PaCa-2 cells, the degradation reached 75% (Hyun and Shin 2021). Nonetheless, the covalent binding of KRAS G12C to MRTX849, along with the recruitment of the E3 ligase CRBN through thalidomide derivatives, did not succeed in degrading endogenous KRAS G12C in cells. (Hyun and Shin 2021). This demonstrated potential E3 ligase-based limitations frequently encountered in TPD.

In a recent promising study, it was demonstrated that small-molecule pan-KRAS degrader, ACBI3, degrades 13 out of the 17 most prevalent oncogenic KRAS alleles (Popow, Farnaby et al. 2024). This includes mutations like G12D and G12V that are currently not targetable by existing inhibitors. Additionally, treatment with ACBI3 resulted in pathway modulation and tumor regressions in a KRAS mutant xenograft mouse model (Popow, Farnaby et al. 2024). Utilizing noncovalent KRAS binders and small molecules targeting the VHL ligase, researchers designed a heterobifunctional VHL-based degrader, forming a KRAS:PROTAC:VHL complex (Popow, Farnaby et al. 2024). This strategy has the capability for addressing the high prevalence of various KRAS mutations in different cancer types. Potential limitations of this study include that ACBI3 is not suitable for further *in vivo* use due to skin lesions observed in mice. Moreover, heterobifunctional degraders are associated with resistance mechanisms. Here, ACBI3 shows high cell efflux which may reduce its effectiveness in KRAS mutant cell lines that have high levels of drug transporters like ABCB1 (Popow, Farnaby et al. 2024). Overall, further research is needed to address efflux-mediated reduction in potency of PROTAC ACBI3.

Covalent Molecules Targeting KRAS

One of the main advantages of molecular glues is their potential to target proteins that are challenging to bind with traditional ligands, such as KRAS. A novel approach for molecular glue discovery, is the development of covalent molecules, containing electrophilic reactive moieties, that can irreversibly bind to target proteins (Geiger, Schäfer et al. 2022). In the pursuit of identifying new molecular glues that target KRAS, particularly the KRAS G12C mutants, researchers screened focused libraries of covalent-reactive FKBP12 and Cyp18 ligands (Geiger, Schäfer et al. 2022). This effort resulted in the extensive optimization of covalent molecules (Geiger, Schäfer et al. 2022) and utilized both the molecular glue and covalent molecule mechanisms. An example of such is RM-018, which has the ability to bind to the GTP-bound (active) KRAS G12C mutant, which could potentially overcome KRAS-

mediated resistance, in cells harboring the KRAS G12C mutant (Tanaka, Lin et al. 2021). These RM-018 molecular glues appear to function by obstructing the docking of KRAS to downstream effector proteins. Precisely, it appears that RM-018 forms a “binary complex” with cyclophilin A when entering the cell. Subsequently, this binary complex can associate with active KRAS G12C, and a covalent bond is formed which results in formation of a “tricomplex”. Consequently, KRAS G12C is blocked from associating with downstream effector proteins (Tanaka, Lin et al. 2021). It further suggestions that it may be effective to combine KRAS G12C inhibitors, like RM-018, with MAPK pathway inhibitors to effectively counter KRAS-driven signaling and resistance (Tanaka, Lin et al. 2021).

Another noteworthy compound is RMC-4998, which has a similar pattern of inhibition as RM-018. Schulze et al. developed a potent and selective inhibitor of GTP-bound (active) KRAS G12C, paving the way for a new drug candidate currently in clinical trials (Schulze, Seamon et al. 2023). It is based on sanglifehrin A, a microbial-derived compound with immunosuppressive properties that inhibits cyclophilin, impacting protein folding and immune responses (Zenke, Strittmatter et al. 2001). The initial design centered around linking a cysteine-reacting component to the cyclophilin A (CypA)-binding motif of sanglifehrin. This compound formed a ternary complex with CypA and KRAS G12C bound to a GTP analog, covalently modifying Cys12. While sotorasib and adagrasib irreversibly inhibit KRAS G12C-GDP, the molecular glue RMC-4998 takes a different approach. It selectively targets KRAS G12C-GTP by forming a ternary complex with the prolyl isomerase CypA, leveraging the high-affinity binding of sanglifehrin A's macrocyclic portion to cyclophilin A (Sedrani, Kallen et al. 2003). Using a high-resolution crystal structure of the complex, they iteratively refined the compound, enhancing its potency and selectivity towards GTP-bound (active) KRAS G12C and effectively blocking GTP-bound (active) KRAS G12C signaling activity (Schulze, Seamon et al. 2023). Further development led to RMC-6291, a drug candidate now in phase 1 clinical trials for patients with advanced stage KRAS G12C-mutant cancers (Liu 2023). The larger binding interface of the CypA-RMC-4998 complex allows for selective targeting of the GTP-bound (active) KRAS G12C state, even in the presence of growth factor stimulation, which desensitizes cells to sotorasib and adagrasib (Liu 2023). This approach presents a compelling strategy for targeting active KRAS, a significant challenge in drug development with few existing solutions.

1.5 Targeting Protein-Protein Interactions in Drug Discovery

In the past, the emphasis of drug discovery campaigns has been placed on the binary interaction between a target protein and a small molecule, to generate inhibitors. However, the necessity for such binary interactions is diminished when targeting protein-protein interactions (PPIs). What we require to target protein-protein interfaces is a protein surface, bound by a molecule, capable of selectively blocking or fostering new PPI's. PPIs play a pivotal role in cellular processes, and aberrant PPIs are linked to various diseases such as cancer, infectious diseases, and neurodegenerative disorders (Oláh, Szénási et al. 2022). However, modulation of PPIs is very challenging. Yet, numerous PPI modulators have progressed into clinical trials in the last years, with some being approved, suggesting promising prospects for PPI-targeting drugs (Lu, Zhou et al. 2020, Oláh, Szénási et al. 2022).

As PPI interfaces are often large, flat, and lack well-defined binding pockets, unlike traditional drug targets attacking enzyme active sites (Lu, Zhou et al. 2020). Consequently, developing PPI-targeting drugs requires powerful screening approaches, including high-throughput screening (HTS), fragment-based screening, computational or virtual screening methodology and structure-guided design. HTS is a fruitful starting point for the identification of initial hits yet may need extensive validation and optimization strategies for the development of new drugs. Interestingly, PPIs contain so called "hot spots", or protein surfaces that contain amino acid residues critical for protein-protein binding, which provide a basis for structural information and rational design of modulators (Vázquez, López et al. 2020). Virtual screens and computations docking are additional innovative strategies when designing PPI modulators, including ligand-based and structure-based models (Vázquez, López et al. 2020). The ligand-based approach relies on the structural data and physicochemical attributes of the scaffold or pharmacophore. The structural-based approach exploits the key structural features of the target protein (Vázquez, López et al. 2020). Overall, the complexity of targeting PPIs necessitates a multifaceted approach, combining innovative screening techniques and rational design strategies to effectively develop modulators.

Generally there are three major classes of PPI modulators: small molecules, peptides and antibodies (Lu, Zhou et al. 2020). PPI interfaces typically exhibit hydrophobic surfaces. Consequently, an effective PPI modulator must engage with a significant surface area that is flat with few grooves and establish numerous hydrophobic interactions. Given that larger hydrophobic molecule don't exhibit desirable drug-like and pharmacokinetic properties, small molecule modulators are typically better suited for confined protein-protein interfaces (Lu, Zhou et al. 2020). One strategy employed for the development of small molecule PPI

modulators is fragment-based drug discovery (FBDD), which is complementary to HTS. FBDD is advantageous for PPIs due to its focus on small chemical fragment libraries that can target discontinuous hot-spots in PPI interfaces, though extensive structural validation, fragment growth and structure-guided potency optimization is required (see further details in section 1.4.1). Peptides are another common strategy for targeting PPIs. Although protein-protein interfaces contain amino acid residues that interact with high-affinity, peptides can act as high specificity and high affinity binders, with the ability to outcompete these amino acid interaction (Oláh, Szénási et al. 2022). Nevertheless, a significant disadvantage of peptides is low membrane permeability and intracellular stability, due to peptide hydrolysis (Oláh, Szénási et al. 2022). Conversely, monoclonal antibodies can cover large surfaces yet have higher molecular weights and can induce potential adverse immune side effects (Oláh, Szénási et al. 2022).

In summary, identifying modulators for PPI's necessitates utilizing different approaches, including HTS, FBDD, structure-based design, and virtual screening. HTS allows for the initial discovery of compounds targeting PPI interfaces, but its success hinges on an extensive and diverse compound library. FBDD is a powerful approach to target typically flat and extensive PPI interfaces (Lu, Zhou et al. 2020). Structure-based design leverages hot-spot information to create small molecules or peptidomimetics based on specific structural motifs, like α-helices. Lastly, virtual screening employs computational methods to predict compounds that fit PPI pharmacophores or target structures, aiding in identifying disease-relevant PPIs (Lu, Zhou et al. 2020).

Protein-protein Interaction Inhibitors

Despite the challenges associated with designing PPI modulators, advancements have not only led to promising preclinical candidates but also some clinical trial entries, underscoring the potential of PPI modulators in modern drug discovery (Lu, Zhou et al. 2020). This section will delineate physiochemical properties and summarize several noteworthy instances of PPI inhibitors, along with covering the clinical progress in the field. Considering that the previous section on molecular glues extensively covered many examples of PPI stabilizers, this section will focus on PPI inhibitors and highlight some of their differences.

PPI inhibitors are typically designed to disrupt specific interactions by binding directly to key hot-spots within the PPI interface, often mimicking the natural binding residues of one protein partner (Lu, Zhou et al. 2020). This direct inhibition requires precise targeting of defined interaction sites. In contrast, PPI modulators may act allosterically, binding at sites other than

the primary interaction interface, thereby altering protein conformation and influencing PPI strength indirectly (Lu, Zhou et al. 2020).

The classic characteristic of drug-like compounds is outlined by Lipinski rule set, where molecules have a molecular weight of ≥ 500 Da, an octanol-water partition coefficient (logP) ≤ 5, hydrogen bond donors ≤ 5 and hydrogen bond acceptors ≤ 10. Compounds that meet at least three out of these four criteria are more likely to have good oral bioavailability and permeability, and are more likely to become successful drug candidates (Santos, Ursu et al. 2017). Since many PPI inhibitors do not fit with the Lipinskis “Rule of 5” profile, this definition has been expanded for PPI inhibitors now including molecules that have a higher molecular weight, increased hydrophobicity and a higher ring complexity (Morelli, Bourgeas et al. 2011).

Due to the distinct challenges associated with developing PPI modulators compared to traditional medicinal chemistry compounds, substantial efforts have been dedicated to establishing a framework of guidelines that can effectively support and streamline the drug development process for these modulators. Over the past decade, medicinal chemistry efforts have been directed to understand favorable physiochemical properties of PPI inhibitors, including the shape-related descriptors, topology and free binding energy exhibited by PPI inhibitors (Nakadai and Tomida 2020). As a result, a positive correlation has been observed between the binding free energy and the diameter of cylindrical 3-dimensional molecules, that could aid in the assessment of protein-protein interface antagonists (Nakadai and Tomida 2020).

Several notable examples of PPI inhibitors have emerged over recent years, one of the most prominent being inhibitors targeting the MDM2/TP53 interaction, which effectively disrupts this critical protein-protein interaction and underscores the therapeutic potential of PPI modulation. TP53 is the central tumor suppressor protein, regulating the expression of genes involved in cell cycle arrest, DNA repair, apoptosis, senescence, and angiogenesis (Shangary and Wang 2008). Thus, TP53 plays a pivotal role in preventing the uncontrolled proliferation of damaged or potentially oncogenic cells. MDM2, on the other hand, is an E3 ligase that negatively regulates TP53 activity, by binding TP53 and subsequently, promoting the rapid degradation of TP53. This inhibits TP53’s transcriptional activity, creating a negative feedback loop that controls cellular TP53 levels (Haupt, Maya et al. 1997). Thus, when TP53 levels increase in response to cellular stress, it induces the expression of MDM2, leading to TP53 degradation and attenuation of its transcriptional activity. The disruption of the MDM2/TP53 PPI leads to significant consequences for cellular homeostasis and TP53 deletions or mutation has been implicated in nearly 50% of human cancers (Shangary and

Wang 2008). Concisely, raised MDM2 levels strongly repress TP53 levels (Haupt, Maya et al. 1997).

Considering that TP53 WT function is important for regulating cell growth, recovering TP53 function in WT or functional TP53 tumor cells offers a potential therapeutic intervention point. Small molecule inhibitors like BI-907828 or RG7112, bind to MDM2, preventing the interaction between MDM2 and TP53 (Lu, Zhou et al. 2020, LoRusso, Yamamoto et al. 2023). In turn this stabilizes TP53 WT to enter the nucleus and drive target gene activation for apoptosis, DNA repair and cell cycle arrest. The rational design for inhibitors like RG7112 involves mimicking the structural "hotspot" residues of TP53, which compete for binding to MDM2 (Konopleva, Martinelli et al. 2020).

Another significant example of PPI inhibitor development centers around BCL2 and BAX. BCL2 is an important regulator of apoptosis, located primarily on the outer mitochondrial membrane (Lu, Zhou et al. 2020). It is part of the BCL2 protein family, that is divided into pro- and anti-apoptotic proteins, where BCL2 acts as anti-apoptotic and BAX as pro-apoptotic factor. The interplay of pro- and anti-apoptotic proteins determines the viability fate of the cell (Souers, Leverson et al. 2013). Mechanistically, the pro-apoptotic protein BAX is blocked when bound to anti-apoptotic proteins like BCL2, by forming a hydrophobic BH3 binding pocket domain to interact with BAX. In hematological cancers, particularly in chronic lymphocytic leukemia (CLL), there is an overexpression of BCL2, which helps cancer cells evade apoptosis and promotes their survival. To counteract the overabundance of BCL2, the selective PPI inhibitor ABT-199 (Venetoclax) was developed to bind BCL2. It contains an indole moiety, with the ability to form a hydrophobic interaction with the BCL2 BH3 domain, thereby blocking the BCL2/BAX interaction (Souers, Leverson et al. 2013). Consequently, BAX is activated and oligomerizes, forming pores in the outer mitochondrial membrane. In turn, cytochrome c is released and activates caspases that drive apoptosis leading to dismantling of the cell (Lu, Zhou et al. 2020). Thus, ABT-199 became one of the first small molecule PPI BCL2 inhibitors and gained its approval in 2016 for the treatment of CLL (Lu, Zhou et al. 2020). The substantial advancements in the development of PPI inhibitors underscore their promising potential in therapeutic applications.

1.6 Fragment-Based Drug Discovery

The two primary approaches used in the early stages of drug discovery to identify drug-like molecules are FBDD and HTS (Mureddu and Vuister 2022). FBDD leverages biophysical and biochemical techniques to detect the binding of small molecules, known as "fragments",

to a protein target (Kirsch, Hartman et al. 2019). Initial identification of a fragment can be accomplished using a variety of biophysical techniques and biochemical assays, just like HTS (Kirsch, Hartman et al. 2019). However, FBDD, unlike HTS, involves smaller and less complex molecule libraries, comprised of only few hundred to thousands of molecules (Singh, Tam et al. 2018). Despite their lower affinity to an intended protein target, they exhibit more efficient binding interactions, making for an effective starting point to subsequent lead optimization (Bon, Bilsland et al. 2022).

In FBDD the term "chemical space" refers to the vast, multidimensional realm of all possible chemical compounds that could potentially be synthesized or exist (Hajduk and Greer 2007). Due to the exponential increase in the number of possible molecules with molecular size, small fragment libraries provide a proportionally greater coverage of the “chemical space”, compared to larger HTS libraries containing high molecular weight compounds (Bon, Bilsland et al. 2022). Astonishingly, the realm of all possible molecular conformations with drug-like properties is estimated to be around ~10^{60} molecules (Reymond, van Deursen et al. 2010). By exploring a subset of this space, typically focusing on small fragments rather than full-sized drug-like compounds, small parts that bind and can be merged or grown to create a “lead compound“ (Hajduk and Greer 2007).

Central to FBDD, is the approach of fragment extension that begins with a small fragment which exhibits some affinity for the target protein of interest (POI), followed by chemical modifications or extensions to enhance its interactions with the POI (Wu, Centorrino et al. 2024). Furthermore, FBDD permits a more efficient search of chemical space, to recover information about the molecules binding mode and functional groups that interact with the POI (Carbery, Skyner et al. 2022). Through the identification of these key chemical interactions, a larger lead compound with greater potency can be designed (Carbery, Skyner et al. 2022). In addition, a common strategy for designing small fragment libraries is to select the most structurally diverse set of fragments from those that fall within the desired chemical space, and in this way effectively generate structural information of targets (Bon, Bilsland et al. 2022). Overall FBDD does not only have significant impact on costs but also reduces the time of data generation and analysis (Mureddu and Vuister 2022).

Over the years, drug-like properties have been defined such as lipophilicity, molecular mass, polar surface area, and combinations of physicochemical properties as well as functional group contributions (Hopkins, Keserü et al. 2014). Lipinski's “Rule of 5” is the traditional set of guidelines used in drug discovery to evaluate the drug-likeness of a compound, meaning properties that make a chemical compound a suitable candidate for development (Benet, Hosey et al. 2016). The rule, formulated by Christopher A. Lipinski helps

predict whether a compound has properties that would make it a prospective candidate for oral bioavailability (Lipinski 2000). Thus, compounds that meet these criteria are more likely to have favorable absorption and cellular permeation properties (Lipinski 2000). Vis-à-vis there is the “rule of 3”, a set of guidelines used in FBDD, to evaluate the potential of fragments as starting points for drug development (Jhoti, Williams et al. 2013). These criteria are framed to ensure that fragments have the right balance of properties for effective optimization into more potent and drug-like compounds. Typically, the molecular weight of fragments lies between 150–250 Da, and below 300 Da, whereas compounds discovered through HTS usually range between 400–500 Da. Table 3 compares drug-like properties of compounds, used in HTS versus lead-like properties of fragments, used in FBDD (Kirsch, Hartman et al. 2019). Importantly, the number of molecules comprising the HTS and FBDD vary greatly. HTS libraries contain up to a million or more compounds, whereas fragment libraries are typically comprised of approximately 2000 fragments (Bon, Bilsland et al. 2022).

Table 3: Comparison of HTS and FBDD. Showing differences in molecular properties between HTS that follow Lipinski’s “Rule of 5” and FBDD that follow the “Rule of 3”, including the size of screening libraries.

HTS (“Rule of 5”)	FBDD (“Rule of 3”)
MW ≤ 500 Da	MW < 300 Da
Number of H-bond acceptors ≤ 10	Number of H-bond acceptors ≤ 3
Number of H-bond donors ≤ 5	Number of H-bond donors ≤ 3
Lipophiliciy logP < 5	Lipophiliciy logP < 3
HTS library size ~ 1 million	Fragment library size ~ 1000-2000
Drug-like behavior	Lead-like behavior

Optimizing ligand efficiency (LE) is a crucial principle in drug development pipelines. It involves balancing pharmacokinetic factors to maximize a drug's success, rather than solely focusing on potency and overemphasizing a compound's affinity for the target (Hopkins, Keserü et al. 2014). LE quantifies how efficiently a ligand utilizes its structural features and functional groups to bind to the target protein (Hopkins, Keserü et al. 2014). Generally, fragments exhibit higher LE than HTS compounds. This is likely the case since it can be

difficult to find larger compounds of the desired shape and diversity for challenging drug targets (Wells and McClendon 2007). Although fragments contain fewer than 17 heavy atoms and have a molecular weight below 300 Da, resulting in low potency (K_d ~ 1-10 mM), their LE values can range from good to excellent and can be further optimized (Hopkins, Keserü et al. 2014). Thereby, LE offers a way to evaluate whether adding atoms to the initial fragment is justified in terms of enhancing binding affinity. Nonetheless, when developing these fragments into lead compounds, approximately 15-20 heavy atoms are typically added to increase potency (Hopkins, Keserü et al. 2014).

FBDD offers notable advantages and has been transformative in the therapeutic development of challenging protein targets, including inhibitors or stabilizers targeting PPIs. Fragments typically bind to “hot spots” within protein-protein interfaces, guiding the design and optimization of fragments towards the advancement of more potent modulators (Wang, Shi et al. 2023). Fragment hits with a novel mode of action, including covalent fragments and molecules binding to allosteric sites, have emerged as significant candidates of PPI modulators (Wang, Shi et al. 2023). Through the advancement of structural technologies such as cryo-EM, covalent tethering and proteomics, and artificial intelligence, FBDD has progressed significantly, with several candidates progressing into clinical trials (Wang, Shi et al. 2023).

An exemplary case of FBDD through fragment extension is the development of molecular glues that enhance the stability of the 14-3-3 protein complex with an Estrogen Receptor alpha-derived peptide (ERα) (Wu, Centorrino et al. 2024). Researchers started with a non-covalent amidine-substituted thiophene fragment, which was already known to interact with the 14-3-3 protein. Subsequently, they investigated ways to extend this fragment by introducing new chemical groups or modifying existing ones, creating a focused library of analogues derived from the initial fragment. The goal was to strengthen the stability of the 14-3-3-ERα complex by optimizing interactions within the binding pocket formed by the 14-3-3 protein and ERα (Wu, Centorrino et al. 2024). Utilizing an fluorescence anisotropy (FA) assay, it was possible to monitor how effectively the compound stabilizes the interaction between 14-3-3 and ERα (Wu, Centorrino et al. 2024). Fragment extension in combination with X-ray crystallography for structural determination, led to the identification of key interacting residues and facilitated the establishment of a structure-activity relationship (SAR). This comprehensive approach identified key features of the analogs that contributed to the binding behavior of the PPI (Wu, Centorrino et al. 2024).

Fragment based methods have additionally been employed to BCL2 family proteins, where a library of fragments was screened for their ability to bind to these proteins thereby

potentially disrupting their PPIs (Wang, Shi et al. 2023). In addition, FBDD has been utilized to develop small-molecule inhibitors that can disrupt the interaction between MDM2 and TP53, thereby reactivating TP53's tumor-suppressive functions. Similarly to BCL2, fragment libraries were screened against MDM2 as a starting point of lead optimization, followed by X-ray crystallography to determine the binding modes of these fragments (Wang, Shi et al. 2023). For BCL2, the discovered drug was Venetoclax, while for MDM2 and TP53, it was Nutlin-3 (Wang, Shi et al. 2023). All these PPIs have been comprehensively discussed in the preceding chapters of this dissertation.

1.6.1 FBDD by NMR

The primary challenge of FBDD is to identify and select promising fragments that bind to the target of interest (Kirsch, Hartman et al. 2019). Moreover, identifying the binding residues of the fragment is critical for successive lead optimization. There are numerous orthogonal validation methods to confirm that a fragment binds to the POI, one of them being Nuclear Magnetic Resonance (NMR) (Kirsch, Hartman et al. 2019). NMR spectroscopy has many applications in FBDD and is widely utilized, ranging for primary screening and identification of a fragment, to the final lead optimization of molecules and the generation of new clinical drug candidates (Mureddu and Vuister 2022). Importantly, NMR spectroscopy plays a crucial role in this process due to its sensitivity and ability to provide detailed information about ligand-protein interactions. It is used to screen libraries of fragments against target proteins and can detect weak molecular interactions that are often missed by other methods, making it highly effective in identifying potential starting candidates for further optimization. Moreover, its ability to characterize protein-ligand interactions across a wide range of affinities, from weak interactions to strong binding, makes it a valuable tool for FBDD (Wang, Gao et al. 2022). The capability to identify interactions and binding modes of fragments with the target protein in turn, aids in the rational design and optimization of fragments into more potent and selective lead molecules (Mureddu and Vuister 2022).

The basic principle of NMR spectroscopy is that it is a powerful method in the field of analytical chemistry, to study the structure and behavior of molecules. NMR applies a strong magnetic field to a sample, which causes hydrogen, nitrogen or carbon nuclei to resonate at specific frequencies (Wang, Gao et al. 2022). This resonance reveals detailed information about the molecular environment of the nuclei and is recorded as peaks in an NMR spectrum. The characterization of protein-ligand interactions occurs through the detection of a chemical shift change, meaning changes in the chemical environment of the nuclei in the protein (Wang,

Gao et al. 2022). Specifically, when a ligand binds to a protein, it causes a chemical shift change, indicating the presence and strength of a binding interaction. By analyzing these chemical shift changes, one can infer where and how well a fragment interacts with its target (Wang, Gao et al. 2022).

