

# Identifying Next-Generation RAS Pathway Inhibitors for the Treatment of High Hyperdiploid B-cell Acute Lymphoblastic Leukemia

**Lander AELBRECHT**

Promoter: Prof. J. Cools  
Department of Human Genetics (Laboratory for  
Molecular Biology of Leukemia)

Mentor: Dr. C. Gonzalez-Gil  
Department of Human Genetics (Laboratory for  
Molecular Biology of Leukemia)

Thesis presented in  
fulfillment of the requirements  
for the degree of Master of Science  
in Molecular Biology & Physiology

Academic year 2025-2026

© Copyright by KU Leuven

Without prior written permission from both the supervisor(s) and the author(s), copying, reproducing, using, or realizing this publication or parts thereof is prohibited. For requests or information regarding the copying and/or use and/or realization of parts of this publication, please contact KU Leuven, Faculty of Science, Celestijnenlaan 200H - box 2100, 3001 Leuven (Heverlee), Telephone +32 16 32 14 01.

Prior written permission from the supervisor(s) is also required for using the (original) methods, products, circuits, and programs described in this work for industrial or commercial purposes, and for submitting this publication for scientific awards or contests.



*Copyright Information:*  
*student paper as part of an academic education*  
*and examination.*  
*No correction was made to the paper*  
*after examination.*

### Contribution statement

The student declares that the submitted thesis text (including graphs) is original work, written using the wording of the student and reflecting the student's English language proficiency. The text acknowledges, to the best of the ability and knowledge of the student, previous ideas and data. All experiments and analyses in this study were conducted by Lander Aelbrecht. Dr. Celia Gonzalez-Gil provided significant contributions through supervision of the project and conceptualization.

NotebookLM, Copilot and ChatGPT were used as research tools to get a broader understanding of the topic and to deliver useful journal articles and other sources. However, generative AI was never utilized to generate or rewrite text and was never directly used as a source of information on its own. These models were used as tools to enhance critical thinking, rather than as writing assistants.

# Acknowledgements

Finalizing and reflecting on the work I have conducted over the past nine months has made me realize how many people have played an important role in this journey. The completion of this project marks one of my final milestones as a master's student, while hopefully many more experiences still lie ahead in my academic journey. Three years ago, when I graduated from my professional bachelor's degree in Agro- and Biotechnology at Thomas More, I would never have dared to imagine reaching this moment. Therefore, I am deeply grateful and would like to sincerely thank everyone who contributed to this journey.

First, I would like to express my sincere gratitude to Prof. Jan Cools for giving me the opportunity to conduct my master's thesis in the Lab of Molecular Biology of Leukemia at the VIB-KU Leuven Center for Cancer Biology. Your ability to make people feel welcome and the supportive environment you create in the lab made it a pleasure to come to work every day.

Foremost, I would like to express my deepest gratitude to Dr. Celia Gonzalez-Gil for the outstanding supervision of this project. Thank you for always being available, for your invaluable advice on both the scientific content and the writing process of this thesis, and for guiding me throughout every stage of this project. I have rarely met someone who works as hard as you while still taking the time to answer questions, provide guidance, and carefully review my work. I truly could not have imagined a better supervisor.

Additionally, I would like to thank everyone from the lab of Prof. Jan Cools and the lab of Prof. Marlies Vanden Bempt. The laughter, supportive atmosphere, and unwavering willingness to help one another made this an unforgettable experience. I sincerely wish each of you the very best with your future projects, and I hope our paths cross again soon.

I would also like to thank Rob Moerkens, who repeatedly encouraged me to pursue a master's degree while I was completing my internship at Biobest three years ago. At the time, I had many doubts, and looking back now, I cannot imagine how different my path might have been otherwise. Lieven Janssens, I do not think you fully realize the impact you have had on my life. Thank you for the invaluable life lessons you shared with me. I will always remember to put on my "*stoute schoenen*" and dare to ask. I deeply admire you, and without your advice, I would never have knocked on the door of Prof. Jan Cools.

Finally, I would like to thank my family, especially my parents, for giving me the opportunity to pursue a master's degree. I know this was not an easy decision, and I will always remain grateful for your support and sacrifices. Last but certainly not least, I would like to thank Zita, my girlfriend, for her unwavering support, patience, and encouragement throughout this journey. Your presence has meant more to me than words can express.

## Summary

High hyperdiploid B-cell acute lymphoblastic leukemia (HeH B-ALL) is one of the most prevalent pediatric cancers. While overall survival rates are approaching 95%, this cancer still accounts for most relapses in absolute numbers across all pediatric cancers. A key characteristic of this leukemia subtype is the presence of an abnormal number of chromosomes. In high hyperdiploidy, a cell contains 51 - 67 chromosomes, instead of the normal 46. A second key characteristic is the presence of at least one subpopulation of leukemic cells with mutations in RAS proteins.

A question that remains unanswered is whether leukemic cells with high hyperdiploidy require these RAS mutations in order to deal with the stress induced by the high number of chromosomes or not. Although some studies already tried to explain the co-occurrence of high hyperdiploidy and RAS mutations, a clear mechanism has not been revealed yet. The hypothesis that high hyperdiploid cells rely on the RAS mutations to overcome chromosomal instability means that the RAS pathway can be explored as a therapeutic target for these patients. In order to test this hypothesis, multiple inhibitors of the RAS pathway needed to be tested in vitro in a more simplified model, before further tests can be conducted.

In the past, only few inhibitors were available to target the RAS pathway, but recently, new compounds have been identified that target KRAS or NRAS wild-type or mutant proteins or interfere indirectly with this signaling. In this work, the potential of various drugs was tested in vitro in Ba/F3 cells expressing mutants of KRAS or NRAS that are frequently observed in HeH B-ALL. Additionally, Ba/F3 cells expressing wild-type KRAS or NRAS were included for comparison. ATPlite assays and Western Blot analyses were used to identify which drugs led to decreases in cell proliferation and in RAS signaling. Also cytotoxicity tests were conducted.

We were able to select multiple next-generation RAS pathway inhibitors with different modes of action that can be taken into a further stage of testing. Direct RAS inhibitors Sotorasib, BI-2865 and Daraxonasib showed promising results in our in vitro tests, as well as downstream inhibitors Naporafenib and Trametinib. Additionally, one RAS degrader, PROTAC K-Ras Degradar-4, and a RAS mislocalization drug, ABD957, showed promising results. In future research, these drugs can be taken into patient-derived xenograft models, to look if they can influence real HeH B-ALL progression. This work brings us one step closer to more targeted, effective and clinically relevant treatments for this pediatric cancer.

## List of Abbreviations and List of Symbols

|           |                                                       |
|-----------|-------------------------------------------------------|
| ALL       | Acute lymphoblastic leukemia/lymphoma                 |
| B-ALL     | B-cell ALL / B-lymphoblastic acute leukemia/lymphoma  |
| BCR       | B-cell receptor                                       |
| CAR-T     | Chimeric Antigen Receptor T-cell                      |
| CD        | Cluster of differentiation                            |
| CIN       | Chromosomal instability                               |
| DDR       | DNA damage response                                   |
| EGF(R)    | Epidermal growth factor (receptor)                    |
| FDA       | Food and Drug Administration                          |
| GAP       | GTPase activating proteins                            |
| GEF       | Guanine nucleotide exchange factor                    |
| GPCR      | G-protein coupled receptor                            |
| GRB2      | Growth factor receptor-bound protein 2                |
| HeH       | High hyperdiploidy                                    |
| HeH B-ALL | High hyperdiploid B-cell acute lymphoblastic leukemia |
| HSC       | Hematopoietic stem cell                               |
| HVR       | Hypervariable region                                  |
| MDP       | Macrophage-dendritic progenitor                       |
| mIL-3     | Murine interleukin-3                                  |
| MHC       | Major histocompatibility complex                      |
| NCI       | National Cancer Institute                             |
| NK cells  | Natural killer cells                                  |
| NSCLC     | Non-small cell lung cancer                            |
| p-ERK     | Phosphorylated ERK                                    |
| PDX       | Patient-derived xenograft                             |
| PROTAC    | Proteolysis targeting chimera                         |
| ROS       | Reactive oxygen species                               |
| RTK       | Tyrosine kinase receptor                              |
| Ser/Thr   | Serine/Threonine                                      |
| SH        | Src homology                                          |
| SOS       | Son of sevenless                                      |
| TCR       | T-cell receptor                                       |
| TF        | Transcription factor                                  |
| Tyr       | Tyrosine                                              |
| UPR       | Unfolded protein response                             |
| WB        | Western Blotting                                      |

# Table of Contents

|                                                 |     |
|-------------------------------------------------|-----|
| Acknowledgements .....                          | IV  |
| Summary .....                                   | V   |
| List of Abbreviations and List of Symbols ..... | VI  |
| Table of Contents .....                         | VII |
| 1 Literature review .....                       | 1   |
| 1.1 Hematopoiesis .....                         | 1   |
| 1.2 Lymphocyte development .....                | 2   |
| 1.3 Leukemogenesis .....                        | 5   |
| 1.4 Aneuploidy .....                            | 8   |
| 1.4.1 Aneuploidy in cancers .....               | 8   |
| 1.4.2 High Hyperdiploidy in B-ALL .....         | 9   |
| 1.5 RAS-pathway .....                           | 10  |
| 1.5.1 Canonical RAS signaling .....             | 10  |
| 1.5.2 RAS proteins .....                        | 14  |
| 1.5.3 RAS mutations in HeH B-ALL .....          | 16  |
| 1.5.4 Aneuploidy & RAS mutations .....          | 17  |
| 1.6 Treatment of ALL .....                      | 19  |
| 1.6.1 Chemotherapies .....                      | 19  |
| 1.6.2 Current next-generation therapies .....   | 20  |
| 1.6.3 Targeting the RAS pathway .....           | 22  |
| 2 Research hypothesis and aims .....            | 24  |
| 3 Materials & Methods .....                     | 25  |
| 3.1 Materials .....                             | 25  |
| 3.2 Drug selection .....                        | 25  |
| 3.2.1 RAS inhibitors .....                      | 26  |
| 3.2.2 RAS degraders .....                       | 27  |
| 3.2.3 Downstream RAS inhibitors .....           | 27  |
| 3.2.4 Mislocalization of RAS proteins .....     | 28  |

|       |                                           |    |
|-------|-------------------------------------------|----|
| 3.3   | Mutating Ba/F3 cells.....                 | 29 |
| 3.3.1 | Mutagenesis of MSCV plasmids.....         | 29 |
| 3.3.2 | Transformation of <i>E. coli</i> .....    | 30 |
| 3.3.3 | Transduction of Ba/F3 cells.....          | 31 |
| 3.4   | In vitro assays.....                      | 33 |
| 3.4.1 | ATPlite assay .....                       | 33 |
| 3.4.2 | Western blotting .....                    | 34 |
| 4     | Results.....                              | 36 |
| 4.1   | Establishing Ba/F3 mutant cell lines..... | 36 |
| 4.2   | RAS inhibitors .....                      | 39 |
| 4.2.1 | Sotorasib.....                            | 39 |
| 4.2.2 | Pan-RAS-IN-1 .....                        | 41 |
| 4.2.3 | BI-2865 .....                             | 43 |
| 4.2.4 | Daraxonrasib.....                         | 44 |
| 4.3   | RAS Degraders.....                        | 46 |
| 4.3.1 | PROTAC K-Ras Degradar-4 .....             | 46 |
| 4.3.2 | ACBI3 .....                               | 48 |
| 4.4   | Downstream RAS inhibitors .....           | 49 |
| 4.4.1 | Naporafenib .....                         | 49 |
| 4.4.2 | Trametinib .....                          | 50 |
| 4.5   | Mislocalization of RAS proteins.....      | 52 |
| 4.5.1 | ABD957 .....                              | 52 |
| 5     | Discussion.....                           | 54 |
| 5.1   | Experimental set-up .....                 | 54 |
| 5.2   | Drugs .....                               | 55 |
| 5.3   | Conclusion .....                          | 59 |
| 6     | References.....                           | 60 |
|       | Addendum.....                             | 1  |
|       | Risk analysis.....                        | 1  |
|       | Supplemental and Extended Data.....       | 2  |

# 1 Literature review

## 1.1 Hematopoiesis

Hematopoiesis is the formation of blood cellular components. This process is driven by pluripotent hematopoietic stem cells (HSCs) and occurs in the bone marrow of humans, as displayed in Figure 1.<sup>1</sup> HSCs can differentiate into any type of blood cell and at the same time renew themselves to prevent depletion. HSCs always divide into two cells. One of them stays an HSC and keeps its pluripotency, whereas the other differentiates into a certain type of multipotent progenitor cell. There are two types of progenitor cells that can be formed, myeloid and lymphoid progenitor cells.<sup>1</sup> Differentiation into a myeloid or lymphoid progenitor cell is a partially stochastic event, depending on the fluctuating levels of transcription factors (TFs), and is partially controlled by gene regulatory networks for on-demand differentiation.<sup>2</sup>

Myeloid progenitor cells can give rise to erythrocytes (red blood cells), megakaryocytes, which will further differentiate into platelets, and a large variety of myeloid cells, including eosinophils, basophils, neutrophils and macrophage-dendritic progenitors (MDPs).<sup>3,4</sup> MDPs can further differentiate into dendritic cells and monocytes, which will form macrophages.<sup>3</sup> All these white blood cells play key roles in the innate immune system.<sup>1</sup> Lymphoid progenitor cells, on the other hand, give rise to three other types of white blood cells. B-cells and T-cells are the backbone of the adaptive immune system, whilst natural killer cells (NK cells) are involved in the innate immune system.<sup>1,5</sup>

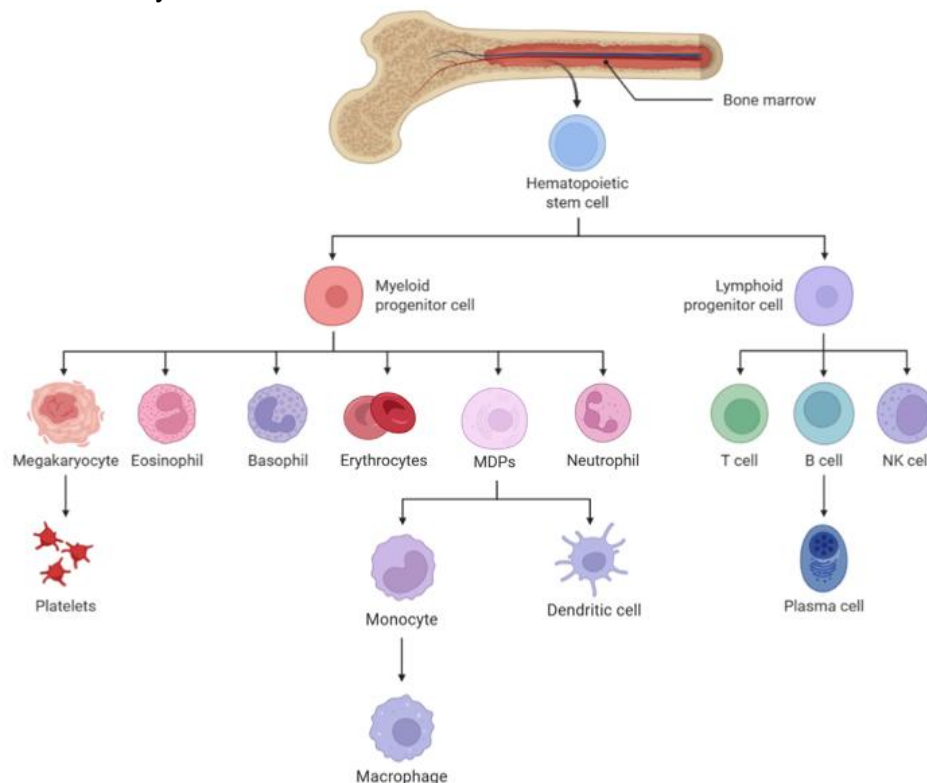


Figure 1: Hematopoiesis: Schematic overview of cell differentiation from hematopoietic stem cells in the bone marrow. Figure template adjusted with BioRender.com

## 1.2 Lymphocyte development

Based on different levels of regulatory changes, lymphoid progenitor cells differentiate into B-cells, T-cells and NK cells. The most important factor is the microenvironment of the progenitor cell. Some progenitors reside in the bone marrow, whilst others are directed towards the thymus. This direction to the thymus is regulated by chemokines, adhesion molecules and the blood circulation.<sup>6</sup> At that point, the fate of the progenitor cells is not yet determined, since they are not yet expressing full T-lineage-specific genes.<sup>7,8</sup>

When they have arrived at the thymus, the progenitor cells receive different environmental signals compared to progenitor cells that reside in the bone marrow. Epithelial cells in the thymus express a Notch ligand, which binds to the Notch1 receptor present on lymphoid progenitor cells. Upon binding, the intracellular domain of Notch1 is cleaved off and relocated to the nucleus. There, it forms a transcription activator complex with other co-activators, inducing transcription of more TFs. Together they activate genes that are involved in downstream programs needed for T-cell receptor (TCR) rearrangement and function. At the same time, Notch signaling suppresses B-cell promoting factors and NK-cell differentiation. This effect is reinforced by epigenetic modifications, like histone methylations and chromatin remodeling, making these genes inaccessible.<sup>7,8</sup>

As shown in Figure 2, T-cells are cluster of differentiation (CD) 4- and CD8-negative in this part of the development. In the thymus, T-cell progenitor cells will undergo rearrangement of the  $\beta$ -chain of TCRs. Recombination activating genes recognize specific signal sequences and cut out and join certain segments. In the end, three gene segments, variable (V), diversity (D) and joining (J) are rearranged, forming a VDJ exon. When this rearrangement is successful, a pre-TCR is expressed on the cell surface. This induces proliferation and survival signaling, driving selection for these pre-TCR T-cells. After  $\beta$ -selection, T-cells upregulate both CD4 and CD8. Double-positive T-cells then rearrange the TCR $\alpha$  chain via the same process as  $\beta$  chain rearrangement, but only containing a V and J gene segment. This enables the ability to recognize self-peptides on major histocompatibility complex (MHC) molecules, expressed by epithelial cells in the thymus. T-cells that bind self-peptide – MHC class I complexes receive signals to only express CD8, whereas binding to an MHC class II leads to CD4 cell fate. Mature CD8<sup>+</sup> T-cells and CD4<sup>+</sup> T-cells are better known as cytotoxic T-cells and helper T-cells respectively.<sup>9–11</sup>

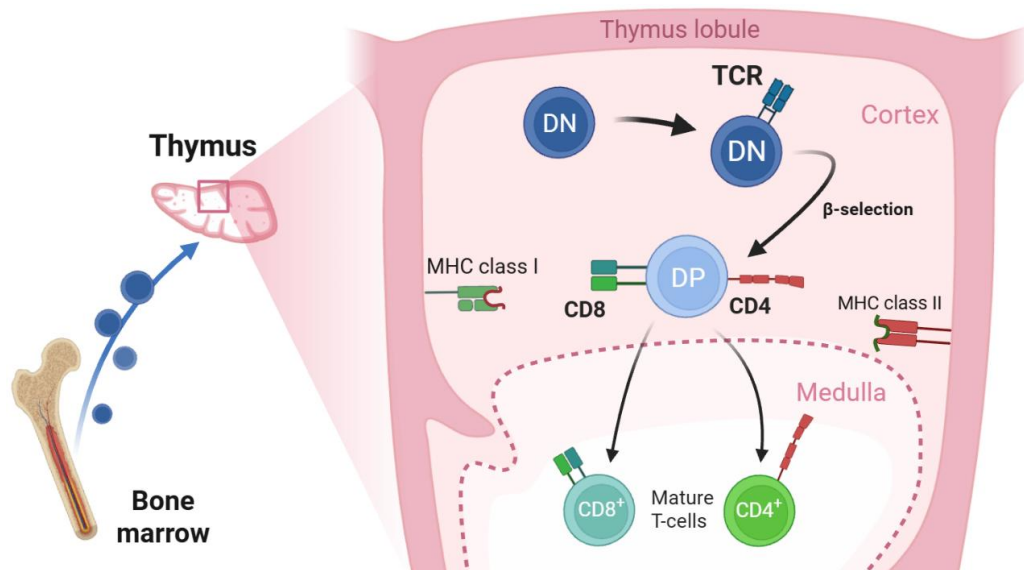


Figure 2: T-cell development in the thymus. Based on the class of MHC in the self-peptide - MHC complex pre-TCR T-cells differentiate into a CD8<sup>+</sup> T-cell or a CD4<sup>+</sup> T-cell. DN = double negative, DP = double positive, TCR = T-cell receptor. Figure was created with BioRender.com.

Lymphoid progenitor cells that stay in the bone marrow do not encounter Notch signaling and will differentiate into either B-cells or NK cells. The bone marrow also has its own microenvironment. Some progenitor cells will be more surrounded by dendritic cells and macrophages, whilst others are more in contact with bone marrow stromal cells. This stochastic process leads to a different fate. Macrophages and dendritic cells produce interleukin-15, which drives NK-cell fate.<sup>12</sup> Bone marrow stromal cells produce interleukin-7 and drive B-cell fate.<sup>13</sup>

Each B-cell expresses its own unique type of B-cell receptor (BCR). These immunoglobulins (Ig) are antibodies bound to the plasma membrane and are unique on each cell, due to VDJ recombination of the Ig heavy and light chain during early B-cell development. Additionally, enzymes like TdT add random nucleotides at the junctions. This creates  $\sim 10^{11}$  theoretical different receptor combinations, more than there are B-cells in the human body. This is enough to cover all the possible antigens that would ever enter the human body.<sup>9,14</sup>

Afterwards, B-cells travel from the bone marrow to the lymph nodes or spleen, where they reside in follicles. They can come across their specific antigen, soluble or presented by follicular dendritic cells. When they bind their antigen, they internalize it and upregulate MHC class II and CD40 by Src kinase signaling. They present the antigen via this MHC class II on their own cell surface and become an antigen-presenting cell. Therefore, this antigen can be recognized by CD4<sup>+</sup> T-cells. Via CD40 – CD40L interactions and cytokine signals the CD4<sup>+</sup> T-cell completely activates the B-cell, visualized in Figure 3. Activated intracellular pathways in B-cells are the RAS and the NF-κB pathways, driving proliferation.<sup>9,15,16</sup>

After their activation, B-cells become either plasma cells or memory cells. This choice happens under the influence of CD4<sup>+</sup> T-cell signals and transcriptional networks inside of the B-cell. Plasma cells will travel back to the bone marrow (long-lived) or will stay in the lymphoid tissue (short-lived). The main function of plasma cells is the production of large amounts of soluble antibodies, without a transmembrane domain. Therefore, the antibodies are not incorporated in the plasma membrane, but secreted into the circulation system. These soluble antibodies circulate throughout the body and perform several tasks. They can directly bind to pathogens, blocking their ability to attach or enter host cells. They also tag pathogens for phagocytosis, activate other proteins, recruit NK cells and form immune complexes that are cleared by macrophages.<sup>9,17</sup>

Memory B-cells stay in the circulation system and in the lymphoid organs to patrol through the body. They express high-affinity BCRs specific for that one antigen they encountered before. Upon re-exposure to the same antigen, they are reactivated rapidly for an early and fast intervention.<sup>9</sup>

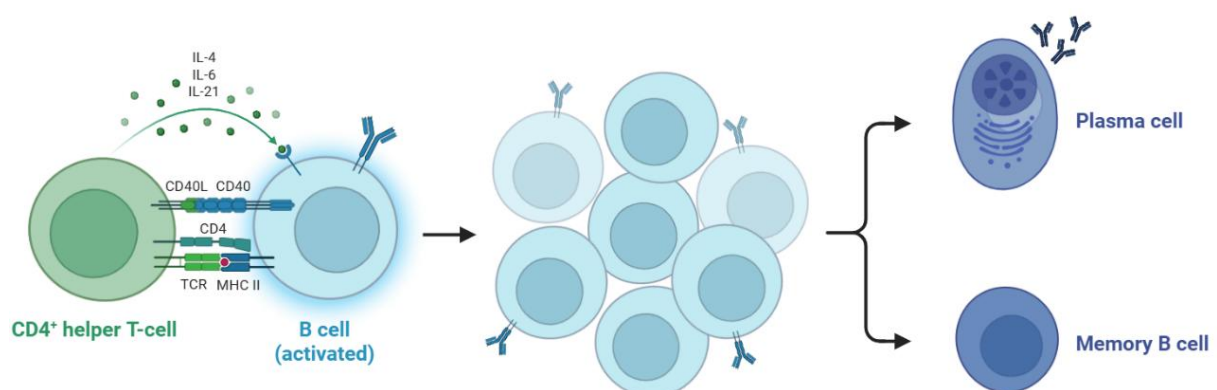


Figure 3: B-cells count on helper T-cells for their activation. Afterwards they start to proliferate and can differentiate into an antibody-secreting plasma cell or in memory B-cells. Figure was created with BioRender.com

## 1.3 Leukemogenesis

The evolution of hematopoietic stem or progenitor cells towards malignant leukemic clones is called leukemogenesis. It is a multistep process that eventually can lead to leukemia.<sup>18</sup> Throughout our lives, hematopoietic stem or progenitor cells can acquire mutations. In some cases, an oncogenic mutation can occur, changing the behavior of that cell.<sup>18,19</sup> These mutations result in an alteration in normal cell homeostasis, leading to abnormal cell development.<sup>20</sup> Over time, more oncogenic mutations might occur, further destabilizing regular cell processes.<sup>18,19</sup> Such altered white blood cells can develop into leukemic cells. Cancerous cells are often kept in an undifferentiated state, resulting in the inability to fight infections properly, due to insufficient mature lymphocytes and myelocytes. Additionally, they impair the production of red blood cells and platelets in the bone marrow.<sup>21</sup>

According to the National Cancer Institute (NCI), four to five children per 100 000 get diagnosed with leukemia.<sup>22</sup> Leukemia, accounting for 30% of cases, is the most prevalent pediatric cancer.<sup>23</sup> Different types of leukemia are classified based on multiple factors. These factors can be the origin of the white blood cancer and/or the progression of the disease. The origin is, in most cases, either lymphoblastic or myelogenous. Exceptions are mixed phenotype leukemias, showing both myeloid and lymphoid markers, and undifferentiated leukemias, showing neither myeloid, nor lymphoid markers.<sup>24</sup> In lymphoblastic leukemia, the cancer develops from lymphocytes, whereas myelogenous leukemia develops from myeloid cells.

The progression of the disease can be slow, called chronic leukemia, or it can be fast, called acute leukemia.<sup>21,25</sup> In acute leukemias, a subset of leukemic cells is often kept in an immature state, keeping the ability to self-renew.<sup>26</sup> In chronic leukemias, where most circulating cells are mature lymphocytes or myeloid cells, the self-renewal property is either retained or reactivated by some of the cells.<sup>27</sup> This classification yields four major subtypes of leukemia, with both lymphoblastic and myeloid leukemia, divided into chronic or acute cases. This classification is a rather simplified version of the reality. As displayed in Table 1, the international consensus classification discriminates between 12 major categories, with a variety of subtypes.<sup>28</sup>

*Table 1: International consensus classification (ICC) of Myeloid Neoplasms and Acute Leukemias, established in 2022. Data retrieved from report publication in Blood.<sup>28</sup>*

|                                                                               |
|-------------------------------------------------------------------------------|
| Myeloproliferative neoplasms (MPNs)                                           |
| Myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions |
| Mastocytosis                                                                  |
| Myelodysplastic/myeloproliferative neoplasms                                  |
| Premalignant clonal cytopenias and myelodysplastic syndromes                  |
| Pediatric and/or germline mutation-associated disorders                       |
| Acute myeloid leukemias (AMLs)                                                |
| Myeloid proliferations associated with Down syndrome                          |
| Blastic plasmacytoid dendritic cell neoplasm                                  |
| Acute leukemia of ambiguous lineage                                           |
| B-lymphoblastic leukemia/lymphoma                                             |
| T-lymphoblastic leukemia/lymphoma                                             |

The American Cancer Society states that 75% of all childhood leukemias are acute lymphoblastic leukemias (ALL).<sup>29</sup> Looking within pediatric ALL cases, 85% are caused by altered B-cells and 15% by altered T-cells.<sup>23</sup> During this study, the focus will be on B-lymphoblastic acute leukemia/lymphoma (B-ALL).

The main symptoms of B-ALL are caused by bone marrow failure. Due to a lack of differentiated mature hematopoietic cells, patients will experience anemia, thrombocytopenia and neutropenia. This manifests as fatigue, shortness of breath, easy bruising, bleeding, recurrent infections and fever. Proliferating bone marrow cells will increase pressure within the bones, leading to bone and joint pain. Organs like lymph nodes, spleen and liver can be infiltrated and can experience functioning difficulties. Also the central nervous system can be infiltrated, resulting in headaches, vomiting and in rare cases even seizures.<sup>30,31</sup>

As shown in Figure 4, B-ALL is divided into subtypes where TFs are altered, kinase-driven cancers and cancers characterized by chromosomal abnormalities. High hyperdiploid B-cell acute lymphoblastic leukemia (HeH B-ALL) is the most common pediatric subtype and accounts for up to 30% of the cases.<sup>32,33</sup> Hyperdiploidy is a form of aneuploidy, meaning there is an abnormal number of chromosomes present in cells. In hyperdiploidy there are more than the regular 46 chromosomes present, typically between 51 and 67 in case of high hyperdiploidy (HeH).<sup>34</sup>

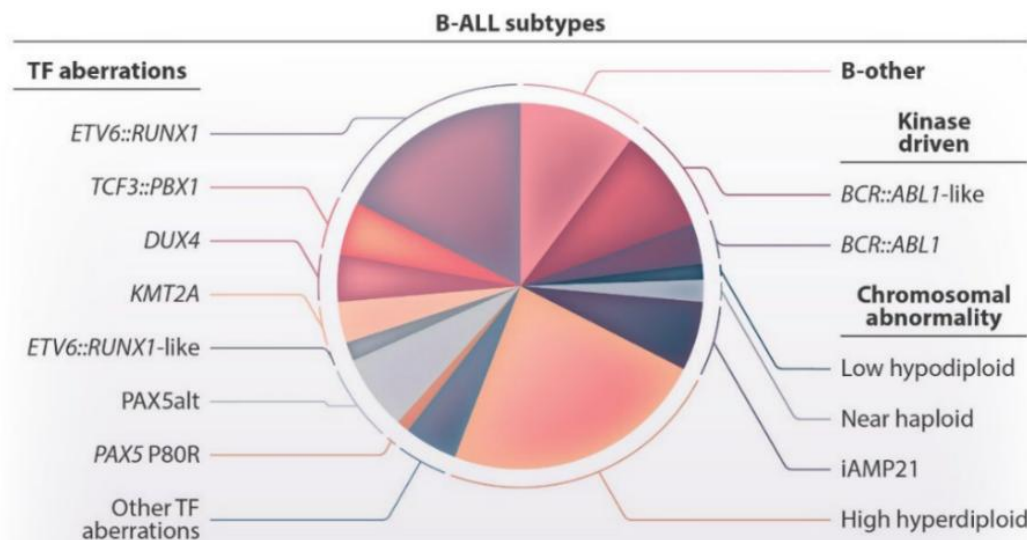


Figure 4: Genetic subtypes of B-ALL, based on a cohort study of 2288 patients with B-ALL. Figure adapted from Springer Nature.

HeH B-ALL arises from a single mitotic segregation error, causing the aneuploidy. Because aneuploidy leads to genomic stress, mutations that help the cell cope with the genomic instability can accumulate and will have a selection advantage. Whereas chromosomal gains occur during a single mitotic segregation error, mutations are acquired in short, rapid bursts separated in time by long periods of genetic stability. This phenomenon is called punctuated equilibrium.<sup>35</sup>

Most mutations will not favor the cancer and are neutral or even detrimental to a cell. However, sporadically, a stochastic mutation might benefit the leukemic cell, leading to more proliferation or higher survival and creating a new subclone of leukemic cells.<sup>36,37</sup> Therefore, multiple subclones of leukemic cells arise within the same patient, all characterized by a different subset of mutations. This results in a high mutational heterogeneity across different clones, although the same chromosomal pattern is observed in HeH B-ALL clones.

In HeH B-ALL these mutations are mostly single nucleotide polymorphisms or indels and occur often in genes involved in the RAS-pathway and in chromatin- and histone-modifying enzymes. These can help the clone to increase proliferation, survive easier and evade therapy. Most common mutations in patients are found in RAS, FLT3, CREBBP, PTPN11 and IKZF1.<sup>38</sup> A recent study highlighted that at least one subclone in each HeH B-ALL patient has a mutation in a RAS protein.<sup>36</sup> Often even multiple different RAS mutations are present in non-overlapping subclones. This suggests that both the aneuploid character as well as the RAS mutations are two important alterations, possibly both required to drive HeH B-ALL.

## 1.4 Aneuploidy

### 1.4.1 Aneuploidy in cancers

Aneuploidy, the presence of an abnormal number of chromosomes or chromosome-arm imbalances, is a nearly universal phenomenon in human cancers.<sup>39</sup> Aneuploidy is initially always a burden to the cell.<sup>37</sup> Due to differences in the number of chromosomes and therefore in DNA content, chromosomal instability (CIN) increases. CIN involves replication stress (due to fork stalling), proteotoxic stress (due to stoichiometric imbalances in protein complexes) and a higher production of reactive oxygen species (ROS) (due to mitochondrial imbalances). Gaining extra chromosomes is a highly unstable process, often leading to apoptosis.<sup>40,41</sup>

Nevertheless, some cells seem to adapt to aneuploidy in the right circumstances. The increase in CIN is initially dealt with at the level of proteins. Dosage compensation is seen through translational control, increased protein degradation and reduced instability of unassembled subunits.<sup>42</sup> Proteomic analyses have shown that a cell can compensate for about 60% to 80% of the protein imbalances. Even with this partial dosage compensation, thousands of proteins are still expressed at abnormal ratios. Subunits of a multiprotein complex cannot fold or integrate properly, leading to misfolded and orphan proteins. These increase the workload for the proteasome and induce the unfolded protein response (UPR). Furthermore, mitochondrial imbalances lead to a higher ROS production, increasing stress in the cell.<sup>40–42</sup>

Besides dosage compensation at the protein level, some clones have additional adaptations. It is shown that CIN has a lower impact in adapted clones, compared to early aneuploid clones. They express fewer proteins involved in the UPR, indicating that proteotoxic stress is no longer induced. Cells that adapt to aneuploidy seem to metabolically rewire their system, as they upregulate peroxisomal activity and glycolysis.<sup>40</sup> Increased peroxisomal activity makes sure that cells can detoxify the elevated ROS and control the NAD<sup>+</sup>/NADH balance, needed to support the higher metabolic turnover.<sup>43</sup> Glycolysis upregulation leads to ATP production independent of mitochondria, to prevent more ROS production. Additionally, glycolysis will provide the cell with more precursors, such as ribose-5-phosphate to support increased RNA and protein synthesis.<sup>40,44</sup>

Transcriptomic analyses revealed even more upregulated genes in aneuploid cells, involved in the cell cycle, translation, mRNA processing, ribosome biogenesis and the DNA damage response (DDR). This is a direct cause of the extrachromosomal gains that lead to more DNA to transcribe and duplicate, more transcripts to process and more proteins to fold, modify and degrade. This implies that aneuploid cancer cells must upregulate growth- and biosynthesis-related pathways to cope with gene-dosage stress, creating a stressed but highly adaptable state that can drive tumor evolution.<sup>39,40,45</sup>

## 1.4.2 High Hyperdiploidy in B-ALL

Even before mutations that are often seen in HeH B-ALL arise, HSCs or lymphoid progenitor cells have already acquired non-random chromosomal gains.<sup>19</sup> These gains are likely the result of a single abnormal mitosis. This hypothesis is generally supported, because all HeH leukemic cells in a patient show the same set of chromosomes. Based on single cell and genomic analyses, chromosomes seem to appear simultaneously, rather than via gradual accumulation. A single abnormal mitosis is thus likely to be the initiation event in many cases of HeH B-ALL.<sup>19,46,47</sup> This cell will lead to a population of HeH clones and can already be established before birth.<sup>48</sup> In other cancers, mutations in genes involved in cytokinesis and mitotic spindle checkpoints are often linked to mis-segregations.<sup>49,50</sup> But, since no such recurrent mutations are found in HeH B-ALL, this is most likely not the driver here.<sup>51</sup>

Other causes of mis-segregation events in HeH B-ALL can vary and there is not one single cause known to be the initiator. Some HeH cases show lower expression in cohesin and condensin.<sup>52</sup> These are two protein complexes that organize the chromosomes during cell division. Cohesin holds sister chromatids together from DNA replication until the anaphase and a defect can lead to improper distribution of sister chromatids across daughter cells. Condensin compresses chromosomes into compact structures as long as the cell is dividing. Without functional condensin, anaphase bridges can be formed, and chromosomes or chromatids remain physically connected. When the mitotic spindle pulls a bridge to one side, an entire chromosome ends up in one cell, leaving the other daughter cell without that chromosome.<sup>47,53</sup>

Another driver of mis-segregation in HeH B-ALL can be replication stress during fetal hematopoiesis. The rapid proliferation of fetal B-cells increases the chance of errors, predominantly by incomplete replication of chromosomes while cell division already occurs and transient spindle defects.<sup>54,55</sup>

Classical HeH B-ALL consists of trisomies and tetrasomies. Trisomies are the result of a duplication of one of the parental chromosomes, whereas tetrasomies are the result of a duplication of both. Chromosomal gains in HeH ALL patients are non-random. Tetrasomy 21, four copies of chromosome 21, is found in nearly all HeH B-ALL patients. These gains come together with gains of chromosomes X, 14, 6, 18, 4, 17 and 10 with decreasing likelihood in that order.<sup>40</sup> Chromosomes X, 14 and 18 can also be found as tetrasomies in patients, despite the fact that they are in most cases trisomies. Chromosomes 4, 6, 10 and 17 are frequently reported as trisomies and almost never as tetrasomies. Although HeH varies between a total number of 51 and 67 chromosomes, the gains usually include the chromosomes mentioned above.<sup>19,46</sup> Because the same pattern is seen across most HeH B-ALL patients, it is likely that only gaining these specific chromosomes gives a cell the advantage to grow and proliferate more compared to normal cells.

## 1.5 RAS-pathway

### 1.5.1 Canonical RAS signaling

The RAS pathway, often called MAPK/ERK pathway, is a signaling cascade in cells, involved in cell cycle regulation, growth and survival. The pathway makes use of different links to transduce and amplify a signal from outside the cell into the nucleus of the cell.<sup>56</sup>

The cascade starts when an extracellular signal binds to its receptor on the plasma membrane. The RAS pathway is activated by tyrosine kinase receptors (RTKs), a class of kinase receptors that phosphorylate tyrosine (Tyr) residues when activated. The epidermal growth factor receptor (EGFR) is the most studied and prevalent receptor that activates RAS signaling. Other RTKs, cytokine receptors, integrins and serpentine receptors are also able to activate RAS proteins.<sup>56</sup> Different ligands can bind to the EGFR, like epidermal growth factor (EGF), TGF- $\alpha$ , amphiregulin, epiregulin, betacellulin, HB-EGF and epigen. The differences between these ligands are their affinity for EGFR, the duration of the signaling and how they are shed from their host membrane into the extracellular space.<sup>57</sup> A recent study shows novel insights into ligands of EGFRs, also proposing families of ribonucleases as ligands for EGFRs.<sup>58</sup>

The extracellular part of an EGFR consists of four protein domains, visible in Figure 5. When no ligand is present, the monomeric EGFR is folded in a conformation where the dimerizing arm in domain II is not exposed to other dimerizing arms. Upon binding of a ligand, the EGFR undergoes a biochemically driven conformational change, now exposing the dimerization arm. This arm can interact with a dimerization arm of another EGFR, forming a dimer.<sup>56,59</sup>

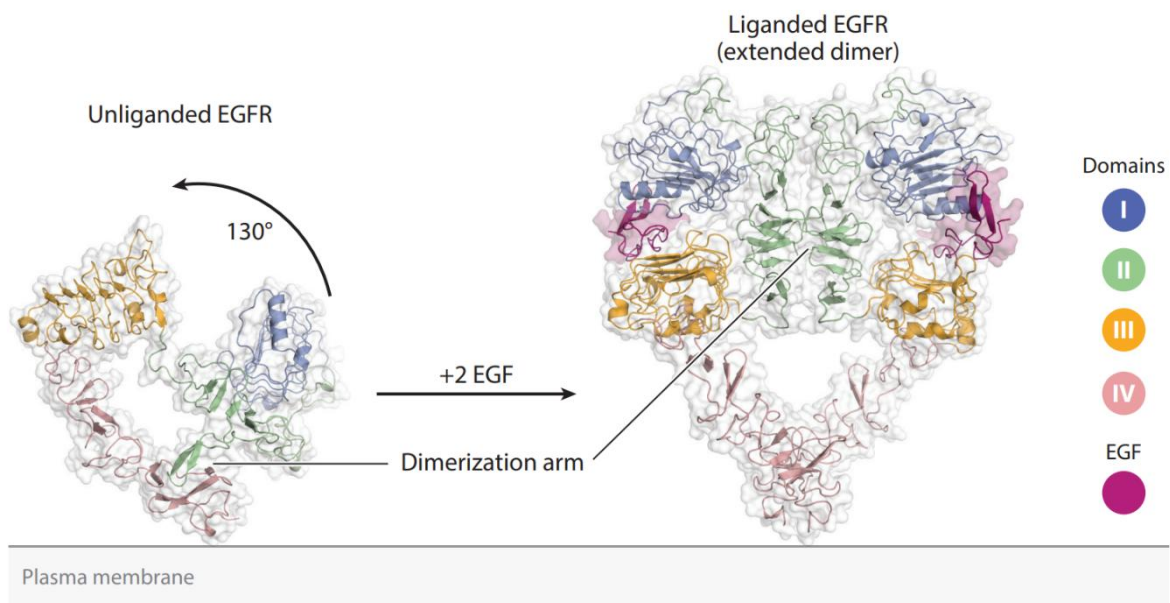
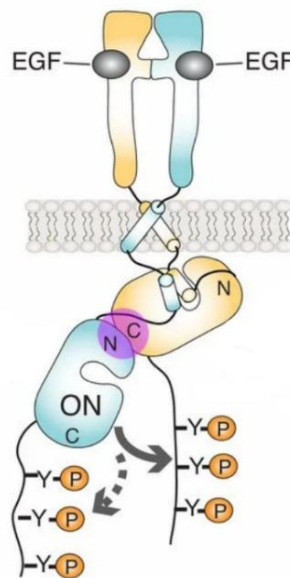


Figure 5: Extracellular part of epidermal growth factor receptor (EGFR). Left: inactive EGFR with unexposed dimerization arm. Right: EGFR bound to ligand and dimer formation. Figure adapted from Kovacs et al. (2015)<sup>59</sup>

EGFR is a kinase receptor, containing a conserved kinase DFG motif, within its activation loop. The DFG motif enables the kinase to phosphorylate substrates.<sup>60</sup> The dimerization of EGFRs also brings the intracellular domains of both protomers close together, including their kinase domains. Unlike other RTKs, EGFRs activate themselves via an asymmetric kinase dimer mechanism, not via trans-autophosphorylation. Here, one kinase domain acts as an activator and the other one as a receiver. The activator kinase binds to the C-lobe of the receiver kinase and activates it. The activated receiver now phosphorylates specific tyrosine residues on its own C-terminal tail as displayed in Figure 6.<sup>59,61</sup>



*Figure 6: Active EGFR. Instead of symmetrically dimerizing, kinase domains align asymmetrically. Blue: activator kinase, binds to C-lobe of receiver kinase. Yellow: receiver protein, gets activated and autophosphorylates itself. Figure adapted from Littlefield & Jura (2013)<sup>61</sup>*

Specific phosphorylated Tyr-residues on EGFR, Tyr1068 and Tyr1086, get recognized by growth factor receptor-bound protein 2 (GRB2). GRB2 is an adaptor protein that has a conserved Src homology (SH) 2 domain. GRB2 recognizes and binds one of the phosphorylated tyrosine residues, via its SH2 domain.<sup>59,62</sup>

GRB2 brings another protein along to the plasma membrane. Via its two SH3 domains, GRB2 binds to proline-rich motifs in Son of Sevenless (SOS). The GRB2 – SOS complex is already formed in the cytosol, but is autoinhibited. The catalytic domain of SOS is partially blocked when residing in the cytosol and the effector of SOS, which is RAS, is anchored in the membrane via post-translational modifications. Therefore, SOS needs to be recruited to the membrane to reach its substrate. GRB2 brings SOS in proximity to RAS, where it will undergo interactions with membrane lipids, such as PIP2 and PIP3. As a result, SOS will slightly change its conformation to a more open state, already able to activate a few RAS proteins. Via a positive feedback loop, RAS will bind to SOS and completely stabilize its open conformation.<sup>63</sup>

RAS proteins are small GTPases. They are binary ON and OFF switches that propagate the signal in the cell. When RAS is inactive, RAS is bound to GDP (RAS•GDP). SOS functions as a guanine nucleotide exchange factor (GEF), driving the dissociation of RAS and GDP. It positions itself over the pocket where GDP is bound and binds to the RAS protein with its CDC25 catalytic domain, visualized in Figure 7. This increases the pressure on structural elements of RAS, needed to stabilize GDP. After GDP is pushed out by SOS, RAS stochastically binds a new guanine nucleotide. Because GTP levels in the cell are higher than GDP levels, the net result will be an exchange between GDP and GTP. In a GTP-bound state, RAS (RAS•GTP), is active and can propagate the signal to RAF.<sup>56,63,64</sup>

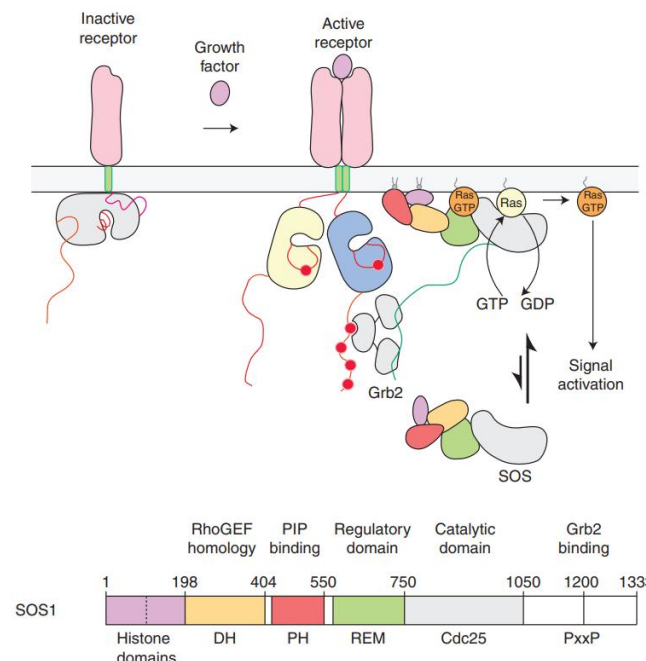


Figure 7: GRB2 - SOS complex linked together via proline-rich motifs of SOS and SH3 of GRB2. The complex is linked to the receiver kinase. Domains of SOS displayed at bottom. CDC25 domain interacts with RAS. Figure adapted from Bandaru et al. (2019)<sup>63</sup>

RAS•GTP activates RAF proteins via a multistep allosteric and localization mechanism. Scaffold proteins, like 14-3-3, hold RAF proteins in their inactivated conformation. When RAS is bound to GTP, it instantly recruits RAF and interacts with its RAS-binding domain. This translocation to the membrane is crucial for the activation of RAF, since it can only dimerize when it is bound to RAS. Binding to RAS exposes the kinase domain, rearranges the binding pattern with 14-3-3 and drives dimerization. Membrane-linked kinases can further activate RAF via phosphorylations. Multiple RAF isoforms are present in the cell, but CRAF and BRAF are the most prevalent ones. CRAF, also known as RAF1, often forms heterodimers with BRAF, but CRAF-CRAF homodimers can also be present in the cell.<sup>65,66</sup> RAS•GTP can be inactivated by GTPase activating proteins (GAPs). They trigger hydrolysis of GTP to GDP, resulting in an inactive RAS•GDP state.

Further down the pathway, activated RAF serine/threonine kinases (Ser/Thr kinases), functioning as a dimer, will phosphorylate both MEK1 and MEK2 on two Ser residues each. Both phosphorylations are needed to fully activate the MEK monomers and enable dimerization into a MEK1 – MEK2 complex, stabilizing their DFG-in state.<sup>56,65</sup>

This MEK complex is a dual specificity kinase, capable of phosphorylating Ser/Thr as well as Tyr residues. Both MEK1 and MEK2 can phosphorylate a Thr and a Tyr residue on ERK1 and ERK2. Phosphorylated ERK (p-ERK) will dimerize and get into the DFG-in state. Dimers of p-ERK are translocated to the nucleus, due to the exposure of their nuclear translocation signal. This signal becomes visible after a conformational change, induced by the phosphorylations. Inside the nucleus, p-ERK acts as a kinase and phosphorylates multiple substrates.<sup>56,66–68</sup>

The most important substrates of p-ERK are the TFs ELK1, c-Fos and c-Myc. ELK1 induces the transcription of multiple TF-complexes, just as c-Fos. c-Myc will act as a master regulator of growth, cell cycle and metabolism. Together with the other substrates of p-ERK, hundreds to thousands of genes are expressed or inhibited upon RAS signaling.<sup>68</sup> Feedback regulation is activated via the transcription of genes that deactivate p-ERK, activate GAPs, lead to internalization of RTKs and inhibit SOS and GRB2 phosphorylation.<sup>56</sup> Figure 8 shows a complete overview of the RAS pathway.<sup>69</sup>

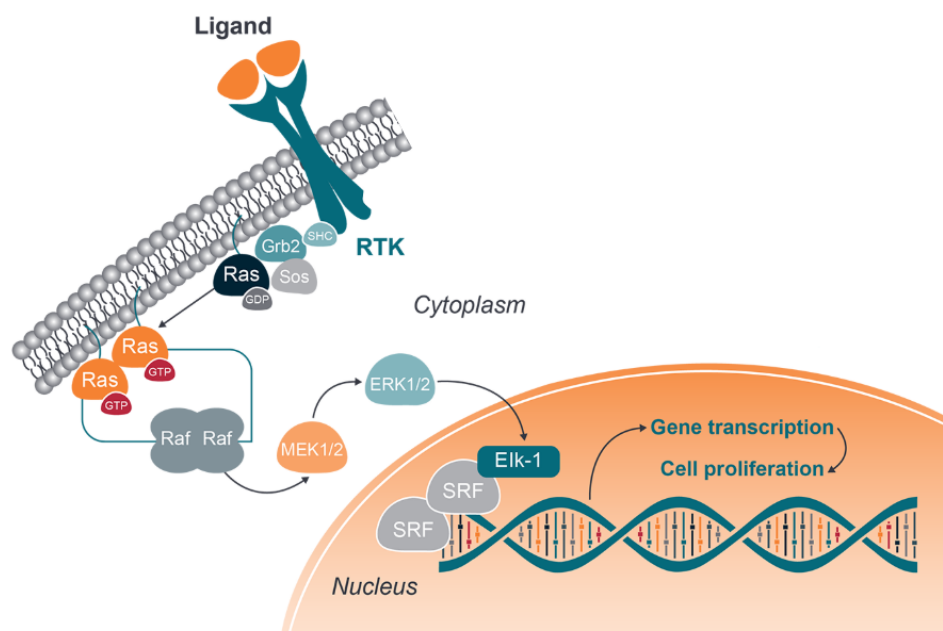


Figure 8: canonical RAS signaling summary. Figure adapted from Hua (2024)<sup>69</sup>

The main cellular response to RAS signaling is cell proliferation. The activated genes promote cell cycle entry and drive DNA synthesis. Furthermore, cell survival is promoted and pro-apoptotic pathways are suppressed. The anabolic metabolism is turned on, leading to cell growth. In normal cells, these responses are needed for multiple mechanisms, including tissue homeostasis, cell survival under stress, normal development and wound healing.<sup>56</sup>

RAS is not exclusively activated via RTKs. Other receptors, such as G-protein coupled receptors (GPCRs) and cell adhesion receptors can also activate RAS. Likewise, RAS can also affect other signaling cascades, such as the PI3K-AKT pathway.<sup>70</sup>

### 1.5.2 RAS proteins

Humans have three different genes coding for RAS proteins, resulting in four different isoforms due to alternative splicing. The different genes, *KRAS*, *NRAS* and *HRAS*, are likely the result of an ancestral *RAS* duplication and divergence in the past. *KRAS* is located on chromosome 12 at the p-arm on position 12.1 (12p12.1) and four exons will eventually produce the protein. *KRAS* can produce two isoforms, because the final exon in the protein can be spliced in two ways, leading to two different proteins, KRAS4A and KRAS4B. *NRAS* is located at 1p13.2 and also has four exons, but only one isoform can be produced, since there is no alternative splicing of exon 4. *HRAS* is located at 11p15.5 and has a similar gene structure as *NRAS*.<sup>65,71,72</sup>

The main difference among the three RAS genes is their expression pattern. They are all activated via the same receptors and they all induce p-ERK signaling, but the location where they are expressed differs. *KRAS* is predominantly present in the pancreas, colon and lungs. *NRAS* is more prevalent in hematopoietic cells, whereas *HRAS* functions more in the bladder, kidneys and thyroid.<sup>56,71,72</sup>

All four proteins consist of a highly conserved G-domain and a hypervariable region (HVR). The G-domain includes multiple subdomains or motifs, needed to bind GTP/GDP, induce a conformational change (switch II pocket) and interact with effectors. Figure 9 shows that, due to its highly specific function, this region is almost identical in all four RAS proteins. The switch II pocket is the most important structure for GTP hydrolysis. It contains amino acids needed to position the  $\gamma$ -phosphate of GTP for hydrolysis and other amino acids that interact with GAPs. The HVR is located at the C-terminus and is needed for membrane targeting and isoform-specific interactions. Deviations between the different proteins are predominantly located in the HVR, enabling the proteins to differentiate in their subcellular location and their effector selectivity.<sup>71</sup>

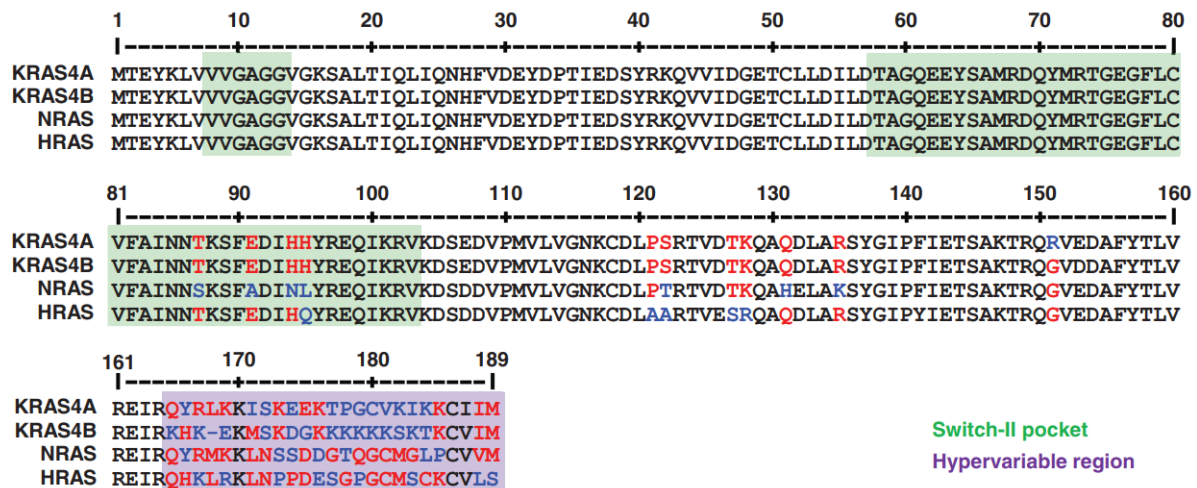


Figure 9: Amino acid sequence alignments of the four RAS isoforms. Amino acid differences are shown in red and blue. Regions involved in the switch-II binding pocket are shaded in green, and the hypervariable region is shaded in purple. Figure copied from Robinson et al. (2024)<sup>103</sup>

Differences in HVR allow for different post-translational modifications. All isoforms get farnesylated at the C-terminus of the protein. The CAAX box is a motif for farnesylation. Farnesyltransferase adds a 15-carbon farnesyl group, a lipid compound, to the cysteine residue. Afterwards the -AAX residues are removed by a protease and a methyl group is added to the cysteine. This farnesylation is required for all isoforms, enabling proper anchoring to membranes.