Similar to other drug discovery methods, the study of NMR-based protein-ligand interactions employs two primary approaches: the ligand-based approach and the protein-based approach. The ligand-based approach focuses on the ligand. This is commonly used in ligand screening and pharmacophore mapping, or characterizing the essential features of ligands that interact with the target protein, and is suitable for early-stage drug discovery (Hiroaki and Kohda 2018). Here techniques such as STD (Saturation Transfer Difference) are employed to identify ligand binding sites. On the contrary, the protein-based approach centers on the protein itself to analyze how it interacts with ligands. Here ^{1}H-^{15}N-HSQC (Heteronuclear Single Quantum Coherence) is primarily used which is an NMR titration method. This approach is suitable for detailed interaction analysis (Hiroaki and Kohda 2018).

Chemical-shift titration can map ligand-binding sites on a target protein, with the ability to estimate the dissociation constant (K_D) of the interaction. In this method, a fragment or ligand is gradually titrated to an isotopically labeled protein sample (typically ^{15}N or ^{13}C) (Furukawa, Konuma et al. 2016, Hobbs, Drant et al. 2022). After each addition of the ligand, an NMR spectrum of the protein is recorded to observe chemical shift changes, indicating a binding event. Thereby, the chemical shift perturbation can be fitted to a binding curve to derive affinities and estimate K_D (Furukawa, Konuma et al. 2016). However, in some instances the resonances may not follow a linear pattern during titration of the ligand, possibly indicative of multiple binding modes between the ligand and the target protein (Furukawa, Konuma et al. 2016).

In conclusion, both ligand-based and protein-based NMR approaches play complementary roles in studying protein-ligand interactions, with ligand-based techniques aiding early-stage screening and protein-based methods providing detailed insights into binding dynamics and affinities, enabling a comprehensive understanding of binding sites and interaction modes.

1.7 Aims of Thesis

In this thesis I wanted to investigate the native substrate- E3 ligase pair, KRAS and $CUL3^{LZTR1}$, and develop a pharmacological strategy to chemically exploit this proteostatic interaction. Recent developments in the field of TPD inspired us to investigate whether a small molecule that acts in a molecular glue-like fashion can potentiate the LZTR1:KRAS PPI, leading to enhanced ubiquitination and degradation of KRAS (WT and/or mutant). Moreover, given the clinical relevance of oncogenic KRAS, novel approaches are urgently needed when it comes to selectively targeting specific mutant versions.

The aims of this thesis were:

1) Develop a cellular screening assay to monitor the interaction of LZTR1 with different substrate RAS GTPases, sensitive enough to detect mutational and chemical perturbations.

2) Screen fragment libraries using the previously established assay for the identification of small molecules that enhance the KRAS-LZTR1 interaction using KRAS WT and the oncogenic mutant KRAS G12D.

3) Chemical optimization of potential hit candidates utilizing fragment-based drug discovery approaches.

4) Validation using orthogonal biochemical and biophysical methods to investigate identified hit candidates that arise from the chemical screening assay.

2. Results

The RAS family of GTPases, particularly KRAS, plays a crucial role in regulating cellular signaling pathways controlling cell growth, survival and differentiation. Mutations in KRAS are among the most frequent oncogenic mutations found across various cancers, making it a key target for therapeutic intervention. However, for many years, KRAS was considered "undruggable" due to its lack of suitable binding sites for conventional small-molecule therapies. This view has shifted significantly in recent years, particularly with the advent and approval of covalent inhibitors targeting specific KRAS mutations, such as G12C. Despite these advances, the clinical impact of these inhibitors has been limited, and resistance mechanisms have emerged, underscoring the demand for novel therapeutic approaches.

In this context, the study presented here explores a new strategy to modulate KRAS activity by enhancing its interaction with LZTR1. LZTR1 serves as a substrate-recruiting adaptor for the CUL3-based E3 ubiquitin ligase complex, playing an essential role in the proteostatic control of RAS GTPase family proteins. Loss of LZTR1 activity has been linked to elevated levels of RAS GTPases and hyperactivation of the MAPK signaling pathway, emphasizing its role in maintaining cellular equilibrium. By utilizing the concept of molecular glues, small molecules that stabilize PPIs, we aim to identify fragments that can enhance the KRAS-LZTR1 interaction, thereby promoting KRAS degradation and dampening its signaling activity.

Here, we wanted to explore the pharmacological strategy of utilizing the proteostatic interaction of LZTR1 with RAS family GTPases, to obtain small molecules with the ability to shift the equilibrium of this native PPI. This research employs a fragment-based screening approach to discover candidate fragments capable of increasing the KRAS-LZTR1 PPI. The first step, presented in this study, was to establish a screening assay based on a split-luciferase reporter assay. We validated the approach through the identification of two small molecules, C53 and Z86, that enhanced the KRAS-LZTR1 interaction, along with evidence for enhanced complex formation detected by BioID, thermal shift assays and NMR spectroscopy. The discovery of these fragments unveils new possibilities for targeted protein degradation strategies to tackle RAS proteins and provides strong evidence supporting the feasibility of modulating this critical interaction with small molecules.

The insights gained from this work advances our understanding and approach to targeting one of the most significant oncogenic drivers. Enhancing KRAS degradation by strengthening the KRAS-LZTR1 PPI interface has the potential to complement existing pharmacological

strategies aimed at hyperactive RAS and MAPK signaling, that may lead to more durable and effective treatment regimens for RAS-driven cancers.

The author of this thesis conceptualized the work together with her supervisors Giulio Superti-Furga and Johannes Bigenzahn and conducted the majority of the experiments. NMR spectroscopy and molecular docking of C53 and KRAS4B G12D were carried out by Sven Brüschweiler. Immunoblotting of transient expression levels of split-luciferase assay (SLA) constructs was performed by Josepha Westphalen, while some SLA experiments were conducted by Julio García Murias. Protein expression and purification of KRAS4B G12D were carried out by Katharina M. Siess. Robert Konrat supervised the NMR spectroscopy and molecular docking part of the project. The published manuscript PDF of Piech *et al. (2024)* is available via open-source access (DOI: 10.1021/acschembio.4c00077).

ACS chemical biology

pubs.acs.org/acschemicalbiology

Article

Identification and Characterization of Novel Small-Molecule Enhancers of the CUL3^{LZTR1} E3 Ligase KRAS Complex

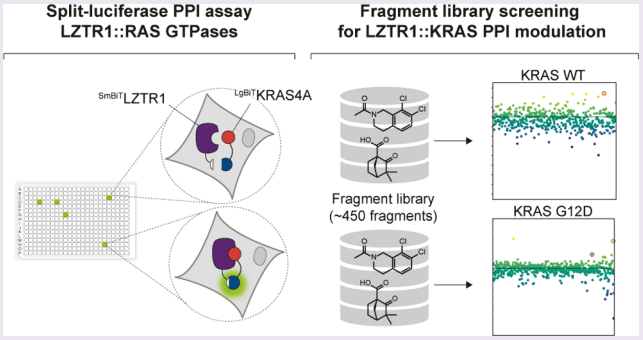
Sophie Piech, Sven Brüschweiler, Josepha Westphalen, Katharina M. Siess, Julio García Murias, Robert Konrat, Johannes W. Bigenzahn,* and Giulio Superti-Furga*

Cite This: https://doi.org/10.1021/acschembio.4c00077

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The RAS family of GTPases is among the most frequently mutated proteins in human cancer, creating a high clinical demand for therapies that counteract their signaling activity. An important layer of regulation that could be therapeutically exploited is the proteostatic regulation of the main RAS GTPases KRAS, NRAS, and HRAS, as well as the closely related members, MRAS and RIT1, by the leucine zipper-like transcriptional regulator 1 cullin 3 RING E3 ubiquitin ligase complex (CUL3^{LZTR1}). Genetic inactivation of *LZTR1*, as observed in different cancer entities and Noonan syndrome leads to enhanced RAS GTPase abundance and altered MAPK pathway activation state. Novel therapeutic approaches to interfere with hyperactive RAS signaling, thereby complementing existing treatments, are highly sought after. Motivated by the growing arsenal of molecular glue degraders, we report the identification of novel chemical fragments that enhance the protein−protein interaction (PPI) of the KRAS-LZTR1 complex. We established a split-luciferase-based reporter assay that monitors the RAS GTPase-LZTR1 interaction in a scalable format, capable of capturing chemical, as well as mutational perturbations. Using this screening system, in combination with a small fragment library, we identified two fragments, C53 and Z86, that enhance the interaction of the KRAS-LZTR1 complex in a dose-dependent manner. Further orthogonal validation experiments using proximity biotinylation (BioID), thermal shift assays, and NMR spectroscopy demonstrated fragment-dependent enhanced recruitment of endogenous LZTR1 and physical engagement of KRAS. The two fragments, which potentiate the KRAS-LZTR1 interaction, serve as starting points for fragment-based drug discovery. Additionally, the assay we introduced is amenable to high-throughput screening to further explore the pharmacological modulation of the CUL3^{LZTR1}-RAS GTPase complex.



INTRODUCTION

RAS family GTPases are known as one of the most significant oncogenes, with the KRAS gene displaying the highest frequency of mutations within the main RAS GTPase group (85%), followed by NRAS (11%) and HRAS (4%).[1] These mutations are genetic drivers in numerous cancers and predominantly occur at the glycine 12 (G12) and 13 (G13) residues, constituting 81% and 14% of all KRAS mutations, respectively.[2,3] The resulting structural changes hinder the arginine finger of associated GTPase activating proteins (GAPs) to hydrolyze GTP to GDP, shifting the KRAS pool toward its active GTP-bound state.[4] Active KRAS facilitates its growth-promoting function by interacting with several downstream effector proteins, with RAF1 being a predominant member, leading to enhanced MAPK signaling.[5] Despite its importance as a cancer driver, and potential as a therapeutic target, KRAS was long considered undruggable because it lacks any large hydrophobic pockets or an active site suitable to classical drug discovery-based approaches.[3,6] In the past decade, a multitude of new drug discovery efforts have been launched to target KRAS, stirred by the discovery, and recent approval of covalent G12C-mutation targeting small molecules.[7,8] However, moderate clinical efficacy, and the high frequency of resistance upon treatment with these agents, demonstrate the need for additional novel targeting approaches.[9,10]

Apart from mutations affecting the RAS GTPase itself, or its associated GAP and GEF interacting partners leading to enhanced RAS activity, alteration of the proteostatic regulation of individual RAS GTPase proteins has been identified as an additional important regulatory layer leading to MAPK pathway hyperactivation.[11−13] The leucine zipper-like transcriptional regulator 1 (LZTR1) protein serves as a substrate

Received: February 2, 2024
Revised: August 2, 2024
Accepted: August 19, 2024

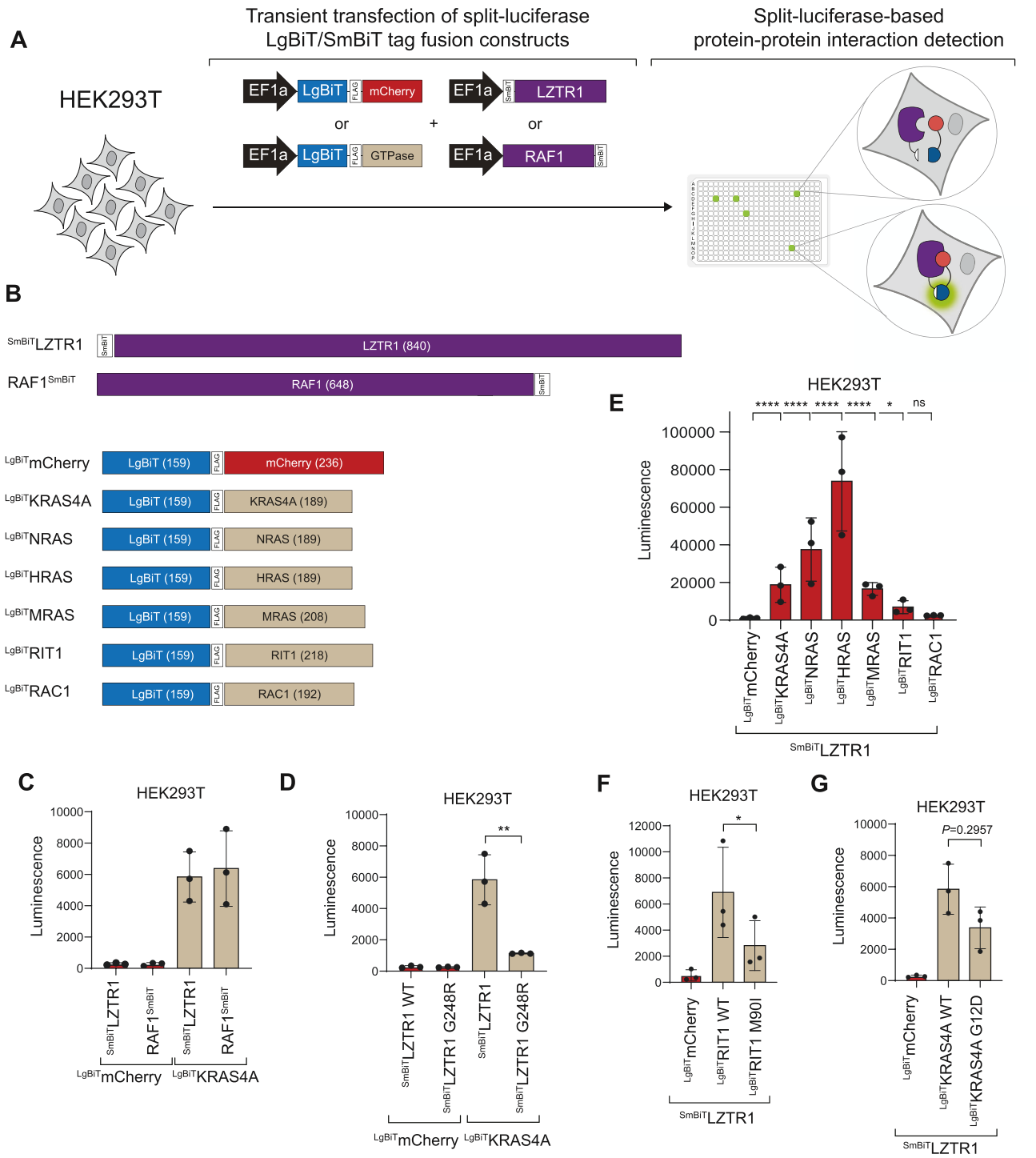


Figure 1. Split-luciferase assay (SLA)-based detection of RAS family GTPase-LZTR1 PPIs. (A) Scheme of SLA assay where two proteins of interest, either KRAS and LZTR1, or KRAS and RAF1, are fused to LgBiT or to SmBiT tags, respectively. After coexpression of the constructs in HEK293T cells and the addition of the live substrate, PPIs are detected. mCherry fused to LgBiT was used as a negative control. (B) Schematic outline of LgBiT- and SmBiT- containing fusion constructs drawn to scale based on their length. Numbering represents amino acid length (excluding stop codon). (C) HEK293T cells were cotransfected with ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1 or RAF1^{SmBiT} and afterwards analyzed by SLA. The expression of the ^{LgBiT}mCherry construct with ^{SmBiT}LZTR1 or RAF1^{SmBiT} served as negative control. $n = 3$. (D) HEK293T cells were cotransfected with ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1 or ^{SmBiT}LZTR1 G248R and analyzed by SLA. ^{LgBiT}mCherry + ^{SmBiT}LZTR1 served as negative control. $n = 3$. (E) HEK293T cells were cotransfected with different RAS GTPase family members (LgBiT-fused KRAS4A, NRAS, HRAS, MRAS, and RIT1) and ^{SmBiT}LZTR1 and analyzed by SLA. ^{LgBiT}mCherry and ^{LgBiT}RAC1 constructs coexpressed with ^{SmBiT}LZTR1 served as negative control. $n = 3$. (F, G) HEK293T cells were cotransfected with ^{LgBiT}KRAS4A WT G12D and ^{SmBiT}LZTR1 (F) as well as ^{LgBiT}RIT1 WT or M90I and ^{SmBiT}LZTR1 (G) and analyzed by SLA. ^{LgBiT}mCherry and ^{SmBiT}LZTR1 cotransfection served negative control. $n = 3$. Statistical significance was calculated with a two-way ANOVA with Dunnett's multiple comparisons test correction (E) or two-tailed t test (D, F, and G). ns, nonsignificant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

receptor (SR) for the cullin 3 RING E3 ubiquitin ligase ($CUL3^{LZTR1}$) complex leading to ubiquitination and altered abundance of the main RAS GTPases KRAS, NRAS, and HRAS as well as the closely related GTPases MRAS and RIT1.[11−13] Structurally, LZTR1 belongs to the BTB/BACK domain-containing protein family, harboring an N-terminal Kelch domain followed by two BTB/BACK domains, whereby the Kelch domain acts as the substrate binding site and the BTB/BACK domains as interaction unit with the CUL3 scaffold.[14] LZTR1-mediated RAS ubiquitination leads to reduced abundance, attenuating the activation and downstream signaling of the MAPK pathway. As a result, LZTR1 acts as a negative modulator of the RAS-MAPK signaling pathway by keeping RAS levels in check. Additionally, mutations of LZTR1 that have been identified in and associated with different human diseases, interfere with $CUL3^{LZTR1}$ complex assembly and/or recruitment of RAS itself.[12] Consequently, the loss of LZTR1 is associated with increased RAS GTPase protein levels, overactivation of RAS, and hyperactivation of the MAPK signaling pathway.[11]

In theory, if one could favor complex formation between LZTR1 and KRAS by using compounds acting like molecular glues, then one would open a new pharmacological strategy for the regulation of KRAS activity. Molecular glue degraders are one embodiment of targeted protein degradation (TPD) drugs that can strengthen preexisting PPIs or induce neo-PPIs with ubiquitin ligases, resulting in desired target protein degradation and therapeutic effects.[15,16] In this study, we aimed to discover fragments that chemically modulate the LZTR1-KRAS PPI in a molecular glue-like manner, resulting in enhanced E3 ligase-substrate complex formation. We hypothesized that such fragments, when optimized for favorable kinetic properties and bioavailability, could lead to reduced RAS abundance and signaling output, as well as decreased downstream MAPK pathway activation. To this end, we aimed to develop a robust assay that could monitor changes in LZTR1-KRAS PPI formation. Through an iterative exploratory and optimization campaign, we designed a high-throughput screening-compatible split-luciferase-based PPI reporter assay to monitor LZTR1-KRAS recruitment in live cells. Screening of a fragment library at high concentrations led to the identification of two candidate fragments able to enhance the natural propensity of LZTR1 to bind KRAS. Orthogonal validation using proximity biotinylation (BioID), thermal shift assays, as well as NMR further corroborated our findings, providing a first proof of concept for the feasibility of small-molecule-based $CUL3^{LZTR1}$-KRAS complex modulation in cells.

RESULTS

Split-Luciferase-Based Assay (SLA) Allows for the Detection of RAS GTPase-LZTR1 PPI. To design a PPI assay that is high-throughput screening-compatible, we tested whether the NanoBiT system was suitable for the detection of the KRAS-LZTR1 interaction. The assay separates the small luciferase NanoLuc into two entities: a small tag (SmBiT, 11 amino acids) and a large tag (LgBiT, 18 kDa).[17] Given that these two subunits have a weak affinity towards each other (K_d ∼ 190 μM),[18] they only come into proximity when fused to two proteins that have sufficient affinity for each other to support complex formation, even if transiently. This PPI-induced proximity results in the complementation of the luciferase resulting in luminescent signal emission upon substrate addition.[17] We tested detecting the interaction of LgBiT-tagged KRAS4A (^{LgBiT}KRAS4A) with SmBiT-tagged LZTR1 (^{SmBiT}LZTR1) or RAF1 (RAF1^{SmBiT}) upon transient transfection of HEK293T cells (Figure 1A,B). Initially, we tried all orientation combinations of the tags, N- and C-terminally, except with RAS, where we solely placed the LgBiT tag on the N-terminus with the understanding that its C-terminal post-translational processing allows for localizing to the cellular membrane required for proper LZTR1 interaction.[19] Cells transfected with a vector expressing the LgBiT-tagged mCherry fluorescent protein (^{LgBiT}mCherry) served as a negative control. Cotransfection of ^{LgBiT}KRAS4A with ^{SmBiT}LZTR1 or RAF1^{SmBiT} led to robust expression and a pronounced increase in luminescence compared to ^{LgBiT}mCherry indicative of successful PPI formation (Figure 1C and Supplementary Figure 1A). Moreover, we observed robust SLA luminescence induction with both KRAS splice isoforms (^{LgBiT}KRAS4A and ^{LgBiT}KRAS4B) upon coexpression with ^{SmBiT}LZTR1 compared to ^{LgBiT}mCherry (Supplementary Figure 1B). LZTR1 mutations identified in Noonan syndrome patients as well as in different cancer entities have been shown to interfere with efficient KRAS proteostatic regulation. We therefore tested whether the LZTR1-KRAS SLA was able to capture mutational interference. Indeed, overexpression of the frequent LZTR1 G248R mutation led to a reduced PPI signal in comparison to WT protein (Figure 1D and Supplementary Figure 1C). Since LZTR1 has been shown to regulate several RAS GTPase family members, we tested whether the SLA was able to capture these interactions (Figure 1B). All tested RAS GTPases displayed a robust interaction signal, with ^{LgBiT}HRAS being the strongest one in our assay, while the negative control ^{LgBiT}mCherry as well as the non-LZTR1 substrate GTPase RAC1 only showed little signal increase above background (Figure 1E and Supplementary Figure 1D). The difference in signal strength in our assay could be at least partially a consequence of different expression levels, attributable to the use of transient transfection (Supplementary Figure 1D). With the understanding that LZTR1 also regulates the ubiquitination and degradation of the small GTPase RIT1, a member of the RAS family of GTPases, and that RIT1 mutations escape LZTR1-mediated proteostatic regulation, we next applied the same method and cotransfected HEK293T cells with ^{LgBiT}RIT1 WT and the M90I mutant (Supplementary Figure 1E).[14] Correspondingly, ^{LgBiT}RIT1 M90I showed a reduced interaction signal compared to that of the WT protein (Figure 1F). Interestingly, we observed a similar pattern comparing ^{LgBiT}KRAS4A WT and the commonly observed oncogenic G12D mutant indicating that at least some KRAS mutants might escape LZTR1-based abundance regulation (Figure 1G and Supplementary Figure 1F). Furthermore, we assessed whether pharmacological perturbation of the CUL3 E3 ligase complex itself would lead to an altered interaction signal between ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1. However, following treatment of transfected HEK293T cells with the E1 inhibitor TAK-243, the COP9 signalosome inhibitor CSN5i-3, or the neddylation inhibitor MLN4924, we did not observe a similar change in the interaction signal compared to that observed with mutational perturbation (Supplementary Figure 1G).