Figure 10 demonstrates that there are also other post-translational modifications. HRAS, NRAS and KRAS4A contain, besides farnesylation sites, additional palmitoylation sites. There, a 16-carbon palmitic acid is added on a cysteine. NRAS and KRAS4A both get palmitoylated once, whereas HRAS gets palmitoylated twice. Unlike farnesylation, palmitoylation is reversible, making dynamic trafficking between the Golgi apparatus and the plasma membrane possible. This trafficking is crucial to get RAS proteins to the right place and to organize them in nanoclusters. Without depalmitoylation, RAS would accumulate in the wrong lipid microdomains, unable to signal properly.<sup>71</sup>

KRAS4B has multiple lysine-rich residues (K) next to each other in its HVR. These positively charged residues allow interactions with negatively charged phospholipids in the plasma membrane, such as PIP2. KRAS4B does not rely on palmitoylation for Golgi cycling, since it can regulate the charge of its K-stretch by phosphorylations that neutralize the positive charges.<sup>71</sup>

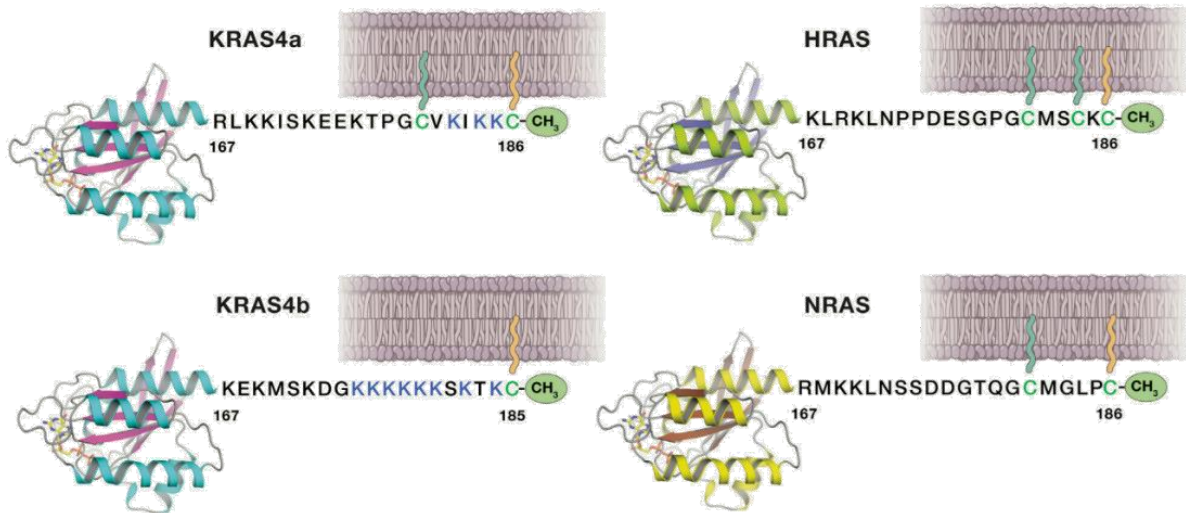


Figure 10: The last 19-20 amino acids located at the C-terminal end show high differences among RAS isoforms and form the hypervariable region (HVR). KRAS4B is not palmitoylated and contains a polybasic region (shown in blue color). Membrane-anchored farnesyl and palmitoyl lipid chains are shown in orange and green colors respectively. Figure copied from Simanshu (2017)<sup>71</sup>

### 1.5.3 RAS mutations in HeH B-ALL

Previous studies, using bulk sequencing techniques, reported that approximately half of the HeH B-ALL cases involve RAS mutations.<sup>37,73</sup> However, a recent single cell characterization showed the presence of at least one subclone with a RAS mutation in each of the patients.<sup>36</sup> The mutations found in RAS proteins always lead to a gain of function, resulting in a constitutively active RAS, driving excessive RAS signaling. KRAS is the most mutated RAS protein in HeH B-ALL patients, followed by NRAS. Interestingly, KRAS and NRAS mutations never occur within the same cell, indicating that they are mutually exclusive.<sup>74</sup> HRAS mutations have not been reported in lymphoid, nor in myeloid neoplasms.<sup>38</sup>

Often reported KRAS mutations are KRAS<sup>G12C</sup>, KRAS<sup>G12V</sup>, KRAS<sup>G13D</sup> and KRAS<sup>A146T</sup>. The glycine at position 12 is located in the P-loop and involved in interactions with GAP proteins. When this glycine is replaced by a cysteine, the GAP protein of KRAS is sterically blocked and GTP hydrolysis cannot happen. RAS will stay stuck in the active RAS•GTP state. Changing this glycine to a valine, gives a similar result. Besides GAP proteins, also catalytic residues will be blocked, additionally impairing intrinsic hydrolysis activity. In KRAS<sup>G13D</sup> mutations, the glycine at position 13 is replaced by an aspartate. This residue is negatively charged, repelling the negatively charged  $\beta$ -phosphate group of GDP. This increases spontaneous GDP release without a GEF, leading to more GTP-bound RAS, independently of SOS. In KRAS<sup>A146T</sup> an alanine in the helix 5 $\alpha$  domain is replaced by a threonine. This increases the flexibility of the helix, destabilizing the nucleotide binding pocket. Therefore, the affinity for GDP is reduced and via the same principle as in KRAS<sup>G13D</sup>, RAS•GTP becomes the default cell setting.<sup>36–38,75,76</sup>

Most reported NRAS mutations are NRAS<sup>G12C</sup>, NRAS<sup>G12D</sup>, NRAS<sup>G12S</sup>, NRAS<sup>G13D</sup> and NRAS<sup>Q61H</sup>. Due to the conserved G-domain, the gain of function in NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup> can be explained by the same mechanism as in KRAS<sup>G12C</sup> and KRAS<sup>G13D</sup>. NRAS<sup>G12D</sup>, where a glycine is replaced by an aspartate, also sterically hinders binding with GAP proteins, impairing hydrolysis of GTP. NRAS<sup>G12S</sup> contains a serine at position 12, blocking GAP interaction and disrupting GDP binding via its polar hydroxyl group. In NRAS<sup>Q61H</sup> the glutamine at position 61 is replaced by a histidine. Glutamine is part of the switch II pocket, where it normally positions the catalytic nucleophile, Mg<sup>2+</sup>, and γ-phosphate. Histidine completely disrupts intrinsic, as well as GAP-stimulated GTP hydrolysis, by preventing correct positioning of these players.<sup>36–38,75,76</sup>

Excessive RAS signaling interferes with the pre-BCR signaling threshold needed for normal early B-cell maturation. B-cell progenitor cells rely on the RAS pathway for their development. The signal is regulated precisely at different time points during maturation.<sup>77</sup> Hyperactive RAS signaling seems to impair normal development of B-cells, although a precise mechanism has not been described yet. It drives the cell into a continuous proliferation cycle and survival condition, keeping the cell in a more stem cell-like state.<sup>76</sup>

#### 1.5.4 Aneuploidy & RAS mutations

Based on the number of RAS mutations found in aneuploid cancers, a correlation between aneuploidy and RAS mutations is expected. Multiple previous studies have already pointed out a correlation between aneuploidy and CRAF activity, the downstream effector of RAS.<sup>45,64,78</sup> In aneuploid cells, CRAF is consistently found in its activated form (p-CRAF).<sup>45</sup> Aneuploid cells also seem more vulnerable to CRAF inhibition than normal cells, suggesting that activated CRAF is needed to cope with aneuploidy-induced stress.<sup>45</sup> Aneuploid cells express more DDR genes compared to normal cells. Interestingly, the activity of CRAF seems to be functionally linked to the DDR. Because aneuploid cells are often under a growth disadvantage compared to euploid cells, they show slower proliferation rates and higher stress levels. Activation of CRAF results in stronger MAPK signaling, which might counterbalance this disadvantage.<sup>45</sup>

It remains unknown if CRAF levels are also upregulated at transcriptional level. However, upon aneuploid stress, posttranslational modifications can promote CRAF activation. Proteotoxic stress induced by aneuploidy, will activate RAC1 and CDC42, via heat shock protein 90 (HSP90). These are direct upstream activators of the kinase PAK1. Other mechanisms, such as increased ROS and replication stress, also activate PAK1. Another stress-activated kinase is Src kinase. Cytoskeletal disruption, caused by aneuploidy, will activate Src kinase, just as DNA damage via the DDR. PAK1 and Src can phosphorylate Ser338 and Tyr341 respectively, activating CRAF and driving dimerization independently of RAS and EGFR signaling.<sup>64,78–81</sup>

The Src kinase family is the same family of proteins that plays a key role in B-cell development. They signal when a pre-BCR binds and internalizes its antigen. Src kinases are responsible for MHC class II upregulation and the formation of activated B-cell clusters. However, defects in Src signaling have not been linked to HeH B-ALL, as Src genes are not mutated in HeH B-ALL patients. Furthermore, the stress signal that induces Src, caused by aneuploidy, is transient and systemic throughout the cell, whereas Src signaling for B-cell development is highly regulated, spatially and temporally. Therefore it seems unlikely that Src signaling, caused by the HeH character, can impair B-cell development and drive leukemogenesis on its own.<sup>81</sup>

CRAF is normally only present in limiting amounts relative to its interactors RAS and MEK.<sup>72</sup> Because CRAF is the bottle neck of the RAS signaling pathway, increasing CRAF activity, via Src and PAK1, will already amplify the signal and elevate the basal signaling level of the RAS pathway.<sup>45</sup> Although CRAF can be activated independent of RAS, its main activator remains RAS. Aneuploid cells that acquire a stochastic RAS mutation, leading to a gain-of-function, could therefore suddenly unlock a growth advantage over non-mutated aneuploid cells. Because of that, the combination of HeH and RAS mutations may form the ideal cell setting to drive abnormal immature B-cell proliferation and leukemogenesis in HeH B-ALL. On top of that, this connection between CRAF and aneuploidy suggests that also aneuploid cells that do not show a gain-of-function mutation in a RAS protein, are vulnerable to inhibiting this signaling pathway.<sup>45</sup>

## 1.6 Treatment of ALL

The current overall survival rate of ALL is approaching 90% and in HeH B-ALL this is already 95%. Despite this number, leukemia is still one of the leading causes of cancer-related deaths in children. This is mostly due to relapses, happening in 15% of the cases. Because HeH B-ALL patients form the largest subgroup of B-ALL, they account for most relapses in absolute numbers.<sup>32,82–85</sup>

### 1.6.1 Chemotherapies

Currently ALL is treated with intensive multi-agent chemotherapy.<sup>86</sup> The treatment consists of different phases and takes up to two years. Induction therapy includes a glucocorticoid, asparaginase, vincristine, intrathecal chemotherapy and the optional use of anthracycline.<sup>33</sup> Glucocorticoids induce pro-apoptotic genes in complex with their glucocorticoid receptor, driving apoptosis.<sup>87</sup> Asparaginase is an enzyme that hydrolyzes extracellular asparagine. ALL cells often lack asparagine synthase, so they cannot produce asparagine themselves, making them dependent on this extracellular asparagine.<sup>88</sup> Vincristine binds to  $\beta$ -tubulin, preventing microtubule polymerization. This will disrupt the mitotic spindle, leaving rapidly dividing cells stuck in mitosis.<sup>89</sup> Intrathecal chemotherapy is often conducted with methotrexate, cytarabine or hydrocortisone. The use of anthracyclines is optional and involves molecules that intercalate into DNA, inhibit topoisomerase II and form free radicals, causing transcription and replication blockage, DNA double-stranded breaks and oxidative DNA damage.<sup>90</sup> All these effects will lead to apoptosis of rapidly dividing cells. The induction therapy takes around five weeks, with the goal of achieving remission.<sup>33</sup>

After remission, patients undergo intensive combinations of chemotherapies designed to consolidate remission for about seven months. This involves repeated courses of methotrexate, a folic acid analog. This drug inhibits dihydrofolate reductase, an enzyme that catalyzes a reaction needed for the production of purines, amino acids and thymidylate (dTMP). By blocking this enzyme the cell will be depleted of purines and dTMP, leading to a DNA synthesis halt. Mostly rapidly dividing cells are affected by this drug, driving apoptosis in proliferating cells.<sup>33,91</sup>

Afterwards, patients receive low-intensity anti-metabolite maintenance therapy. Daily doses of mercaptopurine or thioguanine are administered orally for a period of 18 – 30 months.<sup>33</sup> Mercaptopurine and thioguanine are both purine analogs. Mercaptopurine gets metabolized and eventually misincorporated in DNA, leading to DNA damage and replication stress. It also inhibits de novo synthesis of purines due to enzyme blocking. Thioguanine also gets

metabolized and incorporated in the DNA, triggering mismatch repair pathways leading to DNA damage.<sup>92</sup> Furthermore, weekly oral intake of methotrexate is needed.<sup>33</sup> Sometimes periodic doses of glucocorticoids and vincristine are also added to the maintenance therapy. The optimal composition and duration of chemotherapy for ALL is yet unknown, due to this highly heterogeneous profile and the resulting variety amongst patients.<sup>33</sup>

15 – 20% of children with ALL will relapse. Unfortunately, this comes with a worse prognosis than the first diagnosis. When children relapse after the primary treatment nearly all of them will reach a second remission and the chance at complete cure is around 50%. When relapse already occurs during the treatment, the chance at a second remission drops to 50 – 70% and only 20 – 30% will eventually be cured. Because of the genetic heterogeneity of ALL, certain subclones may be selected and promote resistance against chemotherapy.<sup>33</sup> Therefore, in relapsed patients with high-risk ALL an allogeneic hematopoietic cell transplantation (allo-HCT) is often conducted, where a patient receives healthy HSCs from a competent donor.<sup>93</sup>

Chemotherapies can cause a wide range of side effects on patients, both short term and long term. During treatment, patients can experience fatigue, organ toxicity, infections and overall distress. Nearly all ALL patients struggle with neutropenia, a low number of neutrophils, during chemotherapy. This increases the risk of infection. Other effects are tingling, numbness and pain in feet and hands due to nerve damage caused by certain chemotherapeutics. Memory loss, slower processing of information and difficulties regarding concentration are common issues in pediatric survivors. More downsides of chemotherapy are cardiovascular effects, depression, anxiety, vomiting, diarrhea, inflammation, liver and kidney damage and an increased risk of secondary malignancies.<sup>33,94–96</sup>

## 1.6.2 Current next-generation therapies

The improvements in survival of pediatric ALL over the past decades are rather due to the optimization of chemotherapeutic concentrations and time points of administration, than due to the development of new therapies. Recent discoveries however seem promising. Diving deeper into the genetic basis of ALL allowed for the development of targeted therapies and precision medicine. In the last 10 years, multiple targeted drugs made it from clinical trials into medical use against ALL.<sup>33</sup> All mentioned drugs are approved by the Food and Drug Administration (FDA) and are published on the website of the NCI.<sup>97</sup>

Different tyrosine kinase inhibitors, such as Imatinib and Dasatinib, are already in use against Philadelphia chromosome positive (Ph<sup>+</sup>) ALL. The Ph<sup>+</sup> ALL subtype is characterized by the expression of a constitutive active tyrosine kinase, driving uncontrolled cell proliferation and inhibiting apoptosis. By inhibiting this kinase, the cells arrest in the G1 phase and die.<sup>97,98</sup>

Monoclonal antibodies and antibody-drug conjugates (ADCs) are therapies based on antibodies that bind target receptors on B-cells. ADCs are mostly used in relapsed patients. An example is Blinatumomab, a bispecific antibody, linking T-cells and B-cells via CD3 – CD19 interactions, inducing a cytotoxic synapse. Upon binding, the T-cell releases perforins and granzymes to disrupt the cancerous B-cell.<sup>99</sup>

Chimeric Antigen Receptor T-cell (CAR-T) therapies are advanced immunotherapies, where the T-cells of a patient are used to fight the cancer.<sup>33</sup> In a first stage, the T-cells of the patient are collected and a CAR-gene is introduced into the T-cells via a lentiviral vector. This gene codes for a transmembrane protein, containing an extracellular fragment, derived from an antibody that binds CD19. CD19 is a receptor present on nearly all B-cells. These CAR-T cells are cultured and infused into the patient again. The transduction of these T-cells enables them to recognize CD19 and bind it, without presentation of a peptide-MHC complex. This leads to a cytotoxic response, involving perforins and granzymes.<sup>33,97,100</sup> In a study of 30 relapsed children, CAR-T treatment resulted in remission for 90% of the patients.<sup>101</sup> However, some cancerous B-cells acquire mutations in their CD19 receptor so they are not recognized any longer. Others are CD19-negative or they hide or block a part of their CD19 receptor, called epitope masking, as seen in Figure 11. CD19-negative subclones may already be present before the treatment.<sup>36</sup>

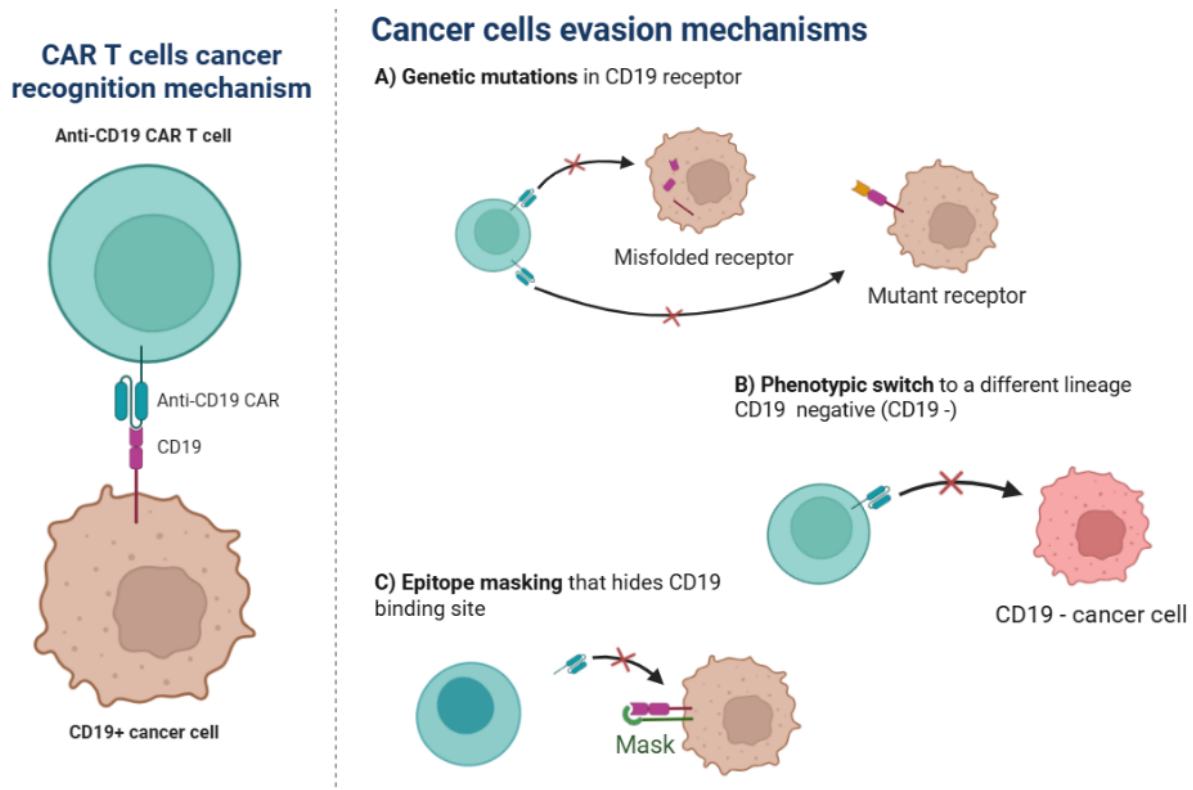


Figure 11: Right: CAR-T system schematically displayed. Left: three main evasion mechanisms of cancer cells: Genetic mutations, phenotypic switches and epitope masking. Figure was created with BioRender.com

### 1.6.3 Targeting the RAS pathway

Although multiple next-generation drugs are already in use against ALL, none of them are particularly designed against HeH B-ALL. Due to the different characteristics between ALL subtypes, looking for more specific targets and drugs remains valuable. Furthermore, only combination therapies are currently administered. This means that chemotherapies, in combination with next-generation drugs, are still applied and the downsides are still experienced by patients. Previous studies showed the reliance of HeH B-ALL on the RAS pathway, indicating that this pathway might be a valuable target to treat HeH B-ALL.<sup>45</sup>

For a long time RAS was called undruggable, due to the lack of any hydrophobic binding pockets and its picomolar affinity for GTP.<sup>102</sup> This made the use of GTP-competitive inhibitors impossible. Over the last years, this view has changed by shifting the approach to mutation-specific pockets, covalent chemistry and degradation of RAS.<sup>102</sup> Now the RAS pathway can be targeted via multiple ways, although none of these novel small molecules have been tested against HeH B-ALL.<sup>97</sup>

Direct RAS inhibitors lock the GTPase in a certain conformation, mostly in its inactive state, impairing GDP release. Other compounds lock RAS in its active state, but prevent interactions of RAS with its downstream effectors. In both cases most small molecules are designed to bind near the switch II pocket.<sup>103,104</sup> Pan-RAS inhibitors are special RAS inhibitors, designed to target all isoforms of RAS.<sup>103</sup> Other targets are downstream proteins involved in the RAS signaling pathway. By inhibiting their function, canonical RAS signaling is also impaired.<sup>65,104</sup> Indirectly inhibiting proper functioning of RAS signaling by targeting enzymes needed for RAS localization, might also be a valuable option to target RAS signaling. Inhibiting the palmitoylation or depalmitoylation prevents dynamic trafficking of RAS isoforms between the Golgi apparatus and the plasma membrane that rely on this posttranslational modification.<sup>105</sup>

Another novel technique is proteolysis targeting chimera (PROTAC). This is based on degradation of the protein, rather than inhibition. Unlike inhibitors, degraders completely erase proteins from the cell. PROTACs are composed of a drug, with affinity for the target protein, and an E3 ligase ligand. They are linked to each other via a linker and the E3 ligase ligand will recruit E3 ligase. E3 ligase is a naturally occurring molecule in cells, where it is part of the protease machinery. Using this mechanism, PROTACs recruit E3 ligase to certain targets in the cell. Once E3 ligase is bound, it recruits the charged E2 enzyme, which carries ubiquitin. Based on the amount of ubiquitin added, the proteins are marked for degradation, signaling or localization. It is a naturally occurring process in healthy cells to degrade malfunctioning proteins or to downregulate certain proteins after expression. Via the use of PROTACs, multiple ubiquitin molecules are placed onto the target protein. Polyubiquitination marks this protein for degradation by the proteasome, depleting the cells of the targeted protein.<sup>104,106</sup>

Drugs that directly inhibit RAS can be specifically designed to target mutated forms of RAS, only targeting cancerous cells. However, most of the available drugs exhibit downstream inhibition and indirect inhibition, and are also detrimental against regular RAS signaling in normal cells. Despite that, the drugs should not affect normal cells as much, because they have redundant survival pathways.<sup>104</sup> HeH B-ALL cells seem to rely more on RAS signaling to overcome their genomic instability, making them possibly more vulnerable to RAS inhibition.

## 2 Research hypothesis and aims

ALL is, unlike some other cancers such as chronic myeloid leukemia (CML) and non-small cell lung cancer (NSCLC), treated with an intensive multi-agent chemotherapy. Additionally, no targeted therapies are used against HeH B-ALL. This lack of specific drugs, together with the genetic heterogeneity of this cancer and variety amongst patients, highlights the need to further investigate new drugs and expand clinical trials. This will enhance the quality of life of patients during treatment, by reducing chemotherapy and shifting towards more targeted therapies and immunotherapies.

Since the last decade, promising candidate small molecules have been developed to target the RAS pathway. However, none of these have been tested or are in clinical trial against HeH B-ALL. Because the hypothesis is that the RAS pathway is important to overcome hyperdiploid stress and drive leukemogenesis, this pathway might be a valuable target in HeH B-ALL treatment. Therefore, testing these drugs is an important step towards improving the life of patients.

The goal of this research is to establish a pro-B cell model, expressing various RAS mutations that are frequently reported in HeH B-ALL patients. Afterwards, an in vitro screen of different small molecules that target different links of the RAS pathway will be conducted. Multiple drugs with different modes of action will be tested. Although these drugs are already established RAS pathway inhibitors, their activity has never been characterized in pro-B cell models. Therefore, their effects in a B-lineage context remain unknown. Evaluating these drugs in a pro-B cell system will allow for the identification of lineage-specific responses or vulnerabilities in a cellular environment close to B-ALL.

Next-generation drugs that show promising responses in the pro-B cell line, can be tested in patient-derived xenograft (PDX) models in the future. These PDX models allow to study the effect of the drugs on HeH B-ALL in an in vivo system. In the end, the goal is to get a better understanding of the role and the importance of the RAS pathway in HeH B-ALL and to propose next-generation candidate drugs to explore further in more HeH B-ALL-like models.

## 3 Materials & Methods

### 3.1 Materials

Different kits were used in this research. The CloneJET PCR Cloning Kit (ThermoFisher) was used to amplify DNA inserts. With the Q5<sup>®</sup> Site-Directed Mutagenesis Kit (New England Biolabs), single nucleotide mutations were induced at specific locations in plasmids. For the isolation of plasmids out of bacteria the CompactPrep Plasmid Midi Kit (Qiagen) was used. DNA extraction was conducted with the Maxwell<sup>®</sup> RSC Cultured Cells DNA Kit and the Maxwell RSC machine (Promega). ATPlite assays were performed with the ATPlite 1step Luminescence Assay System (Revvity).

DNA inserts used in this research were ordered from GenScript, and primers were ordered from Integrated DNA Technologies (IDT). Subcloning Efficiency<sup>™</sup> DH5 $\alpha$  Competent Cells (ThermoFisher) were used to conduct the transformations and were grown in Super Optimal broth with Catabolite repression (SOC) medium (New England Biolabs). Transductions were conducted with FuGENE HD transfection reagent (Promega E2312). Medium for cell culture was acquired from Gibco<sup>™</sup> and culturing flasks from ThermoFisher. Sanger sequencing was performed by Eurofins Genomics. Drugs were bought from MedChemExpress.

Primary antibodies used for Western Blotting: **Vinculin** (Sigma-Aldrich, Anti-Vinculin Antibody V9131-2ML), **ERK** (Santa Cruz Biotechnology, ERK 2 Antibody (D-2): sc-1647), **p-ERK** (Cell Signaling Technology, Phospho-p44/42 MAPK Erk1/2 Thr202/Tyr204 Antibody #9101), **KRAS** (Proteintech, KRAS Polyclonal antibody 12063-1-AP) and **pan-RAS** (Cell Signaling Technology, K-Ras Antibody #33197). Secondary antibodies used for Western Blotting: **mouse** (Cytiva, Anti-mouse IgG antibody NA931V), **rabbit** (Cytiva, Anti-mouse IgG antibody NA934V).

### 3.2 Drug selection

A large literature screen was performed, looking for drugs that target different links within the RAS-pathway. The selection consists of already known drugs that show promising results against other RAS-dependent cancers and promising novel drugs described in recent studies. Drugs were dissolved in DMSO, so a concentration of 10 mM was obtained. Working solutions were kept at -20°C and stock aliquots were kept at -80°C.

### 3.2.1 RAS inhibitors

**Sotorasib** (Cat. No.: HY-114277) is a covalent inhibitor that is designed to specifically target KRAS<sup>G12C</sup> by irreversibly locking the mutated RAS protein in the inactive GDP bound state (Figure 12A). The drug binds to the sulfur atom in the cysteine, present in KRAS<sup>G12C</sup>, preventing GDP release. In Europe and in the USA it is approved as a clinical drug for non-small cell lung cancer (NSCLC), and in the USA it is also approved for Metastatic Colorectal Cancer (mCRC). This drug was chosen because it is a well known mutation-specific covalent inhibitor, whereas most other small molecules are not mutation-specific.<sup>103,107</sup>

**Pan-RAS-IN-1** (Cat. No.: HY-101295) is a small molecule that targets all three RAS isoforms (Figure 12B). It binds to a conserved region near the switch II pocket and thereby interrupts the interaction between RAS proteins and their downstream effectors. Currently, Pan-RAS-IN-1 is not approved into clinical trials. This small molecule was selected due to its capability to target all RAS isoforms.<sup>107</sup>

**BI-2865** (Cat. No.: HY-153724) is a non-covalent pan-KRAS inhibitor that targets WT KRAS, KRAS<sup>G12C</sup>, KRAS<sup>G12D</sup>, KRAS<sup>G12V</sup> & KRAS<sup>G13D</sup> (Figure 12C). BI-2865 binds in an allosteric pocket in the Switch II region of KRAS, preventing GDP release. This drug is not approved for clinical use in humans. BI-2865 was selected, since it should have a higher potency towards the mutant RAS-proteins, compared to the WT.<sup>107</sup>

**Daraxonrasib** (Cat. No.: HY-148439) is a pan-RAS inhibitor that binds within a conserved pocket around the Switch II pocket. RAS gets locked in its active state, but cannot signal because of the impaired interaction with its effectors (Figure 12D). Currently Daraxonrasib is in clinical trial against NSCLC and several RAS-mutated ALL subtypes. This drug was selected, because it targets RAS in its active form, which is often the state of RAS in cancers. It is supposed to have a higher potency than Pan-RAS-IN-1.<sup>107</sup>

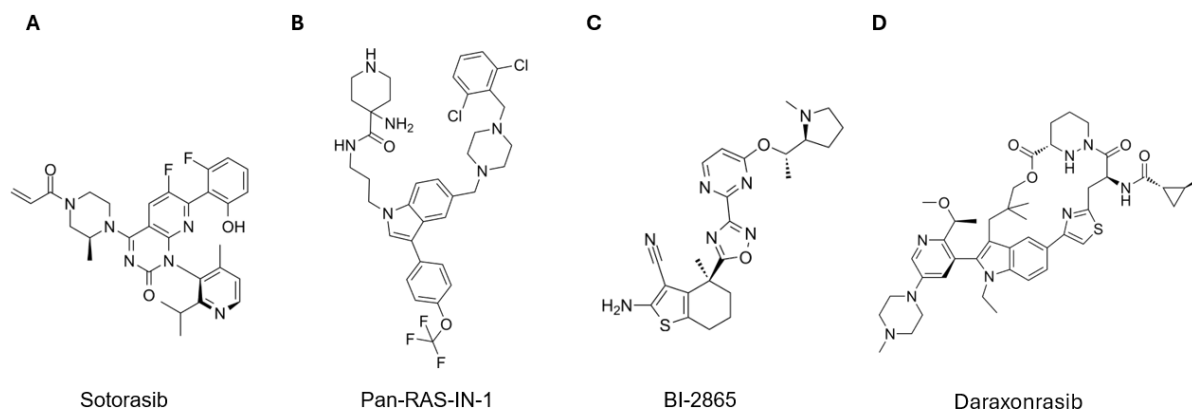


Figure 12: Chemical structure of selected RAS inhibitors. A: Sotorasib; B: Pan-RAS-IN-1; C: BI-2865; D: Daraxonrasib. Structures retrieved from MedChem.com

### 3.2.2 RAS degraders

**PROTAC K-Ras Degradar-4** (Cat. No.: HY-159788) is a novel drug making use of the PROTAC technique (Figure 13A). It targets possibly all KRAS proteins, but should be more efficient against KRAS<sup>G12D</sup> and KRAS<sup>G12V</sup>. This difference in potency between mutant and WT RAS proteins, is the reason this drug was selected. Since the novelty of the drug, it has not yet been approved into any clinical trials.<sup>106,107</sup>

**ACBI3** (Cat. No.: HY-157228) is another PROTAC targeting KRAS proteins (Figure 13B). ACBI3 binds nearly all KRAS mutants, but with more affinity towards KRAS<sup>G12D</sup>, KRAS<sup>G12V</sup> and KRAS<sup>G13D</sup>. It is also able to degrade WT KRAS, although with lower potency, which is why this drug was selected. Also ACBI3 is currently not in any clinical trial.<sup>106,107</sup>

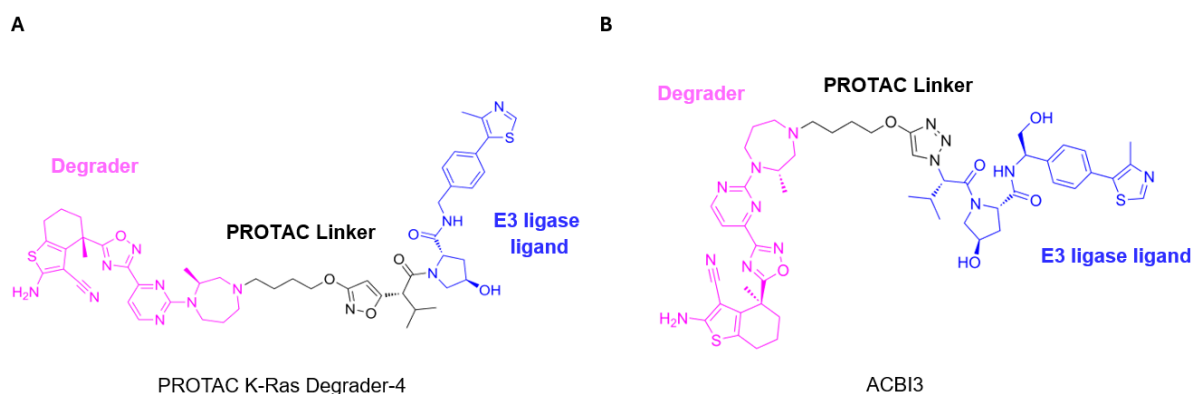


Figure 13: Chemical structure of selected RAS degraders. A: PROTAC K-Ras Degradar-4; B: ACBI3. Both function as PROTACs. Structures retrieved from MedChem.com

### 3.2.3 Downstream RAS inhibitors

**Naporaafenib** (Cat. No.: HY-112089) is a type II RAF inhibitor, binding the inactive form of RAF and occupying the ATP-binding site (Figure 14A). An advantage of Naporaafenib is that it binds both protomers in a RAF dimer, thereby preventing transactivation, which was a problem with other RAF inhibitors. The binding to two protomers, together with the suggestions that RAF is a possible target in aneuploid cells, led to the selection of Naporaafenib.<sup>45,107</sup>

**Trametinib** (Cat. No.: HY-10999) is an allosteric inhibitor that targets MEK1 and MEK2, two proteins downstream of RAS (Figure 14B). It binds in a hydrophobic pocket that becomes available when MEK is in its inactive conformation, preventing activation. Trametinib is already used in treatment of metastatic melanoma, NSCLC and anaplastic thyroid cancer with specific mutations, which is why it was selected as a possible downstream inhibitor.<sup>107</sup>

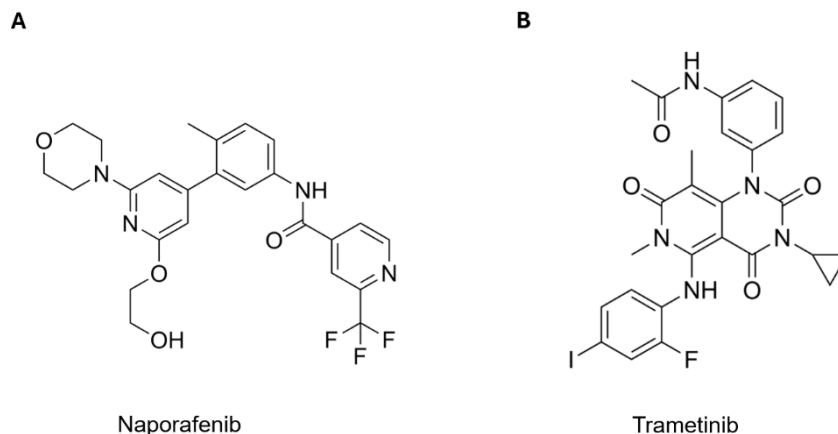


Figure 14: Chemical structure of selected inhibitors of downstream RAS inhibitors. A: Naporafenib; B: Trametinib. Structures retrieved from MedChem.com

### 3.2.4 Mislocalization of RAS proteins

**ABD957** (Cat. No.: HY-142161) selectively inhibits APT1 and APT2, the main acyl-protein thioesterases responsible for depalmitoylation of S-palmitoylated proteins, such as NRAS, HRAS and KRAS4A (Figure 15). ABD957 forms a covalent adduct with the serine where the palmitic acid is placed, irreversibly blocking enzymatic activity. Most available small molecules are designed against KRAS, since this is the most mutated RAS protein in cancers. Therefore, this indirect NRAS targeting molecule was taken along.<sup>107</sup>

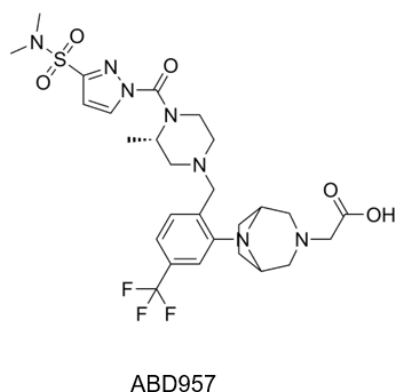


Figure 15: Chemical structure of selected drug, ABD957, that leads to the mislocalization of RAS. Structure retrieved from MedChem.com

### 3.3 Mutating Ba/F3 cells

The selected drugs were tested against RAS mutations in a murine interleukin-3 (mIL-3) dependent pro-B cell line (Ba/F3 cells). The cells are derived from mouse bone marrow, acting as pro B-lymphocytes growing in culture. This cell line is often used in drug discovery, because they can survive without mIL-3, when expressing an oncogene. Since they depend on the oncogene from that moment on, it is a good model to test inhibitors of that oncogene. Here, mutated forms of RAS genes were transduced into the Ba/F3 cell line.

#### 3.3.1 Mutagenesis of MSCV plasmids

Prior to this research, DNA coding for KRAS<sup>G13D</sup>, KRAS<sup>G12C</sup>, KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup> was already inserted in separate MSCV-IRES-GFP plasmids (Figure 16). This plasmid was first modified to include a BglII restriction site. At the DNA inserts, coding for each of the mutations, restriction sites for BglII, at the beginning of the sequence, and NotI, at the end of the sequence, were included. The CloneJET PCR cloning kit was used to amplify the DNA insert. By cutting out the DNA sequence with BglII and NotI, the inserts were compatible to insert in the MSCV plasmid. The produced MSCV plasmids were kept in the freezer.

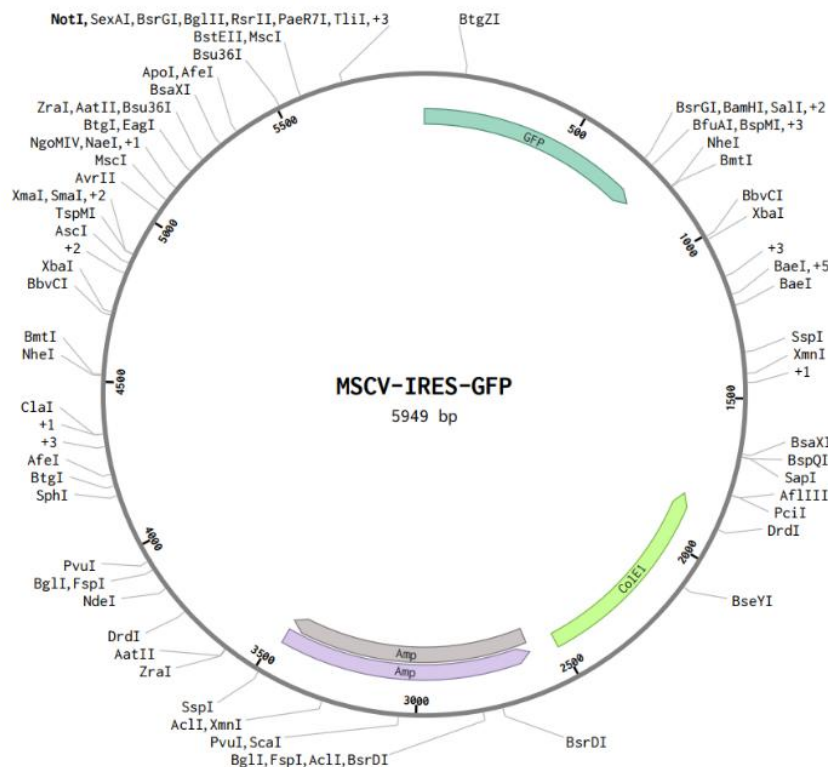


Figure 16: Modified MSCV-IRES-GFP plasmid, including restriction sites for both BglII and NotI. Annotations: gene for Green Fluorescent Protein (GFP), gene for resistance against ampicillin (Amp) and the origin of replication, based on a ColE1 plasmid (ColE1).

Based on data from the PeCan database and literature, involving bulk sequencing techniques and single cell approaches, additional mutations KRAS<sup>A146T</sup>, NRAS<sup>G12D</sup>, NRAS<sup>G12S</sup> and NRAS<sup>Q61H</sup> were selected to investigate.<sup>36,38,76,108</sup> Also WT KRAS and WT NRAS plasmids were synthesized to look at the effects of the drugs on non-mutated RAS proteins.

Plasmids already containing *RAS* gene mutations, (KRAS<sup>G13D</sup>, KRAS<sup>G12C</sup>, KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup>) were used as a template to synthesize the additional mutations and WT cells. Using the Q5<sup>®</sup> Site-Directed Mutagenesis Kit, single nucleotide or dinucleotide mutations were applied to the already existing plasmids. The protocol, which can be consulted online, is based on a PCR method, and makes use of whole plasmid amplification. Specific primers were designed, complementary to a piece of the inserted DNA in the MSCV plasmid, except for one or two nucleotides. These nucleotides were specifically chosen so the newly synthesized strand contained a single nucleotide or dinucleotide substitution compared to the original plasmid, creating the desired mutations or WT *RAS* gene. These specific primers were generated with the NEBaseChanger™ tool, as advised by New England Biolabs (Table S1).

Some deviations from the Q5<sup>®</sup> Site-Directed Mutagenesis Kit Protocol were made. Plasmids were diluted to a concentration of 15 ng/μL before adding 1 μL to the PCR master mix. An elongation time of three minutes was applied, based on the length of the plasmid, which was approximately 6,6 kbp. The annealing temperature, which is primer dependent, was calculated by the NEBaseChanger™ tool (Table S1). All other thermocycling conditions can be found in the protocol. Based on previous experiences, the incubation time of the KDL reaction was increased to 30 – 40 minutes, instead of 5 minutes.

### 3.3.2 Transformation of *E. coli*

To conduct the transformation, recommendations from the Q5<sup>®</sup> Site-Directed Mutagenesis Kit Protocol were followed. A deviation was the use of Subcloning Efficiency™ DH5α Competent Cells, instead of NEB 5-alpha Competent *E. coli* cells. The transformation was performed by a heat shock for 25 seconds at 42°C and quickly placing the *E. coli* on ice afterwards. 950 μL of pre-warmed SOC medium was added to the *E. coli* and after an hour of incubation at 37°C, they were plated in a 10% and 90% concentration on an agar plate containing ampicillin. These plates were incubated at 37°C overnight.

Due to the presence of a resistance gene for ampicillin in the plasmid, only transformed *E. coli* were able to grow on the plates containing this antibiotic. For each desired mutation three colonies were picked from the plate and grown in 5 mL lysogeny broth (LB) medium. From this step on, ampicillin was always added to LB medium in a concentration of 1 mg/mL. To check whether the mutagenesis worked, a PCR was performed to amplify the DNA of interest. The

forward primer was one universal primer for all the MSCV plasmids and the reverse primers were specific for *KRAS* or *NRAS*, targeting them at exon 2 (Table S2). This allowed for the amplification of a part of the *RAS* DNA, which should contain the mutation. For the *KRAS*<sup>A146T</sup> mutation, two universal MSCV primers were used, because the mutation is localized further down in the *RAS* sequence. For these PCR reactions the GoTaq® DNA Polymerase (M300) Protocol was followed. The length of the fragment was expected to be ±300 bp, resulting in an elongation step of 30 seconds. 30 cycles of amplification were applied and the annealing temperature was set to 54°C, based on the melting temperature of the primers (Table S2). Other thermocycling conditions are stated in the protocol, that can be consulted online.

These amplified pieces of DNA were checked for the right mutation based on gel electrophoresis and Sanger sequencing. As a negative control the whole process was performed with LB medium, containing no transformed *E. coli*, for the specific *KRAS* primer, as well as for the *NRAS* specific primer. For Sanger sequencing two tubes per colony were prepared. In each tube 5 µL of the PCR product was added, together with either the forward primer or the reverse primer (Table S2). Only when both of these checkpoints gave a positive result, that colony was kept for further use. For each mutation of interest, one colony containing the plasmid with the correct mutation was expanded to 50 mL LB medium. Additionally, a back-up agar plate was prepared with all the original picked colonies and kept in 4°C.

### 3.3.3 Transduction of Ba/F3 cells

Once confirmed that the plasmids contained the right mutation and the *E. coli* was expanded in 50 mL LB medium, the plasmids were recovered to perform a transduction. The recovery of the plasmids was done by following the Quick-Start Protocol from the CompactPrep Plasmid Midi Kit, available online. The starting volume was 50 mL LB medium containing *E. coli* and the volumes of reagents for high-copy plasmid DNA were followed. The plasmids were resuspended in nuclease free water, quantified via a nanodrop and stored in 4°C.

To start the transduction, HEK293T cells were seeded in 2 mL RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) in treated 6-well plates. These cells were used to produce the virus, containing the desired plasmid. For each plasmid two wells with HEK293T cells were prepared. When the confluency of HEK293T cells attached to the bottom of the plate was around 80%, the medium was refreshed and 2 µg plasmids, 2 µg Ecopac and 6 µL FuGENE HD transfection reagent (Promega E2312) were brought in 150 µL serum-free RPMI medium. Ecopac is a retroviral packaging construct containing the necessary genetic sequences *gag*, *pol* and *env*, coding for structural proteins and enzymes needed to assemble the replication-incompetent retrovirus. FuGENE is a mix of

transfection reagents, used to efficiently introduce the foreign DNA of the virus into cultured cells. The plasmid-retrovirus mixture was incubated at room temperature. After 15 minutes it was dropwise added to the HEK293T cells and incubated at 37°C and 5% CO<sub>2</sub>.

After 24 hours the medium was aspirated and 1,2 mL fresh RPMI 10% FBS medium was added. After an additional 24h of incubation at 37°C the supernatant was isolated with a syringe and pushed through a 0,45 µm filter. This allowed the viral particles to go through, whilst cells and other debris stayed behind. In clean untreated 6-well plates, 1 mL of Ba/F3 cells in a concentration of 600 000 cells/mL in RPMI 10% FBS medium was seeded. 1 mL of the viral supernatant was added to the Ba/F3 cells. Also protamine sulphate was added in a final concentration of 5 µL/mL, improving transduction efficiency by neutralizing negative charges on cells and viruses. Because Ba/F3 cells are dependent on mIL-3 to grow, mIL-3 was added in a final concentration of 10 ng/mL per well. Again the cells were incubated at 37°C and 5% CO<sub>2</sub>.

24 hours later, the cells were centrifuged and resuspended in PBS, to wash away the protamine sulphate. PBS was removed after centrifuging and new RPMI 10% FBS medium was added. After 48 hours of incubation, the viable cells were counted with the MACSQuant® VYB Flow Cytometer (Miltenyi Biotec) and the percentage of Ba/F3 GFP positive cells was determined. Cells that were GFP positive contained the plasmid, since a GFP gene was present in the MSCV-plasmid. Cells were diluted to a concentration of 500 000 cells/mL and two flasks per mutant were made. One flask contained regular RPMI 10% FBS medium in order to see whether expressing the oncogene enabled them to grow independent of mIL-3. A back-up flask containing RPMI 10% FBS medium, complemented with mIL-3 (10 ng/mL), was kept for each mutation until a full mutation cell line was established. The cells were monitored every day of the week, to evaluate the development of %GFP.

Established cell lines were kept in RPMI 1640 medium supplemented with 10% FBS. Living cells were counted with the MACSQuant® VYB Flow Cytometer each Monday, Wednesday and Friday. Afterwards they were centrifuged and diluted in fresh medium to a concentration of 400 000 cells/mL. Depending on the number of cells needed for experiments they were kept in 8mL medium in T-25 flasks or in 15mL medium in T-75 flasks. DNA was extracted from 3 000 000 cells per established cell lines, using the Maxwell® RSC Cultured Cells DNA Kit. A PCR was conducted on these genes, using the universal MSCV forward and reverse primer (table S2). Genomic DNA was added in a concentration of 100 ng/µL, annealing temperature was set to 54°C and an elongation time of 40 seconds was used. This PCR product was subjected to Sanger sequencing in order to check whether the correct mutation was present in the Ba/F3 cells. Figure 17 shows an overview of the process and the results of the transduction.

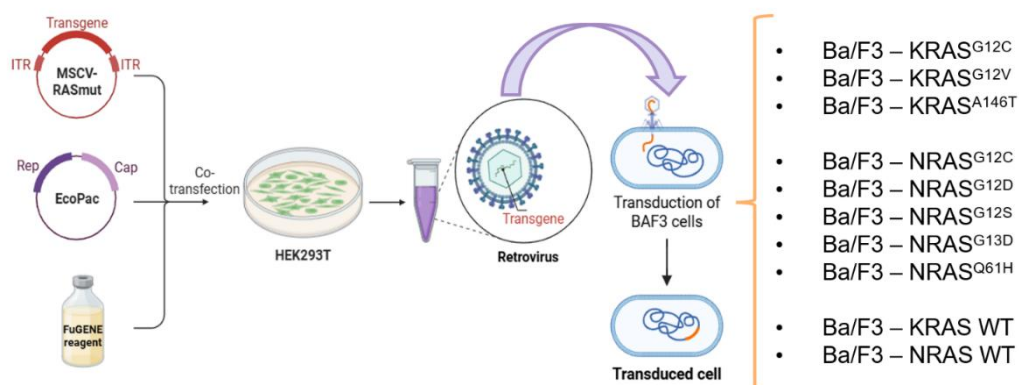


Figure 17: Visual representation of the pipeline to transduce Ba/F3 cells. Figure was created with BioRender.com

To obtain p-values for cell viability after the transduction, a dataframe with the absolute cell counts was constructed in R. All data was normalized to the start values for each mutation directly after the transduction, called day 0. Afterwards, a mixed linear model was made, with each timepoint as a random factor (data was non-independent) in R studio (R 4.5.1). For this linear model, results were grouped into mutant Ba/F3 cells and WT Ba/F3 cells. With an emmeans post-hoc comparison, p-values were calculated.

## 3.4 In vitro assays

To propose next-generation drugs to take into a next testing stage against HeH B-ALL, the drugs were tested in an in vitro screen. The effect of the drugs on RAS signaling in the transduced Ba/F3 cells was tested by conducting an ATPlite assay and western blotting analysis (WB). All the selected drugs, listed before, were taken into the screen. ATPlite assays were performed to construct dose-response curves. These results were validated by looking at the phosphorylation of ERK and the degradation of RAS in WB (Figure 18).

### 3.4.1 ATPlite assay

For each mutation, 100  $\mu$ L of cells were seeded in a sterile 96-well plate (200 000 cells/mL) with fresh RPMI 10% FBS. To avoid evaporation, outer wells were filled with 200  $\mu$ L PBS. Via the Tecan D300e Digital Dispenser, drugs were added in concentrations of 0, 10, 100, 500, 1000, 1500, 2500, 3500, 5000 and 10 000 nM in triplicate. Drugs were randomized across the plate and DMSO volumes were normalized to the highest total volume. Because the plates needed to be opened out of the laminar flow in order to add the drugs with the Tecan Dispenser, primocin, an antibiotic, was added in a concentration of 2  $\mu$ L/mL medium. Afterwards, the cells with the drugs were incubated for 48 hours at 37°C and 5% CO<sub>2</sub>.

After 48 hours, ATPlite solution from the ATPlite 1step Luminescence Assay System was diluted 1:2 with PBS and 100  $\mu$ L was added to each well. After 5 minutes of incubation at room temperature the absorbance was measured with the CLARIOstar® *Plus* plate reader (BMG Labtech). As a day 0 control, the absorbance of cells was measured before incubation (200 000 cells/mL) in triplicate. This value was subtracted from the absorbance values after 24 hours, to look solely at proliferation after adding the drugs. An R-script was written to merge files from the plate reader, together with the randomized template of the Tecan Dispenser. Graphs were made with GraphPad Prism 11.0.0 (84).

To test cytotoxic effects of the drugs on non-RAS dependent cells, ATPlite assays were conducted on different human cell lines. Four different cell lines were selected, based on previous findings that when RAS signaling is blocked, these cell models are able to adapt and have rebalancing mechanisms to cope with the impaired RAS signaling. The selected cell lines were CCRF-CEM (T-ALL), containing a KRAS<sup>G12D</sup> mutation, KE37 (T-ALL), containing a NRAS<sup>G12D</sup> mutation, NOMO-1 (AML), containing a KRAS<sup>G13D</sup> mutation and HL-60 (AML), containing a NRAS<sup>Q61L</sup> mutation. Since these cell lines have redundant pathways and do not solely rely on RAS signaling for survival, the drugs should not lead to major drops in cell viability. Thus, when drugs have cytotoxic effects, this will be visible in the cell viability. For these assays, the same concentrations and protocol were used as for the Ba/F3 cells.

For Daraxonrasib, Naporafenib and ABD957, only CCRF-CEM was used to test for cytotoxicity, since this was the only cell line in culture and limited time. Additional cytotoxicity tests for all drugs were conducted on JURKAT cells, which contain a BRAF oncogenic mutation. Since this mutation is located downstream of the RAS pathway, these cells are better to test cytotoxic effects of direct RAS inhibiting drugs. For all these drugs, concentrations of 0, 10, 100, 1000 and 10 000 nM were taken to do the cytotoxicity tests.

### 3.4.2 Western blotting

Before WB analysis, 2mL of cells were seeded in a 6-well plate at a concentration of 500 000 cells/mL and incubated at 37°C and 5% CO<sub>2</sub>. After 24 hours, drugs were added to obtain a final concentration of 0, 1000 and 10 000 nM, based on the ATPlite results. After another 4h of incubation, the cells were harvested and proteins were recovered conducting a standard cell lysis protocol. Cells were lysed in 1X Cell Lysis Buffer (Cell Signaling Technology) supplemented with 5 mM Sodium Orthovanadate (Na<sub>3</sub>VO<sub>4</sub>) (New England Biolabs) and Complete EDTA-free Protease Inhibitor Cocktail (Roche) at 4°C. These cell lysates were kept in -80°C for further use.

On the day that the WB was performed, the protein concentration of the cell lysates was determined, using the Bio-Rad Protein Assay Dye Reagent Concentrate. The CLARIOstar® Plus plate reader (BMG Labtech) was used to determine the absorbance at 595 nm, measured in triplicate. Based on a standard curve from known BSA concentrations, the protein concentration was calculated. For every sample, 25 µg of protein was taken, and a total volume of 30 µL was obtained, including NuPAGE reducing agent and NuPAGE sample buffer. After 10 minutes of denaturation at 70°C, each sample was loaded on a gel. The first well was preserved for a protein ladder.