Fragment-Based Screen Identifies C53 as a Modulator of the LZTR1-KRAS Interaction. Fragment-based drug discovery (FBDD) aims at the identification of low-molecular-weight molecules (MW < 300 g/mol) and is particularly applicable for challenging target classes, including modulation of PPIs.[20,21] In contrast to high-throughput compound

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

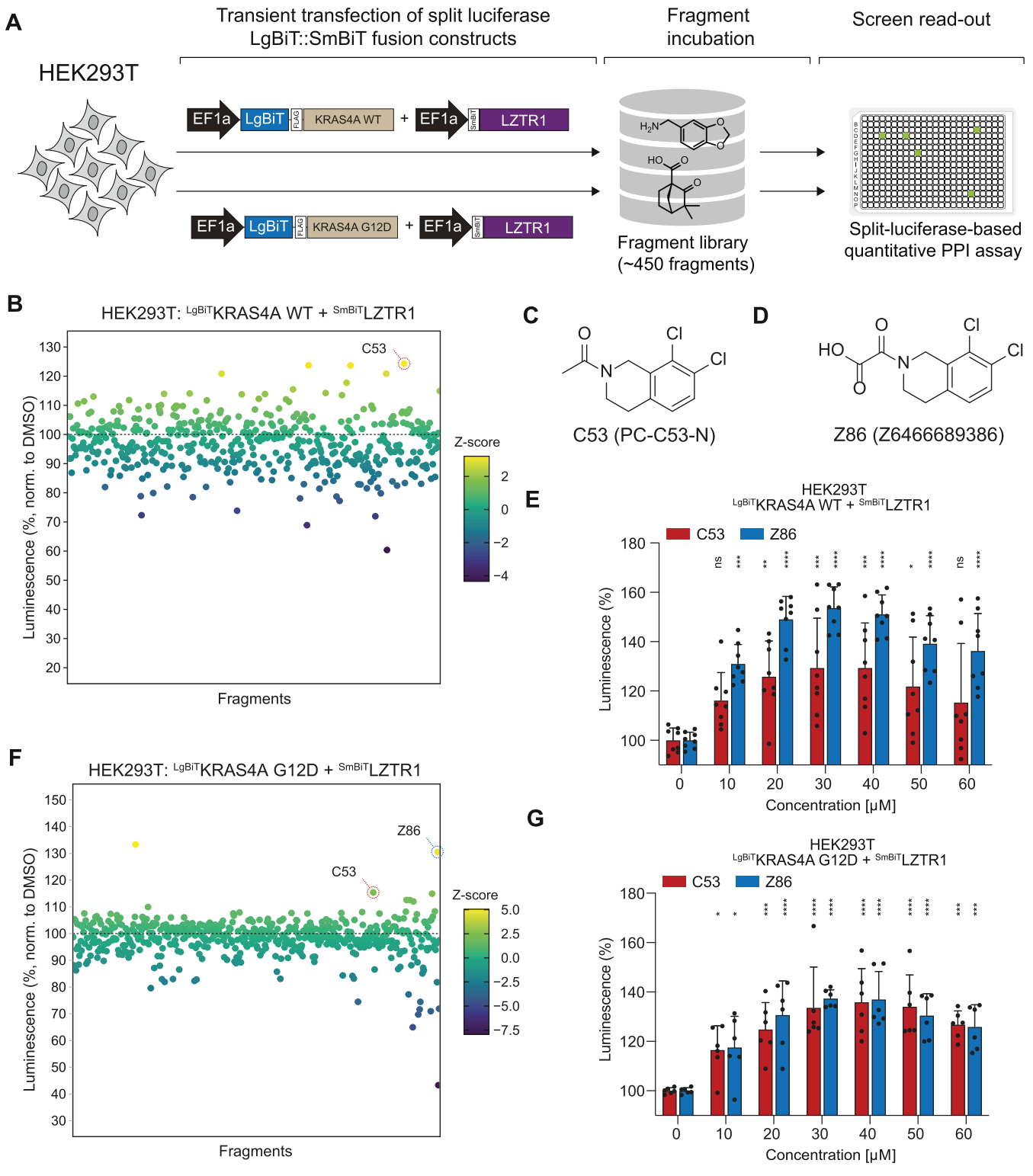


Figure 2. Identification of fragments that enhance the KRAS-LZTR1 interaction. (A) Schematic of fragment screen using SLA in a 384-well plate format. (B) Luminescence for all fragments screened in cells coexpressing ^{LgBiT}KRAS4A WT and ^{SmBiT}LZTR1 using the SLA. Each dot represents an individual fragment with the hit C53 highlighted. (C, D) Chemical structure of fragment hits C53 and Z86. (E) Dose−response bar graph in HEK293T cells coexpressing ^{LgBiT}KRAS4A WT and ^{SmBiT}LZTR1 upon treatment with increasing concentrations of C53 or Z86 (10, 20, 30, 40, 50, and 60 μM, 0 μM corresponds to DMSO negative control treatment). $n = 4$. (F) Luminescence for all fragments and analogues screened in cells coexpressing ^{LgBiT}KRAS4A G12D and ^{SmBiT}LZTR1 using the SLA with C53 and Z86 highlighted. (G) Dose−response bar graph in HEK293T cells coexpressing ^{LgBiT}KRAS4A G12D and ^{SmBiT}LZTR1 upon treatment with increasing concentrations of C53 or Z86 (10, 20, 30, 40, 50, and 60 μM, 0 μM corresponds to DMSO negative control treatment). $n = 3$. Statistical significance was calculated with a two-way ANOVA with Dunnett's multiple comparisons test correction. ns, nonsignificant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

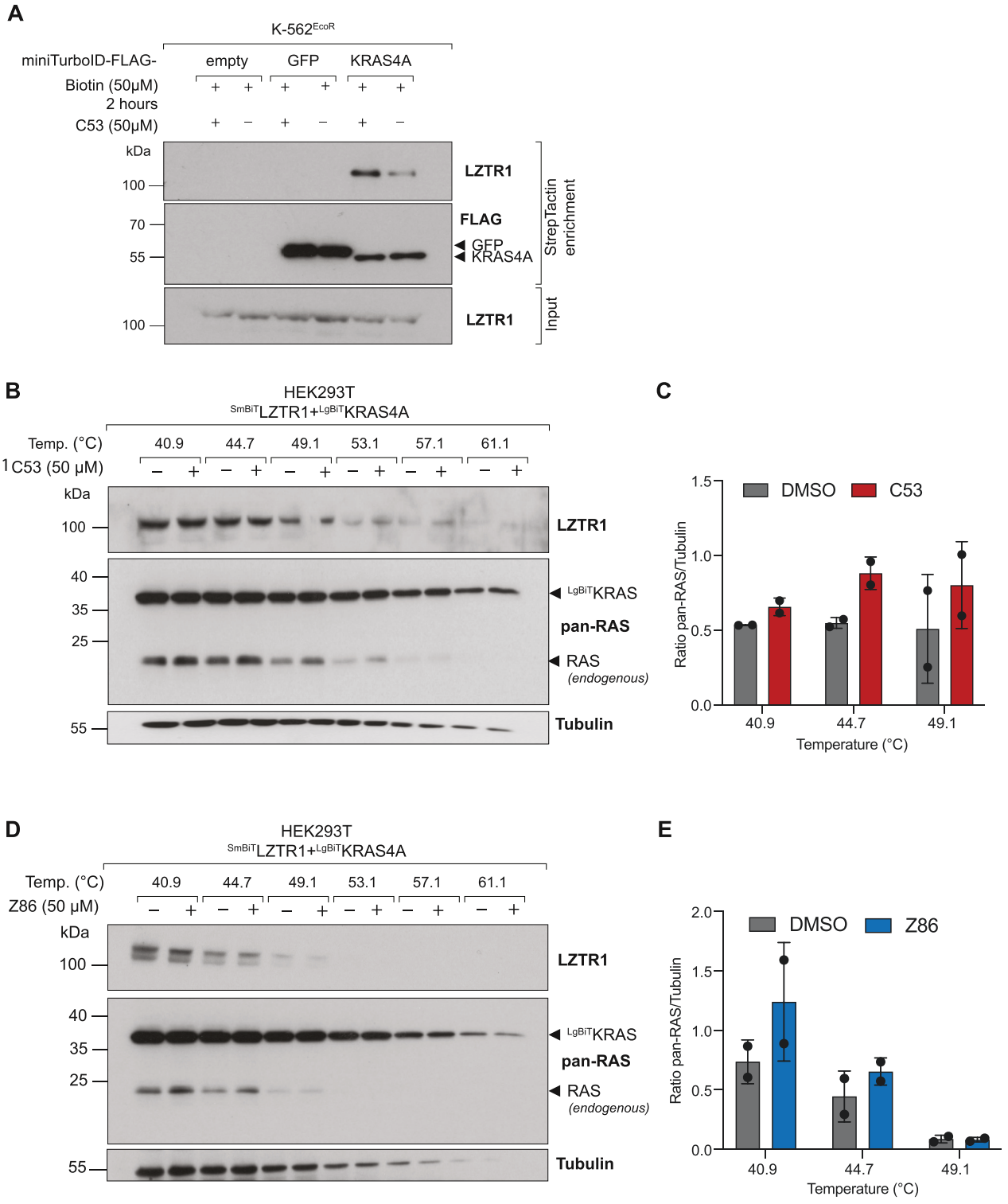


Figure 3. Orthogonal validation experiments detect enhanced recruitment and fragment engagement. (A) Proximity biotinylation (miniTurboID) analysis in K-562 cells lentivirally transduced with the indicated miniTurboID-FLAG constructs. Cells were treated with 50 μM of C53 (+) or DMSO (−) overnight, followed by 50 μM biotin treatment for 2 h, cell lysis, StrepTactin enrichment, and subsequent SDS-PAGE and immunoblot analysis. Immunoblots are probed with the indicated antibodies. miniTurboID-GFP fusion protein served as negative control. BioID results shown are representative of two independent biological experiments (n = 2). (B) Thermal shift assay analysis of C53. HEK293T cells coexpressing ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1 were lysed and treated with C53 (50 μM) for 1 h. The samples were separated by SDS-PAGE following immunoblotting analysis. Immunoblots show the thermostability of endogenous RAS and ^{LgBiT}KRAS4A monitored with anti-pan-RAS antibody staining, following heat treatment at a temperature range of 40.9−61.1 °C, in the absence (−) or presence (+) of C53. (C) Band intensity of the thermal shift immunoblot films was quantified and normalized to the band intensity of the α-tubulin control. The fold change of endogenous RAS signal is displayed in the presence and absence of C53 between 40.9 and 49.1 °C. (D−E) Same as (B) and (C) except treatment with Z86. Thermal shift assay quantifications are based on two independent and representative sets (n = 2).

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

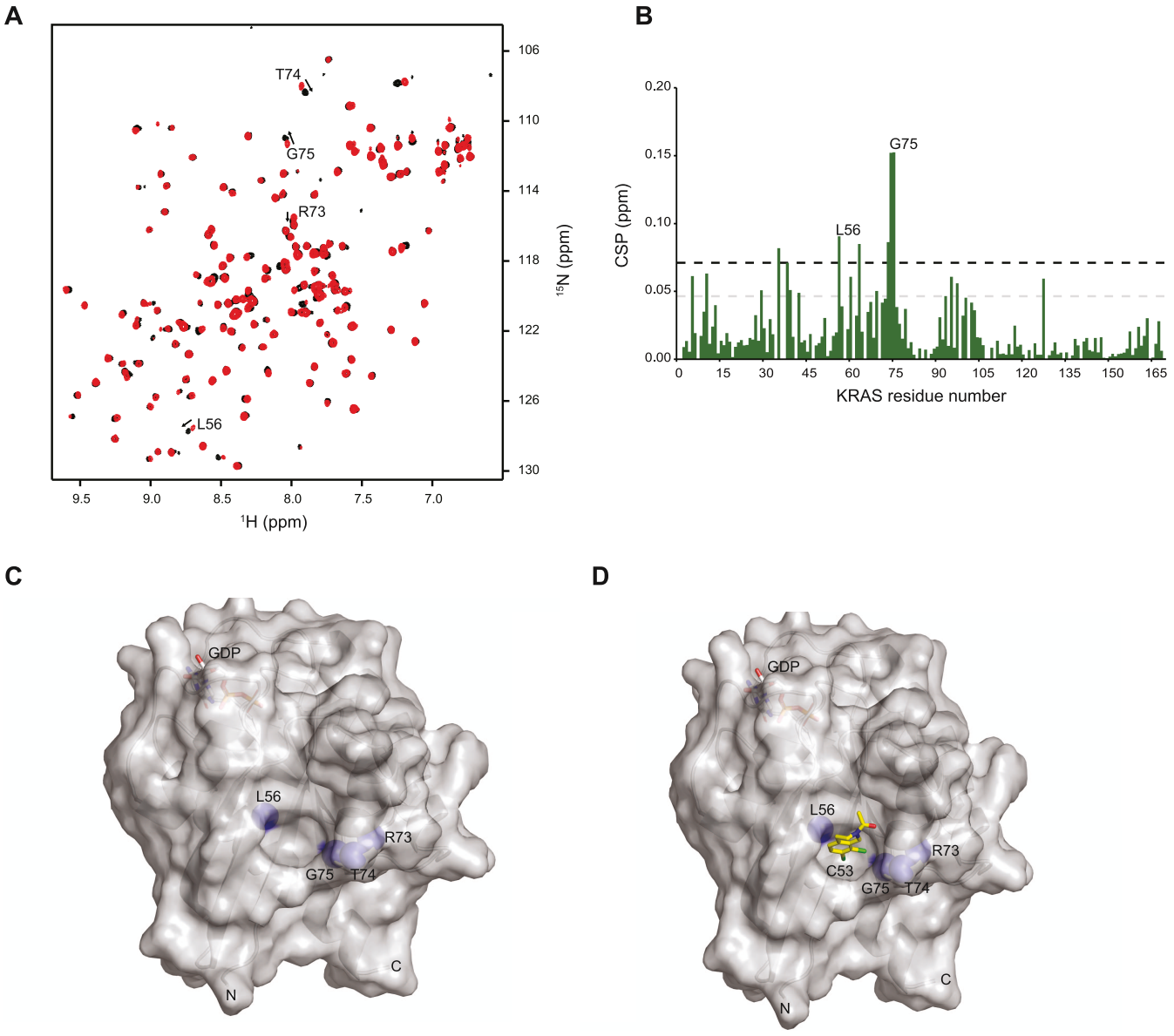


Figure 4. NMR-based C53 binding validation and characterization. (A) Overlay of ^{1}H−^{15}N HSQC spectra of 100 μM GDP-KRAS G12D in the absence (red) and presence (black) of 2.5 mM C53. The four largest amide backbone CSPs are indicated by black arrows. (B) Histogram showing CSP values for backbone amide groups of ^{15}N-labeled GDP-KRAS G12D in the presence of 2.5 mM C53. Horizontal dotted gray and black lines indicate one and two standard deviations of the CSP values, respectively. Residues without a bar were not assigned or overlapped in the 2D spectra. (C) Ribbon and surface representation of KRAS GDP (PDB ID 4EPW) with nitrogen atoms shown as blue spheres for residues that showed CSP values larger than 2 standard deviations of the mean value of all CSPs in the NMR titration experiment and are located in the SI/II pocket. (D) Binding site docking pose of C53, the compound is shown in yellow sticks representation and the protein (PDB ID 4EPW) is represented as in (C).

libraries, fragment libraries are smaller, with low-potency molecules that can then be grown and optimized to form larger lead compounds.[22] Based on the observation that the LZTR1-KRAS SLA can monitor mutation-induced interaction changes, we aimed to identify fragments that could increase the signal between ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1, and thus potentially act as enhancers of this PPI in a molecular glue-like manner (Figure 2A). We screened a library of around 450 commercially available fragments at a concentration of 50 μM and identified fragment C53 (PC−C53-N; *Z*-score = 3.20), which showed a prominent increase in luminescence signal compared to the DMSO control (Figure 2B). Structurally, C53 is an *N*-acetyl-7,8-dichlorotetrahydroisoquinoline (Figure 2C).

As the initial library that we screened was designed for diversity, with as little structural similarity among the fragments as possible, we wanted to further explore 50 analogues of C53. For this purpose, we designed a virtual make-on-demand library from Enamine, utilizing its REAL Space structure−activity relationship (SAR) search tool for small-molecule characterization and scaffold-hopping.[23] The chosen analogues were screened in the same cellular model configuration, and we detected fragment Z86 (Z6466689386) displaying a modest improvement in PPI-modulating propensity (Supplementary Figure 2A). Z86 differed from C53, by

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

containing a carboxylic acid (Figure 2D). Further single-well validation experiments of C53 and Z86 confirmed our initial observations from the screen revealing a dose-dependent signal increase of cells coexpressing ^{SmBiT}LZTR1 and ^{LgBiT}KRAS4A (Figure 2E). Interestingly, we observed that several of the selected analogues, contrary to C53 and Z86, led to a reduced interaction signal. To rule out cytotoxicity as a potential confounding factor in the analogue library screen, we evaluated the impact of our analogues on cell viability. Reassuringly, most of the screened analogues did not demonstrate any substantial antiproliferative effect, apart from fragment Z6739291816 (Supplementary Figure 2B). We speculate that analogues decreasing the luminescent signal in our SLA likely represent fragments that interact with one of the two targets but are sterically not tolerated in the PPI interface and thereby prevent ^{SmBiT}LZTR1 from engaging with ^{LgBiT}KRAS4A.

Following our observation that the KRAS G12D mutant showed reduced binding propensity to LZTR1, we rescreened both fragment libraries (original 450 fragments and 50 C53 analogs) in the context of ^{LgBiT}KRAS4A G12D and ^{SmBiT}LZTR1, to test whether the mutant KRAS protein would display a similar or altered fragment preference (Figure 2F). Interestingly, C53 (Z-score = 2.46) and Z86 (Z-score = 4.64) showed again a marked increase in luminescence signal compared to the DMSO control (Figure 2F). Moreover, C53 and Z86 also showed a dose-dependent increase in the signal in single-well validation experiments, corroborating the screen result (Figure 2G). Given that we were able to validate fragment activity in our SLA for both ^{SmBiT}LZTR1-^{LgBiT}KRAS4A WT and ^{SmBiT}LZTR1-^{LgBiT}KRAS4A G12D cells, we decided to pursue orthogonal validation experiments.

Enhanced Recruitment of the LZTR1-KRAS Complex in the Presence of C53. To test whether C53 would be able to enhance the recruitment of endogenous LZTR1, we used the cellular proximity biotinylation assay (BioID). Previously, we successfully used BioID to map the association of the four main RAS GTPases (fused to BirA*) with endogenously expressed LZTR1 in chronic myeloid leukemia (CML) cells.[11] We stably expressed KRAS4A fused to miniTurboID, a BirA* derivative enzyme with shorter labeling times and improved sensitivity, in the CML cell line K-562.[24] Empty vector-transduced cells, as well as cells expressing miniTurboID-GFP, served as a negative control. Cells were treated with C53 overnight, followed by the addition of biotin labeling for 2 h. As expected, we found an association of miniTurboID-fused KRAS4A with endogenous LZTR1 in the unperturbed state. Treatment with C53 led to enhanced recruitment and labeling of LZTR1, strongly supporting the notion that C53 acted by favoring the formation of the LZTR1-KRAS complex (Figure 3A).

Fragment Engagement with KRAS. A ligand-target interaction can be monitored by thermal shift assay, where ligand-engagement thermodynamically stabilizes its target, delaying temperature-dependent denaturation.[25] We tested whether the thermal shift assay would allow target engagement detection of the fragments with either LZTR1 or KRAS. For this purpose, we lysed HEK293T cells coexpressing ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1, treated the lysates with 50 μM C53, Z86, or DMSO for 1 h, and subsequently incubated the samples at an increasing temperature range before immunoblotting analysis. Compared to vehicle control, we detected thermal stabilization of the endogenous RAS pool within the applied temperature range (40.9 to 53.1 °C) in C53 or Z86 fragment-treated cell lysates (C53, Figure 3B and C; Z86, Figure 3D and E). Interestingly, we did not observe a thermal shift to the same extent for the ^{LgBiT}KRAS4A fusion protein, which could potentially be attributed to an altered thermal stabilization property of the LgBiT-tagged KRAS4A. These data provide further evidence for target engagement of both fragments with endogenous (K-)RAS. However, in contrast to our BioID findings, we failed to detect any stabilization of endogenous and SmBiT-tagged LZTR1 upon fragment incubation.

NMR Reveals Binding of Fragments to the Switch I/II Pocket of KRAS G12D. ^{15}N-labeled GDP-bound KRAS G12D was utilized to further validate fragment binding with 2D NMR spectroscopy. To determine ligand binding, we monitored the changes in the ^{1}H and ^{15}N chemical shifts in ^{1}H−^{15}N HSQC spectra. The addition of compounds C53 and Z86 to GDP-bound KRAS G12D led to chemical shift perturbations (CSPs) of a similar set of peaks, indicative of an identical binding site for the two compounds (Figure 4A and Supplementary Figure 3A). Amide backbone chemical shifts were available for GDP-KRAS G12D, and the chemical shift changes upon fragment addition were used to map the binding site (Figure 4B and Supplementary Figure 3B). Among the peaks that showed no overlap in the 2D spectra and could be unambiguously assigned, L56, R73, T74, and G75 showed CSPs more than twice the standard deviation of the mean upon C53 addition (Figure 4B). These residues coincide with a conserved RAS surface pocket referred to as the switch I/II pocket.[26−28] The magnitudes of CSPs induced by Z86 were smaller; however, for residues S39, T74, and G75, significant CSPs were observed (Supplementary Figure 3B).

Binding Pose Prediction of C53 by Molecular Docking. To generate a model of the potential binding pose of C53, a binding site docking simulation, based on the CSP data, was performed using AutoDock Vina.[29] In two of the docking poses, the benzene ring and the chloro groups of C53 are inserted into the SI/II pocket, which is formed by V7, L56, and Y71, as well as the aliphatic parts of the K5 and T74 side chains (Figure 4D and Supplementary Figure 3C). These ligand orientations are similar to the ligand poses in the X-ray structures determined by Maurer et al. and Sun et al. (Supplementary Figure 3D,E). These docking poses suggest that the piperidine and propanone motifs of C53 may be mediating the interaction with LZTR1.

DISCUSSION

In this study, we aimed to design a high-throughput chemical screening method to exploit the proteostatic interaction between the CUL3^{LZTR1} complex and the RAS family of GTPases. This approach seeks to identify small molecules capable of altering the equilibrium of the naturally occurring PPI between these proteins, providing new therapeutic avenues to mitigate cancerous RAS signaling. Our first step was to establish a screening modality that, once scaled up to an industrial level, could warrant a drug discovery campaign. We validated this approach by identifying two small-molecule fragments, C53 and Z86, that enhanced the KRAS-LZTR1 interaction, with evidence for enhanced complex formation using BioID, thermal shift assays as well as NMR spectroscopy.

Diverse therapeutic strategies have been pursued in the past to target RAS for cancer therapy. Given the difficulty of targeting RAS itself, the initial focus has been on interfering

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

with its post-translational processing, a prerequisite for its efficient signaling activity, as exemplified by the development of farnesyl transferase inhibitors. However, following farnesyl transferase inhibition, especially KRAS and NRAS mutated cancers failed to demonstrate encouraging activity due to alternative geranylgeranyl transferase modification.[30] Other non-covalent small binding molecules have been discovered by means of NMR-based fragment screening, yielding some promising compounds.[6] Unfortunately, these compounds also prevent the normal function of RAS and might have little effect on RAS mutants, as well as potentially result in enhanced toxicities within healthy tissues.[6] Only recently, notable milestones have been reached with the development of Sotorasib (AMG-510), a covalent ligand, that binds to the distinct KRAS mutant allele G12C, showing the first promising results in clinical trials of KRAS mutant cancers.[31] Nevertheless, clinical data has illustrated that even direct targeting of KRAS G12C only provides an intermittent control of tumor growth, requiring potential additional combinatorial treatments for sufficient long-term disease control.[31]

Considering that an imbalance of protein turnover is associated with cancer development and can create selective therapeutic vulnerabilities,[32] the growing field of targeted protein degradation (TPD) offers a compelling new approach for targeting RAS-driven cancers. First TPD methods have attempted to degrade KRAS with bifunctional molecules (PROTACs) hijacking the E3 ligase adapter proteins such as Cereblon (CRBN)[33] and Von Hippel-Lindau Tumor Suppressor (VHL).[34,35] Despite the promise of PROTAC-based strategies, their clinical utility can be limited by three key factors: their large size, poor drug-like properties, and the fact that they do not exploit endogenous substrate-ligase pairs as molecular glue-like small molecules do.

Therefore, as an alternative strategy, we directed our efforts toward developing proximity-inducing fragments to chemically target the native substrate-ligase interaction of KRAS, specifically the KRAS-LZTR1 PPI interface. Similar efforts to chemically enhance the existing affinity of a PPI interface have been made, such as the identification of the small molecule NRX-252114 by Simonetta et al. NRX-252114 strengthens the interaction between the oncogenic transcription factor β-Catenin and its associated E3 ligase, $SCF^{\beta\text{-TrCP}}$, resulting in enhanced ubiquitination and degradation of mutant β-Catenin.[36] Interestingly, recent efforts to identify novel molecular glue degraders with antiproliferative effects in transformed cells have revealed several candidates that potentiate the weak affinity between substrate proteins and E3 ligase complexes. Notably, Kozicka et al. identified small-molecule degraders that enhance the minimal affinity between CDK12-cyclin K and the DDB1-CUL4-RBX1 E3 ligase complex, leading to substrate degradation.[37]

Despite the robustness and reproducibility of the screening setup presented here, several aspects warrant further investigation and improvement. First, although we successfully employed the SLA constructs using transient transfection, we were unable to achieve stable expression of these constructs in cells with a screening-compatible signal-to-noise ratio. Additionally, the assay variability observed in some instances might be due to expression variability resulting from transient transfection. Second, we have not detected any proteostatic consequences of fragment action, potentially due to the low affinity of our current fragments. Finally, our study has been limited by the lack of physical structural data on the LZTR1 protein. Despite our efforts, we have not been able to obtain suitable amounts of soluble, properly folded LZTR1 protein to sufficiently assess ternary complex formation, evaluate direct LZTR1 target engagement *in vitro*, and enable further hit expansion to increase the potency of our fragments.