After running the gel and blotting the protein from the gel onto a membrane, the membrane was blocked with 5% non-fat milk for 1 hour. Afterwards, the membrane was cut into three pieces based on the location of the proteins. Vinculin (116 kDa) was used as a loading control, since it is a housekeeping protein, and should therefore always be present in each sample. ERK and p-ERK should be present at the same height (ERK1: 44 kDa, ERK2: 42 kDa), so they were separately visualized at different time points. RAS, the smallest protein (21 kDa) was at the third piece of membrane. Each membrane was separately incubated with its primary antibody (vinculin, p-ERK or RAS, diluted 1:1000 in 5% non-fat milk) overnight at 4°C. After washing away the non-bound antibodies with TBS-T, the membranes were incubated with their secondary antibody (mouse or rabbit, diluted 1:5000 in 5% non-fat milk) for 1 hour at room temperature. To detect the proteins, Western Lightning™ Plus Chemiluminescence Reagent (Revvity) was used and immunodetection was done with the Vilber Fusion FX6 Edge Spectra. Afterwards, the membranes were stripped using stripping buffer (Revvity) and everything was repeated with the primary antibodies (Vinculin or ERK, diluted 1:1000 in 5% non-fat milk) to detect non-phosphorylated ERK levels.

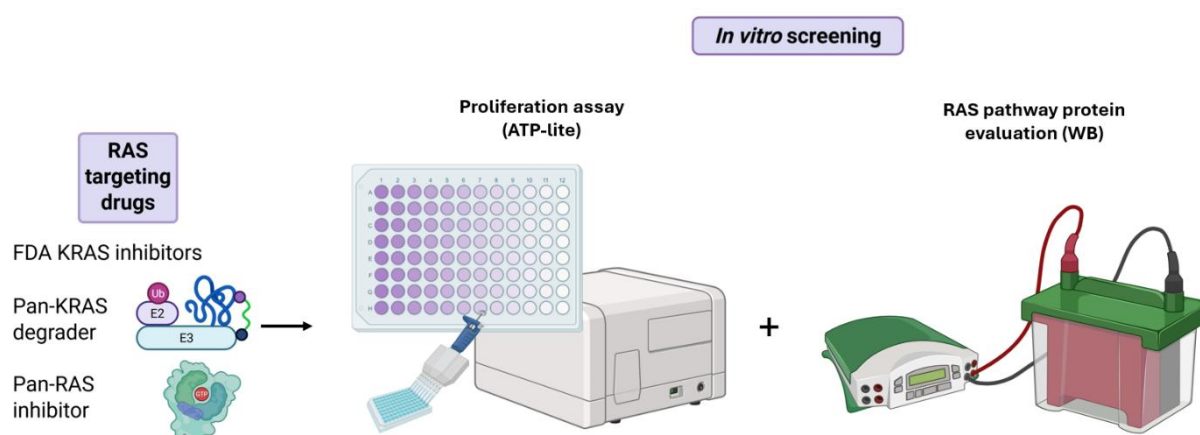


Figure 18: Overview of the *in vitro* assays. Multiple drugs were tested against different RAS mutated Ba/F3 cells. Figure was created with BioRender.com

## 4 Results

### 4.1 Establishing Ba/F3 mutant cell lines

Automated gel electrophoresis with the QIAxcel machine, confirmed that the desired length of the amplified DNA was present in all of the picked colonies after transforming Subcloning Efficiency™ DH5α Competent Cells (Extended Data Figure 1). This showed that the MSCV plasmid containing the *RAS* gene sequence was present in the *E. coli*. Additionally, Sanger sequencing gave a 100% match between the mutated or WT reference sequences in the picked colonies. This confirmed that the desired mutation was indeed present inside the *RAS* sequence in the plasmid, as is shown for *NRAS* WT (Extended Data Figure 2). These plasmids were purified and used to generate retroviral vectors to transduce Ba/F3 cells.

To see if the Ba/F3 cells were able to grow solely upon excessive RAS signaling, the cells were depleted of mIL-3. Flow cytometry analysis showed that the cells surviving this depletion were predominantly transduced cells. Figure 19 shows that, after a few days, mutant cells were nearly 100% GFP positive, confirming that a RAS dependent Ba/F3 cell line was established (displayed for *KRAS*<sup>G12V</sup>, other mutants showed the same result). When the GFP percentage exceeded 90%, a mutant cell line was considered fully established and the back-up mIL-3 supplemented flasks were discarded.

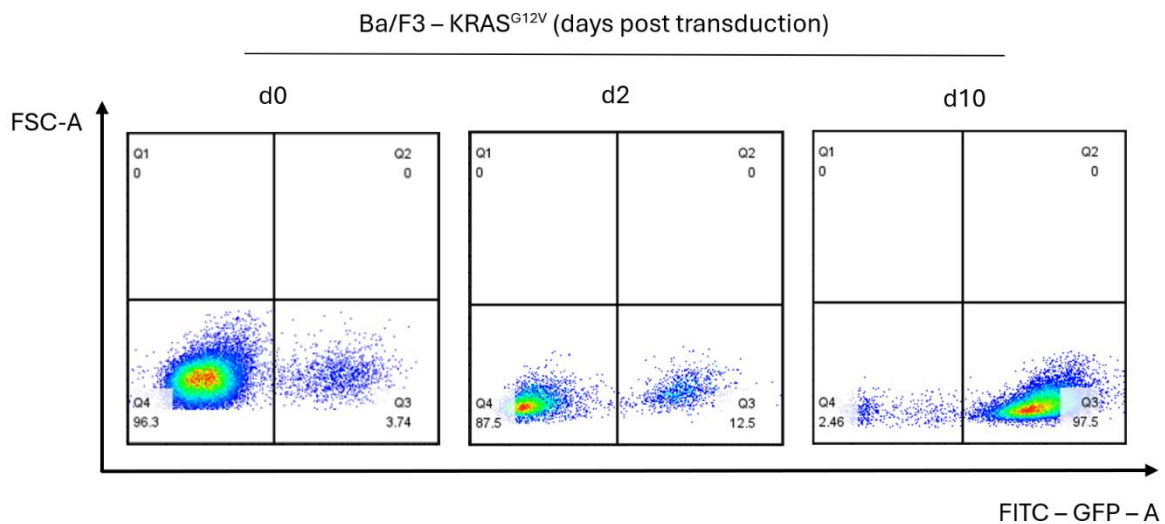


Figure 19: Flow cytometry data of *KRAS*<sup>G12V</sup> mutant Ba/F3 cells. Upon mIL-3 depletion, the percentage of GFP positive cells was monitored each day. Results are shown for the first measurement after the transduction, 2 days and 10 day post-transduction. GFP positive cells are located in the right quadrant and GFP negative cells in the left quadrant.

Figure 20A shows that depleting the Ba/F3 cells of mIL-3, selectively gives a growth advantage to oncogene expressing Ba/F3 cells, since GFP positive cells take over the population (displayed for KRAS<sup>G12V</sup>, all data in Extended Data Figure 3). Transduced Ba/F3 cells with WT versions of the RAS protein were also able to survive without mIL-3, but it took longer to recover from mIL-3 withdrawal, probably due to the fact that WT RAS is less potent compared to mutant RAS (Figure 20B, all data in Extended Data Figure 3). It took around 9 days to reach 90% GFP, compared to 5 days for mutated forms. However, overexpressing a WT RAS protein in Ba/F3 cells was sufficient to drive proliferation independent of mIL-3.

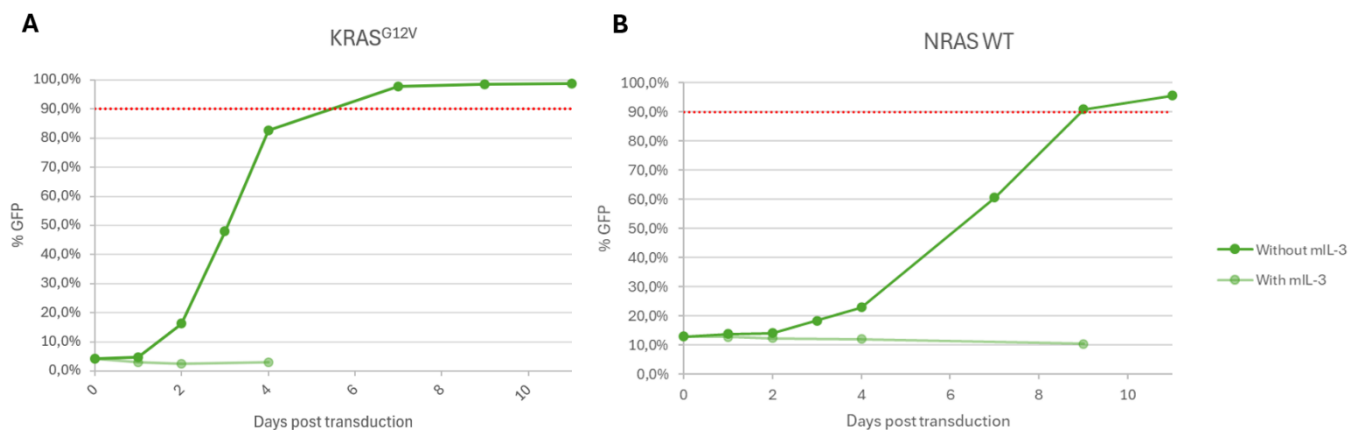


Figure 20: GFP development over time shows an established RAS Ba/F3 cell line. Red line: 90% GFP, a fully established RAS mutant or RAS WT Ba/F3 cell line. **A:** GFP evaluation over time for KRAS<sup>G12V</sup> **B:** GFP evaluation over time for NRAS WT.

In Figure 21, it is demonstrated that all transduced cells need time to recover from, and adapt to, the mIL-3 depletion, which can be seen by the initial drop in viability. All mutant RAS Ba/F3 cells hit a minimum viability 3 days post mIL-3 depletion, with a log fold change of 0,0667 being the lowest for NRAS<sup>G12S</sup>. After 3 days, some mutant cells were already able to adapt and proliferate again, increasing the viability to a fold change of 3 by day 9. From that moment on, cells were split into the desired concentration of 400 000 cells/mL and excess cells were discarded.

Moreover, Figure 21 shows that the number of viable cells was initially lower for the Ba/F3 cells expressing WT RAS, compared to cells expressing mutant RAS proteins (p-value <0.0001; comparison against a few mutants, all data in Extended Data Figure 4). WT RAS Ba/F3 cells continue to drop in viability until 7 days after mIL-3 depletion to a log fold change of 0,004 for KRAS WT. At that point in time, there were only 28 000 and 78 000 living cells remaining for KRAS WT and NRAS WT respectively, although there were 6 000 000 living cells at day 0. After 7 days, the few surviving WT RAS cells start to proliferate, most likely indicating that only cells with the highest RAS expression levels were able to survive.

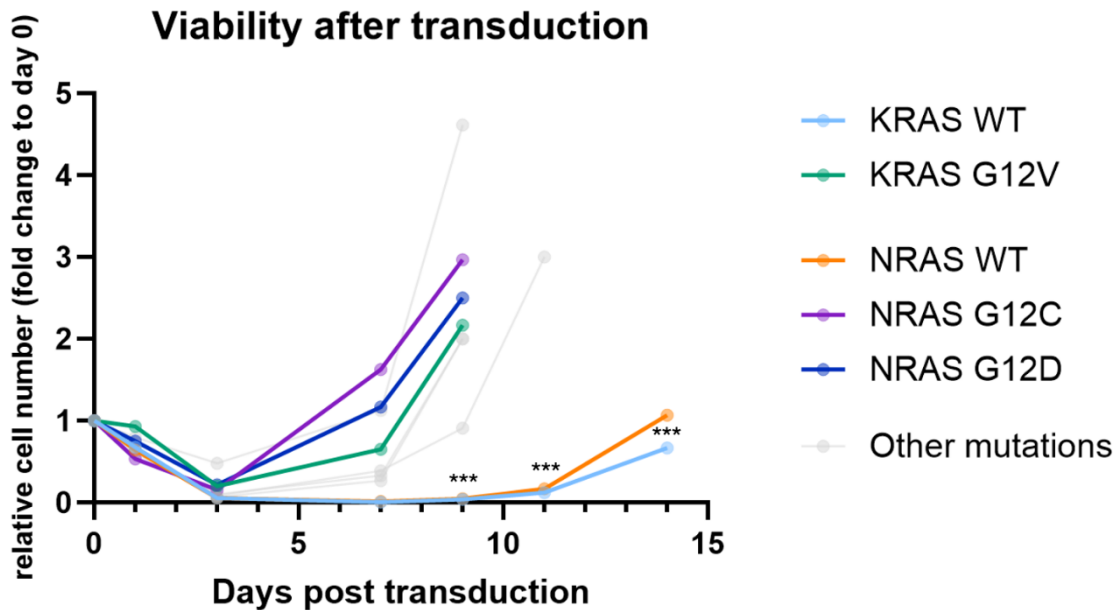


Figure 21: Viability after transduction. Data is normalized for the total number of cells per mutation at day 0. For significance testing, all mutations were grouped and the two WT transductions were grouped. A linear mixed model was produced in R and via Post-hoc comparisons, p-values were obtained. p-value < 0.05 \*, < 0.01 \*\*, < 0.001 \*\*\*

A more intensive GFP positive signal, compared to mutant cells, suggests that there is selection for Ba/F3 cells that have incorporated the WT RAS GFP construct more than once. Figure 22 shows that four distinct populations of GFP intensities can be observed for WT KRAS, where a subset of cells do not even fit the plot, and two for NRAS WT. Taken together, this confirms that cells expressing WT RAS protein, have more difficulties, but are able to grow independent of mL-3.

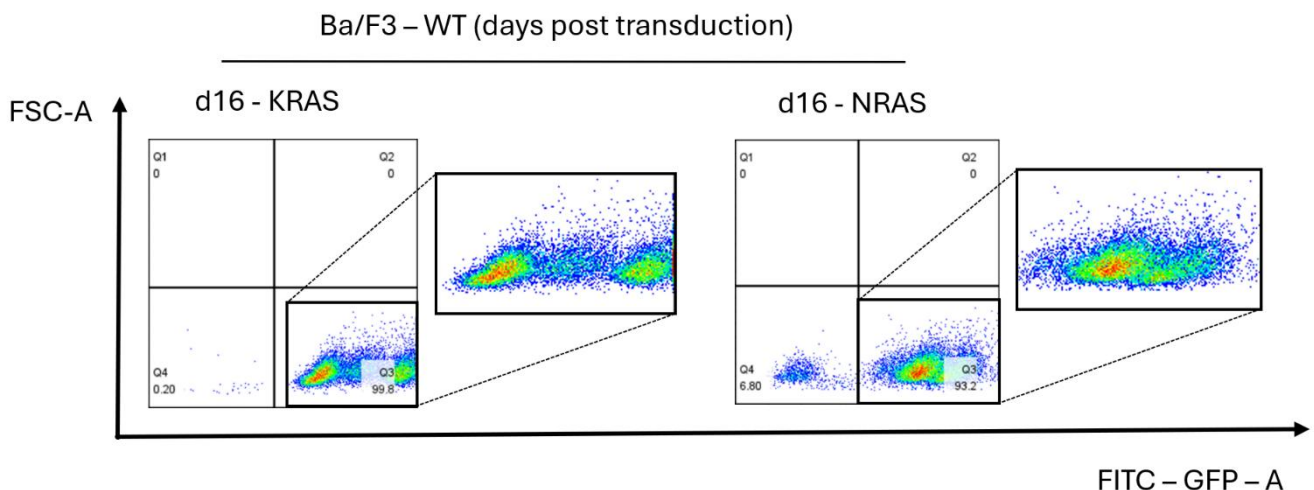


Figure 22: Flow cytometry data of WT RAS protein transduced Ba/F3 cells. GFP positive cells are located in the right quadrant and GFP negative cells in the left quadrant. Different populations of GFP positive clusters can be seen. Something which is not observed in mutant transduced cells.

## 4.2 RAS inhibitors

### 4.2.1 Sotorasib

Figure 23 shows that Sotorasib can effectively block the RAS pathway in cells with G12C mutated RAS proteins. In KRAS<sup>G12C</sup>, as well as in NRAS<sup>G12C</sup>, the phosphorylation of ERK was inhibited and the productivity of the cells was decreased drastically. ATPlite analysis estimated an IC<sub>50</sub> of 12,8 nM against KRAS<sup>G12C</sup> and 13,1 nM against NRAS<sup>G12C</sup>. Sotorasib was also tested against WT and non-G12C mutated forms of RAS proteins, but had no effect on the proliferation or survival of the cells.

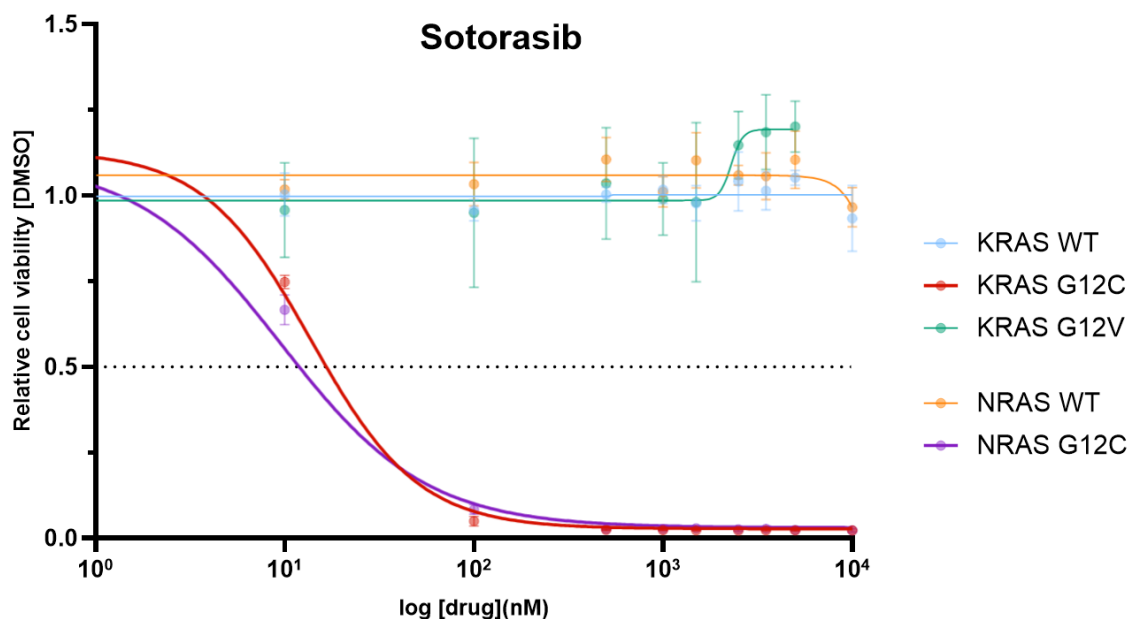


Figure 23: Dose-response curve for Sotorasib. Cells were incubated for 48h with different concentrations of Sotorasib. Viability after 48h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. When the drug was effective against a certain mutation, the line is shown in bold (1pt), and when there was no effect the line is thinner (1/2pt).

WB analysis confirmed these results, visible in Figure 24, where clearly decreased levels of p-ERK were detected in KRAS<sup>G12C</sup> or NRAS<sup>G12C</sup> expressing cells, when Sotorasib was administered in concentrations of 1000 nM and 10 000 nM.

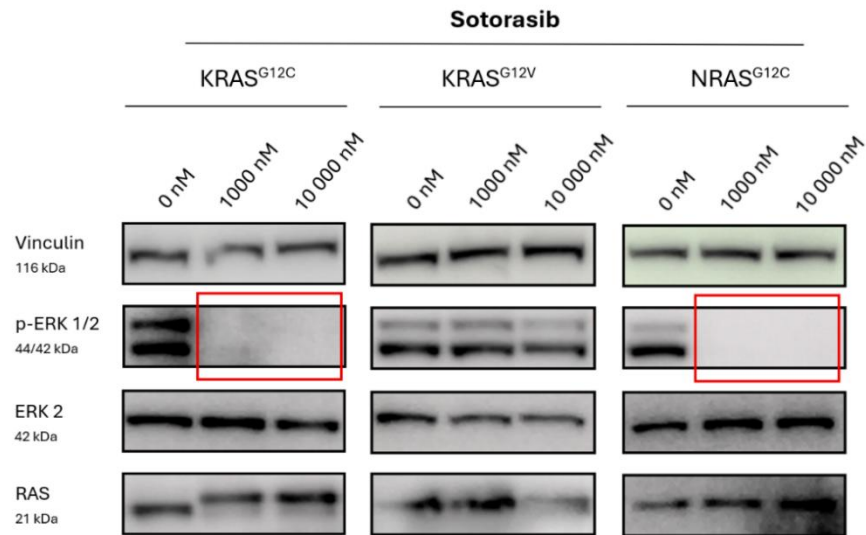


Figure 24: Western blotting analysis for Sotorasib. Cells were incubated with Sotorasib for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control. Red boxes indicate no detectable p-ERK levels in the cell lysate.

**Note:** Sotorasib is the only drug that was tested against KRAS<sup>G12C</sup> and NRAS<sup>G12C</sup> mutations in a Cas9-expressing Ba/F3 cell line. Due to an error and time limitations, these are the only available results for Sotorasib. Since it was noticed that the metabolism of the cells is slightly different from regular Ba/F3 cells, these results should not be compared to the efficacy of other drugs. There was no day 0 data for this model in this assay, so here the graph is based on viability, rather than proliferation.

This drug was also tested against human cell lines. Since these cells are not completely dependent on the RAS pathway and have redundant survival pathways, this drug should not lead to major drops in cell viability. Figure 25 shows that Sotorasib indeed does not lead to cell death in the tested human cell lines. This suggests that there are no cytotoxic effects of Sotorasib on non-RAS dependent cells. Also cytotoxicity testing against JURKAT cells yielded the same result (Extended Data Figure 5A).

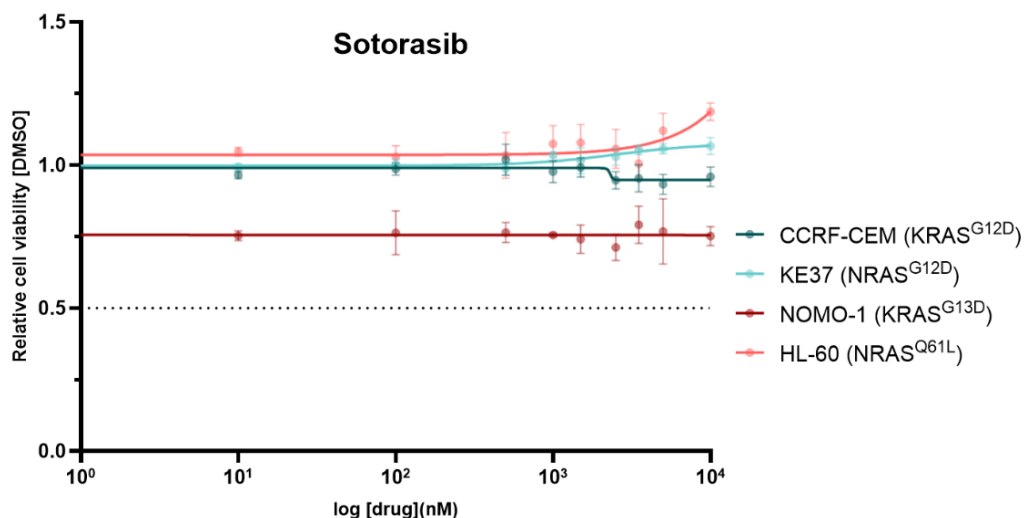


Figure 25: Dose-response curve for Sotorasib against human cell lines. Cells were incubated for 24h with different concentrations of Sotorasib. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

## 4.2.2 Pan-RAS-IN-1

The next drug that was tested, Pan-RAS-IN-1 inhibitor, seems to be effective in blocking RAS signaling, but only at higher concentrations. Figure 26 shows it effectively inhibits proliferation, with  $IC_{50}$  values around 4000 nM for the mutants.

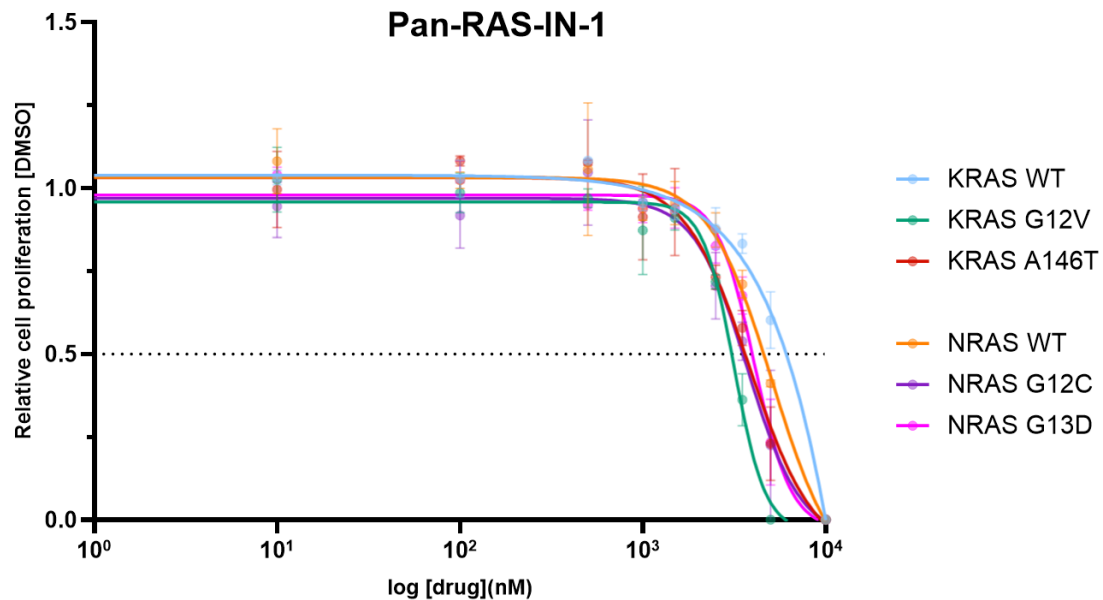


Figure 26: Dose-response curve for Pan-RAS-IN-1. Cells were incubated for 48h with different concentrations of Pan-RAS-IN-1. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

WB analyses confirmed these findings and showed decreased levels of p-ERK at a concentration of 10 000 nM, as displayed in Figure 27. However, p-ERK levels were not reduced to undetectable levels.

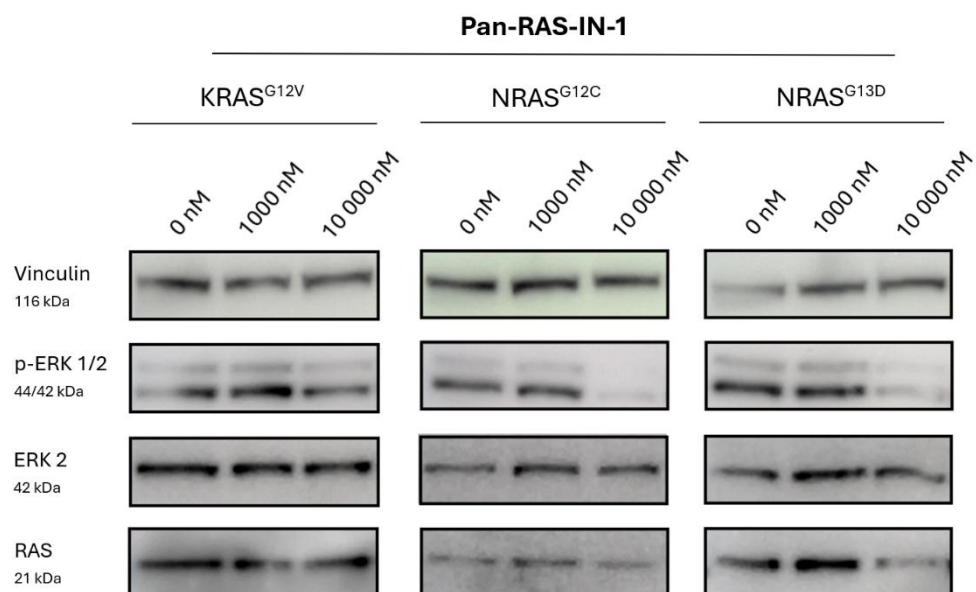


Figure 27: Western blotting analysis for Pan-RAS-IN-1. Cells were incubated with Pan-RAS-IN-1 for 4h. Vinculin is a housekeeping gene (always present) and is used as a loading control.

A striking difference between Pan-RAS-IN-1 and other drugs was, that from 5000 nM on, absorbance levels during the ATPlite assay nearly hit 0. This implies that cell activity was absent and all cells were dead. Extended Data Figure 6 shows the result of cell viability without subtracting day 0 values for Pan-RAS-IN-1 in comparison to some other drugs. Although the  $IC_{50}$  values differ for each drug, all drugs result into a 10% absorbance compared to the DMSO treated cells when they hit their maximum efficacy. This percentage is in line with all other results. However, for Pan-RAS-IN-1 the relative viability drops to 0,1%, indeed confirming that almost all cells were dead (KRAS<sup>G12V</sup> taken as an example, but the same was observed for all other tested cells).

To confirm the hypothesis of cytotoxicity, this drug was also tested against the human cell lines with RAS mutations. Figure 28 shows that when Pan-RAS-IN-1 reaches concentrations above 5000 nM, all these cells die, although they should not rely solely on the RAS pathway for survival. This confirmed that the drug has a cytotoxic effect on all cells, which is an unwanted characteristic for a drug that will need to treat human diseases. These findings were validated when Pan-RAS-IN-1 was tested against JURKAT cells (Extended Data Figure 5A).

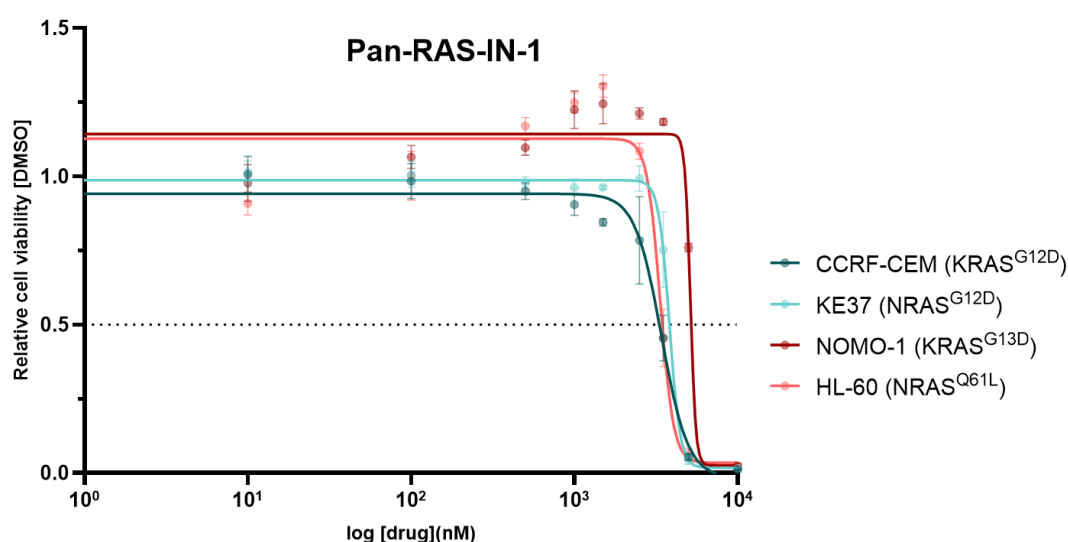


Figure 28: Dose-response curve for Pan-RAS-IN-1 against human cell lines. Cells were incubated for 24h with different concentrations of Pan-RAS-IN-1. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

### 4.2.3 BI-2865

As can be seen in Figure 29, BI-2865 showed potent inhibition of cell proliferation for KRAS mutated Ba/F3 cells. All estimated  $IC_{50}$  values are around 100 nM. Against NRAS, there is no noteworthy effect on cell proliferation.

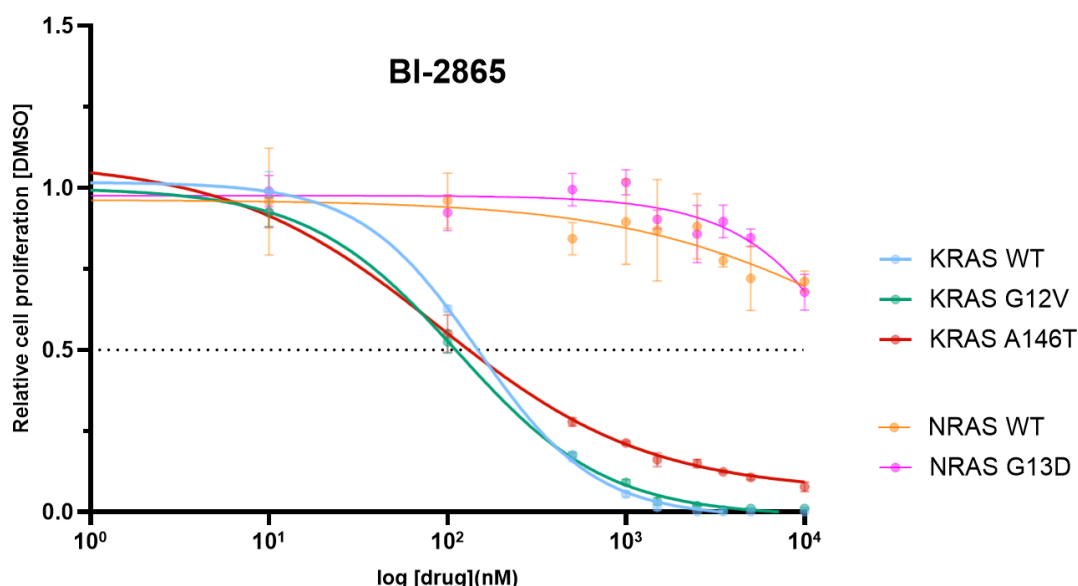


Figure 29: Dose-response curve for BI-2865. Cells were incubated for 48h with different concentrations of BI-2865. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. When the drug was effective against a certain mutation, the line is shown in bold (1pt), and when there was no effect the line is thinner (1/2pt).

These findings were validated by WB analysis, shown in Figure 30. KRAS<sup>G12V</sup> mutated Ba/F3 cells showed very low p-ERK levels at 1000 nM and undetectable levels of p-ERK at 10 000 nM. As expected, this was not seen in NRAS mutated Ba/F3 cells, although at 10 000 nM, a slight drop in p-ERK seems to appear. This is in line with the slightly visible drop in the dose-response curve at 10 000 nM.

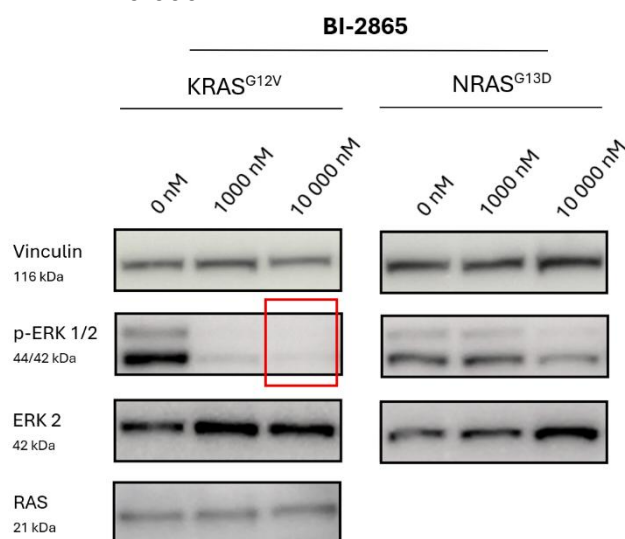


Figure 30: Western blotting analysis for BI-2865. Cells were incubated with BI-2865 for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control. Red boxes indicate no detectable p-ERK levels in the cell lysate.

Also this drug was tested for cytotoxic effects against human cell lines. Results of these tests can be seen in Figure 31A. Here BI-2865 had no effect on the cell proliferation, but a treatment of NOMO-1 with BI-2865, followed by WB imaging, confirmed that there is still inhibition of the RAS pathway (Figure 31B). Additionally, there was no effect of BI-2865 on the viability of JURKAT cells (Extended Data Figure 5A). Therefore, BI-2865 might be a good drug to target possibly KRAS-dependent HeH B-ALL cells.

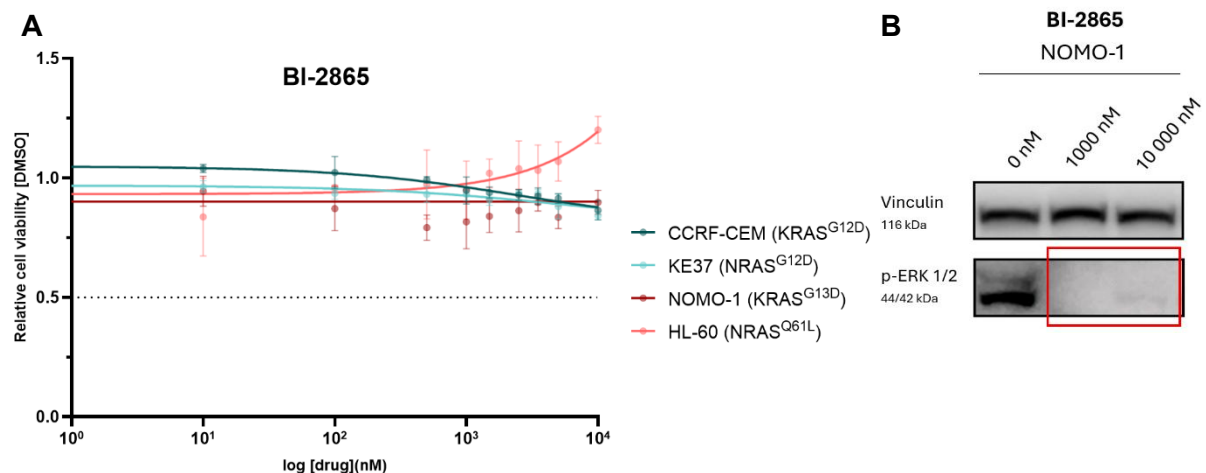


Figure 31: **A:** Dose-response curve for BI-2865 against human cell lines. Cells were incubated for 24h with different concentrations of BI-2865. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. **B:** Western blotting analysis for BI-2865 against NOMO-1 human cell line. Cells were incubated with BI-2865 for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control. Red boxes indicate no detectable p-ERK levels in the cell lysate.

#### 4.2.4 Daraxonrasib

In Figure 32, Daraxonrasib was found to be a potent RAS inhibitor. This drug completely blocked proliferation of all tested KRAS and NRAS mutant Ba/F3 cells, as well as the WT NRAS and KRAS expressing cells. Since proliferation was already completely reduced at a concentration of 10 nM, graphs for KRAS WT, NRAS WT and KRAS<sup>G12V</sup> overlap. Because there were no datapoints before 10 nM, no reliable IC<sub>50</sub> values could be obtained. Nevertheless, Daraxonrasib inhibited proliferation already at very low concentrations. For NRAS<sup>G13D</sup>, one of the few cell models with lower sensitivity, the estimated IC<sub>50</sub> is 18,38 nM, the highest of all the tested cell models (all data in Extended Data Figure 8A).

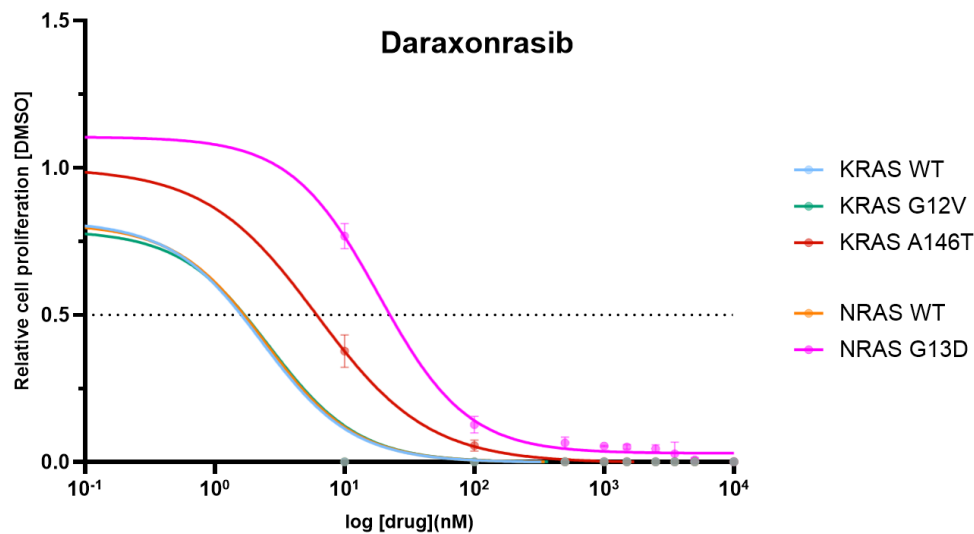


Figure 32: Dose-response curve for Daraxonrasib. Cells were incubated for 48h with different concentrations of Daraxonrasib. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

Also WB analysis confirmed the potent inhibition of the RAS pathway. No levels of p-ERK were detected in KRAS<sup>G12V</sup> or NRAS<sup>G12C</sup> cells 4h after administration of Daraxonrasib (Figure 33). Cytotoxicity testing resulted in a slight drop in viability against human cell line CCRF-CEM, possibly due to the high potency of the drug and the presence of a RAS mutation in the cell line (Extended Data Figure 7). However, viability did not decrease below 50% and in JURKAT cells, where no RAS mutation is present, this drug had no effect on the viability (Extended Data Figure 5A). These findings indicate that Daraxonrasib has no cytotoxic effects on cells.

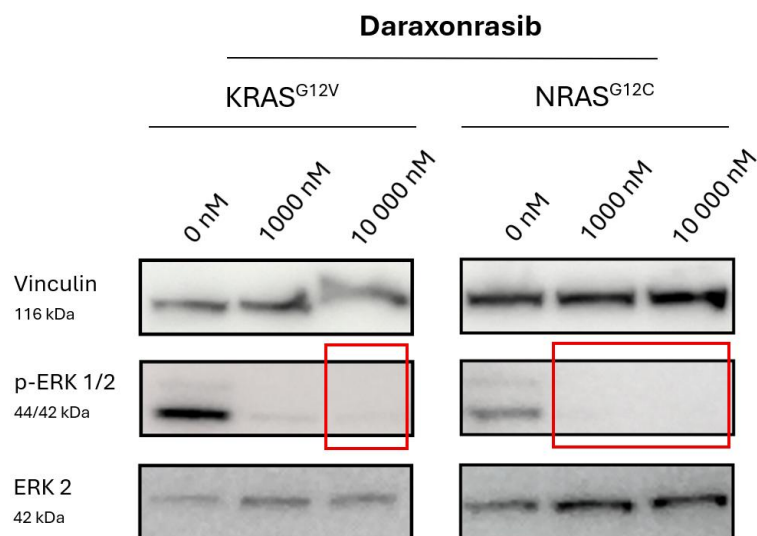


Figure 33: Western blotting analysis for Daraxonrasib. Cells were incubated with Daraxonrasib for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control. Red boxes indicate no detectable p-ERK levels in the cell lysate.

## 4.3 RAS Degraders

### 4.3.1 PROTAC K-Ras Degradar-4

Figure 34 shows that PROTAC K-Ras Degradar-4 inhibited the cell proliferation for KRAS mutant and WT Ba/F3 cells, but it shows no effect on NRAS mutant or WT Ba/F3 cells, as expected. The effect on KRAS signaling is within the same range for all tested cells, with  $IC_{50}$  values varying between 168 nM for KRAS WT and 260 nM for KRAS<sup>A146T</sup>.

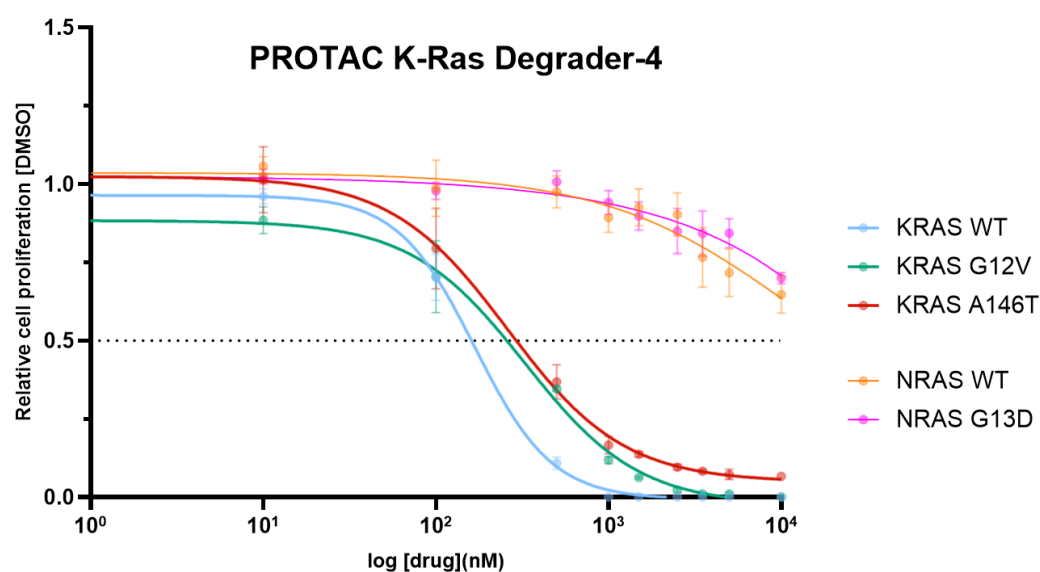


Figure 34: Dose-response curve for PROTAC K-Ras Degradar-4. Cells were incubated for 48h with different concentrations of PROTAC K-Ras Degradar-4. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. When the drug was effective against a certain mutation, the line is shown in bold (1pt), and when there was no effect the line is thinner (1/2pt).

These results were validated by WB analyses and displayed in Figure 35. The levels of p-ERK decrease with increasing concentration of PROTAC K-Ras Degradar-4 for KRAS overexpressing Ba/F3 cells, but not for NRAS. Although PROTAC K-Ras Degradar-4 is able to inhibit cell proliferation, the degradation of KRAS is not observed. Additional tests on other mutations yielded the same results. This suggests that even when degradation/linking to the proteasome fails, the inhibitor part can still block RAS signaling.

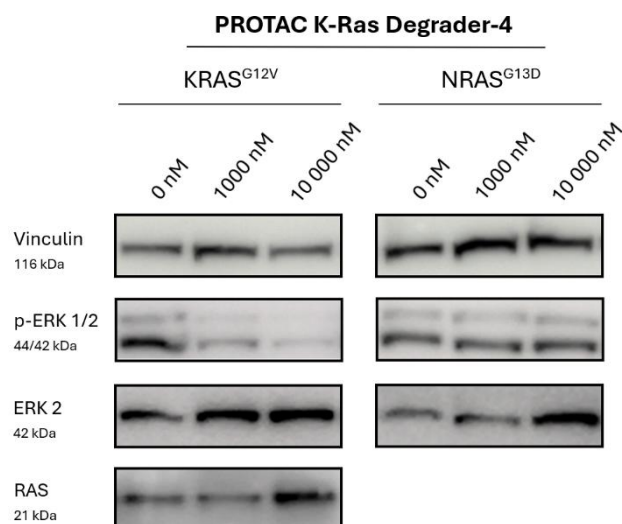


Figure 35: Western blotting analysis for PROTAC K-Ras Degradator-4. Cells were incubated with PROTAC K-Ras Degradator-4 for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control.

This drug was also tested against CCRF-CEM, KE37, NOMO-1 and HL60, showing similar results as BI-2865, which makes this a valuable candidate for KRAS mutated HeH B-ALL subclones. Figure 36 shows a slight drop in cell viability towards higher concentrations (5000 nM). In JURKAT cells the same was seen for a concentration of 10 000 nM, but with viability still over 80%, there is no indication of cytotoxicity (Extended Data Figure 5B). On top of that, PROTAC K-Ras Degradator-4 is already effective at concentrations below 260 nM, as was seen in the KRAS mutated Ba/F3 cells.

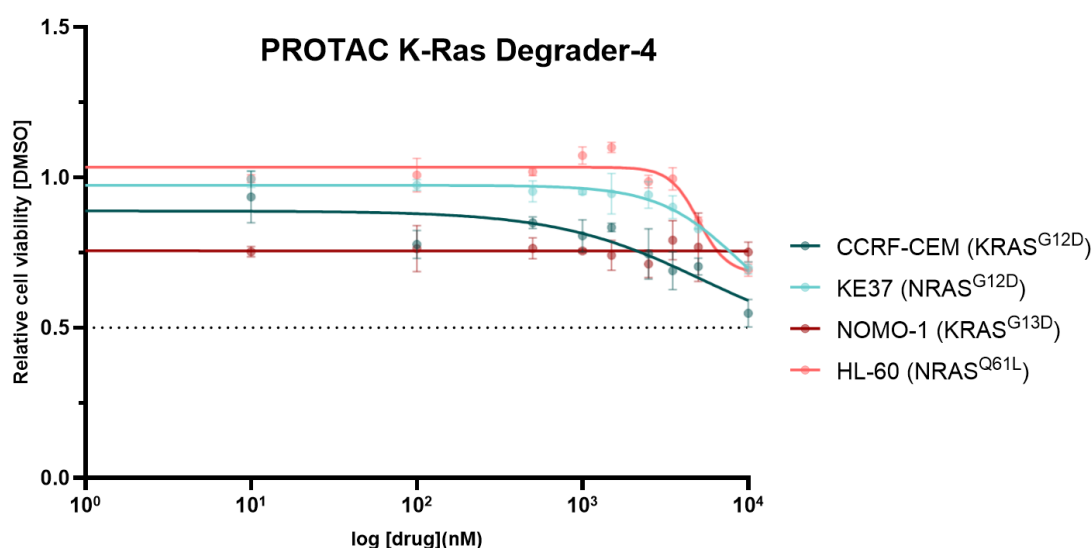


Figure 36: Dose-response curve for PROTAC K-Ras Degradator-4 against human cell lines. Cells were incubated for 24h with different concentrations of PROTAC K-Ras Degradator-4. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

### 4.3.2 ACBI3

ACBI3 treatment did not show an effect on the proliferation of KRAS<sup>G12V</sup> Ba/F3 cells and WB analysis showed no effect on p-ERK levels, as is shown in Figure 37A and B. Also previous tests against other transduced Ba/F3 cells yielded no effect on the cell proliferation or p-ERK levels (Extended Data Figure 9). Therefore, there was no point in testing the effect against WT RAS proteins. As expected, there was no effect on NRAS mutants. Taken together, ACBI3 seems not to work against RAS signaling in this model.

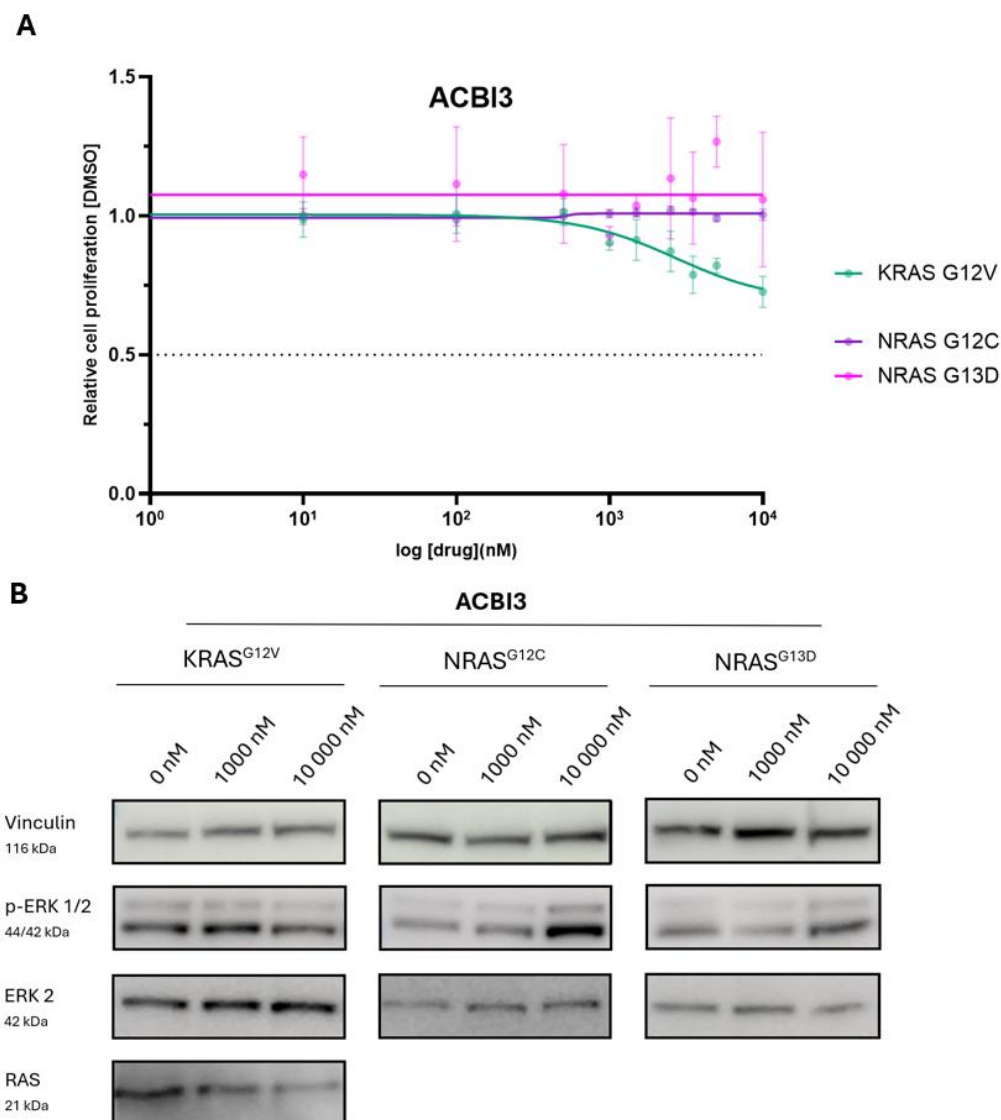


Figure 37: **A:** Dose-response curve for ACBI3. Cells were incubated for 48h with different concentrations of ACBI3. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. **B:** Western blotting analysis for ACBI3. Cells were incubated with ACBI3 for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control.

## 4.4 Downstream RAS inhibitors

### 4.4.1 Naporafenib

Naporafenib inhibits RAF dimers and was able to decrease proliferation in KRAS as well as in NRAS overexpressing Ba/F3 cells. Figure 38 shows that all mutated forms of RAS proteins were sensitive to Naporafenib, but slightly less compared to the WT RAS cells. This resulted in a more gradual decrease in proliferation with increasing concentrations. (shown for KRAS<sup>A146T</sup> and NRAS<sup>G12C</sup>, other data in Extended Data Figure 8B).

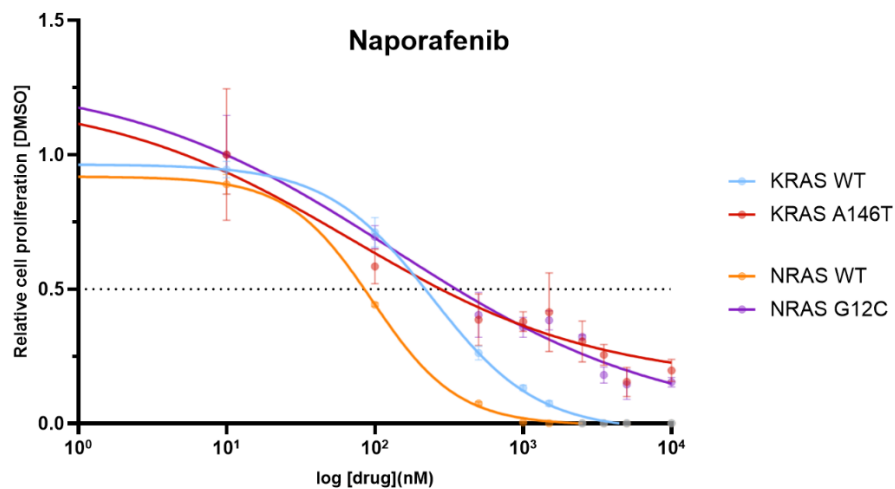


Figure 38: Dose-response curve for Naporafenib. Cells were incubated for 48h with different concentrations of Naporafenib. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

WB analysis on NRAS<sup>G12C</sup> shows the same gradual decrease in p-ERK levels, corresponding to the gradual drop in proliferation. These results are visualized in Figure 39. Cytotoxicity testing against CCFR-CEM and JURKAT cells yielded a 50% decrease in viability in both cases compared to non-treated cells (Extended Data Figure 5B and Extended Data Figure 7).