Nevertheless, the effects of C53 and Z86 observed in living cells demonstrate the engagement of both endogenous LZTR1 and RAS as well as the frequently mutated KRAS G12D variant. This provides an attractive starting ground for developing a novel class of RAS GTPase-targeting agents that engage the CUL3-based E3 ligase complex $CUL3^{LZTR1}$. We are convinced that our study will advance the biochemical toolbox for screening proximity-inducing drugs and pave the way for optimizing fragments that could proteostatically regulate KRAS. Ultimately, enhanced degradation of KRAS, facilitated by potentiating the KRAS-LZTR1 PPI interface, could bolster existing RAS and MAPK pathway-focused pharmacological interventions, leading to a more durable treatment of RAS-driven cancers.

MATERIALS AND METHODS

Chemicals. Compound libraries and fragments C53 (PC-C53-N, Z1861995405) and Z86 (Z6466689386) were obtained from Enamine. The structures of C53 and Z86 are shown in Figure 2C and D. Additional reagents were used as follows: TAK-243 (S8341, Selleckchem, Houston, TX), CSN5i-3 (HY-112134, MedChemExpress, Monmouth Junction, NJ), and MLN4924 (S7109, Selleckchem). All chemicals were dissolved in DMSO (D5879, Sigma-Aldrich, St. Louis, MI).

Cell Lines. HEK293T cells were obtained from ATCC (Manassas, VA) and K-562 cells were from DSMZ (Braunschweig, Germany). HEK293T cells were cultured in DMEM (D5796, Sigma-Aldrich, St. Louis, MO) and K-562 cells were cultured in RPMI1640 medium (R8758, Sigma) both supplemented with 10% (v/v) FCS (S1810-500, Biowest, Riverside, MO) and antibiotics (100 U/mL penicillin and 100 mg mL^{-1} streptomycin; P4333, Sigma). Cells were cultured at 37 °C and 5% CO_2, authenticated by STR profiling, and checked for mycoplasma infection by PCR or ELISA regularly.

Plasmids. For split-luciferase-based reporter assays, the LgBiT cDNA, derived from pBiT1.1-N (Promega, Madison), containing a short linker sequence and a FLAG tag, was cloned in frame with mCherry or the indicated human GTPase-encoding cDNAs into the LEIH (pRRL-EF1a-IRES-HygroR) expression vector using the NEBuilder HiFi DNA Assembly Master Mix (NEB, Ipswich, MA). The SmBiT tag sequence was added at the N-terminus of LZTR1 and the C terminus of RAF1 using the Q5 Site-Directed Mutagenesis Kit (NEB). SmBiT-LZTR1 and RAF1-SmBiT cDNAs in gateway-compatible pDONR221 or pDONR223 vectors were transferred by LR recombination (11791100, Thermo Fisher Scientific, Waltham, MA) into the LEgwSHIB (pRRL-EF1a-gateway-StrepHA-IRES-BlastR) expression vector. pDONR221 or pDONR223 entry plasmids containing the coding sequence of mCherry or human KRAS4A, KRAS4B, NRAS, HRAS, RIT1, RAC1, LZTR1, and RAF1 have been described previously.[11] KRAS4A G12D, RIT1 M90I, and LZTR1 G248R mutations were performed using the Q5 Site-Directed Mutagenesis Kit (NEB).

For proximity biotinylation experiments, stable lentiviral expression vectors were generated by insertion of the miniTurboID cDNA, derived from pcDNA3-V5 miniTurbo-NES (Addgene plasmid #107170), with a FLAG tag-gateway (gw) cassette into the LEIH (pRRL-EF1a-IRES-HygroR) vector using the NEBuilder HiFi DNA Assembly Master Mix (NEB). GFP and KRAS4A cDNAs in gateway-compatible pDONR221 were transferred into LEmTIDFgwIH (pRRL-EF1A-miniTurboID-FLAG-gateway-IRES-HygroR) by LR recombination.

Lentiviral Transduction. To generate ecotropic receptor (EcoR)-expressing K-562 cells, HEK293T were transiently transfected

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

with LERZIE (pRRL-EF1a-rtTA3-P2A-ZeoR-IRES-EcoR) as well as psPAX2 (Addgene plasmid #12260) and pMD2.G (Addgene plasmid #12259) packaging plasmids using polyethylenimine (PEI) as previously described.[11] 24 h post transfection, medium was replaced, and virus-containing supernatant was harvested after 48 h, filtered through 0.45 μm sterile filters (6780-2504, Cytiva, Marlborough, MA), supplemented with 8 μg/mL protamine sulfate (Sigma) and added to K-562 target cells followed by selection of transduced cells using zeocin (ant-zn-1, InvivoGen, San Diego, CA).

For proximity biotinylation experiments, lentiviral supernatant was prepared as mentioned above using the respective empty vector, LEmTIDFgwIH-GFP or -KRAS4A lentiviral expression vectors, and pEcoEnv envelope plasmid instead of pMD2.G. Virus-containing supernatant was applied to K-562^{EcoR} cells followed by selection of transduced cells using hygromycin (ant-hg-1, InvivoGen).

Split-Luciferase Assay (SLA). HEK293T cells were seeded into 6-well plates. After 24 h, SmBiT- and LgBiT-fusion constructs were transiently cotransfected using polyethylenimine (PEI). SmBiT- and LgBiT-constructs were cotransfected in equal amounts (3 μg total plasmid DNA). 48 h after transfection, cells were washed with phosphate-buffer saline (PBS) (D8537, Sigma), detached using trypsin (T3924, Sigma), and harvested in DMEM supplemented with 5% (v/v) FCS and antibiotics. Following cell counting-based normalization, equal cell amounts were seeded in 384-well flat clear-bottom white assay plates (3765, Corning, Corning, NY), Nano-Glo Live Cell Reagent (N2014, Promega, Madison, WI) was added, and after a 10 min incubation time, luminescence was recorded on a SpectraMax i3x microplate reader (Molecular Devices, San José, CA).

Fragment-Based High-Throughput Screen. HEK293T were transiently transfected with either ^{LgBiT}KRAS4A WT and ^{SmBiT}LZTR1 or ^{LgBiT}KRAS4A G12D and ^{SmBiT}LZTR1 as described above and seeded at a concentration of 20,000 cells per well in 384-well flat clear-bottom white assay plates containing the spotted compounds at 50 μM or DMSO as negative control. Following incubation for 18 h, Nano-Glo Live Cell Reagent was added and after 10 min incubation, luminescence was recorded on a SpectraMax i3x microplate reader (Molecular Devices). Hit thresholds were defined by >20% (^{LgBiT}KRAS4A WT+^{SmBiT}LZTR1) and >15% (^{LgBiT}KRAS4A G12D +^{SmBiT}LZTR1) enhancement of interaction compared to DMSO control as well as a Z-score threshold of ≥2.5 for mean percent luminescence normalized to DMSO control.

Immunoblotting. Cells were lysed using Nonidet-40 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, and one tablet of Roche EDTA-free protease inhibitor cocktail (Sigma-Aldrich) per 50 mL) for 10 min on ice. Lysates were cleared by centrifugation (13000 rpm, 10 min, 4 °C), and proteins were subsequently quantified and normalized with Bradford assay using γ-globin as a standard (Bio-Rad, Hercules, CA). Cell lysates were resolved by SDS-PAGE and transferred to Protran BA 85 nitrocellulose membranes (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).

Antibodies used were: mouse monoclonal anti-RAS clone RAS10 (Millipore, 05-516, 1:500 dilution), mouse monoclonal anti-LZTR1 (E-12) (Santa Cruz, SACSC-390166, 1:200 dilution), rabbit monoclonal anti-c-RAF (Cell Signaling, 9422S, 1:1000 dilution), mouse monoclonal anti-FLAG M2 (Sigma, F1804, 1:1000 dilution), and mouse monoclonal anti-α-tubulin (Abcam, ab7291, 1:10000 dilution). The secondary antibodies used were goat anti-mouse HRP (115-035-003, Jackson ImmunoResearch, West Grove, PA) and goat anti-rabbit HRP (111-035-003, Jackson ImmunoResearch).

Thermal Shift Assay. LgBiT and SmBiT constructs were transiently expressed in HEK293T cells, as described above. After 48 h, cells were harvested and washed with PBS prior to lysis in Nonidet-40 lysis buffer and lysates were prepared as described above. Equal amounts of cell lysates were incubated with 50 μM of the respective fragment or DMSO as negative control for 1 h on ice. After compound incubation, 30 μL of the lysates were heated in individual tubes in a thermocycler at different temperatures for 6 min and then cooled for 3 min on ice. Subsequently, the samples were centrifuged at 13 000 rpm for 40 min at 4 °C, and supernatants were transferred to new tubes, mixed with 4× Laemmli buffer, and analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

Proximity Biotinylation (miniTurboID). K-562^{EcoR} cells expressing empty vector, miniTurboID-eGFP or -KRAS4A were treated with 50 μM C53 for 18 h followed by treatment with 50 μM biotin for 2 h, washed with PBS, and subsequently lysed with Nonidet-40 lysis buffer. Protein concentration was determined using Bradford assay and 15 mg of cell lysates were incubated with StrepTactin sepharose beads (2-1201-010, IBA Lifesciences, Göttingen, Germany) for 2 h at 4 °C. Beads were recovered by centrifugation and washed three times with lysis buffer, bound proteins were eluted by addition of 4x Laemmli buffer and boiling for 5 min before analysis by SDS-PAGE and immunoblotting.

Protein Expression and Purification of KRAS4B G12D. Uniformly ^{15}N-labeled KRAS4B G12D (residues 1−169) was expressed in *Escherichia coli* BL21 (DE3) with an N-terminal His6-tag followed by a TEV (tobacco etch virus) protease cleavage site. Cells were grown at 37 °C in M9 minimal media containing $^{15}NH_4Cl$ as a sole nitrogen source in the presence of kanamycin until $OD_{600} \approx 0.6$, then the temperature was lowered to 18 °C, and after 45 min protein synthesis was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Expression was carried out overnight. Cells were lysed by sonication in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and afterward clarified by centrifugation. Subsequently, the supernatant was loaded onto a HisTrap FF crude (Cytiva, Marlborough, MA) column. After elution, the buffer was exchanged on a HiPrep Desalting (Cytiva) column to 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM TCEP, then the His−tag was cleaved by incubation with TEV. The His-tag was removed by passing the sample through a second HisTrap FF crude (Cytiva) column. The protein solution was afterward supplemented with 1 mg GDP per 20 mg protein and 5 mM $MgCl_2$. In a final purification step, GDP-KRAS4B^{G12D} was purified to homogeneity by size exclusion chromatography using a Superdex 75 (Cytiva).

NMR Spectroscopy. NMR spectra were recorded at 25 °C on an Avance III HD 800 MHz (18.8 T) spectrometer. Data were processed using TopSpin 4 (Bruker BioSpin) and analyzed using CcpNmr.[38] C53 titration experiments were carried out on a 100 μM ^{15}N uniformly labeled GDP-KRAS G12D solution in PBS, pH 7.4, 1 mM TCEP in 5% D_2O/95% H_2O with 50 mM C53 stock solutions in dimethyl sulfoxid-d6 (DMSO-d6). Z86 titration experiments were carried out on a 50 μM ^{15}N uniformly labeled GDP-KRAS G12D solution in 100 mM HEPES buffer, pH 7.4, 150 mM NaCl, 1 mM TCEP in 5% D_2O/95% H_2O with 50 mM Z86 stock solutions in dimethyl sulfoxid-d6 (DMSO-d6). The ligands were titrated to final concentrations of 0.500, 1.0, 1.5, and 2.5 mM. Total DMSO-d6 concentration was kept constant at 5% for all NMR measurements. At each titration step chemical shift changes upon ligand addition were monitored with ^{1}H−^{15}N HSQC experiments. The combined chemical shift perturbation of $^1H^N$ and $^{15}N^H$ was calculated as $\Delta\sigma = \sqrt{(\Delta 1H)^2 + 0.20(\Delta 15N)^2}$. The GDP-KRAS G12D assignment with BMRB ID 27719 was used for the CSP analysis.

Molecular Docking Simulations of C53. In the docking calculation, the crystal structure of GDP-KRAS (PDB ID 4EPW) was employed as the receptor. Before protonation at physiological pH and conversion to the PDBQT file format using AutoDockTools,[39] all HETATM lines were removed from the PDB file. The docking search space in the simulation was centered on the switch I/II pocket, encompassing the residues that exhibited chemical shift perturbations in the NMR experiment. C53 was prepared using ChemDraw 22.2.0 (Revvity Signals) and converted to the PDBQT format with AutoDockTools. It was then docked into the rigid receptor using AutoDock Vina 1.20.[29] The calculation utilized default settings with an exhaustiveness level of 32. The docking poses aligned best with the

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

X-ray structures from Sun et al. (PDB ID 4EPW) and Maurer et al. (PDB ID 4DST), featured the benzyl group and chlorine atoms inserted into the switch I/II pocket and exhibited the second and fourth lowest estimated free binding energies.

Data Analysis. The band intensity of the thermal shift immunoblots was analyzed using Image Lab Software (version 6.1, Bio-Rad). Data organization and calculations were performed using Microsoft Excel (Microsoft, Redmond, WA) unless otherwise stated. Fragment screen analysis was performed using the R programming environment within RStudio (Posit PBC, Boston, MA). Luminescent signal bar graphs were analyzed in GraphPad Prism 9 (version 9.4.0). Experiments were carried out in typical independent triplicate sets (n = 3) unless otherwise stated. Statistical significance of individual split-luciferase assay results was calculated with a two-tailed *t* test or for comparison of multiple conditions a two-way ANOVA with Dunnett's multiple comparisons test correction. ns, nonsignificant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.4c00077.

Additional immunoblot and fragment screening as well as NMR results (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Johannes W. Bigenzahn − *CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria; Department of Laboratory Medicine, Medical University of Vienna, 1090 Vienna, Austria*; Email: johannes.bigenzahn@meduniwien.ac.at

Giulio Superti-Furga − *CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria; Center for Physiology and Pharmacology, Medical University of Vienna, 1090 Vienna, Austria*; orcid.org/0000-0002-0570-1768; Phone: +43 1 40160 70 001; Email: gsuperti@cemm.oeaw.ac.at; Fax: +43 1 40160 970 000

Authors

Sophie Piech − *CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria*

Sven Brüschweiler − *MAG-LAB GmbH, 1030 Vienna, Austria*

Josepha Westphalen − *CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria*

Katharina M. Siess − *MAG-LAB GmbH, 1030 Vienna, Austria*

Julio García Murias − *CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria*

Robert Konrat − *MAG-LAB GmbH, 1030 Vienna, Austria; Department of Structural and Computational Biology, University of Vienna, 1030 Vienna, Austria*

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.4c00077

Author Contributions

S.P., J.W.B., and G.S.-F. designed research; S.P., J.W.B., J.W., and J.G.M. performed research; S.B., K.M.S., and R.K. designed, performed, and analyzed the NMR research; S.P., J.W., J.G.M., J.W.B., and G.S.-F. analyzed and interpreted the data; S.P., J.W.B., and G.S.-F. wrote the paper with contributions from all other coauthors.

Notes

The authors declare the following competing financial interest(s): G.S.-F. is co-founder and owns shares of Proxygen GmbH and Solgate GmbH. G.S.-F. receives research funding from Pfizer. R.K. is co-founder and scientific head of MAG-LAB GmbH. The other authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the members of the Superti-Furga laboratory for critical discussions and suggestions. They thank the Molecular Discovery Platform at CeMM for assistance with the fragment-based screen. They are grateful to G. Winter and his laboratory for reading the manuscript, providing critical feedback, and providing reagents. This work was supported by the Austrian Academy of Sciences (to G.S.-F., S.P., J.W., J.G.M.), the Medical University of Vienna (to J.W.B.), the Austrian Science Fund (FWF SFB F4711 to G.S.-F.), and the Medical Scientific Fund of the Mayor of the City of Vienna (MUW-AP21005MWF to J.W.B.). Plasmids obtained through Addgene were a gift from D. Trono and A. Ting.

■ REFERENCES

(1) Hobbs, G. A.; Der, C. J.; Rossman, K. L. RAS isoforms and mutations in cancer at a glance. *J. Cell Sci.* **2016**, *129* (7), 1287−1292.

(2) Moore, A. R.; Rosenberg, S. C.; McCormick, F.; Malek, S. RAS-targeted therapies: is the undruggable drugged? *Nat. Rev. Drug Discovery* **2020**, *19* (8), 533−552.

(3) Uras, I. Z.; Moll, H. P.; Casanova, E. Targeting KRAS Mutant Non-Small-Cell Lung Cancer: Past, Present and Future. *Int. J. Mol. Sci.* **2020**, *21* (12), 4325.

(4) Jiao, D.; Yang, S. Overcoming Resistance to Drugs Targeting KRAS(G12C) Mutation. *Innovation* **2020**, *1* (2), No. 100035.

(5) Herdeis, L.; Gerlach, D.; McConnell, D. B.; Kessler, D. Stopping the beating heart of cancer: KRAS reviewed. *Curr. Opin. Struct. Biol.* **2021**, *71*, 136−147.

(6) McCormick, F. Progress in targeting RAS with small molecule drugs. *Biochem. J.* **2019**, *476* (2), 365−374.

(7) Hong, D. S.; Fakih, M. G.; Strickler, J. H.; Desai, J.; Durm, G. A.; Shapiro, G. I.; et al. KRAS(G12C) Inhibition with Sotorasib in Advanced Solid Tumors. *N. Engl. J. Med.* **2020**, *383* (13), 1207−1217.

(8) Skoulidis, F.; Li, B. T.; Dy, G. K.; Price, T. J.; Falchook, G. S.; Wolf, J.; et al. Sotorasib for Lung Cancers with KRAS p.G12C Mutation. *N. Engl. J. Med.* **2021**, *384* (25), 2371−2381.

(9) Awad, M. M.; Liu, S.; Rybkin, I. I.; Arbour, K. C.; Dilly, J.; Zhu, V. W.; et al. Acquired Resistance to KRASG12C Inhibition in Cancer. *N. Eng. J. Med.* **2021**, *384* (25), 2382−2393.

(10) Weinberg, R. A. It took a long, long time: Ras and the race to cure cancer. *Cell* **2024**, *187* (7), 1574−1577.

(11) Bigenzahn, J. W.; Collu, G. M.; Kartnig, F.; Pieraks, M.; Vladimer, G. I.; Heinz, L. X.; et al. LZTR1 is a regulator of RAS ubiquitination and signaling. *Science* **2018**, *362* (6419), 1171−1177.

(12) Steklov, M.; Pandolfi, S.; Baietti, M. F.; Batiuk, A.; Carai, P.; Najm, P.; et al. Mutations in LZTR1 drive human disease by dysregulating RAS ubiquitination. *Science* **2018**, *362* (6419), 1177−1182.

(13) Castel, P.; Cheng, A.; Cuevas-Navarro, A.; Everman, D. B.; Papageorge, A. G.; Simanshu, D. K.; et al. RIT1 oncoproteins escape LZTR1-mediated proteolysis. *Science* **2019**, *363* (6432), 1226−1230.

(14) Abe, T.; Umeki, I.; Kanno, S.-i.; Inoue, S.-i.; Niihori, T.; Aoki, Y. LZTR1 facilitates polyubiquitination and degradation of RAS-GTPases. *Cell Death Differentiation* **2020**, *27* (3), 1023−1035.

(15) Stanton, B. Z.; Chory, E. J.; Crabtree, G. R. Chemically induced proximity in biology and medicine. *Science* **2018**, *359* (6380), No. eaao5902.
(16) Du, X.; Li, Y.; Xia, Y. L.; Ai, S. M.; Liang, J.; Sang, P.; et al. Insights into Protein-Ligand Interactions: Mechanisms, Models, and Methods. *Int. J. Mol. Sci.* **2016**, *17* (2), 144.
(17) Cooley, R.; Kara, N.; Hui, N. S.; Tart, J.; Roustan, C.; George, R.; et al. Development of a cell-free split-luciferase biochemical assay as a tool for screening for inhibitors of challenging protein-protein interaction targets. *Wellcome Open Res.* **2020**, *5*, 20.
(18) Dixon, A. S.; Schwinn, M. K.; Hall, M. P.; Zimmerman, K.; Otto, P.; Lubben, T. H.; et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.* **2016**, *11* (2), 400−408.
(19) Hancock, J. F.; Parton, R. G. Ras plasma membrane signalling platforms. *Biochem. J.* **2005**, *389* (Pt 1), 1−11.
(20) Erlanson, D. A.; McDowell, R. S.; O'Brien, T. Fragment-Based Drug Discovery. *J. Med. Chem.* **2004**, *47* (14), 3463−3482.
(21) Wang, Z. Z.; Shi, X. X.; Huang, G. Y.; Hao, G. F.; Yang, G. F. Fragment-based drug discovery supports drugging 'undruggable' protein-protein interactions. *Trends Biochem. Sci.* **2023**, *48* (6), 539−552.
(22) Murray, C. W.; Rees, D. C. The rise of fragment-based drug discovery. *Nat. Chem.* **2009**, *1* (3), 187−192.
(23) Grygorenko, O. O.; Radchenko, D. S.; Dziuba, I.; Chuprina, A.; Gubina, K. E.; Moroz, Y. S. Generating Multibillion Chemical Space of Readily Accessible Screening Compounds. *iScience* **2020**, *23* (11), No. 101681.
(24) Branon, T. C.; Bosch, J. A.; Sanchez, A. D.; Udeshi, N. D.; Svinkina, T.; Carr, S. A.; et al. Efficient proximity labeling in living cells and organisms with TurboID. *Nat. Biotechnol.* **2018**, *36* (9), 880−887.
(25) Molina, D. M.; Jafari, R.; Ignatushchenko, M.; Seki, T.; Larsson, E. A.; Dan, C.; et al. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science* **2013**, *341* (6141), 84−87.
(26) Maurer, T.; Garrenton, L. S.; Oh, A.; Pitts, K.; Anderson, D. J.; Skelton, N. J.; et al. Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109* (14), 5299−5304.
(27) Sun, Q.; Burke, J. P.; Phan, J.; Burns, M. C.; Olejniczak, E. T.; Waterson, A. G.; et al. Discovery of small molecules that bind to K-Ras and inhibit Sos-mediated activation. *Angew. Chem., Int. Ed.* **2012**, *51* (25), 6140−6143.
(28) Kessler, D.; Gmachl, M.; Mantoulidis, A.; Martin, L. J.; Zoephel, A.; Mayer, M.; et al. Drugging an undruggable pocket on KRAS. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (32), 15823−15829.
(29) Eberhardt, J.; Santos-Martins, D.; Tillack, A. F.; Forli, S. AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. *J. Chem. Inf. Model.* **2021**, *61* (8), 3891−3898.
(30) Gilardi, M.; Wang, Z.; Proietto, M.; Chillà, A.; Calleja-Valera, J. L.; Goto, Y.; et al. Tipifarnib as a Precision Therapy for HRAS-Mutant Head and Neck Squamous Cell Carcinomas. *Mol. Cancer Ther.* **2020**, *19* (9), 1784−1796.
(31) Skoulidis, F.; Li, B. T.; Dy, G. K.; Price, T. J.; Falchook, G. S.; Wolf, J.; et al. Sotorasib for Lung Cancers with KRAS p.G12C Mutation. *N. Engl. J. Med.* **2021**, *384* (25), 2371−2381.
(32) Schlierf, A.; Altmann, E.; Quancard, J.; Jefferson, A. B.; Assenberg, R.; Renatus, M.; et al. Targeted inhibition of the COP9 signalosome for treatment of cancer. *Nat. Commun.* **2016**, *7* (1), No. 13166.
(33) Zeng, M.; Xiong, Y.; Safaee, N.; Nowak, R. P.; Donovan, K. A.; Yuan, C. J.; et al. Exploring Targeted Degradation Strategy for Oncogenic KRAS(G12C). *Cell Chem. Biol.* **2020**, *27* (1), 19−31.e6.
(34) Bond, M. J.; Chu, L.; Nalawansha, D. A.; Li, K.; Crews, C. M. Targeted Degradation of Oncogenic KRAS(G12C) by VHL-Recruiting PROTACs. *ACS Cent Sci.* **2020**, *6* (8), 1367−1375.
(35) Popow, J.; Farnaby, W.; Gollner, A.; Kofink, C.; Fischer, G.; Wurm, M.et al. Targeting cancer with small molecule pan-KRAS degraders *bioRxiv*, 2023.
(36) Simonetta, K. R.; Taygerly, J.; Boyle, K.; Basham, S. E.; Padovani, C.; Lou, Y.; et al. Prospective discovery of small molecule enhancers of an E3 ligase-substrate interaction. *Nat. Commun.* **2019**, *10* (1), No. 1402.
(37) Kozicka, Z.; Suchyta, D. J.; Focht, V.; Kempf, G.; Petzold, G.; Jentzsch, M.; et al. Design principles for cyclin K molecular glue degraders. *Nat. Chem. Biol.* **2024**, *20* (1), 93−102.
(38) Vranken, W. F.; Boucher, W.; Stevens, T. J.; Fogh, R. H.; Pajon, A.; Llinas, M.; et al. The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* **2005**, *59* (4), 687−696.
(39) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009**, *30* (16), 2785−2791.

https://doi.org/10.1021/acschembio.4c00077
ACS Chem. Biol. XXXX, XXX, XXX−XXX

Supporting Information

Identification and Characterization of Novel Small-Molecule Enhancers of the CUL3^{LZTR1} E3 Ligase KRAS Complex

Sophie Piech[1], Sven Brüschweiler[2], Josepha Westphalen[1], Katharina M. Siess[2], Julio García Murias[1], Robert Konrat[2,5], Johannes W. Bigenzahn[1,3,*], Giulio Superti-Furga[1,4,*]

[1] CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria.