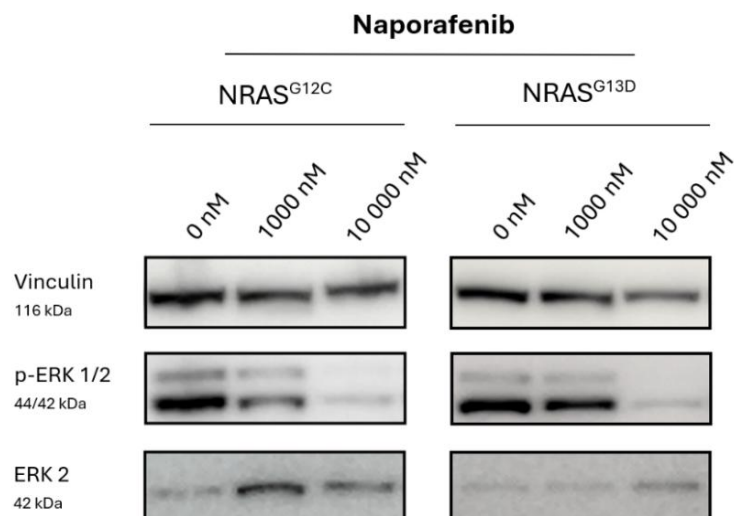


Figure 39: Western blotting analysis for Naporafenib. Cells were incubated with Naporafenib for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control.

## 4.4.2 Trametinib

Trametinib was found to be effective in blocking RAS signaling by inhibiting MEK. Cell proliferation was blocked at relatively low concentrations as is shown in Figure 40. For all mutations as well as for the WT proteins, this is the case (displayed for WT proteins, KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup>, all data in Extended Data Figure 8C). All estimated IC<sub>50</sub> values are below 10 nM, except for NRAS<sup>G12D</sup> and NRAS<sup>G12S</sup>, where they are 12 nM and 28 nM respectively.

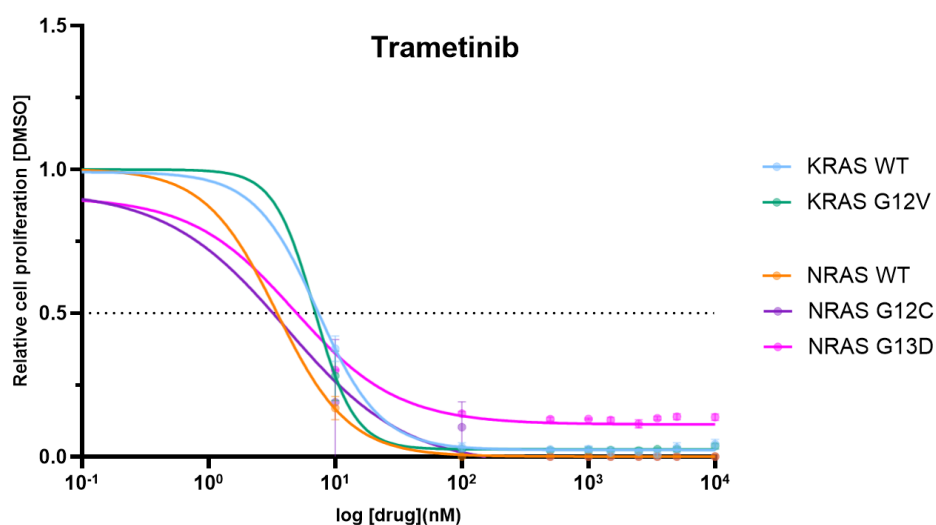


Figure 40: Dose-response curve for Trametinib. Cells were incubated for 48h with different concentrations of Trametinib. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

This inhibition was also confirmed by WB analysis for KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup>, shown in Figure 41. Levels of p-ERK were undetectable at 1000 nM Trametinib.

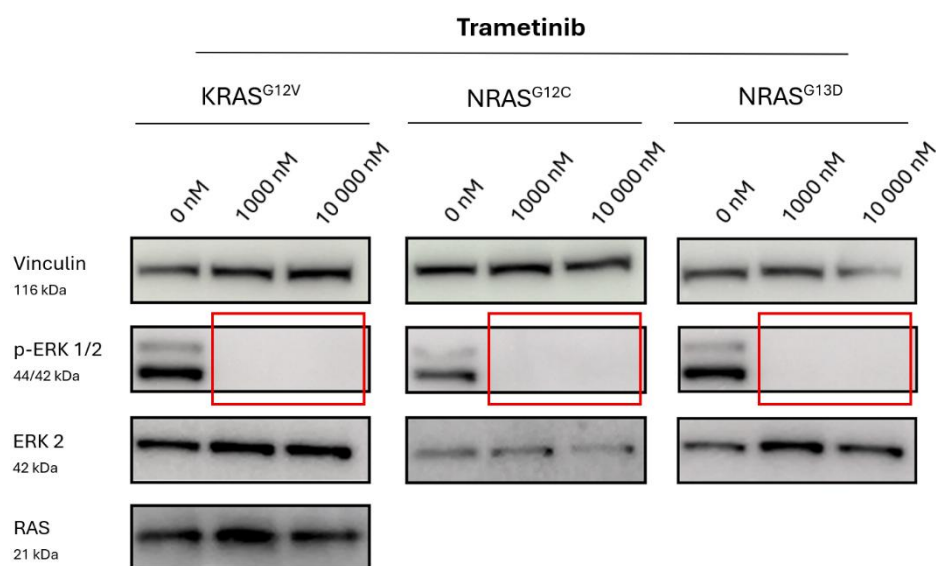
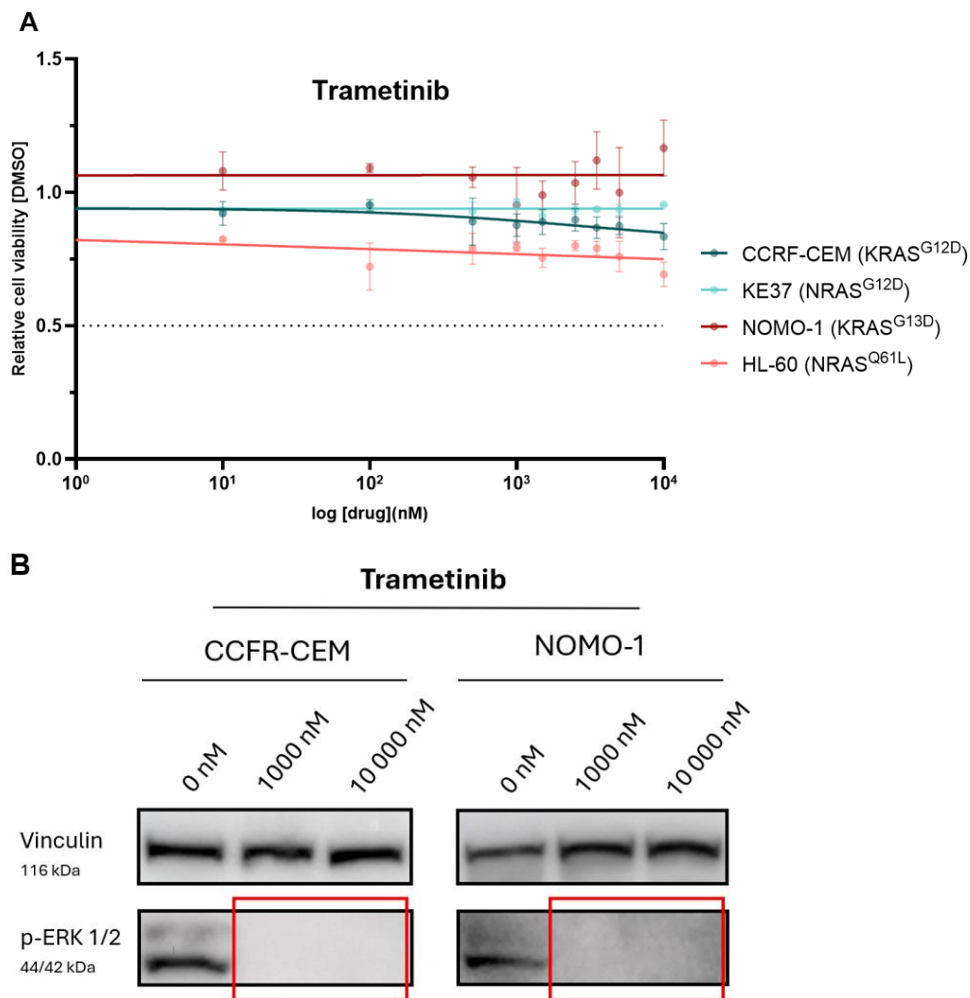


Figure 41: Western blotting analysis for Trametinib. Cells were incubated with Trametinib for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control. Red boxes indicate no detectable p-ERK levels in the cell lysate.

Trametinib was also tested in human cell lines and showed no effect on cell viability (Figure 42A). However, WB analysis revealed that the inhibition of the RAS pathway is still present (Figure 42B). JURKAT cells also showed no decrease in viability after Trametinib administration (Extended Data Figure 5B). This again supports that Trametinib might be a valuable drug against the RAS-dependent HeH B-ALL cells, without harming healthy cells.



**Figure 42: A:** Dose-response curve for Trametinib against human cell lines. Cells were incubated for 24h with different concentrations of Trametinib. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. **B:** Western blotting analysis for Trametinib against human cell lines. Cells were incubated with Trametinib for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control. Red boxes indicate no detectable p-ERK levels in the cell lysate.

## 4.5 Mislocalization of RAS proteins

### 4.5.1 ABD957

Figure 43 shows the result of treatment with ABD957, an indirect inhibitor of the RAS signaling pathway. As expected, the results against KRAS WT and KRAS<sup>G12V</sup> were limited, since they do not rely on depalmitoylation for proper signaling. NRAS mutants were more affected by the drug, with most proliferation rates decreased by 50 – 75%. For NRAS WT, the proliferation was completely blocked (all data in Extended Data Figure 8D). Between NRAS<sup>G13D</sup> and all other NRAS mutants, there was a considerable difference in results. NRAS<sup>G13D</sup> had an IC<sub>50</sub> of 52 nM, whilst NRAS<sup>G12C</sup> for example had one of 3500 nM.

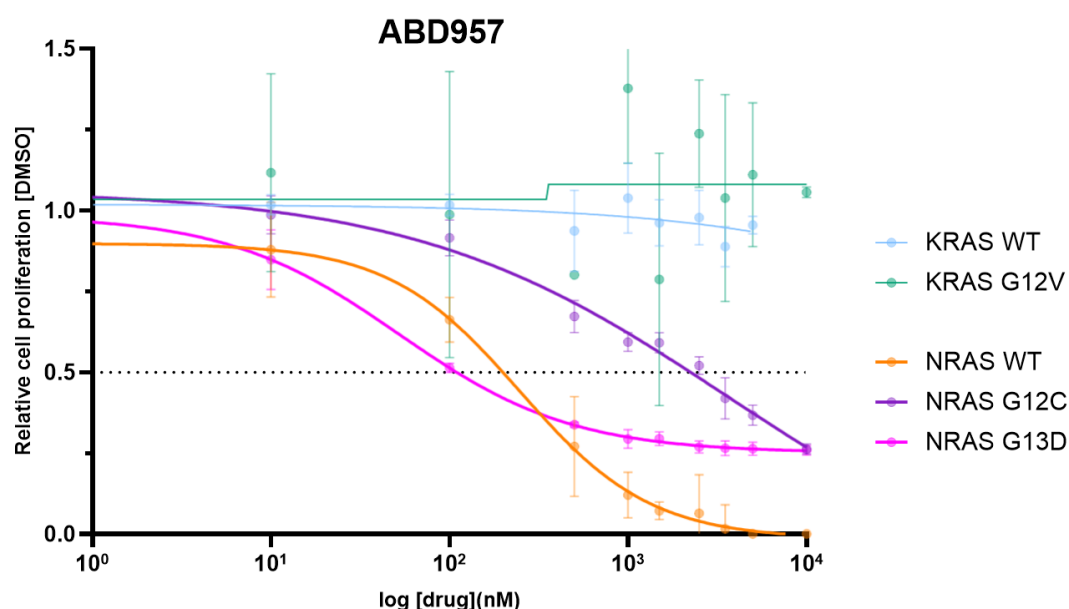


Figure 43: Dose-response curve for ABD957. Cells were incubated for 48h with different concentrations of ABD957. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. When the drug was effective against a certain mutation, the line is shown in bold (1pt), and when there was no effect the line is thinner (1/2pt).

Figure 44 validates that p-ERK levels were decreased for NRAS<sup>G13D</sup> upon treatment with ABD957. In comparison with previous tested drugs, p-ERK levels were not reduced to undetectable levels and can be compared to the efficacy of Naporafenib. However, since this is an indirect NRAS targeting drug, this is in line with the expectations.

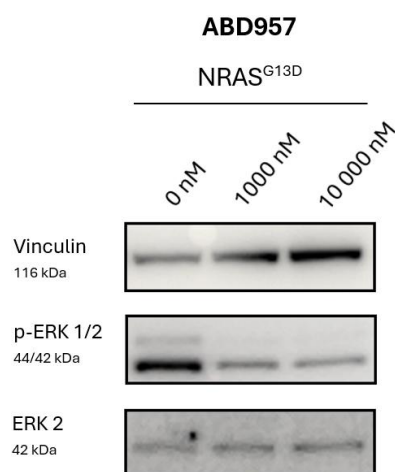


Figure 44: Western blotting analysis for ABD957. Cells were incubated with ABD957 for 4h. Vinculin is a housekeeping gene (always present) and used as a loading control.

Since ABD957 does not directly block RAS signaling and works via mislocalization, a new hypothesis was raised. Since it took longer for this drug to achieve visible effects and only works optimally 18h after administration, proliferation might have been completely blocked between 24h and 48h. Therefore, new ATPlite assays were performed, taking not only a day 0, but also a day 1 value into account. These results are shown in Figure 45. This indeed showed that the efficacy of ABD957 was higher than initially thought. After 24 hours of administration, ABD957 is able to drop proliferation rates to 0. For NRAS<sup>G13D</sup>, the IC<sub>50</sub> shifts to 10 nM, and for NRAS<sup>G12C</sup>, this becomes 1183 nM. This difference between NRAS<sup>G13D</sup> and NRAS<sup>G12C</sup> is in line with the previous results. Cytotoxicity tests yielded similar results for CCFR-CEM and JURKAT cells, where ABD957 did not have any effect on cell viability (Extended Data Figure 5B and Extended Data Figure 7).

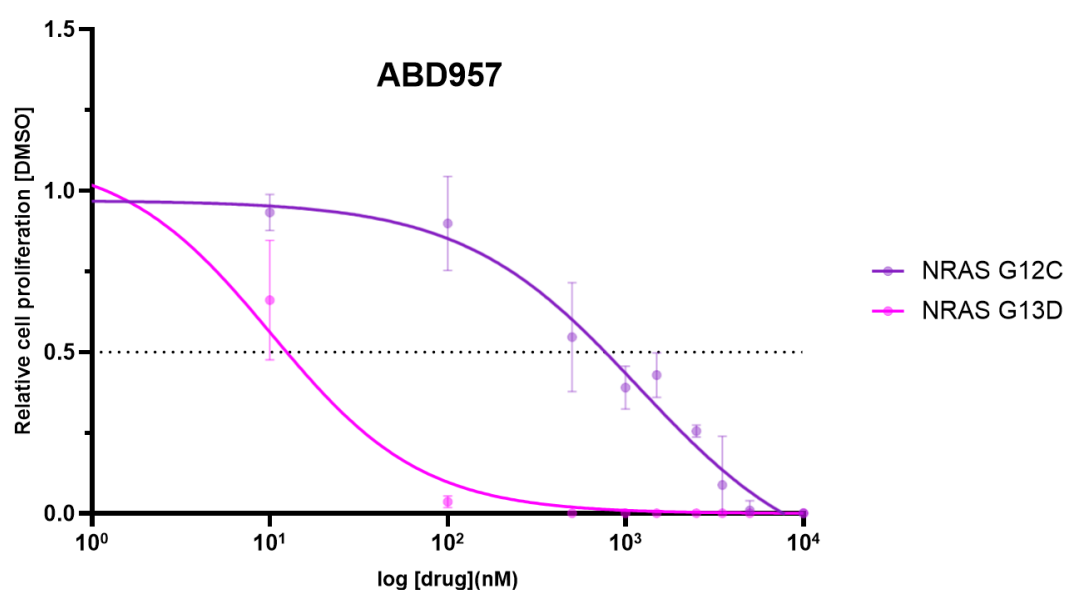


Figure 45: Dose-response curve for ABD957. Cells were incubated for 48h with different concentrations of ABD957. Proliferation between 24h and 48h after administration, was measured by subtracting the absorbance at 24 hours. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.

## 5 Discussion

HeH B-ALL is characterized by the presence of NRAS or KRAS mutations, but these proteins have not yet been explored in detail as therapeutic targets. In the past, only few inhibitors were available to target the RAS/MAPK pathway, but recently, new compounds have been identified that target KRAS or NRAS WT or mutant proteins or interfere indirectly with this signaling. In this work, the potency of various drugs in Ba/F3 cells expressing wild type NRAS or KRAS or expressing mutants of NRAS or KRAS that are frequently observed in HeH ALL was tested.

### 5.1 Experimental set-up

By the establishment of the Ba/F3 cells, it was seen that WT transduced Ba/F3 cells struggled more to become mIL-3 independent. This result was expected, since they do not express an oncogene. Interestingly, KRAS WT seems to have more difficulties, resulting in fewer cells that survived the initial mIL-3 depletion and more selection for cells that had taken up the construct multiple times compared to NRAS WT. This might be explained by the fact that NRAS is naturally more important in hematopoietic signaling compared to KRAS.<sup>56,71,72</sup>

The in vitro tests in this research were conducted on Ba/F3 cells, a pro-B cell model, to see the effects of the drugs in B-cells. However, this model shows no abnormalities in chromosome numbers, so outcomes in HeH B-ALL cells might slightly differ. Whilst specific IC<sub>50</sub> values might vary due to differences in metabolism, HeH B-ALL cells are hypothesized to also be sensitive to the same drugs, due to their shared RAS-dependence.

Other mutations that are often found in HeH B-ALL patients are KRAS<sup>G12C</sup> and KRAS<sup>G13D</sup>. The goal was to include these mutations in the screen, but due to an unfortunate error, these mutations were transduced into a Cas9 expressing Ba/F3 cell line, just as KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup>. Before it was noticed that these cells were not transduced in regular Ba/F3 cells, WB and ATPlite assays were already performed. Because these cells seem to have a different metabolism, these results are not shown in this study, unless mentioned otherwise. KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup> were fortunately newly transduced into regular Ba/F3 cells, after a contamination occurred. It was only this new transduction in regular Ba/F3 cells that led to the discovery that the original transductions happened in Cas9-Ba/F3 cells. At that moment, not enough time was left to perform new transductions for KRAS<sup>G12C</sup> and KRAS<sup>G13D</sup> and conduct all analyses. This is the reason why the results for Sotorasib are from KRAS<sup>G12C</sup> and NRAS<sup>G12C</sup> in the Cas9-Ba/F3 cell line and cannot be compared to the results of other drugs in terms of IC<sub>50</sub> values.

Because of the coincidence that KRAS<sup>G12V</sup>, NRAS<sup>G12C</sup> and NRAS<sup>G13D</sup> were transduced twice, direct comparisons of drug effects could be made between regular and Cas9 Ba/F3 cells. The potency was in both cell models the same, just as the effects on p-ERK levels. Therefore, all the drugs would have probably yielded similar results, against KRAS<sup>G12C</sup> and KRAS<sup>G13D</sup> in regular Ba/F3 cells as they did in Cas9-Ba/F3 cells. The differences that were seen between both cell models were predominantly proliferation rate, medium color and cell viability.

The experimental construct used for ATPlite assays with fixed concentrations, results in a lack of datapoints closely around the IC<sub>50</sub> of each drug. Therefore, multiple drugs have no IC<sub>50</sub> value against certain mutations and when they have one, the error margins are high. Due to limited time, there was no option to redo the assays with concentrations closer to the IC<sub>50</sub>. If this would have been done, it would have allowed to make boxplots of IC<sub>50</sub> values and compare mutations and drugs statistically to each other. Additionally, when time was not a determining factor, KRAS<sup>G12C</sup> and KRAS<sup>G13D</sup> mutations would have been transduced into regular Ba/F3 cells and drugs would have been tested against these two mutations as well. Another valuable experiment would be to test the drugs against a complete RAS independent cell line. It would have been better to test cytotoxic effects in a complete RAS pathway independent cell line, but due to time limitations, the choices were limited to cell lines that were in culture.

All suggestions above can enhance the quality of this research. Nevertheless, the data this study has yielded remains valuable. The goal was to get an overview of possible next-generation drugs and make a first selection of candidates that can be taken into PDX model testing. Taken everything together, some additions could be made, although this data already indicates confidently which drugs are good candidates and which drugs are less qualified.

## 5.2 Drugs

|                           | Ba/F3 - KRAS |      |      |       | Ba/F3 - NRAS |      |      |      |      |      |                         |
|---------------------------|--------------|------|------|-------|--------------|------|------|------|------|------|-------------------------|
| IC <sub>50</sub> (nM)     | WT           | G12C | G12V | A146T | WT           | G12C | G12D | G12S | G13D | Q61H | Cytotoxic               |
| RAS Inhibitors            |              |      |      |       |              |      |      |      |      |      |                         |
| Sotorasib                 |              | 14   |      |       |              | 9,5  |      |      |      |      | no                      |
| Pan-RAS-IN-1              | 7000         |      | 4000 | 4000  | 5000         | 4000 | 4000 | 4000 | 4000 | 4000 | yes                     |
| BI-2865                   | 150          |      | 120  | 89    |              |      |      |      |      |      | no                      |
| Daraxonrasib              | 2,3          |      | 2,6  | 6,1   | 2,5          | 2,8  | 3,8  | 2,9  | 18   | 1,6  | no                      |
| RAS Degraders             |              |      |      |       |              |      |      |      |      |      |                         |
| PROTAC K-Ras Degradar-4   | 170          |      | 330  | 260   |              |      |      |      |      |      | no                      |
| ACBI3                     |              |      |      |       |              |      |      |      |      |      | no                      |
| Downstream RAS inhibitors |              |      |      |       |              |      |      |      |      |      |                         |
| Naprafenib                | 240          |      | 200  | 210   | 96           | 130  | 290  | 740  | 290  | 100  | Possibly<br>(1000 nM +) |
| Trametinib                | 7,2          |      | 6,8  | 5,6   | 3,4          | 4,1  | 13   | 28   | 4,7  | 3,7  | no                      |
| RAS mislocalization       |              |      |      |       |              |      |      |      |      |      |                         |
| ABD957                    |              |      |      |       | 250          | 4000 | 480  | 7000 | 52   | 320  | no                      |

no data

no effect

IC<sub>50</sub> (nM)

< 10 nM

10 - 100 nM

100 - 1000 nM

> 1000 nM

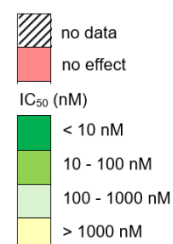


Figure 46: Summary of all drugs. Estimated IC<sub>50</sub> values are given, for which the IC<sub>50</sub> is reached with a high probability. Numbers are rounded up, according to their value. Also cytotoxicity results are mentioned.

**Sotorasib** was specifically designed to target KRAS<sup>G12C</sup>, and was later also found to be able to inhibit NRAS<sup>G12C</sup>.<sup>103</sup> Indeed, also in RAS<sup>G12C</sup> mutated Ba/F3 cells, Sotorasib was able to decrease p-ERK levels and inhibit proliferation. Thus, Sotorasib is a potent inhibitor, but only against these mutations. Currently this is the only RAS mutation-specific drug, besides Adagrasib (also against RAS<sup>G12C</sup>), that is approved for therapy. Sotorasib is currently used against NSCLC and mCRC, but further studying this drug in PDX models, might expand clinical trials and eventually get Sotorasib approved to treat RAS<sup>G12C</sup>-positive HeH B-ALL patients.

In contrast to Sotorasib, **Pan-RAS-IN-1** was a potent inhibitor of all Ba/F3 cells expressing various RAS mutants. Although IC<sub>50</sub> values were higher compared to other drugs, Pan-RAS-IN-1 is known to work in the range of multiple µM with a determined IC<sub>50</sub> of 8,8 µM in a DLD-1 cell line.<sup>109</sup> Additionally, it is not mutation-specific and also targets WT RAS proteins. However, none of the tested drugs, besides Sotorasib, make a clear distinction between WT or mutated RAS isoforms, leaving Sotorasib the only specific drug. A previous study mentions possible cytotoxicity of Pan-RAS-IN-1 in mutant cells.<sup>110</sup> However, they found a distinction between mutations and WT proteins, where no cytotoxicity was observed when adding 5 µM of Pan-RAS-IN-1 to WT cells, but when repeated with NRAS mutant human cell lines, viability dropped to 80%.<sup>110</sup> Here, Pan-RAS-IN-1 had cytotoxic effects in all cell models that were tested. Taken together, this drug might not be the best candidate to go into in vivo trials.

**BI-2865** on the other hand, was a potent inhibitor of Ba/F3 cells expressing various KRAS mutants. This was in line with the expectations from literature, where also IC<sub>50</sub> values around 100 nM were established for different KRAS mutations.<sup>111</sup> A downside of BI-2865 is that it is effective against KRAS, but not against NRAS. This suggests that BI-2865 might be a good candidate for treating KRAS mutated subclones in HeH B-ALL patients. Compared to other RAS inhibitors, BI-2865 has a lower IC<sub>50</sub> than Pan-RAS-IN-1, but higher than Daraxonrasib.

Based on this data, **Daraxonrasib** seems to be the most promising out of the three tested Pan-RAS inhibitors. Previous research on Daraxonrasib determined half-maximal effective concentration (EC<sub>50</sub>) values between 0,3 nM and 3,6 nM for different KRAS mutations in human cancer cell lines.<sup>112</sup> Our pro-B cell model yielded similar results. IC<sub>50</sub> values were lower than 10 nM, with NRAS<sup>G13D</sup> cells as the only exception. The lower sensitivity of NRAS<sup>G13D</sup> might be attributed to the fact that Daraxonrasib locks RAS proteins in their active conformation. While RAS<sup>G12</sup> and RAS<sup>Q61</sup> mutations keep RAS constantly ON, RAS<sup>G13D</sup> drives rapid dissociation of GDP, leaving RAS cycling between their ON and OFF state. That Daraxonrasib is the most promising inhibitor, also translates in current clinical trials, where this drug is tested against previously treated pancreatic ductal adenocarcinoma (PDAC) with RAS mutations.<sup>113</sup> Progressive-free responses were seen in 35% of the patients that received Daraxonrasib as a second-line therapy and this response had a median duration of 8,1 months.<sup>113</sup>

Of the two PROTACs used to target the RAS pathway, **PROTAC K-Ras Degradar-4** gave the most promising results, being able to block proliferation in all KRAS mutated cells. Although multiple studies highlight the novelty of the technique and underline the degradation as the main strength, this degradation could not be visualized or observed in our study.<sup>106,114</sup> Reasons for this might be the timing of the drug treatment and the higher levels of KRAS due to the overexpression in Ba/F3 cells. It is unlikely the drugs were inactive, since potent inhibition of proliferation was observed.

Indeed, a key parameter in the efficacy of PROTACs is the halftime life of the proteins they degrade. Since all proteins are transduced into Ba/F3 cells and under control of a strong promotor, the levels of RAS proteins are highly elevated in these cells. Moreover, the depletion of mIL-3, is likely driving selection for cells with higher or more stable KRAS expression. However, PROTACs are designed to degrade even highly expressed proteins and previous studies with PROTACs already showed effective degradation in overexpression models.<sup>114,115</sup> To completely rule out that the absence of degradation is due to the overexpression of the proteins, the drug could have been tested against WT Ba/F3 cells that still grow in the presence of mIL-3, or in the human ALL cell lines. More time points after drug administration and more concentrations of the PROTACs could be taken into account. Via WB, the effect of the duration of the treatment and the halftime of the proteins could be visualized.