[2] MAG-LAB GmbH, 1030 Vienna, Austria.

[3] Department of Laboratory Medicine, Medical University of Vienna, 1090 Vienna, Austria.

[4] Center for Physiology and Pharmacology, Medical University of Vienna, 1090 Vienna Austria.

[5] Department of Structural and Computational Biology, University of Vienna, 1030 Vienna, Austria.

* Correspondence: gsuperti@cemm.oeaw.ac.at , johannes.bigenzahn@meduniwien.ac.at

Requests for materials should be addressed to: Giulio Superti-Furga, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, AKH BT25.3, 1090 Vienna, Austria; Email: gsuperti@cemm.oeaw.ac.at; Telephone: +43 1 40160 70 001; Fax: +43 1 40160 970 000

S1

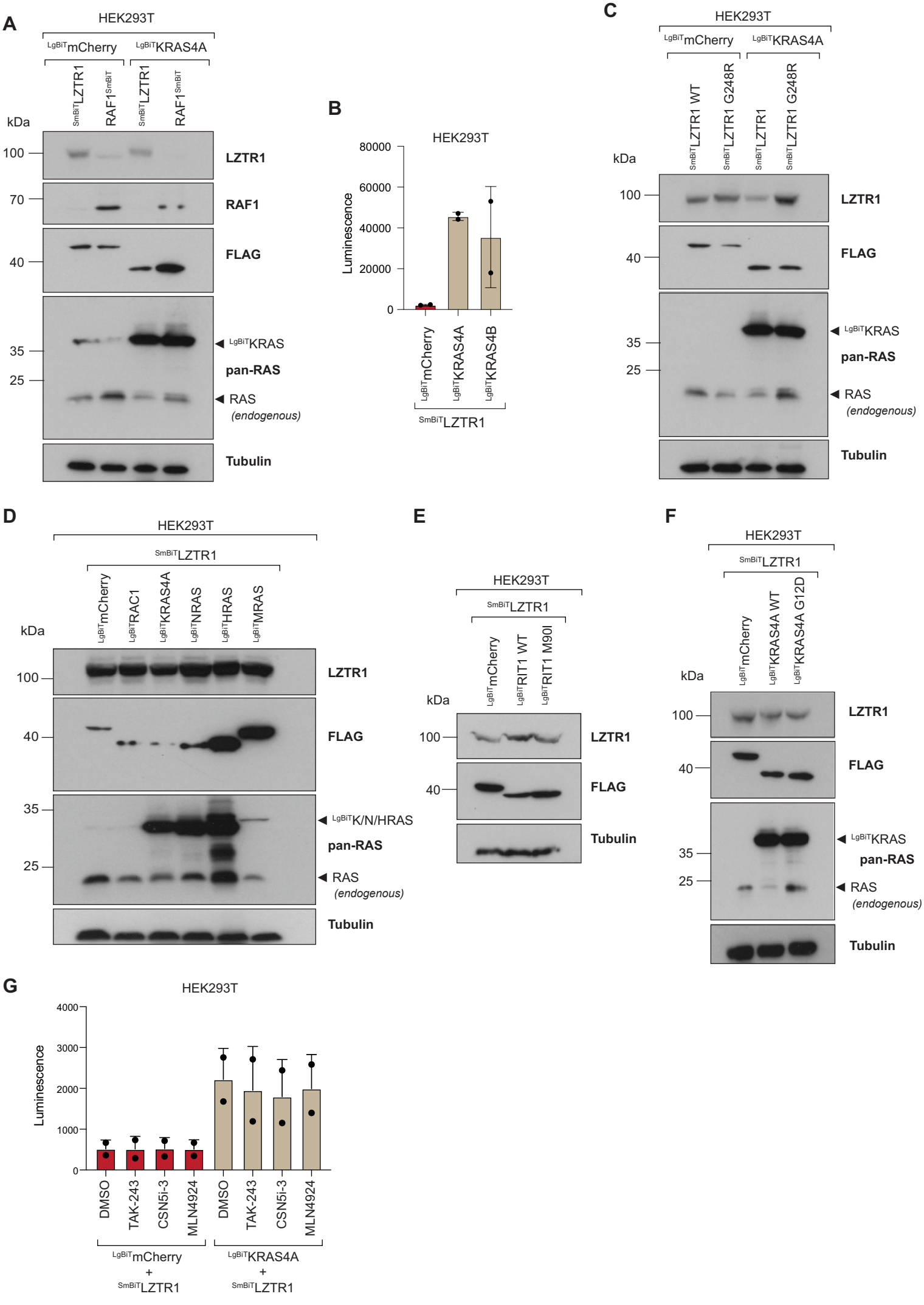
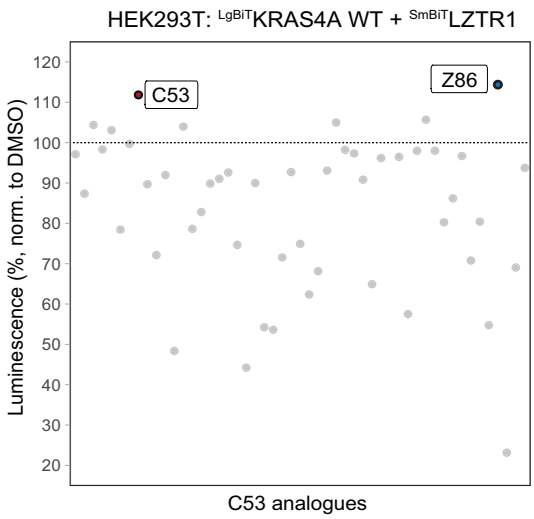

A
HEK293T
LgBiTmCherry
LgBiTKRAS4A
SmBiTLZTR1
RAF1SmBiT
kDa
100
70
40
35
25
LZTR1
RAF1
FLAG
LgBiTKRAS
pan-RAS
RAS
(endogenous)
Tubulin
B
HEK293T
Luminescence
80000
60000
40000
20000
0
LgBiTmCherry
LgBiTKRAS4A
LgBiTKRAS4B
SmBiTLZTR1
C
HEK293T
LgBiTmCherry
LgBiTKRAS4A
SmBiTLZTR1 WT
SmBiTLZTR1 G248R
SmBiTLZTR1
SmBiTLZTR1 G248R
kDa
100
40
35
25
LZTR1
FLAG
LgBiTKRAS
pan-RAS
RAS
(endogenous)
Tubulin
D
HEK293T
SmBiTLZTR1
LgBiTmCherry
LgBiTRAC1
LgBiTKRAS4A
LgBiTNRAS
LgBiTHRAS
LgBiTMRAS
kDa
100
40
35
25
LZTR1
FLAG
LgBiTK/N/HRAS
pan-RAS
RAS
(endogenous)
Tubulin
E
HEK293T
SmBiTLZTR1
LgBiTmCherry
LgBiTRIT1 WT
LgBiTRIT1 M90I
kDa
100
40
LZTR1
FLAG
Tubulin
F
HEK293T
SmBiTLZTR1
LgBiTmCherry
LgBiTKRAS4A WT
LgBiTKRAS4A G12D
kDa
100
40
35
25
LZTR1
FLAG
LgBiTKRAS
pan-RAS
RAS
(endogenous)
Tubulin
G
HEK293T
Luminescence
4000
3000
2000
1000
0
DMSO
TAK-243
CSN5i-3
MLN4924
DMSO
TAK-243
CSN5i-3
MLN4924
LgBiTmCherry
+
SmBiTLZTR1
LgBiTKRAS4A
+
SmBiTLZTR1

Figure S1. Transient expression levels of SLA constructs. (A and C-F) HEK293T cells transiently co-expressing LgBiT and SmBiT fusion constructs were lysed, and samples were run on SDS-PAGE. The expression levels were then analyzed by immunoblotting analysis and anti-RAS antibody was used to detect ^{LgBiT}KRAS4A WT, ^{LgBiT}KRAS4A G12D and endogenous KRAS levels, anti-LZTR1 was used to detect ^{SmBiT}LZTR1 and ^{SmBiT}LZTR1 G248R, anti-RAF1 was used to detect RAF1^{SmBiT}, anti-FLAG was used to detect the FLAG peptide sequence and anti-α-tubulin antibody to detect Tubulin. **(B)** Constructs co-expressing ^{LgBiT}KRAS4A and ^{LgBiT}KRAS4B with ^{SmBiT}LZTR1 were tested by transient expression in HEK293T cells and SLA analysis. The expression of ^{LgBiT}mCherry construct with ^{SmBiT}LZTR1 served as negative control. n=2. **(G)** Treatment of cells co-expressing ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1 or as negative control ^{LgBiT}mCherry and ^{SmBiT}LZTR1 with the E1 inhibitor TAK-243 (1μM), the COP9 signalosome inhibitor CSN5i-3 (2μM), or the neddylation inhibitor MLN4924 (10μM) for 6 hours. n=2.

S2

A


HEK293T: LgBiTKRAS4A WT + SmBiTLZTR1
C53
Z86
Luminescence (%, norm. to DMSO)
C53 analogues

B

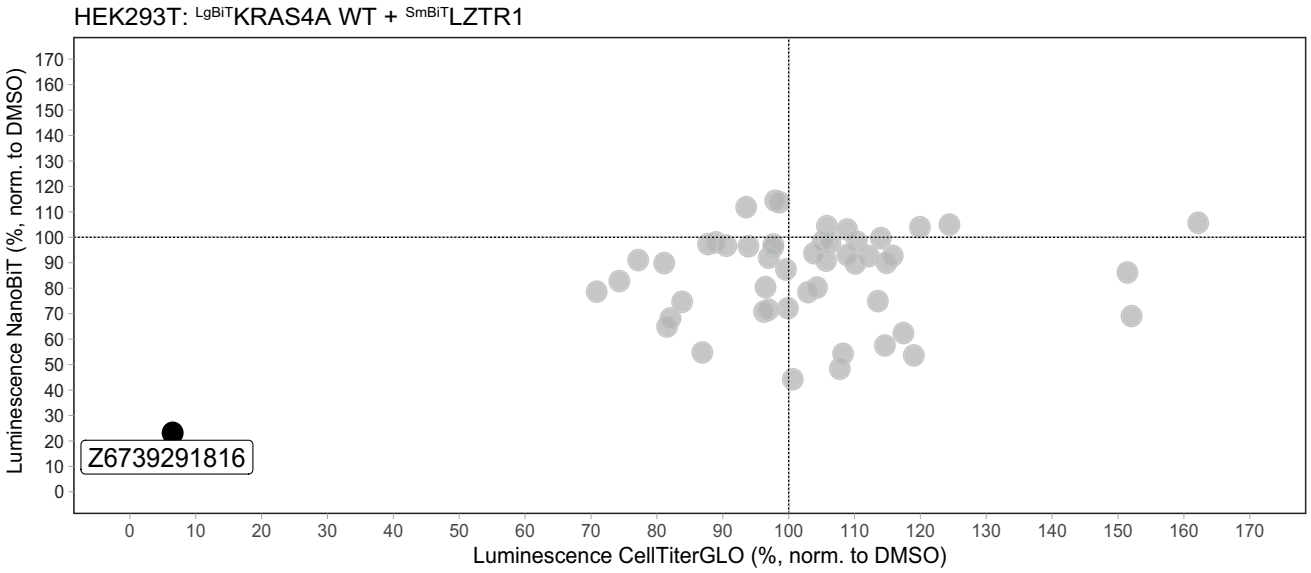
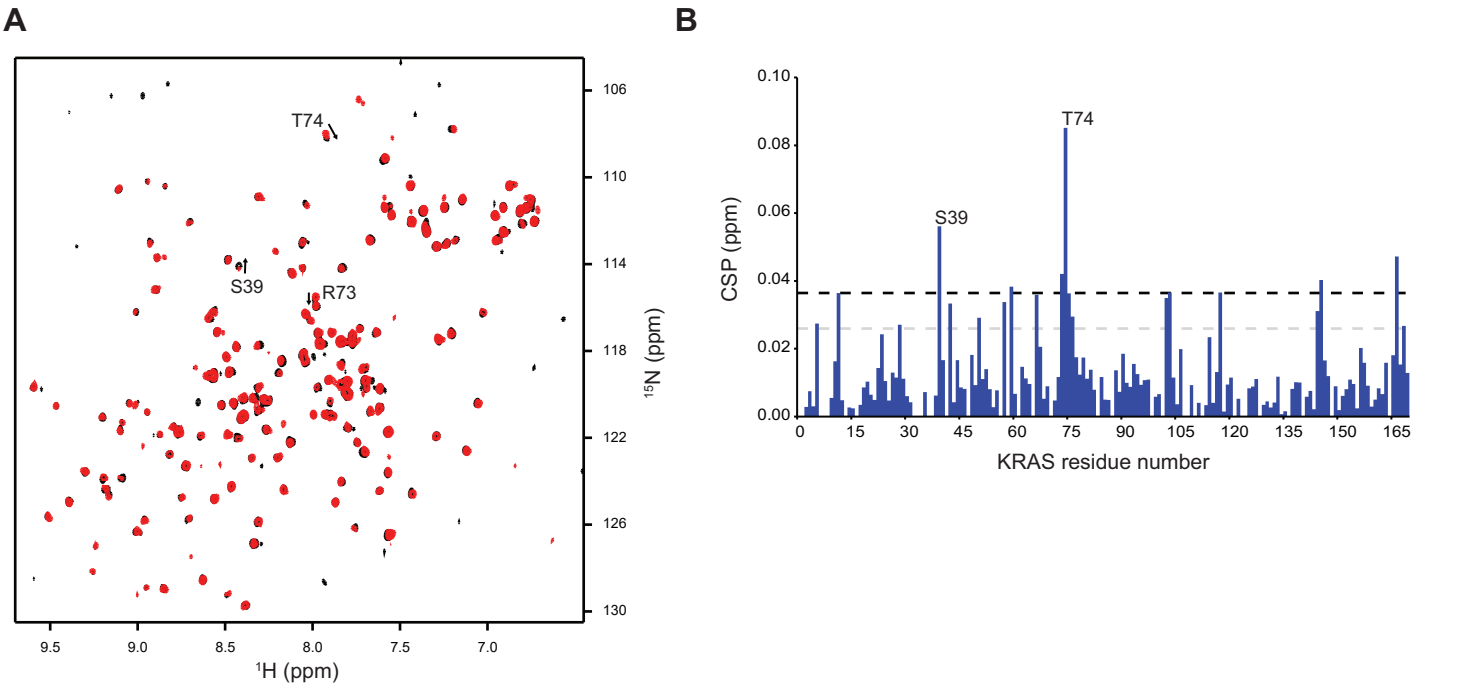

HEK293T: LgBiTKRAS4A WT + SmBiTLZTR1
Z6739291816
Luminescence NanoBiT (%, norm. to DMSO)
Luminescence CellTiterGLO (%, norm. to DMSO)

Figure S2. Screening analog library for characterization and optimization of C53. (A) Luminescence for analog library screened in cells co-expressing ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1 using the SLA assay with C53 and Z86 highlighted. **(B)** Cell viability blotted against SLA luminescence of HEK293T cells co-expressing ^{LgBiT}KRAS4A and ^{SmBiT}LZTR1 treated with the analog library, with Z6739291816 highlighted. Cell viability was analyzed by the CellTiter Glo® assay.

S3


A
T74
S39
R73
106
110
114
118
122
126
130
9.5
9.0
8.5
8.0
7.5
7.0
^{1}H (ppm)
^{15}N (ppm)
B
0.10
0.08
0.06
0.04
0.02
0.00
CSP (ppm)
T74
S39
0
15
30
45
60
75
90
105
120
135
150
165
KRAS residue number

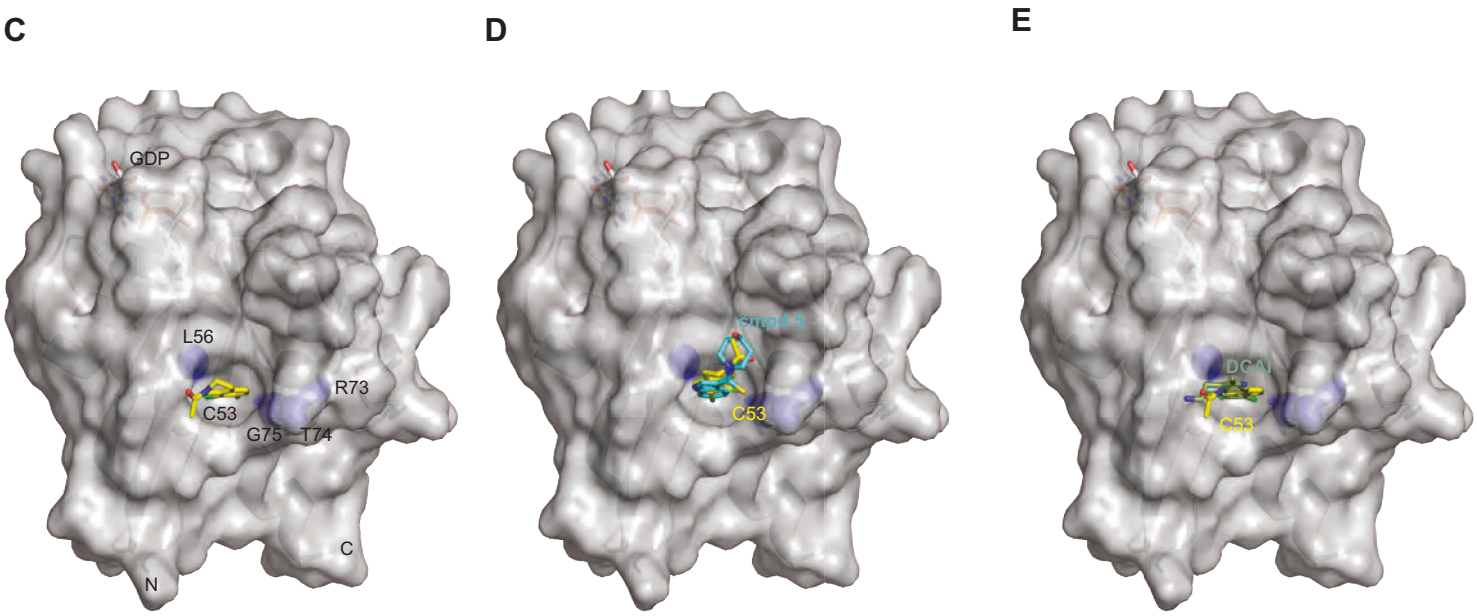

C
GDP
L56
C53
R73
G75
T74
C
N
D
cmpd-5
C53
E
DCAI
C53

Figure S3. NMR-based Z86 binding validation and characterization as well as docking poses of C53. (A) Overlay of ^{1}H-^{15}N HSQC spectra of 50 μM GDP-KRAS G12D in the absence (red) and presence (black) of 2.5 mM Z86. The three largest amide backbone CSPs are indicated by black arrows. **(B)** Histogram showing CSP values for backbone amide groups of ^{15}N labelled GDP-KRAS G12D in the presence of 2.5 mM Z86. Horizontal dotted grey and black lines indicate one and two standard deviations of the CSP values, respectively. Residues without a bar were not assigned or overlapped in the 2D spectra. **(C)** Molecular docking pose of C53, the compound is shown in yellow sticks representation and the protein (PDB ID 4EPW) is represented as grey ribbon and surface representation with nitrogen atoms shown as blue spheres for residues that showed CSP values larger than 2 standard deviations of the mean value of all CSPs in the NMR titration experiment. **(D)** Comparison of the original 4EPW ligand, compound 5, shown as cyan sticks and a docking pose of C53 shown as yellow sticks. **(E)** Comparison of the ligand DCAI (PDB ID 4DST) shown as green sticks and a docking pose of C53 shown as yellow sticks.

3. Discussion

3.1 General Discussion

Given that conventional HTS methods are typically designed to identify molecules that directly inhibit or activate single protein targets, the identification and development of molecular glues in drug discovery remains a challenge. Considering that PPIs are highly complex and molecular glues need to promote interactions between two proteins, standard screening assays may miss potential hits. Moreover, access to structures of target proteins is crucial for structure-guided screening approaches and lead optimization, allowing for the rational design and refinement of compounds that bind specifically and effectively to the desired target. Ultimately, the lack of precedent in the molecular glue field makes it difficult to derive a methodology for development of new agents. This thesis aimed to explore whether small molecules can enhance the interaction between LZTR1 and KRAS, ultimately promoting the degradation of oncogenic KRAS. Specifically, the research aimed to develop a cellular screening assay, identify small molecule modulators, and validate potential compounds that could selectively target and degrade KRAS mutants, such as G12D. Although there are several drugs targeting KRAS and numerous efforts focused on designing PROTACs and identifying molecular glues to target KRAS, no drug discovery efforts to date, as far as we know, have sought to enhance the native interaction between the substrate KRAS and its destined E3 ligase $CUL3^{LZTR1}$. Making use of PPI-dedicated techniques such as the SLA NanoBiT screening assay, thermal shift assay, BioID-based proximity biotinylation and NMR spectroscopy, this thesis offers an experimental primer for the identification of molecular glue-like agents and proximity inducing fragments for native E3 ligase-substrate pairs. Collectively, we were able to identify small fragments, C53 and Z86, that potentiate the LZTR1-KRAS PPI.

Nevertheless, while successful in identifying two fragments, there are several limitations associated with the presented findings that will need further work. This includes the limited potency of the identified small molecules, C53 and Z86, as well as the lack of any observable degradation effect on KRAS. The current limitation of obtaining sufficient purified, properly folded LZTR1 protein and the lack of an available structural model for LZTR1, creates a significant bottle neck and prevent us to conduct any structure-guided fragment optimization and expansion. Therefore future drug discovery efforts, building on the presented work, will require a more comprehensive screening of a larger library of analogs to identify molecules with significantly higher potency. Only with such potent molecules would it be feasible to

assess whether enhanced interactions could not only strengthen the KRAS-LZTR1 PPI, but also induce KRAS degradation.

Overall, this research provides a valuable framework to guide the development of proximity-inducing drugs that could address the significant pool of proteins so far deemed "undruggable". Additionally, aside from NMR spectroscopy, the methodologies employed can be utilized in drug discovery projects involving proteins with unsolved structures, broadening the range of potential drug targets. The next sections will discuss in more detail the implications of the experimental findings outlined in the manuscript.

3.2 SLA for the Identification of PPI Enhancers and Molecular Glues

Quantitative analysis of PPIs is essential for studying protein interactions dynamics and assessing pharmacological effects. The SLA NanoBiT system leverages the small size and bright luminescence of NanoLuc and enables sensitive detection with minimal interference on target proteins. Moreover, the tags are designed for high conformational stability and low intrinsic affinity, minimizing disruption of natural protein interactions (Dixon, Schwinn et al. 2016). SLA NanoBiT has been shown to accurately reflect interaction dynamics, including those of weak or transient interactions, and is effective for studying dynamic processes and drug-induced interactions. To study native protein interactions, such as those between KRAS and LZTR1, and to screen for molecules that enhance this interaction, it's crucial that the tags used have low intrinsic affinity. This allows for an accurate assessment of whether their interaction can be pharmacologically enhanced (Dixon, Schwinn et al. 2016). Overall, the assay has broad potential applications in studying PPIs and protein interaction studies and is suitable for screening molecules that modulate interactions. However, given that many diseases, including cancer, are driven by the dysregulation of PPIs, there are several noteworthy assays available that enable the monitoring and stabilization of these interactions. In this section of the discussion, I will outline and compare several alternative biochemical assays.

The NanoBRET, like the SLA NanoBiT assays, is a NanoLuc Luciferase-based method to study PPIs, yet they differ significantly in their mechanisms. NanoBRET measures energy transfer between two proteins tagged with NanoLuc (donor) and HaloTag (acceptor), allowing it to detect proximity and subtle changes in protein interaction dynamics (Machleidt, Woodroofe et al. 2015). In contrast, NanoBiT relies on the reconstitution of the NanoLuc enzyme, the LgBiT protein body and SmBiT tag, when proteins interact. Additionally,

NanoBRET is more sensitive to small changes in distance between proteins, while NanoBiT is designed for robust detection of direct and binary interactions (Lay, Kilpatrick et al. 2023).

In terms of drug discovery applications, NanoBRET excels in measuring dynamic interactions and the stabilization of PPIs, including molecular glues. This makes it particularly useful for studying complex interactions, real-time monitoring and protein kinetics (Vickery, Virta et al. 2024). NanoBiT, being a binary system, is more suited for high-throughput screening of compounds, as it provides clear, robust signals when proteins bind. However, it lacks the sensitivity to detect dynamic changes that NanoBRET offers. For instance, the NanoBRET system is highly valuable for therapeutic interventions targeting hub proteins like 14-3-3σ, which interact with numerous client proteins (Vickery, Virta et al. 2024). Vickery et al. evaluate molecular glues using the NanoBRET assay, focusing on their ability to target 14-3-3σ and enhance the stability of its client proteins, including CRAF, TAZ, and ERα (Vickery, Virta et al. 2024). This includes the calculation of kinetic parameters, such as binding affinity (EC_{50} values) and fold changes, providing a quantitative assessment of how these interactions evolve over time in response to the respective molecular glues (CRAF-02, TAZ-02, and ERα-02) (Vickery, Virta et al. 2024). This example highlights the suitability of NanoBRET for studying binding affinities, kinetics, and monitoring changes in protein conformation or interactions over time. Furthermore, when utilizing live-cell assays like NanoBiT and NanoBRET, it is important to note that these assays are unsuitable for non-cell membrane-permeable compounds (Cooley, Kara et al. 2020). As a result, these assays are capable of selectively filtering out molecules that are non-cell permeable.

Nevertheless, when comparing the SLA NanoBiT system to the NanoBRET system for studying the interaction between LZTR1 and KRAS, several important factors must be considered. Unpublished data suggests that attaching larger tags to both the RAS and LZTR1 proteins can significantly disrupt functional RAS proteostatic regulation via the $CUL3^{LZTR1}$ E3 ligase complex. In the case of KRAS, we observed that degradation is highly sensitive to the presence of larger tags, which impairs the degradation process. Although the interaction with LZTR1 is maintained, it no longer results in KRAS degradation. Additionally, attaching larger tags to LZTR1 interferes not only with protein degradation but also with PPI. Given this, the NanoBRET system, which uses NanoLuc (19 kDa) and HaloTag (36 kDa) (Machleidt, Woodroofe et al. 2015), may be less suitable for studying the LZTR1-KRAS interaction due to the tag sizes. It can be argued that the NanoBiT system, with its smaller tags, would currently be a better fit for studying this protein interface.

Another prominent assay utilized in the TPD drug discovery field is the HiBiT protein tagging system. This system employs an 11-amino-acid peptide tag, that in contrast to the

SmBiT tag utilized in the SLA, shows high binding affinity for its complementary NanoLuc partner, LgBiT (Landreman 9/2017). Optimized specifically for quantifying protein degradation, the HiBiT tag enables precise luminescent measurements by linking target protein abundance to luminescence loss, which directly correlates with degradation (Landreman 9/2017). Given the strong affinity between the HiBiT and LgBiT components, this system is particularly effective for monitoring degradation dynamics without interference from additional protein interactions (Landreman 9/2017). Both NanoBiT and HiBiT are based on based on luciferase complementation, with HiBiT using the high affinity HiBiT tag and the other one the low affinity SmBiT tag. However, HiBiT is optimized for detecting PPIs, thus, serving primarily as a tool for real-time interaction studies (Nagashima, Primadharsini et al. 2023). For future directions of this project, the NanoBiT and HiBiT assays could be employed in tandem to achieve two primary objectives: to generate comprehensive data on PPIs, and to support lead optimization efforts by assessing the potential of optimized fragments to induce KRAS degradation.

Another noteworthy proximity assay for studying PPIs and screening for molecular glues is the Homogeneous Time-Resolved Fluorescence (HTRF) assay. This well-established technique allows for the investigation of molecular interactions by measuring the proximity between two labeled molecules. HTRF is based on fluorescence resonance energy transfer (FRET) with time-resolved measurement, where energy is transferred from a donor to an acceptor fluorophore when they are in proximity, typically as a result of molecular binding (Degorce, Card et al. 2009). In the context of molecular glue screening, HTRF is particularly valuable because it can be used in high-throughput screening (HTS) to identify small molecules that stabilize protein-protein interactions. Additionally, the HTRF assay is typically highly sensitive and robust, making it suitable for miniaturization into 384- and 1536-well plate formats (Degorce, Card et al. 2009). Soini and colleagues utilized the HTRF assay to screen 20,000 small molecules, resulting in the identification of 16 novel stabilizers of the 14-3-3/SLP76 interaction (Soini, Redhead et al. 2021). These molecules represent potential molecular glues that could be further developed to modulate T-cell receptor signaling by enhancing SLP76 degradation, offering a new avenue for treating autoimmune and inflammatory conditions (Soini, Redhead et al. 2021). One of the primary limitations of the HTRF assay is the potential for false positives, due to the absence of positive controls, as there were no previously identified stabilizers for the 14-3-3/SLP76 interaction (Soini, Redhead et al. 2021). The study, therefore, had to introduce a matched FRET pair counter-screen to mitigate this issue by reducing hits that did not specifically stabilize the PPI (Soini, Redhead et al. 2021). This represents a similar limitation encountered in the scientific project discussed within this thesis. Given the absence of a positive control, meaning a LZTR1/KRAS interaction enhancer, it remains challenging to evaluate whether the identified enhancers are

approaching saturation in this assay setting or if further optimization could yield more potent candidates.

Another significant example is the screening assay outlined by Simonetta and colleagues, who used a fluorescence polarization (FP)-based binding assay to characterize the interaction between the ubiquitin ligase β-TrCP with its native substrate, β-catenin. Successively, they identified the small molecule, NRX-252114, which enhance the β-catenin/ β-TrCP interaction (Simonetta, Taygerly et al. 2019). Specifically, it utilized β-catenin phosphodegron peptides (residues 17–48) and recombinant β-TrCP/Skp1 complex (Simonetta, Taygerly et al. 2019). It is important to note that phosphorylation of specific serine residues, such as Ser33 and Ser37, allows β-catenin to be recognized by β-TrCP, which tags it for degradation. Mutations in these phosphorylation sites, such as Ser37 mutations, impair the interaction with β-TrCP, leading to β-catenin stabilization and potential oncogenic signaling (Simonetta, Taygerly et al. 2019). Accordingly, the FP assay enabled the quantitative monitoring of the interaction, with phosphodegron peptides showing varying affinities depending on their phosphorylation status (Simonetta, Taygerly et al. 2019). Given that the FP assay is sensitive to small changes in molecular interactions, such as phosphorylation states, it is very well suited when studying proteins like β-catenin with multiple post-translational modifications. Consequently, this approach does not only indicate whether proteins are binding, but it also provides quantitative information about the strength and affinity of the PPI. This adds a crucial layer of detail, by monitoring changes in conditions beyond just detecting binding events, vis-à-vis the NanoBiT assay. However, a limitation of the assay is that it typically requires purified proteins to ensure that the interaction between the target proteins or peptides is measured accurately, free from interference by other cellular components. Therefore, purified recombinant β-TrCP/Skp1 complexes and β-catenin phosphodegron peptides were used for the FP assay (Simonetta, Taygerly et al. 2019).

Overall, the NanoBiT assay has several limitations, including the fact that its readout primarily captures the binding event (binary outcome: ON/ OFF) between two proteins, making it less suitable for quantitative assessment of small molecules or kinetic studies. Additionally, it does not monitor changes in conditions such as phosphorylation or other post-translational modifications. Nevertheless, the small tag size and the ability to conduct the assay in live cells make it highly compatible for studying KRAS and LZTR1.

3.3 Techniques and Approaches for Validating Target Engagement in the Absence of Protein Structures

In drug discovery, validating target engagement is a critical step to confirm that a molecule effectively interacts with its intended protein target. Based on a 3D protein structure, via methods like X-ray crystallography, NMR, or cryo-EM, it is possible to gain insight into which parts of the protein interact with the ligand, including the mapping of binding sites. However, when protein structures are not available, alternative methods must be employed to ensure reliable validation. Due to the absence of structural data on the LZTR1 protein, we were unable to adequately assess ternary complex formation or directly evaluate LZTR1 target engagement with C53 or Z86 *in vitro*. As a result, evidence for target engagement predominantly relies on cellular biochemical techniques, including cellular thermal shift assay (also known as CETSA). To this end, we assessed fragment engagement with KRAS and LZTR1 by monitoring the thermostability of proteins in the presence or absence of C53 and Z86. The assay demonstrated that treatment with C53 or Z86 stabilized endogenous RAS at specific temperature ranges, indicative of direct target engagement. Interestingly, no such stabilization was observed for LZTR1.

The thermal shift is used to assess the interaction of small molecules with their protein targets in a cellular environment. It operates on the principle that ligand binding to a protein increases the protein's thermal stability, preventing it from denaturing and aggregating when heated. The assay then measures the amount of soluble (non-aggregated) protein at different temperatures, using techniques like western blotting, luminescence assays or unbiased mass spectrometry-based proteomics (Molina, Jafari et al. 2013, Jafari, Almqvist et al. 2014). The resulting thermal shift, indicating increased thermal stability, provides evidence of ligand binding to the target protein (Jafari, Almqvist et al. 2014). Consequently, this experimental approach is highly accessible and straightforward, requiring only cells expressing the POI, along with a relatively simple detection method, such as immunoblotting. Additionally, the assay can be applied to various biological contexts, including cell lysates, intact cells, and even tissues, offering a direct measure of target engagement within the natural cellular environment (Jafari, Almqvist et al. 2014). Nevertheless, there are several limitations including limited quantitative affinity estimation. Although it provides qualitative insights into ligand binding and protein stabilization, it does not directly allow for quantitative determination of ligand affinity or binding constants (Jafari, Almqvist et al. 2014). Moreover, it is challenging for proteins that require interaction partners or allosteric regulators to bind ligands effectively. This is particularly true for membrane proteins, which may require complex optimization and screening for appropriate detergents to solubilize without disturbing the protein structure

(Jafari, Almqvist et al. 2014). Furthermore, a notable limitation of this approach is that protein stabilization alone may not directly indicate binding of the ligand, as it could be an indirect result of a biological process triggered by ligand addition (Hashimoto, Girardi et al. 2018).

Some examples from the literature where the thermal shift assay has been successfully applied include studies by Hashimoto *et al.* and Dvorak *et al.* On account of high-resolution structural information being limited or unavailable, Hashimoto and colleagues, utilized the thermal shift assay to investigate small molecule interactions with solute carrier proteins (SLCs) (Hashimoto, Girardi et al. 2018). Specifically, they applied thermal shift assay to two SLCs, SLC16A1 (MCT1) and SLC1A2 (EAAT2), using inhibitors like AZD3965 and AR-C155858 to demonstrate stabilization of the target proteins (Hashimoto, Girardi et al. 2018). This approach allowed to assess chemical engagement with SLCs without needing detailed structural information, making it a powerful method for studying SLC-ligand interactions under physiological conditions. Similarly, Dvorak *et al.* employed thermal shift assay to confirm the binding of identified small molecule, slCeMM1, to the SLC16A3 transporter (Dvorak, Casiraghi et al. 2023). Specifically, cell lysates expressing a version of SLC16A3 tagged with the afore discussed HiBiT tag were treated with the test compounds (Dvorak, Casiraghi et al. 2023). When a small molecule bound to SLC16A3, it caused a thermal stabilization of the protein, which was detected by an increase in luminescence from the reconstituted luciferase enzyme (Dvorak, Casiraghi et al. 2023). This luminescence indicated a successful thermal shift, indicating ligand binding to the transporter, which was crucial for orthogonally validating their engagement with the target transporter (Dvorak, Casiraghi et al. 2023). This serves as a representative example of how thermal shift can be employed not only through immunoblotting but also in conjunction with the HiBiT luciferase system.

Wang et al. studied erianin, a dual inhibitor of MEK1/2 and CRAF, that suppresses activation of the MAPK signaling cascade. Thermal shift assay experiments confirmed that erianin binds to and stabilizes its target proteins within cells by increasing their thermal resistance (Wang, Jia et al. 2023). The assay involved heating transfected HEK293T cells expressing these proteins and measuring protein stability using immunoblotting. Mutations at key residues (MEK1: Lys97, Met146; MEK2: Lys101, Met150; CRAF: Lys375) reduced this stabilizing effect, demonstrating that these residues are crucial for erianin’s binding. This validated erianin's role in inhibiting MEK1/2 and CRAF (Wang, Jia et al. 2023). In summary, the thermal shift assay is a useful tool for assessing ligand binding and protein stabilization in a cellular environment, its limitations, such as difficulties with quantitative affinity measurements and the need for optimization with complex proteins, highlight the need for complementary methods to fully understand ligand-protein interactions.

To date, there are no known studies that utilize CETSA to evaluate the target engagement or binding of a compound to endogenous or overexpressed KRAS in a cellular context. However, there is a study that employed thermal shift assays using purified recombinant KRAS proteins, including both WT and mutant forms (KRAS G12D, G12V, G13D and Q61H), to assess compound binding and stability. The assay was designed to study the binding and thermal stability effects of ACA-14 on KRAS in a controlled, *in vitro* system rather than within the cellular environment (Pagba, Gupta et al. 2022).

In the case of LZTR1, the challenge of obtaining sufficient quantities of soluble protein for *in vitro* biochemical analyses led us to adopt CETSA as the most practical and effective approach for evaluating interactions. This method leveraged the availability of cell lines that overexpressed both LZTR1 and KRAS. Additionally, the presence of specific antibodies capable of detecting changes in protein thermal stability via immunoblotting further facilitated this approach. CETSA proved to be invaluable for assessing target engagement within the cellular environment, providing qualitative insights into the binding and stabilization effects of candidate molecules on KRAS. However, the reliance on this method also posed certain limitations. Specifically, the absence of purified protein precluded us from conducting quantitative pharmacokinetic and thermodynamic assessments of the interactions of molecules C53 and Z86, such as determining precise binding affinities or dissociation rates.

3.4 BioID to evaluate chemically induced protein and E3 ligase interactions

Proximity biotinylation technology (BioID) has successfully been used in the past to identify canonical and novel interaction partners of the main RAS GTPase family. BioID was used by fusing the BirA* enzyme, a mutant biotin ligase, to various RAS proteins, including KRAS4A, KRAS4B, NRAS, and HRAS, and express these fusion proteins in an inducible manner in K-562 CML cells (Bigenzahn, Collu et al. 2018). This approach enabled the biotinylation of nearby proteins, which were subsequently identified through mass spectrometry, and led to the discovery of LZTR1 as a proteostatic regulator of all four RAS proteins (Bigenzahn, Collu et al. 2018). In recent years, BioID has also become a powerful technique in molecular glue drug discovery. BioID can be utilized to assess the action of molecular glues or PROTACs by enabling the identification of drug-induced interactions between a target protein and an E3 ligase (Yamanaka, Horiuchi et al. 2022). Specifically, a modified form of the BirA biotin ligase can be fused to an E3 ligase, which allows for proximity-dependent biotinylation of any protein that comes into close contact due to the action of molecular glues or PROTACs (Yamanaka, Horiuchi et al. 2022). These biotinylated proteins

can then be pulled down and identified via mass spectrometry or immunoblotting, revealing neo-substrate interactions that are mediated by the molecule. This approach is particularly useful for detecting transient, weak, or drug-induced PPIs, helping to elucidate how molecular glues or PROTACs facilitate targeted protein degradation (Yamanaka, Horiuchi et al. 2022). In this manner, Yamanaka et al. employed BioID combined with mass spectrometry to identify proteins that interact with the E3 ubiquitin ligase CRBN in the presence of molecular glues like IMiDs and PROTACs (Yamanaka, Horiuchi et al. 2022). By fusing AirID (ancestral BirA) to CRBN, the study enabled selective biotinylation of proteins in close proximity to CRBN when molecular glues facilitated their interaction (Yamanaka, Horiuchi et al. 2022). These biotinylated proteins were then enriched and identified using mass spectrometry, revealing both known and novel CRBN substrates, such as IKZF1, IKZF3, and ZMYM2 (Yamanaka, Horiuchi et al. 2022). This approach provides a powerful method and can be broadly applied to other E3 ligases and molecular glues to study drug-induced PPIs.

For this work, I adapted the miniTurboID system, which offers more rapid and efficient proximity biotinylation compared to first generation BioID. BioID utilizes the BirA* enzyme to biotinylate nearby proteins requiring prolonged biotin incubation times (usually around 18-24 hours), making it useful for detecting weak or transient interactions, though it may lead to nonspecific labeling (Branon, Bosch et al. 2018). In contrast, miniTurboID is a faster and more efficient version of BirA*, capable of labeling proteins within minutes (10-60 minutes), which reduces nonspecific background and is ideal for capturing rapid or dynamic interactions (Branon, Bosch et al. 2018). Specifically, these features allowed us to capture the enhanced interaction of miniTurboID-tagged KRAS with endogenous LZTR1 via C53.

BioID-tagged KRAS demonstrated the ability to recruit endogenous LZTR1 in the presence of C53, providing strong validation that the compound's action is effective within the endogenous LZTR1 setting. However, the lack of observed degradation presents a notable discrepancy that warrants further investigation. BioID could be instrumental in addressing this inconsistency by enabling a detailed proteomic analysis to identify whether additional components, such as CUL3 or other E3 ligase-associated factors, are similarly recruited in the presence of C53. Additionally, a time-course treatment could be employed to monitor the kinetics of recruitment, while treatments with MLN4924 could elucidate the impact on recruitment of UPS components. Thereby proteomics could provide deeper insights into the molecular mechanisms and clarify whether there is a ternary complex formation in the presence of C53. Together, these approaches could help rationalize the observed outcomes and refine our understanding of the LZTR1/KRAS interaction in response to C53.

For instance, Namura and colleagues utilized chemoproteomics to discover and validate a covalent molecular glue degrader, EN450, that induces proximity between the E2 ubiquitin-conjugating enzyme UBE2D1 and the oncogenic transcription factor NFKB1 (King, Cho et al. 2023). EN450 bears a cysteine-reactive warhead and through proximity-labeling proteomics, they identified key interaction sites and demonstrated that EN450 covalently engages UBE2D1, forming a ternary complex with NFKB1. Precisely, the study identified the Cullin E3 ligase complex CUL4A/RBX1/NEDD8 as being involved in the mechanism of action. Moreover, quantitative proteomics confirmed the degradation of NFKB1 as the primary target, illustrating EN450's mechanism as a molecular glue and its anti-proliferative effects in leukemia cells (King, Cho et al. 2023). Although covalent ligands can enhance the BioID approach by offering deeper insights into protein interactions, the method remains highly effective on its own, relying solely on biotinylation.

Incorporating the split-Turbo or split-BioID method could be a valuable additional experiment. Split-Turbo, composed of an N-terminal and C-terminal fragment, and -BioID is divided into two inactive fragments that only become active when brought together by specific PPI’s, offering higher specificity in labeling. Thereby, it labels exclusively in areas where the two split fragments are reconstituted, reduces off-target labeling (Cho, Branon et al. 2020). This makes split-Turbo and -BioID more suitable for applications needing higher specificity in terms of protein interactions or subcellular compartments (Cho, Branon et al. 2020).

This technique is particularly valuble at identifying complexes with defined localization and composition, as described in Schopp and colleagues. Here, researchers fused one half of the split-BioID enzyme to the FKBP protein and the other half to FRB (Schopp, Amaya Ramirez et al. 2017). In the absence of rapamycin, these two halves remain separated and inactive. However, upon adding rapamycin, FKBP and FRB bind tightly, bringing the split halves of the BioID enzyme into close proximity and activating biotinylation (Schopp, Amaya Ramirez et al. 2017). This example highlights how split-BioID serves as a conditional proteomics method to identify dynamic protein complexes with precise spatial and temporal definition (Schopp, Amaya Ramirez et al. 2017).

In a similar approach, we could attempt to fuse the N-terminal fragment (8 kDa) to LZTR1 and the C-terminal fragment (27 kDa) (Cho, Branon et al. 2020) to KRAS, expressing these constructs stably within cells to assess potential enhancement of biotinylation in the presence of C53. However, since LZTR1 is known to exhibit limited tolerance for tags, it is essential to verify that the tag size does not interfere with the LZTR1-KRAS PPI.

3.5 Conclusion and Future Prospects

In summary, this thesis identified novel small-molecule fragments, C53 and Z86, that enhance the PPI between KRAS and LZTR1. Using a high-throughput split-luciferase-based reporter assay, the study showed that these fragments increase LZTR1's natural tendency to bind KRAS in a dose-dependent manner. Further validation through BioID, thermal shift assays, and NMR spectroscopy confirmed the physical interaction with KRAS and enhanced recruitment of endogenous LZTR1. These results indicate that small molecules can chemically enhance the LZTR1-KRAS interaction and, with further optimization, may improve the activity of the $CUL3^{LZTR1}$ E3 ligase complex. By strengthening the native KRAS-LZTR1 protein-protein interface, this approach holds potential for promoting KRAS degradation, presenting a promising new strategy to target RAS-driven cancers and addressing issues related to hyperactive RAS signaling.

Future research should focus on optimizing the identified fragments, C53 and Z86, to enhance their potency, and potentially identify additional new fragments. This endeavor would necessitate a substantially expanded drug discovery campaign, including the screening of a broader range of C53 analogs and fragment collections. Ultimately, obtaining a structural model of LZTR1 will be crucial for performing SAR optimization on these fragments, as it will provide deeper insights into the KRAS-LZTR1 interaction interface and allows for more tailored fragment improvement and growth.

The continued exploration and development of molecular glues hold immense promise for transforming the landscape of targeting RAS mutant cancers, offering innovative strategies to modulate challenging PPI and unlock new therapeutic opportunities.

References

Abe, T., I. Umeki, S.-i. Kanno, S.-i. Inoue, T. Niihori and Y. Aoki (2020). "LZTR1 facilitates polyubiquitination and degradation of RAS-GTPases." Cell Death & Differentiation **27**(3): 1023-1035.

Ahearn, I., M. Zhou and M. R. Philips (2018). "Posttranslational Modifications of RAS Proteins." Cold Spring Harb Perspect Med **8**(11).

Alabi, S. B. and C. M. Crews (2021). "Major advances in targeted protein degradation: PROTACs, LYTACs, and MADTACs." J Biol Chem **296**: 100647.

An, J., C. M. Ponthier, R. Sack, J. Seebacher, M. B. Stadler, K. A. Donovan and E. S. Fischer (2017). "pSILAC mass spectrometry reveals ZFP91 as IMiD-dependent substrate of the CRL4CRBN ubiquitin ligase." Nature Communications **8**(1): 15398.

Azmi, A. S. and P. A. Philip (2017). Chapter 14 - Targeting Rho, Rac, CDC42 GTPase Effector p21 Activated Kinases in Mutant K-Ras-Driven Cancer. Conquering RAS. A. S. Azmi. Boston, Academic Press**:** 251-270.

Bahar, M. E., H. J. Kim and D. R. Kim (2023). "Targeting the RAS/RAF/MAPK pathway for cancer therapy: from mechanism to clinical studies." Signal Transduction and Targeted Therapy **8**(1): 455.

Bard, J. A. M., E. A. Goodall, E. R. Greene, E. Jonsson, K. C. Dong and A. Martin (2018). "Structure and Function of the 26S Proteasome." Annu Rev Biochem **87**: 697-724.

Basso, A. D., P. Kirschmeier and W. R. Bishop (2006). "Lipid posttranslational modifications. Farnesyl transferase inhibitors." J Lipid Res **47**(1): 15-31.

Békés, M., D. R. Langley and C. M. Crews (2022). "PROTAC targeted protein degraders: the past is prologue." Nature Reviews Drug Discovery **21**(3): 181-200.

Benet, L. Z., C. M. Hosey, O. Ursu and T. I. Oprea (2016). "BDDCS, the Rule of 5 and drugability." Adv Drug Deliv Rev **101**: 89-98.

Bhela, I. P., A. Ranza, F. C. Balestrero, M. Serafini, S. Aprile, R. M. C. Di Martino, F. Condorelli and T. Pirali (2022). "A Versatile and Sustainable Multicomponent Platform for the Synthesis of Protein Degraders: Proof-of-Concept Application to BRD4-Degrading PROTACs." Journal of Medicinal Chemistry **65**(22): 15282-15299.

Bigenzahn, J. W., G. M. Collu, F. Kartnig, M. Pieraks, G. I. Vladimer, L. X. Heinz, V. Sedlyarov, F. Schischlik, A. Fauster, M. Rebsamen, K. Parapatics, V. A. Blomen, A. C. Müller, G. E. Winter, R. Kralovics, T. R. Brummelkamp, M. Mlodzik and G. Superti-Furga (2018). "LZTR1 is a regulator of RAS ubiquitination and signaling." Science **362**(6419): 1171-1177.

Bon, M., A. Bilsland, J. Bower and K. McAulay (2022). "Fragment-based drug discovery-the importance of high-quality molecule libraries." Mol Oncol **16**(21): 3761-3777.

Bond, M. J., L. Chu, D. A. Nalawansha, K. Li and C. M. Crews (2020). "Targeted Degradation of Oncogenic KRAS(G12C) by VHL-Recruiting PROTACs." ACS Cent Sci **6**(8): 1367-1375.

Branon, T. C., J. A. Bosch, A. D. Sanchez, N. D. Udeshi, T. Svinkina, S. A. Carr, J. L. Feldman, N. Perrimon and A. Y. Ting (2018). "Efficient proximity labeling in living cells and organisms with TurboID." Nature Biotechnology **36**(9): 880-887.

Bricelj, A., C. Steinebach, R. Kuchta, M. Gütschow and I. Sosič (2021). "E3 Ligase Ligands in Successful PROTACs: An Overview of Syntheses and Linker Attachment Points." Front Chem **9**: 707317.

Carbery, A., R. Skyner, F. von Delft and C. M. Deane (2022). "Fragment Libraries Designed to Be Functionally Diverse Recover Protein Binding Information More Efficiently Than Standard Structurally Diverse Libraries." Journal of Medicinal Chemistry **65**(16): 11404-11413.

Castel, P., A. Cheng, A. Cuevas-Navarro, D. B. Everman, A. G. Papageorge, D. K. Simanshu, A. Tankka, J. Galeas, A. Urisman and F. McCormick (2019). "RIT1 oncoproteins escape LZTR1-mediated proteolysis." Science **363**(6432): 1226-1230.

Chamberlain, P. P. and L. G. Hamann (2019). "Development of targeted protein degradation therapeutics." Nat Chem Biol **15**(10): 937-944.

Chen, S., R. S. Vedula, A. Cuevas-Navarro, B. Lu, S. J. Hogg, E. Wang, S. Benbarche, K. Knorr, W. J. Kim, R. F. Stanley, H. Cho, C. Erickson, M. Singer, D. Cui, S. Tittley, B. H. Durham, T. S. Pavletich, E. Fiala, M. F. Walsh, D. Inoue, S. Monette, J. Taylor, N. Rosen, F. McCormick, R. C. Lindsley, P. Castel and O. Abdel-Wahab (2022). "Impaired Proteolysis of Noncanonical RAS Proteins Drives Clonal Hematopoietic Transformation." Cancer Discov **12**(10): 2434-2453.

Chenette, E. J. and C. J. Der (2011). 5 - Lipid Modification of Ras Superfamily GTPases: Not Just Membrane Glue. The Enzymes. F. Tamanoi, C. A. Hrycyna and M. O. Bergo, Academic Press. **29:** 59-95.

Cho, K. F., T. C. Branon, N. D. Udeshi, S. A. Myers, S. A. Carr and A. Y. Ting (2020). "Proximity labeling in mammalian cells with TurboID and split-TurboID." Nature Protocols **15**(12): 3971-3999.

Cieślak, M. and M. Słowianek (2023). "Cereblon-Recruiting PROTACs: Will New Drugs Have to Face Old Challenges?" Pharmaceutics **15**(3).

Cooley, R., N. Kara, N. S. Hui, J. Tart, C. Roustan, R. George, D. C. Hancock, B. F. Binkowski, K. V. Wood, M. Ismail and J. Downward (2020). "Development of a cell-free split-luciferase biochemical assay as a tool for screening for inhibitors of challenging protein-protein interaction targets." Wellcome Open Res **5**: 20.

Cowan, A. D. and A. Ciulli (2022). "Driving E3 Ligase Substrate Specificity for Targeted Protein Degradation: Lessons from Nature and the Laboratory." Annual Review of Biochemistry **91**(1): 295-319.

Degorce, F., A. Card, S. Soh, E. Trinquet, G. P. Knapik and B. Xie (2009). "HTRF: A technology tailored for drug discovery - a review of theoretical aspects and recent applications." Curr Chem Genomics **3**: 22-32.

Dixon, A. S., M. K. Schwinn, M. P. Hall, K. Zimmerman, P. Otto, T. H. Lubben, B. L. Butler, B. F. Binkowski, T. Machleidt, T. A. Kirkland, M. G. Wood, C. T. Eggers, L. P. Encell and K. V. Wood (2016). "NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells." ACS Chem Biol **11**(2): 400-408.

Dvorak, V., A. Casiraghi, C. Colas, A. Koren, T. Tomek, F. Offensperger, A. Rukavina, G. Tin, E. Hahn, S. Dobner, F. Frommelt, A. Boeszoermenyi, V. Bernada, J. T. Hannich, G. F. Ecker, G. E. Winter, S. Kubicek and G. Superti-Furga (2023). "Paralog-dependent isogenic cell assay cascade generates highly selective SLC16A3 inhibitors." Cell Chemical Biology **30**(8): 953-964.e959.

Evans, D. G., L. M. Messiaen, W. D. Foulkes, R. E. A. Irving, A. J. Murray, C. Perez-Becerril, B. Rivera, D. M. McDonald-McGinn, D. A. Stevenson and M. J. Smith (2021). "Typical 22q11.2 deletion syndrome appears to confer a reduced risk of schwannoma." Genet Med **23**(9): 1779-1782.

Fang, Z., C. B. Marshall, J. C. Yin, M. T. Mazhab-Jafari, G. M. Gasmi-Seabrook, M. J. Smith, T. Nishikawa, Y. Xu, B. G. Neel and M. Ikura (2016). "Biochemical Classification of Disease-associated Mutants of RAS-like Protein Expressed in Many Tissues (RIT1)." J Biol Chem **291**(30): 15641-15652.

Fanigliulo, D., P. E. Lazzerini, P. L. Capecchi, C. Ulivieri, C. T. Baldari and F. Laghi-Pasini (2015). "Clinically-relevant cyclosporin and rapamycin concentrations enhance regulatory T cell function to a similar extent but with different mechanisms: an in-vitro study in healthy humans." Int Immunopharmacol **24**(2): 276-284.

Flick, K. and P. Kaiser (2012). "Protein degradation and the stress response." Semin Cell Dev Biol **23**(5): 515-522.

Frattini, V., V. Trifonov, J. M. Chan, A. Castano, M. Lia, F. Abate, S. T. Keir, A. X. Ji, P. Zoppoli, F. Niola, C. Danussi, I. Dolgalev, P. Porrati, S. Pellegatta, A. Heguy, G. Gupta, D. J. Pisapia, P. Canoll, J. N. Bruce, R. E. McLendon, H. Yan, K. Aldape, G. Finocchiaro, T. Mikkelsen, G. G. Privé, D. D. Bigner, A. Lasorella, R. Rabadan and A. Iavarone (2013). "The integrated landscape of driver genomic alterations in glioblastoma." Nat Genet **45**(10): 1141-1149.

Fuchs, O. (2023). "Targeting cereblon in hematologic malignancies." Blood Rev **57**: 100994.

Furukawa, A., T. Konuma, S. Yanaka and K. Sugase (2016). "Quantitative analysis of protein–ligand interactions by NMR." Progress in Nuclear Magnetic Resonance Spectroscopy **96**: 47-57.

Gaali, S., R. Gopalakrishnan, Y. Wang, C. Kozany and F. Hausch (2011). "The chemical biology of immunophilin ligands." Curr Med Chem **18**(35): 5355-5379.

Gadd, M. S., A. Testa, X. Lucas, K. H. Chan, W. Chen, D. J. Lamont, M. Zengerle and A. Ciulli (2017). "Structural basis of PROTAC cooperative recognition for selective protein degradation." Nat Chem Biol **13**(5): 514-521.

Geiger, T. M., S. C. Schäfer, J. K. Dreizler, M. Walz and F. Hausch (2022). "Clues to molecular glues." Current Research in Chemical Biology **2**: 100018.

Gilardi, M., Z. Wang, M. Proietto, A. Chillà, J. L. Calleja-Valera, Y. Goto, M. Vanoni, M. R. Janes, Z. Mikulski, A. Gualberto, A. A. Molinolo, N. Ferrara, J. S. Gutkind and F. Burrows (2020). "Tipifarnib as a Precision Therapy for HRAS-Mutant Head and Neck Squamous Cell Carcinomas." Mol Cancer Ther **19**(9): 1784-1796.

Golan, T., E. Z. Khvalevsky, A. Hubert, R. M. Gabai, N. Hen, A. Segal, A. Domb, G. Harari, E. B. David, S. Raskin, Y. Goldes, E. Goldin, R. Eliakim, M. Lahav, Y. Kopleman, A. Dancour, A. Shemi and E. Galun (2015). "RNAi therapy targeting KRAS in combination with chemotherapy for locally advanced pancreatic cancer patients." Oncotarget **6**(27): 24560-24570.

Hajduk, P. J. and J. Greer (2007). "A decade of fragment-based drug design: strategic advances and lessons learned." Nature Reviews Drug Discovery **6**(3): 211-219.

Haluska, P., G. K. Dy and A. A. Adjei (2002). "Farnesyl transferase inhibitors as anticancer agents." European Journal of Cancer **38**(13): 1685-1700.

Hashimoto, M., E. Girardi, R. Eichner and G. Superti-Furga (2018). "Detection of Chemical Engagement of Solute Carrier Proteins by a Cellular Thermal Shift Assay." ACS Chemical Biology **13**(6): 1480-1486.

Haupt, Y., R. Maya, A. Kazaz and M. Oren (1997). "Mdm2 promotes the rapid degradation of p53." Nature **387**(6630): 296-299.

Hausch, F., C. Kozany, M. Theodoropoulou and A. K. Fabian (2013). "FKBPs and the Akt/mTOR pathway." Cell Cycle **12**(15): 2366-2370.

Henneberg, L. T., J. Singh, D. M. Duda, K. Baek, D. Yanishevski, P. J. Murray, M. Mann, S. S. Sidhu and B. A. Schulman (2023). "Activity-based profiling of cullin–RING E3 networks by conformation-specific probes." Nature Chemical Biology **19**(12): 1513-1523.

Hillig, R. C., B. Sautier, J. Schroeder, D. Moosmayer, A. Hilpmann, C. M. Stegmann, N. D. Werbeck, H. Briem, U. Boemer, J. Weiske, V. Badock, J. Mastouri, K. Petersen, G. Siemeister, J. D. Kahmann, D. Wegener, N. Böhnke, K. Eis, K. Graham, L. Wortmann, F. von Nussbaum and B. Bader (2019). "Discovery of potent SOS1 inhibitors that block RAS activation via disruption of the RAS-SOS1 interaction." Proc Natl Acad Sci U S A **116**(7): 2551-2560.

Hiroaki, H. and D. Kohda (2018). Protein–Ligand Interactions Studied by NMR. Experimental Approaches of NMR Spectroscopy: Methodology and Application to Life Science and Materials Science. J. The Nuclear Magnetic Resonance Society of. Singapore, Springer Singapore**:** 579-600.

Hobbs, B., J. Drant and M. P. Williamson (2022). "The measurement of binding affinities by NMR chemical shift perturbation." J Biomol NMR **76**(4): 153-163.

Hobbs, G. A., C. J. Der and K. L. Rossman (2016). "RAS isoforms and mutations in cancer at a glance." Journal of Cell Science **129**(7): 1287-1292.

Hofmann, M. H., M. Gmachl, J. Ramharter, F. Savarese, D. Gerlach, J. R. Marszalek, M. P. Sanderson, D. Kessler, F. Trapani, H. Arnhof, K. Rumpel, D. A. Botesteanu, P. Ettmayer, T. Gerstberger, C. Kofink, T. Wunberg, A. Zoephel, S. C. Fu, J. L. Teh, J. Böttcher, N. Pototschnig, F. Schachinger, K. Schipany, S. Lieb, C. P. Vellano, J. C. O'Connell, R. L. Mendes, J. Moll, M. Petronczki, T. P. Heffernan, M. Pearson, D. B. McConnell and N. Kraut (2021). "BI-3406, a Potent and Selective SOS1-KRAS Interaction Inhibitor, Is Effective in KRAS-Driven Cancers through Combined MEK Inhibition." Cancer Discov **11**(1): 142-157.

Hopkins, A. L., G. M. Keserü, P. D. Leeson, D. C. Rees and C. H. Reynolds (2014). "The role of ligand efficiency metrics in drug discovery." Nature Reviews Drug Discovery **13**: 105.

Hughes, S. J. and A. Ciulli (2017). "Molecular recognition of ternary complexes: a new dimension in the structure-guided design of chemical degraders." Essays Biochem **61**(5): 505-516.

Hyun, S. and D. Shin (2021). "Small-Molecule Inhibitors and Degraders Targeting KRAS-Driven Cancers." Int J Mol Sci **22**(22).

Ito, T., H. Ando, T. Suzuki, T. Ogura, K. Hotta, Y. Imamura, Y. Yamaguchi and H. Handa (2010). "Identification of a primary target of thalidomide teratogenicity." Science **327**(5971): 1345-1350.

Jaeger, M. G. and G. E. Winter (2020). "Expanding the Degradable Proteome: Designing PROTACs by the Book." Cell Chem Biol **27**(1): 14-16.

Jafari, R., H. Almqvist, H. Axelsson, M. Ignatushchenko, T. Lundbäck, P. Nordlund and D. M. Molina (2014). "The cellular thermal shift assay for evaluating drug target interactions in cells." Nature Protocols **9**(9): 2100-2122.

Jaumot, M., J. Yan, J. Clyde-Smith, J. Sluimer and J. F. Hancock (2002). "The Linker Domain of the Ha-Ras Hypervariable Region Regulates Interactions with Exchange Factors, Raf-1 and Phosphoinositide 3-Kinase*." Journal of Biological Chemistry **277**(1): 272-278.

Jhoti, H., G. Williams, D. C. Rees and C. W. Murray (2013). "The 'rule of three' for fragment-based drug discovery: where are we now?" Nature Reviews Drug Discovery **12**(8): 644-644.

Khan, Z. M., A. M. Real, W. M. Marsiglia, A. Chow, M. E. Duffy, J. R. Yerabolu, A. P. Scopton and A. C. Dar (2020). "Structural basis for the action of the drug trametinib at KSR-bound MEK." Nature **588**(7838): 509-514.

Khrenova, M. G., B. L. Grigorenko, A. B. Kolomeisky and A. V. Nemukhin (2015). "Hydrolysis of Guanosine Triphosphate (GTP) by the Ras·GAP Protein Complex: Reaction Mechanism and Kinetic Scheme." The Journal of Physical Chemistry B **119**(40): 12838-12845.

King, E. A., Y. Cho, N. S. Hsu, D. Dovala, J. M. McKenna, J. A. Tallarico, M. Schirle and D. K. Nomura (2023). "Chemoproteomics-enabled discovery of a covalent molecular glue degrader targeting NF-κB." Cell Chemical Biology **30**(4): 394-402.e399.

Kirsch, P., A. M. Hartman, A. K. H. Hirsch and M. Empting (2019). "Concepts and Core Principles of Fragment-Based Drug Design." Molecules **24**(23).

Kleiger, G. and T. Mayor (2014). "Perilous journey: a tour of the ubiquitin-proteasome system." Trends Cell Biol **24**(6): 352-359.

Ko, J. M., J.-M. Kim, G.-H. Kim and H.-W. Yoo (2008). "PTPN11, SOS1, KRAS, and RAF1 gene analysis, and genotype–phenotype correlation in Korean patients with Noonan syndrome." Journal of Human Genetics **53**(11): 999-1006.

Konopleva, M., G. Martinelli, N. Daver, C. Papayannidis, A. Wei, B. Higgins, M. Ott, J. Mascarenhas and M. Andreeff (2020). "MDM2 inhibition: an important step forward in cancer therapy." Leukemia **34**(11): 2858-2874.

Kozicka, Z., D. J. Suchyta, V. Focht, G. Kempf, G. Petzold, M. Jentzsch, C. Zou, C. Di Genua, K. A. Donovan, S. Coomar, M. Cigler, C. Mayor-Ruiz, J. L. Schmid-Burgk, D. Häussinger, G. E. Winter, E. S. Fischer, M. Słabicki, D. Gillingham, B. L. Ebert and N. H. Thomä (2023). "Design principles for cyclin K molecular glue degraders." Nat Chem Biol.

Kozicka, Z. and N. H. Thomä (2021). "Haven't got a glue: Protein surface variation for the design of molecular glue degraders." Cell Chem Biol.

Ladygina, N., B. R. Martin and A. Altman (2011). Chapter 1 - Dynamic Palmitoylation and the Role of DHHC Proteins in T Cell Activation and Anergy. Advances in Immunology. F. W. Alt, Academic Press. **109:** 1-44.

Landreman, A. a. E., C. (9/2017). "Quantifying Protein Abundance at Endogenous Levels."

Latif, T., N. Chauhan, R. Khan, A. Moran and S. Z. Usmani (2012). "Thalidomide and its analogues in the treatment of Multiple Myeloma." Exp Hematol Oncol **1**(1): 27.

Lay, C. S., L. E. Kilpatrick, P. D. Craggs and S. J. Hill (2023). "Use of NanoBiT and NanoBRET to characterise interleukin-23 receptor dimer formation in living cells." Br J Pharmacol **180**(11): 1444-1459.

Lipinski, C. A. (2000). "Drug-like properties and the causes of poor solubility and poor permeability." J Pharmacol Toxicol Methods **44**(1): 235-249.

Liu, J. O. (2023). "Targeting cancer with molecular glues." Science **381**(6659): 729-730.

Longo, J. F. and S. L. Carroll (2022). Chapter Ten - The RASopathies: Biology, genetics and therapeutic options. Advances in Cancer Research. J. P. O'Bryan and G. A. Piazza, Academic Press. **153:** 305-341.

LoRusso, P., N. Yamamoto, M. R. Patel, S. A. Laurie, T. M. Bauer, J. Geng, T. Davenport, M. Teufel, J. Li, M. Lahmar and M. M. Gounder (2023). "The MDM2-p53 Antagonist Brigimadlin (BI 907828) in Patients with Advanced or Metastatic Solid Tumors: Results of a Phase Ia, First-in-Human, Dose-Escalation Study." Cancer Discov **13**(8): 1802-1813.

Lu, G., R. E. Middleton, H. Sun, M. Naniong, C. J. Ott, C. S. Mitsiades, K. K. Wong, J. E. Bradner and W. G. Kaelin, Jr. (2014). "The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins." Science **343**(6168): 305-309.

Lu, G., L. Wang, J. Zhou, W. Liu and H. M. Shen (2021). "A Destiny for Degradation: Interplay between Cullin-RING E3 Ligases and Autophagy." Trends Cell Biol **31**(6): 432-444.

Lu, H., Q. Zhou, J. He, Z. Jiang, C. Peng, R. Tong and J. Shi (2020). "Recent advances in the development of protein–protein interactions modulators: mechanisms and clinical trials." Signal Transduction and Targeted Therapy **5**(1): 213.

Machleidt, T., C. C. Woodroofe, M. K. Schwinn, J. Méndez, M. B. Robers, K. Zimmerman, P. Otto, D. L. Daniels, T. A. Kirkland and K. V. Wood (2015). "NanoBRET—A Novel BRET Platform for the Analysis of Protein–Protein Interactions." ACS Chemical Biology **10**(8): 1797-1804.

Mao, Z., H. Xiao, P. Shen, Y. Yang, J. Xue, Y. Yang, Y. Shang, L. Zhang, X. Li, Y. Zhang, Y. Du, C.-C. Chen, R.-T. Guo and Y. Zhang (2022). "KRAS(G12D) can be targeted by potent inhibitors via formation of salt bridge." Cell Discovery **8**(1): 5.

Marais, R., Y. Light, H. F. Paterson and C. J. Marshall (1995). "Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation." Embo j **14**(13): 3136-3145.

Martinez Molina, D., R. Jafari, M. Ignatushchenko, T. Seki, E. A. Larsson, C. Dan, L. Sreekumar, Y. Cao and P. Nordlund (2013). "Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay." Science **341**(6141): 84-87.

Maurer, T., L. S. Garrenton, A. Oh, K. Pitts, D. J. Anderson, N. J. Skelton, B. P. Fauber, B. Pan, S. Malek, D. Stokoe, M. J. Ludlam, K. K. Bowman, J. Wu, A. M. Giannetti, M. A. Starovasnik, I. Mellman, P. K. Jackson, J. Rudolph, W. Wang and G. Fang (2012). "Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity." Proc Natl Acad Sci U S A **109**(14): 5299-5304.

McCormick, F. (2019). "Progress in targeting RAS with small molecule drugs." Biochem J **476**(2): 365-374.

McGregor, L. M., M. L. Jenkins, C. Kerwin, J. E. Burke and K. M. Shokat (2017). "Expanding the Scope of Electrophiles Capable of Targeting K-Ras Oncogenes." Biochemistry **56**(25): 3178-3183.

Melchert, M. and A. List (2007). "The thalidomide saga." Int J Biochem Cell Biol **39**(7-8): 1489-1499.

Metzger, M. B., J. N. Pruneda, R. E. Klevit and A. M. Weissman (2014). "RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination." Biochim Biophys Acta **1843**(1): 47-60.

Molina, D. M., R. Jafari, M. Ignatushchenko, T. Seki, E. A. Larsson, C. Dan, L. Sreekumar, Y. Cao and P. Nordlund (2013). "Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay." Science **341**(6141): 84-87.

Molina, J. R. and A. A. Adjei (2006). "The Ras/Raf/MAPK pathway." J Thorac Oncol **1**(1): 7-9.

Moore, A. R., S. C. Rosenberg, F. McCormick and S. Malek (2020). "RAS-targeted therapies: is the undruggable drugged?" Nat Rev Drug Discov **19**(8): 533-552.

Morelli, X., R. Bourgeas and P. Roche (2011). "Chemical and structural lessons from recent successes in protein–protein interaction inhibition (2P2I)." Current Opinion in Chemical Biology **15**(4): 475-481.

Motta, M., M. Fidan, E. Bellacchio, F. Pantaleoni, K. Schneider-Heieck, S. Coppola, G. Borck, L. Salviati, M. Zenker, I. C. Cirstea and M. Tartaglia (2019). "Dominant Noonan syndrome-causing LZTR1 mutations specifically affect the Kelch domain substrate-recognition surface and enhance RAS-MAPK signaling." Hum Mol Genet **28**(6): 1007-1022.

Mukhopadhyay, S., M. G. Vander Heiden and F. McCormick (2021). "The Metabolic Landscape of RAS-Driven Cancers from biology to therapy." Nat Cancer **2**(3): 271-283.

Mureddu, L. G. and G. W. Vuister (2022). "Fragment-Based Drug Discovery by NMR. Where Are the Successes and Where can It Be Improved?" Front Mol Biosci **9**: 834453.

Nacak, T. G., K. Leptien, D. Fellner, H. G. Augustin and J. Kroll (2006). "The BTB-kelch protein LZTR-1 is a novel Golgi protein that is degraded upon induction of apoptosis." J Biol Chem **281**(8): 5065-5071.

Nagashima, S., P. P. Primadharsini, T. Nishiyama, M. Takahashi, K. Murata and H. Okamoto (2023). "Development of a HiBiT-tagged reporter hepatitis E virus and its utility as an antiviral drug screening platform." J Virol **97**(9): e0050823.

Nakadai, M. and S. Tomida (2020). "Diameter Is a Key 3D Characteristic for Assessments of Efficient Inhibitors of Protein–Protein Interactions." Journal of Chemical Information and Modeling **60**(10): 4785-4790.

Nakayama, K. I. and K. Nakayama (2006). "Ubiquitin ligases: cell-cycle control and cancer." Nature Reviews Cancer **6**(5): 369-381.

Nandi, D., P. Tahiliani, A. Kumar and D. Chandu (2006). "The ubiquitin-proteasome system." J Biosci **31**(1): 137-155.

Nussinov, R., C. J. Tsai, M. Chakrabarti and H. Jang (2016). "A New View of Ras Isoforms in Cancers." Cancer Res **76**(1): 18-23.

Oláh, J., T. Szénási, A. Lehotzky, V. Norris and J. Ovádi (2022). "Challenges in Discovering Drugs That Target the Protein-Protein Interactions of Disordered Proteins." Int J Mol Sci **23**(3).

Ostrem, J. M., U. Peters, M. L. Sos, J. A. Wells and K. M. Shokat (2013). "K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions." Nature **503**(7477): 548-551.

Pagba, C. V., A. K. Gupta, A. K. Naji, D. van der Hoeven, K. Churion, X. Liang, J. Jakubec, M. Hook, Y. Zuo, M. Martinez de Kraatz, J. A. Frost and A. A. Gorfe (2022). "KRAS Inhibitor that Simultaneously Inhibits Nucleotide Exchange Activity and Effector Engagement." ACS Bio & Med Chem Au **2**(6): 617-626.

Petroski, M. D. and R. J. Deshaies (2005). "Function and regulation of cullin–RING ubiquitin ligases." Nature Reviews Molecular Cell Biology **6**(1): 9-20.

Pickart, C. M. (2001). "Mechanisms underlying ubiquitination." Annu Rev Biochem **70**: 503-533.

Piotrowski, A., J. Xie, Y. F. Liu, A. B. Poplawski, A. R. Gomes, P. Madanecki, C. Fu, M. R. Crowley, D. K. Crossman, L. Armstrong, D. Babovic-Vuksanovic, A. Bergner, J. O. Blakeley, A. L. Blumenthal, M. S. Daniels, H. Feit, K. Gardner, S. Hurst, C. Kobelka, C. Lee, R. Nagy, K. A. Rauen, J. M. Slopis, P. Suwannarat, J. A. Westman, A. Zanko, B. R. Korf and L. M. Messiaen (2014). "Germline loss-of-function mutations in LZTR1 predispose to an inherited disorder of multiple schwannomas." Nat Genet **46**(2): 182-187.

Popow, J., W. Farnaby, A. Gollner, C. Kofink, G. Fischer, M. Wurm, D. Zollman, A. Wijaya, N. Mischerikow, C. Hasenoehrl, P. Prokofeva, H. Arnhof, S. Arce-Solano, S. Bell, G. Boeck, E. Diers, A. B. Frost, J. Goodwin-Tindall, J. Karolyi-Oezguer, S. Khan, T. Klawatsch, M. Koegl, R. Kousek, B. Kratochvil, K. Kropatsch, A. A. Lauber, R. McLennan, S. Olt, D. Peter, O. Petermann, V. Roessler, P. Stolt-Bergner, P. Strack, E. Strauss, N. Trainor, V. Vetma, C. Whitworth, S. Zhong, J. Quant, H. Weinstabl, B. Kuster, P. Ettmayer and A. Ciulli (2024). "Targeting cancer with small-molecule pan-KRAS degraders." Science **385**(6715): 1338-1347.

Prior, I. A. and J. F. Hancock (2012). "Ras trafficking, localization and compartmentalized signalling." Semin Cell Dev Biol **23**(2): 145-153.

Prior, I. A., P. D. Lewis and C. Mattos (2012). "A comprehensive survey of Ras mutations in cancer." Cancer Res **72**(10): 2457-2467.

Rehman, W., L. M. Arfons and H. M. Lazarus (2011). "The rise, fall and subsequent triumph of thalidomide: lessons learned in drug development." Ther Adv Hematol **2**(5): 291-308.

Reymond, J.-L., R. van Deursen, L. C. Blum and L. Ruddigkeit (2010). "Chemical space as a source for new drugs." MedChemComm **1**(1): 30-38.

Roberts, P. J. and C. J. Der (2007). "Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer." Oncogene **26**(22): 3291-3310.

Ross, S. J., A. S. Revenko, L. L. Hanson, R. Ellston, A. Staniszewska, N. Whalley, S. K. Pandey, M. Revill, C. Rooney, L. K. Buckett, S. K. Klein, K. Hudson, B. P. Monia, M. Zinda, D. C. Blakey, P. D. Lyne and A. R. Macleod (2017). "Targeting KRAS-dependent tumors with AZD4785, a high-affinity therapeutic antisense oligonucleotide inhibitor of KRAS." Sci Transl Med **9**(394).

Santos, R., O. Ursu, A. Gaulton, A. P. Bento, R. S. Donadi, C. G. Bologa, A. Karlsson, B. Al-Lazikani, A. Hersey, T. I. Oprea and J. P. Overington (2017). "A comprehensive map of molecular drug targets." Nature reviews. Drug discovery **16**(1): 19-34.

Scheffzek, K., M. R. Ahmadian, W. Kabsch, L. Wiesmüller, A. Lautwein, F. Schmitz and A. Wittinghofer (1997). "The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants." Science **277**(5324): 333-338.

Schlessinger, J. (2000). "Cell signaling by receptor tyrosine kinases." Cell **103**(2): 211-225.

Schmaler, T. and W. Dubiel (2010). "Control of Deneddylation by the COP9 Signalosome." Subcell Biochem **54**: 57-68.

Schopp, I. M., C. C. Amaya Ramirez, J. Debeljak, E. Kreibich, M. Skribbe, K. Wild and J. Béthune (2017). "Split-BioID a conditional proteomics approach to monitor the composition of spatiotemporally defined protein complexes." Nature Communications **8**(1): 15690.

Schulze, C. J., K. J. Seamon, Y. Zhao, Y. C. Yang, J. Cregg, D. Kim, A. Tomlinson, T. J. Choy, Z. Wang, B. Sang, Y. Pourfarjam, J. Lucas, A. Cuevas-Navarro, C. Ayala-Santos, A. Vides, C. Li, A. Marquez, M. Zhong, V. Vemulapalli, C. Weller, A. Gould, D. M. Whalen, A. Salvador, A. Milin, M. Saldajeno-Concar, N. Dinglasan, A. Chen, J. Evans, J. E. Knox, E. S. Koltun, M. Singh, R. Nichols, D. Wildes, A. L. Gill, J. A. M. Smith and P. Lito (2023). "Chemical remodeling of a cellular chaperone to target the active state of mutant KRAS." Science **381**(6659): 794-799.

Sedrani, R. C., J. Kallen, L. M. Martín Cabrejas, C. D. Papageorgiou, F. Senia, S. Rohrbach, D. Wagner, B. Thai, A.-M. Jutzi Eme, J. France, L. Oberer, G. Rihs, G. Zenke and J. Wagner (2003). "Sanglifehrin-cyclophilin interaction: degradation work, synthetic macrocyclic analogues, X-ray crystal structure, and binding data." Journal of the American Chemical Society **125 13**: 3849-3859.

Shangary, S. and S. Wang (2008). "Targeting the MDM2-p53 interaction for cancer therapy." Clin Cancer Res **14**(17): 5318-5324.

Simanshu, D. K., D. V. Nissley and F. McCormick (2017). "RAS Proteins and Their Regulators in Human Disease." Cell **170**(1): 17-33.

Simonetta, K. R., J. Taygerly, K. Boyle, S. E. Basham, C. Padovani, Y. Lou, T. J. Cummins, S. L. Yung, S. K. von Soly, F. Kayser, J. Kuriyan, M. Rape, M. Cardozo, M. A. Gallop, N. F. Bence,

P. A. Barsanti and A. Saha (2019). "Prospective discovery of small molecule enhancers of an E3 ligase-substrate interaction." Nat Commun **10**(1): 1402.

Singh, M., B. Tam and B. Akabayov (2018). "NMR-Fragment Based Virtual Screening: A Brief Overview." Molecules **23**(2).

Skoulidis, F., B. T. Li, G. K. Dy, T. J. Price, G. S. Falchook, J. Wolf, A. Italiano, M. Schuler, H. Borghaei, F. Barlesi, T. Kato, A. Curioni-Fontecedro, A. Sacher, A. Spira, S. S. Ramalingam, T. Takahashi, B. Besse, A. Anderson, A. Ang, Q. Tran, O. Mather, H. Henary, G. Ngarmchamnanrith, G. Friberg, V. Velcheti and R. Govindan (2021). "Sotorasib for Lung Cancers with KRAS p.G12C Mutation." N Engl J Med **384**(25): 2371-2381.

Słabicki, M., H. Yoon, J. Koeppel, L. Nitsch, S. S. Roy Burman, C. Di Genua, K. A. Donovan, A. S. Sperling, M. Hunkeler, J. M. Tsai, R. Sharma, A. Guirguis, C. Zou, P. Chudasama, J. A. Gasser, P. G. Miller, C. Scholl, S. Fröhling, R. P. Nowak, E. S. Fischer and B. L. Ebert (2020). "Small-molecule-induced polymerization triggers degradation of BCL6." Nature **588**(7836): 164-168.

Soini, L., M. Redhead, M. Westwood, S. Leysen, J. Davis and C. Ottmann (2021). "Identification of molecular glues of the SLP76/14-3-3 protein–protein interaction." RSC Medicinal Chemistry **12**(9): 1555-1564.

Soucy, T. A., P. G. Smith, M. A. Milhollen, A. J. Berger, J. M. Gavin, S. Adhikari, J. E. Brownell, K. E. Burke, D. P. Cardin, S. Critchley, C. A. Cullis, A. Doucette, J. J. Garnsey, J. L. Gaulin, R. E. Gershman, A. R. Lublinsky, A. McDonald, H. Mizutani, U. Narayanan, E. J. Olhava, S. Peluso, M. Rezaei, M. D. Sintchak, T. Talreja, M. P. Thomas, T. Traore, S. Vyskocil, G. S. Weatherhead, J. Yu, J. Zhang, L. R. Dick, C. F. Claiborne, M. Rolfe, J. B. Bolen and S. P. Langston (2009). "An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer." Nature **458**(7239): 732-736.

Souers, A. J., J. D. Leverson, E. R. Boghaert, S. L. Ackler, N. D. Catron, J. Chen, B. D. Dayton, H. Ding, S. H. Enschede, W. J. Fairbrother, D. C. Huang, S. G. Hymowitz, S. Jin, S. L. Khaw, P. J. Kovar, L. T. Lam, J. Lee, H. L. Maecker, K. C. Marsh, K. D. Mason, M. J. Mitten, P. M. Nimmer, A. Oleksijew, C. H. Park, C. M. Park, D. C. Phillips, A. W. Roberts, D. Sampath, J. F. Seymour, M. L. Smith, G. M. Sullivan, S. K. Tahir, C. Tse, M. D. Wendt, Y. Xiao, J. C. Xue, H. Zhang, R. A. Humerickhouse, S. H. Rosenberg and S. W. Elmore (2013). "ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets." Nat Med **19**(2): 202-208.

Spradlin, J. N., E. Zhang and D. K. Nomura (2021). "Reimagining Druggability Using Chemoproteomic Platforms." Acc Chem Res **54**(7): 1801-1813.

Steklov, M., S. Pandolfi, M. F. Baietti, A. Batiuk, P. Carai, P. Najm, M. Zhang, H. Jang, F. Renzi, Y. Cai, L. Abbasi Asbagh, T. Pastor, M. De Troyer, M. Simicek, E. Radaelli, H. Brems, E. Legius, J. Tavernier, K. Gevaert, F. Impens, L. Messiaen, R. Nussinov, S. Heymans, S. Eyckerman and A. A. Sablina (2018). "Mutations in LZTR1 drive human disease by dysregulating RAS ubiquitination." Science **362**(6419): 1177-1182.

Stevers, L. M., E. Sijbesma, M. Botta, C. MacKintosh, T. Obsil, I. Landrieu, Y. Cau, A. J. Wilson, A. Karawajczyk, J. Eickhoff, J. Davis, M. Hann, G. O'Mahony, R. G. Doveston, L. Brunsveld and C. Ottmann (2018). "Modulators of 14-3-3 Protein-Protein Interactions." Journal of medicinal chemistry **61**(9): 3755-3778.

Struntz, N. B., A. Chen, A. Deutzmann, R. M. Wilson, E. Stefan, H. L. Evans, M. A. Ramirez, T. Liang, F. Caballero, M. H. E. Wildschut, D. V. Neel, D. B. Freeman, M. S. Pop, M. McConkey, S. Muller, B. H. Curtin, H. Tseng, K. R. Frombach, V. L. Butty, S. S. Levine, C. Feau, S. Elmiligy, J. A. Hong, T. A. Lewis, A. Vetere, P. A. Clemons, S. E. Malstrom, B. L. Ebert, C. Y. Lin, D. W.

Felsher and A. N. Koehler (2019). "Stabilization of the Max Homodimer with a Small Molecule Attenuates Myc-Driven Transcription." Cell Chem Biol **26**(5): 711-723.e714.

Takahashi, K., M. Nakagawa, S. G. Young and S. Yamanaka (2005). "Differential membrane localization of ERas and Rheb, two Ras-related proteins involved in the phosphatidylinositol 3-kinase/mTOR pathway." J Biol Chem **280**(38): 32768-32774.

Tan, X., L. I. A. Calderon-Villalobos, M. Sharon, C. Zheng, C. V. Robinson, M. Estelle and N. Zheng (2007). "Mechanism of auxin perception by the TIR1 ubiquitin ligase." Nature **446**(7136): 640-645.

Tanaka, N., J. J. Lin, C. Li, M. B. Ryan, J. Zhang, L. A. Kiedrowski, A. G. Michel, M. U. Syed, K. A. Fella, M. Sakhi, I. Baiev, D. Juric, J. F. Gainor, S. J. Klempner, J. K. Lennerz, G. Siravegna, L. Bar-Peled, A. N. Hata, R. S. Heist and R. B. Corcoran (2021). "Clinical Acquired Resistance to KRAS(G12C) Inhibition through a Novel KRAS Switch-II Pocket Mutation and Polyclonal Alterations Converging on RAS-MAPK Reactivation." Cancer Discov **11**(8): 1913-1922.

Tang, D. and R. Kang (2023). "Glimmers of hope for targeting oncogenic KRAS-G12D." Cancer Gene Therapy **30**(3): 391-393.

Taveras, A. G., S. W. Remiszewski, R. J. Doll, D. Cesarz, E. C. Huang, P. Kirschmeier, B. N. Pramanik, M. E. Snow, Y. S. Wang, J. D. del Rosario, B. Vibulbhan, B. B. Bauer, J. E. Brown, D. Carr, J. Catino, C. A. Evans, V. Girijavallabhan, L. Heimark, L. James, S. Liberles, C. Nash, L. Perkins, M. M. Senior, A. Tsarbopoulos, A. K. Ganguly, R. Aust, E. Brown, D. Delisle, S. Fuhrman, T. Hendrickson, C. Kissinger, R. Love, W. Sisson, E. Villafranca and S. E. Webber (1997). "Ras oncoprotein inhibitors: The discovery of potent, ras nucleotide exchange inhibitors and the structural determination of a drug-protein complex." Bioorganic & Medicinal Chemistry **5**(1): 125-133.

Tidyman, W. E. and K. A. Rauen (2009). "The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation." Curr Opin Genet Dev **19**(3): 230-236.

Van, Q. N., C. A. López, M. Tonelli, T. Taylor, B. Niu, C. B. Stanley, D. Bhowmik, T. H. Tran, P. H. Frank, S. Messing, P. Alexander, D. Scott, X. Ye, M. Drew, O. Chertov, M. Lösche, A. Ramanathan, M. L. Gross, N. W. Hengartner, W. M. Westler, J. L. Markley, D. K. Simanshu, D. V. Nissley, W. K. Gillette, D. Esposito, F. McCormick, S. Gnanakaran, F. Heinrich and A. G. Stephen (2020). "Uncovering a membrane-distal conformation of KRAS available to recruit RAF to the plasma membrane." Proc Natl Acad Sci U S A **117**(39): 24258-24268.

Vargesson, N. (2015). "Thalidomide-induced teratogenesis: history and mechanisms." Birth Defects Res C Embryo Today **105**(2): 140-156.

Vázquez, J., M. López, E. Gibert, E. Herrero and F. J. Luque (2020). "Merging Ligand-Based and Structure-Based Methods in Drug Discovery: An Overview of Combined Virtual Screening Approaches." Molecules **25**(20).

Vickery, H. R., J. M. Virta, M. Konstantinidou and M. R. Arkin (2024). "Development of a NanoBRET assay for evaluation of 14-3-3σ molecular glues." SLAS Discov **29**(5): 100165.

Wang, C., Y. Zhang, J. Wang and D. Xing (2022). "VHL-based PROTACs as potential therapeutic agents: Recent progress and perspectives." Eur J Med Chem **227**: 113906.

Wang, C., Y. Zhang, S. Yang, W. Chen and D. Xing (2022). "PROTACs for BRDs proteins in cancer therapy: a review." J Enzyme Inhib Med Chem **37**(1): 1694-1703.

Wang, L., J. Gao, R. Ma, Y. Liu, M. Liu, F. Zhong, J. Hu, S. Li, J. Wu, H. Jiang, J. Zhang and K. Ruan (2022). "Recent progress in fragment-based drug discovery facilitated by NMR spectroscopy." Magnetic Resonance Letters **2**(2): 107-118.

Wang, P., X. Jia, B. Lu, H. Huang, J. Liu, X. Liu, Q. Wu, Y. Hu, P. Li, H. Wei, T. Liu, D. Zhao, L. Zhang, X. Tian, Y. Jiang, Y. Qiao, W. Nie, X. Ma, R. Bai, C. Peng, Z. Dong and K. Liu (2023). "Erianin suppresses constitutive activation of MAPK signaling pathway by inhibition of CRAF and MEK1/2." Signal Transduct Target Ther **8**(1): 96.

Wang, X., S. Allen, J. F. Blake, V. Bowcut, D. M. Briere, A. Calinisan, J. R. Dahlke, J. B. Fell, J. P. Fischer, R. J. Gunn, J. Hallin, J. Laguer, J. D. Lawson, J. Medwid, B. Newhouse, P. Nguyen, J. M. O'Leary, P. Olson, S. Pajk, L. Rahbaek, M. Rodriguez, C. R. Smith, T. P. Tang, N. C. Thomas, D. Vanderpool, G. P. Vigers, J. G. Christensen and M. A. Marx (2022). "Identification of MRTX1133, a Noncovalent, Potent, and Selective KRASG12D Inhibitor." Journal of Medicinal Chemistry **65**(4): 3123-3133.

Wang, Z. Z., X. X. Shi, G. Y. Huang, G. F. Hao and G. F. Yang (2023). "Fragment-based drug discovery supports drugging 'undruggable' protein-protein interactions." Trends Biochem Sci **48**(6): 539-552.

Weinmann, H. and E. Ottow (2007). 7.09 - Recent Development in Novel Anticancer Therapies. Comprehensive Medicinal Chemistry II. J. B. Taylor and D. J. Triggle. Oxford, Elsevier**:** 221-251.

Wells, J. A. and C. L. McClendon (2007). "Reaching for high-hanging fruit in drug discovery at protein–protein interfaces." Nature **450**(7172): 1001-1009.

Wu, Q., F. Centorrino, X. Guillory, M. Wolter, C. Ottmann, P. J. Cossar and L. Brunsveld (2024). "Discovery of 14-3-3 PPI Stabilizers by Extension of an Amidine-Substituted Thiophene Fragment." Chembiochem **25**(1): e202300636.

Wurz, R. P., H. Rui, K. Dellamaggiore, S. Ghimire-Rijal, K. Choi, K. Smither, A. Amegadzie, N. Chen, X. Li, A. Banerjee, Q. Chen, D. Mohl and A. Vaish (2023). "Affinity and cooperativity modulate ternary complex formation to drive targeted protein degradation." Nat Commun **14**(1): 4177.

Yamamoto, G. L., M. Aguena, M. Gos, C. Hung, J. Pilch, S. Fahiminiya, A. Abramowicz, I. Cristian, M. Buscarilli, M. S. Naslavsky, A. C. Malaquias, M. Zatz, O. Bodamer, J. Majewski, A. A. Jorge, A. C. Pereira, C. A. Kim, M. R. Passos-Bueno and D. R. Bertola (2015). "Rare variants in SOS2 and LZTR1 are associated with Noonan syndrome." J Med Genet **52**(6): 413-421.

Yamanaka, S., Y. Horiuchi, S. Matsuoka, K. Kido, K. Nishino, M. Maeno, N. Shibata, H. Kosako and T. Sawasaki (2022). "A proximity biotinylation-based approach to identify protein-E3 ligase interactions induced by PROTACs and molecular glues." Nature Communications **13**(1): 183.

Zenke, G., U. Strittmatter, S. Fuchs, V. F. Quesniaux, V. Brinkmann, W. Schuler, M. Zurini, A. Enz, A. Billich, J. J. Sanglier and T. Fehr (2001). "Sanglifehrin A, a novel cyclophilin-binding compound showing immunosuppressive activity with a new mechanism of action." J Immunol **166**(12): 7165-7171.

Zhao, L., J. Zhao, K. Zhong, A. Tong and D. Jia (2022). "Targeted protein degradation: mechanisms, strategies and application." Signal Transduct Target Ther **7**(1): 113.

Zheng, Z.-Y. and E. C. Chang (2014). Chapter Two - A Bimolecular Fluorescent Complementation Screen Reveals Complex Roles of Endosomes in Ras-Mediated Signaling. Methods in Enzymology. P. M. Conn, Academic Press. **535:** 25-38.

Curriculum Vitae

Name: Sophie Piech

Date of Birth: 25th of April, 1994

Citizenship: Austrian

Address: Hohe Warte 28/4, 1190 Vienna, Austria

Email: spiech@cemm.oeaw.ac.at

n11947237@students.meduniwien.ac.at

Education

2020-present	Center for Molecular Medicine (CeMM), Austria Predoctoral Fellow at the Giulio Superti-Furga Lab
2018-2019	University College London (UCL), United Kingdom Master of Science in Drug Design and Synthesis with Merit, thesis conducted at: The Alzheimer's Research UK, Paul Fish Lab
2013-2018	University of Zurich, Switzerland Bachelor of Science UZH in Biochemistry, thesis conducted at: The Plückthun Lab

Conferences, Posters and Oral Presentations

2023	EUROPIN Summer School on Drug Design, Vienna, Austria
2023	IRB Barcelona BioMed Conference on "Proximity-inducing pharmacology: Targeted protein degradation and beyond", Barcelona, Spain, poster presentation
2023	Ubiquitin & Friends Symposium, Vienna, Austria, poster presentation
2023	18th YSA PhD-Symposium, Vienna, Austria, poster presentation
2022	ACS Medicinal Chemistry Conference, New York, USA, poster presentation

Languages

German: Native Speaker

English: Fluent

French: Intermediate

Technical skills

Programming Languages: R, MATLAB

Tools: OriginLab, MS-Office (Word, Excel, Power Point, Access), MOE, PyMOL, ChemDraw, ASTRA 6, Mnova, SnapGene, GraphPad Prism, Maestro Schrödinger (Course Certificate: Introduction to Molecular Modeling in Drug Discovery)