An exciting advantage about PROTAC K-Ras Degradar-4 is that, even when degradation is not observed, inhibition of KRAS signaling is still present. Previous tests with PROTACs also came to the same conclusion. In a study from 2019, six different PROTAC structures targeting KRAS were tested. The results yielded only more than 50% degradation in concentrations above 1  $\mu$ M for two out of the six structures.<sup>116</sup> In most cases degradation was below 25%.<sup>116</sup> A reason for the inhibition, but the absence of degradation could be a low proteasome capacity in pro-B cells or low expression of the E3 ligase. Since PROTAC K-Ras Degradar-4 recruits the E3-ligase Cereblon, this protein needs to be present in order for the degradation to work. Larger studies across multiple tumor types, showed that the expression of Cereblon can be highly context dependent and is a common hurdle that PROTACs face.<sup>117</sup> A good follow-up experiment would be to quantify the amount of degradation in these Ba/F3 cells, via capillary Western Blotting or proteomic approaches. Nevertheless, because the inhibition of the RAS pathway is still observed, PROTAC K-Ras Degradar-4 is still a promising candidate to target KRAS mutated HeH B-ALL subclones.

**ACBI3** does not even show inhibition of the RAS pathway. All reasons mentioned above are also applicable to why ACBI3 might not work. Interestingly, ACBI3 recruits the Von Hippel-Lindau E3 ligase, which should be less prone to context-dependent expression.<sup>118</sup> This should make the drug more robust than PROTAC K-Ras Degradar-4. Interestingly, a researcher

mentioned at a forum for chemical compounds that ACBI3 had difficulties with high efflux in certain cell systems.<sup>119</sup> Other research groups at the VIB KU-Leuven Center for Cancer Biology have experience with ACBI3 and confirmed detection of KRAS degradation via WB in other cell models. Unfortunately, due to time limitations, there was no time left to try and validate whether there was something wrong with the aliquots that were used or if ACBI3 was really unable to degrade RAS. Remarkably, all other drugs that were tested in this study worked as expected and only ACBI3 had no effect at all.

Both **Naporafenib** and **Trametinib**, the two downstream inhibitors, showed similar efficacy in the various Ba/F3 cells. However, Trametinib had higher potency, already blocking proliferation in a range of 10 nM, while Naporafenib only achieved this around 300 nM. This high potency makes Trametinib a good candidate to take into further research. Nonetheless, Naporafenib can still be a very useful drug, knowing that CRAF is thought to be a critical player in overcoming aneuploidy stress.<sup>45</sup> On top of that, studies show that CRAF might be activated independent of RAS signaling, highlighting the need of an inhibitor downstream of RAS.<sup>45</sup> This hypothesis suggests that Naporafenib might work even better in a real HeH B-ALL-like model. Therefore, both Naporafenib and Trametinib are good drugs to try in HeH B-ALL therapies.

Based on these assays, it is not clear whether Naporafenib shows cytotoxic effects at high concentrations, or whether the cells rely too much on RAF signaling for their survival. JURKAT cells have an oncogenic mutation in BRAF, the protein that directly gets inhibited by Naporafenib. Additionally, viability was not decreased to 0%, indicating cytostatic, rather than cytotoxic effects. However, an earlier study suggests that Naporafenib does exhibit off-target kinase inhibition in concentrations above 1000 nM, although no changes in viability were mentioned.<sup>120</sup> Luckily, the IC<sub>50</sub> in this study was already reached at 300 nM for most mutations.

Since most RAS mutations in cancer are driven by KRAS mutations, almost all RAS inhibitors are designed to target KRAS. Just as BI-2865 and PROTAC K-Ras Degradier-4 as examples from this study. This leaves the field with a lack of specific NRAS mutation targeting drugs.<sup>121</sup> When patients have only NRAS-mutated HeH B-ALL subclones, targeting NRAS specifically might provide a broader therapeutic window.

Here, a novel compound, **ABD957**, was selected as a specific NRAS targeting drug. Despite its novelty, it showed promising effects, mostly for NRAS<sup>G13D</sup> mutations. Remarkably, a large difference between the IC<sub>50</sub> of NRAS<sup>G13D</sup> and other NRAS mutations was seen. Multiple studies compare KRAS mutants and RAS biochemistry, showing that RAS<sup>Q61</sup> and RAS<sup>G12</sup> mutations are generally more activating than RAS<sup>G13</sup> mutations.<sup>71,102,122</sup> Because most mutations only show a complete block of proliferation when ABD957 is administered in a concentration of 5 µM or higher, this might not be an ideal candidate. However, for patients showing only NRAS mutations and predominantly have NRAS<sup>G13D</sup> mutations, this can still be a good drug.

## 5.3 Conclusion

Ba/F3 cells can be used as an excellent model to test various drugs that target the RAS pathway. Overexpression of RAS mutants or WT proteins was sufficient to drive cell proliferation independent of mIL-3. This resulted in a pro-B cell line, solely depending on RAS signaling for their proliferation, which is ideal for RAS targeting drug testing.

Taken everything together, next-generation drugs that are ready for testing in PDX models of HeH B-ALL patients are Daraxonrasib and Trametinib. Daraxonrasib was the most potent Pan-RAS inhibitor. Also Trametinib showed to be a potent downstream inhibitor, with a low IC<sub>50</sub>. Sotorasib, BI-2865 and ABD957 are also advised to be taken into further PDX model testing, but only when patients show a certain mutation profile. Sotorasib can be taken along, when a patient has RAS<sup>G12C</sup> mutated subclones. BI-2865 on the other hand, can be particularly valuable when patients only show KRAS mutated subclones, leaving NRAS undisturbed. ABD957 can be selected when a patient shows predominantly NRAS<sup>G13D</sup> mutations and no KRAS mutated subclones.

Drugs that are suggested to take into further testing, but require more validations, are PROTAC K-Ras Degradar-4 and Naporafenib. PROTAC K-Ras Degradar-4 could be a valuable drug, since it is beneficial that it can still inhibit proliferation independently of its degradation success. However, new PROTACs could be screened to find drugs that are able to degrade RAS. All tests indicate that Naporafenib might give positive results in HeH B-ALL PDX models. However, its possible cytotoxic effects should be validated, although all findings here point into the direction of cytostatic, rather than cytotoxic effects. Nevertheless, to be completely sure, a clear therapeutic window must be determined, before advancing into further testing stages. Pan-RAS-IN-1 and ACBI3 are not recommended to take into further studies due to the cytotoxicity and the inability to target RAS respectively.

An interesting future perspective, besides in vivo experiments, might be to explore dual therapies with distinct modes of action. Based on this research, the suggestion would be to take Daraxonrasib together with Trametinib, the most potent direct RAS inhibitor and downstream RAS inhibitor respectively.

Taken together, these findings identify promising next-generation therapeutic candidates to treat HeH B-ALL and also lay the foundation for in vivo validation in PDX-models. This brings us one step closer to more targeted, effective and clinically relevant treatments for this pediatric cancer.

## 6 References

1. Cumano, A., & Godin, I. (2007). Ontogeny of the Hematopoietic System. *Annual Review of Immunology*, 25, 745–785. <https://doi.org/10.1146/annurev.immunol.25.022106.141538>
2. Kato, H., & Igarashi, K. (2019). To be red or white: Lineage commitment and maintenance of the hematopoietic system by the “inner myeloid.” *Haematologica*, 104(10), 1919–1927. <https://doi.org/10.3324/haematol.2019.216861>
3. Puhr, S., Lee, J., Zvezdova, E., et al. (2015). Dendritic Cell Development – History, Advances, and Open Questions. *Seminars in Immunology*, 27(6), 388–396. <https://doi.org/10.1016/j.smim.2016.03.012>
4. Rieger, M. A., & Schroeder, T. (2012). Hematopoiesis. *Cold Spring Harbor Perspectives in Biology*, 4(12), a008250. <https://doi.org/10.1101/cshperspect.a008250>
5. Orakpoghenor, O., Avazi, D. O., Markus, T. P., et al. (2019). Lymphocytes: A Brief Review. *Scientific Journal of Immunology & Immunotherapy*, 5.
6. Zlotoff, D. A., & Bhandoola, A. (2011). Hematopoietic progenitor migration to the adult thymus. *Annals of the New York Academy of Sciences*, 1217(1), 122–138. <https://doi.org/10.1111/j.1749-6632.2010.05881.x>
7. Lai, A. Y., & Kondo, M. (2008). T and B lymphocyte differentiation from hematopoietic stem cell. *Seminars in Immunology*, 20(4), 207–212. <https://doi.org/10.1016/j.smim.2008.05.002>
8. Rothenberg, E. V. (2011). T cell lineage commitment: Identity and renunciation. *Journal of Immunology (Baltimore, Md. : 1950)*, 186(12), 6649–6655. <https://doi.org/10.4049/jimmunol.1003703>
9. Kenneth M. Murphy, Casey Weaver, & Leslie J. Berg. (2022). *Janeway's Immunobiology*.
10. Germain, R. N. (2002). T-cell development and the CD4–CD8 lineage decision. *Nature Reviews Immunology*, 2(5), 309–322. <https://doi.org/10.1038/nri798>
11. Chann, A. S., Charnley, M., Newton, L. M., et al. (2022). Stepwise progression of  $\beta$ -selection during T cell development involves histone deacetylation. *Life Science Alliance*, 6(1), e202201645. <https://doi.org/10.26508/lsa.202201645>
12. Abe, S., Asahi, T., Hara, T., et al. (2023). Hematopoietic cell-derived IL-15 supports NK cell development in scattered and clustered localization within the bone marrow. *Cell Reports*, 42(9), 113127. <https://doi.org/10.1016/j.celrep.2023.113127>
13. Stephan, R. P., Reilly, C. R., & Witte, P. L. (1998). Impaired Ability of Bone Marrow Stromal Cells to Support B-Lymphopoiesis With Age. *Blood*, 91(1), 75–88. <https://doi.org/10.1182/blood.V91.1.75>
14. Jung, D., Giallourakis, C., Mostoslavsky, R., et al. (2006). MECHANISM AND CONTROL OF V(D)J RECOMBINATION AT THE IMMUNOGLOBULIN HEAVY CHAIN LOCUS. *Annual Review of Immunology*, 24(Volume 24, 2006), 541–570. <https://doi.org/10.1146/annurev.immunol.23.021704.115830>
15. Chen, D., Ireland, S. J., Remington, G., et al. (2016). CD40-mediated NF $\kappa$ B activation in B cells is increased in multiple sclerosis and modulated by therapeutics. *Journal of Immunology (Baltimore, Md. : 1950)*, 197(11), 4257–4265. <https://doi.org/10.4049/jimmunol.1600782>
16. Rossetti, R. A. M., Lorenzi, N. P. C., Yokochi, K., et al. (2018). B lymphocytes can be activated to act as antigen presenting cells to promote anti-tumor responses. *PLOS ONE*, 13(7), e0199034. <https://doi.org/10.1371/journal.pone.0199034>
17. Forthal, D. N. (2014). Functions of Antibodies. *Microbiology Spectrum*, 2(4), 1–17.
18. Ding, L., Ley, T. J., Larson, D. E., et al. (2012). Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*, 481(7382), 506–510. <https://doi.org/10.1038/nature10738>
19. Aertgeerts M. (2025). *Clonal Heterogeneity and Evolution During Treatment in Pediatric Acute Lymphoblastic Leukemia—A Single-Cell Characterization* [Doctoral thesis, KU Leuven].
20. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, 144(5), 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>

21. *Leukemia—Hematology.org.* (n.d.). Retrieved October 13, 2025, from <https://www.hematology.org/education/patients/blood-cancers/leukemia>
22. *Childhood Acute Lymphoblastic Leukemia—NCI.* (2026, February 10). <https://www.cancer.gov/types/leukemia/patient/child-all-treatment-pdq>
23. Inaba, H., & Pui, C.-H. (2021). Advances in the Diagnosis and Treatment of Pediatric Acute Lymphoblastic Leukemia. *Journal of Clinical Medicine*, 10(9), 1926. <https://doi.org/10.3390/jcm10091926>
24. Khoury, J. D., Solary, E., Abla, O., et al. (2022). The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. *Leukemia*, 36(7), 1703–1719. <https://doi.org/10.1038/s41375-022-01613-1>
25. Davis, A. S., Viera, A. J., & Mead, M. D. (2014). Leukemia: An overview for primary care. *American Family Physician*, 89(9), 731–738.
26. Thomas, D., & Majeti, R. (2017). Biology and relevance of human acute myeloid leukemia stem cells. *Blood*, 129(12), 1577–1585. <https://doi.org/10.1182/blood-2016-10-696054>
27. Holyoake, T. L., & Vetrie, D. (2017). The chronic myeloid leukemia stem cell: Stemming the tide of persistence. *Blood*, 129(12), 1595–1606. <https://doi.org/10.1182/blood-2016-09-696013>
28. Arber, D. A., Orazi, A., Hasserjian, R. P., et al. (2022). International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: Integrating morphologic, clinical, and genomic data. *Blood*, 140(11), 1200–1228. <https://doi.org/10.1182/blood.2022015850>
29. *Childhood Leukemia.* (n.d.). Retrieved May 13, 2026, from <https://www.cancer.org/cancer/types/leukemia-in-children.html>
30. *Signs and Symptoms of Acute Lymphocytic Leukemia.* (n.d.). Retrieved November 30, 2025, from <https://www.cancer.org/cancer/types/acute-lymphocytic-leukemia/detection-diagnosis-staging/signs-symptoms.html>
31. *Acute lymphoblastic leukemia (ALL) signs and symptoms | Blood Cancer United.* (n.d.). Retrieved November 30, 2025, from <https://bloodcancerunited.org/blood-cancer/leukemia/acute-lymphoblastic-leukemia-all/signs-and-symptoms>
32. Reismüller, B., Steiner, M., Pichler, H., et al. (2017). High hyperdiploid acute lymphoblastic leukemia (ALL)—A 25-year population-based survey of the Austrian ALL-BFM (Berlin-Frankfurt-Münster) Study Group. *Pediatric Blood & Cancer*, 64(6), e26327. <https://doi.org/10.1002/pbc.26327>
33. Hunger, S. P., & Mullighan, C. G. (2015). Acute Lymphoblastic Leukemia in Children. *New England Journal of Medicine*, 373(16), 1541–1552. <https://doi.org/10.1056/NEJMra1400972>
34. Paulsson, K., & Johansson, B. (2009). High hyperdiploid childhood acute lymphoblastic leukemia. *Genes, Chromosomes and Cancer*, 48(8), 637–660. <https://doi.org/10.1002/gcc.20671>
35. Greaves, M., & Maley, C. C. (2012). Clonal Evolution In Cancer. *Nature*, 481(7381), 306–313. <https://doi.org/10.1038/nature10762>
36. Aertgeerts, M., Meyers, S., Gielen, O., et al. (2025). Single-cell DNA and surface protein characterization of high hyperdiploid acute lymphoblastic leukemia at diagnosis and during treatment. *HemaSphere*, 9(2), e70085. <https://doi.org/10.1002/hem3.70085>
37. Paulsson, K., Lilljebjörn, H., Biloglav, A., et al. (2015). The genomic landscape of high hyperdiploid childhood acute lymphoblastic leukemia. *Nature Genetics*, 47(6), 672–676. <https://doi.org/10.1038/ng.3301>
38. *PeCan | St. Jude Cloud.* (n.d.). Retrieved October 21, 2025, from <https://pecan.stjude.cloud/>
39. Taylor, A. M., Shih, J., Ha, G., et al. (2018). Genomic and Functional Approaches to Understanding Cancer Aneuploidy. *Cancer Cell*, 33(4), 676–689.e3. <https://doi.org/10.1016/j.ccell.2018.03.007>
40. Hintzen, D. C., Schubert, M., Soto, M., et al. (2024). Reduction of chromosomal instability and inflammation is a common aspect of adaptation to aneuploidy. *EMBO Reports*, 25(11), 5169–5193. <https://doi.org/10.1038/s44319-024-00252-0>

41. Santaguida, S., & Amon, A. (2015). Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nature Reviews Molecular Cell Biology*, 16(8), 473–485. <https://doi.org/10.1038/nrm4025>
42. Ippolito, M. R., Zerbib, J., Eliezer, Y., et al. (2024). Increased RNA and Protein Degradation Is Required for Counteracting Transcriptional Burden and Proteotoxic Stress in Human Aneuploid Cells. *Cancer Discovery*, 14(12), 2532–2553. <https://doi.org/10.1158/2159-8290.CD-23-0309>
43. He, A., Dean, J. M., & Lodhi, I. J. (2021). Peroxisomes as cellular adaptors to metabolic and environmental stress. *Trends in Cell Biology*, 31(8), 656–670. <https://doi.org/10.1016/j.tcb.2021.02.005>
44. Newman, D. L., & Gregory, S. L. (2019). Co-Operation between Aneuploidy and Metabolic Changes in Driving Tumorigenesis. *International Journal of Molecular Sciences*, 20(18), 4611. <https://doi.org/10.3390/ijms20184611>
45. Zerbib, J., Ippolito, M. R., Eliezer, Y., et al. (2024). Human aneuploid cells depend on the RAF/MEK/ERK pathway for overcoming increased DNA damage. *Nature Communications*, 15(1), 7772. <https://doi.org/10.1038/s41467-024-52176-x>
46. *Hyperdiploidy: The longest known, most prevalent, and most enigmatic form of acute lymphoblastic leukemia in children | Leukemia*. (n.d.). Retrieved October 30, 2025, from <https://www.nature.com/articles/s41375-022-01720-z>
47. Woodward, E. L., Yang, M., Moura-Castro, L. H., et al. (2023). Clonal origin and development of high hyperdiploidy in childhood acute lymphoblastic leukaemia. *Nature Communications*, 14(1), 1658. <https://doi.org/10.1038/s41467-023-37356-5>
48. Maia, A. T., van der Velden, V. H. J., Harrison, C. J., et al. (2003). Prenatal origin of hyperdiploid acute lymphoblastic leukemia in identical twins. *Leukemia*, 17(11), 2202–2206. <https://doi.org/10.1038/sj.leu.2403101>
49. Bargiela-Iparraguirre, J., Prado-Marchal, L., Pajuelo-Lozano, N., et al. (2014). Mad2 and BubR1 modulates tumourigenesis and paclitaxel response in MKN45 gastric cancer cells. *Cell Cycle*, 13(22), 3590–3601. <https://doi.org/10.4161/15384101.2014.962952>
50. Fujiwara, T., Bandi, M., Nitta, M., et al. (2005). Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. *Nature*, 437(7061), 1043–1047. <https://doi.org/10.1038/nature04217>
51. Molina, O., Vinyoles, M., Granada, I., et al. (2020). Impaired Condensin Complex and Aurora B kinase underlie mitotic and chromosomal defects in hyperdiploid B-cell ALL. *Blood*, 136(3), 313–327. <https://doi.org/10.1182/blood.2019002538>
52. Moura-Castro, L. H., Peña-Martínez, P., Castor, A., et al. (2021). Sister chromatid cohesion defects are associated with chromosomal copy number heterogeneity in high hyperdiploid childhood acute lymphoblastic leukemia. *Genes, Chromosomes & Cancer*, 60(6), 410–417. <https://doi.org/10.1002/gcc.22933>
53. Moura-Castro, L. H., Peña-Martínez, P., Castor, A., et al. (2021). Sister chromatid cohesion defects are associated with chromosomal copy number heterogeneity in high hyperdiploid childhood acute lymphoblastic leukemia. *Genes, Chromosomes and Cancer*, 60(6), 410–417. <https://doi.org/10.1002/gcc.22933>
54. Wang, N., Xu, S., & Egli, D. (2023). Replication stress in mammalian embryo development, differentiation, and reprogramming. *Trends in Cell Biology*, 33(10), 872–886. <https://doi.org/10.1016/j.tcb.2023.03.015>
55. Flach, J., & Milyavsky, M. (2018). Replication stress in hematopoietic stem cells in mouse and man. *Mutation Research*, 808, 74–82. <https://doi.org/10.1016/j.mrfmmm.2017.10.001>
56. Molina, J. R., & Adjei, A. A. (2006). The Ras/Raf/MAPK Pathway. *Journal of Thoracic Oncology*, 1(1), 7–9. [https://doi.org/10.1016/S1556-0864\(15\)31506-9](https://doi.org/10.1016/S1556-0864(15)31506-9)
57. Singh, B., Carpenter, G., & Coffey, R. J. (2016). EGF receptor ligands: Recent advances. *F1000Research*, 5, F1000 Faculty Rev-2270. <https://doi.org/10.12688/f1000research.9025.1>
58. Wang, Y.-N., Lee, H.-H., & Hung, M.-C. (2018). A novel ligand-receptor relationship between families of ribonucleases and receptor tyrosine kinases. *Journal of Biomedical Science*, 25(1), 83. <https://doi.org/10.1186/s12929-018-0484-7>

59. Kovacs, E., Zorn, J. A., Huang, Y., et al. (2015). A Structural Perspective on the Regulation of the Epidermal Growth Factor Receptor. *Annual Review of Biochemistry*, 84(Volume 84, 2015), 739–764. <https://doi.org/10.1146/annurev-biochem-060614-034402>
60. Jura, N., Zhang, X., Endres, N. F., et al. (2011). Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. *Molecular Cell*, 42(1), 9–22. <https://doi.org/10.1016/j.molcel.2011.03.004>
61. Littlefield, P., & Jura, N. (2013). EGFR lung cancer mutants get specialized. *Proceedings of the National Academy of Sciences*, 110(38), 15169–15170. <https://doi.org/10.1073/pnas.1314719110>
62. Wang, D., Liu, G., Meng, Y., et al. (2024). The Configuration of GRB2 in Protein Interaction and Signal Transduction. *Biomolecules*, 14(3), 259. <https://doi.org/10.3390/biom14030259>
63. Bandaru, P., Kondo, Y., & Kuriyan, J. (2019). The Interdependent Activation of Son-of-Sevenless and Ras. *Cold Spring Harbor Perspectives in Medicine*, 9(2), a031534. <https://doi.org/10.1101/cshperspect.a031534>
64. Herman, J. A., Romain, R. R., Hoellerbauer, P., et al. (2022). Hyper-active RAS/MAPK introduces cancer-specific mitotic vulnerabilities. *Proceedings of the National Academy of Sciences*, 119(41), e2208255119. <https://doi.org/10.1073/pnas.2208255119>
65. Jeon, H., Tkacik, E., & Eck, M. J. (2024). Signaling from RAS to RAF: The Molecules and Their Mechanisms. *Annual Review of Biochemistry*, 93(Volume 93, 2024), 289–316. <https://doi.org/10.1146/annurev-biochem-052521-040754>
66. Peyssonnaud, C., & Eychène, A. (2001). The Raf/MEK/ERK pathway: New concepts of activation. *Biology of the Cell*, 93(1–2), 53–62. [https://doi.org/10.1016/S0248-4900\(01\)01125-X](https://doi.org/10.1016/S0248-4900(01)01125-X)
67. Shaul, Y. D., & Seger, R. (2007). The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, Mitogen-Activated Protein Kinases: New Insights on Regulation, Function and Role in Human Disease*, 1773(8), 1213–1226. <https://doi.org/10.1016/j.bbamcr.2006.10.005>
68. Maik-Rachline, G., Hacohen-Lev-Ran, A., & Seger, R. (2019). Nuclear ERK: Mechanism of Translocation, Substrates, and Role in Cancer. *International Journal of Molecular Sciences*, 20(5), 1194. <https://doi.org/10.3390/ijms20051194>
69. *RAS: Targeting the Impossible*. (n.d.). Retrieved May 23, 2026, from <https://blog.crownbio.com/targeting-ras-oncology>
70. Castellano, E., & Downward, J. (2011). RAS Interaction with PI3K: More Than Just Another Effector Pathway. *Genes & Cancer*, 2(3), 261–274. <https://doi.org/10.1177/1947601911408079>
71. Simanshu, D. K., Nissley, D. V., & McCormick, F. (2017). RAS Proteins and Their Regulators in Human Disease. *Cell*, 170(1), 17–33. <https://doi.org/10.1016/j.cell.2017.06.009>
72. Santos, E., & Nebreda, A. R. (1989). Structural and functional properties of ras proteins. *The FASEB Journal*, 3(10), 2151–2163. <https://doi.org/10.1096/fasebj.3.10.2666231>
73. Brady, S. W., Roberts, K. G., Gu, Z., et al. (2022). The genomic landscape of pediatric acute lymphoblastic leukemia. *Nature Genetics*, 54(9), 1376–1389. <https://doi.org/10.1038/s41588-022-01159-z>
74. Paulsson, K., Horvat, A., Strömbeck, B., et al. (2008). Mutations of FLT3, NRAS, KRAS, and PTPN11 are frequent and possibly mutually exclusive in high hyperdiploid childhood acute lymphoblastic leukemia. *Genes, Chromosomes and Cancer*, 47(1), 26–33. <https://doi.org/10.1002/gcc.20502>
75. Hunter, J. C., Manandhar, A., Carrasco, M. A., et al. (2015). Biochemical and Structural Analysis of Common Cancer-Associated KRAS Mutations. *Molecular Cancer Research*, 13(9), 1325–1335. <https://doi.org/10.1158/1541-7786.MCR-15-0203>
76. Case, M., Matheson, E., Minto, L., et al. (2008). Mutation of Genes Affecting the RAS Pathway Is Common in Childhood Acute Lymphoblastic Leukemia. *Cancer Research*, 68(16), 6803–6809. <https://doi.org/10.1158/0008-5472.CAN-08-0101>
77. Iritani, B. M., Forbush, K. A., Farrar, M. A., et al. (1997). Control of B cell development by Ras-mediated activation of Raf. *The EMBO Journal*, 16(23), 7019–7031. <https://doi.org/10.1093/emboj/16.23.7019>

78. Kamata, T., Hussain, J., Giblett, S., et al. (2010). BRAF Inactivation Drives Aneuploidy by Deregulating CRAF. *Cancer Research*, 70(21), 8475–8486. <https://doi.org/10.1158/0008-5472.CAN-10-0603>
79. Zang, M., Gong, J., Luo, L., et al. (2008). Characterization of Ser338 Phosphorylation for Raf-1 Activation\*. *Journal of Biological Chemistry*, 283(46), 31429–31437. <https://doi.org/10.1074/jbc.M802855200>
80. Zang, M., Hayne, C., & Luo, Z. (2002). Interaction between Active Pak1 and Raf-1 Is Necessary for Phosphorylation and Activation of Raf-1\*. *Journal of Biological Chemistry*, 277(6), 4395–4405. <https://doi.org/10.1074/jbc.M110000200>
81. Dandoulaki, M., Petsalaki, E., Sumpton, D., et al. (2018). Src activation by Chk1 promotes actin patch formation and prevents chromatin bridge breakage in cytokinesis. *Journal of Cell Biology*, 217(9), 3071–3089. <https://doi.org/10.1083/jcb.201802102>
82. *Genetic variations may predict relapse risk in childhood ALL*. (n.d.). BSH. Retrieved October 15, 2025, from <https://b-s-h.org.uk/about-us/news/genetic-variations-may-predict-relapse-risk-in-childhood-all>
83. Bertuccio, P., Alicandro, G., Malvezzi, M., et al. (2020). Childhood cancer mortality trends in Europe, 1990–2017, with focus on geographic differences. *Cancer Epidemiology*, 67, 101768. <https://doi.org/10.1016/j.canep.2020.101768>
84. Rheingold, S. R., Bhojwani, D., Ji, L., et al. (2024). Determinants of survival after first relapse of acute lymphoblastic leukemia: A Children's Oncology Group study. *Leukemia*, 38(11), 2382–2394. <https://doi.org/10.1038/s41375-024-02395-4>
85. Jeha, S., Choi, J., Roberts, K. G., et al. (2021). Clinical Significance of Novel Subtypes of Acute Lymphoblastic Leukemia in the Context of Minimal Residual Disease–Directed Therapy. *Blood Cancer Discovery*, 2(4), 326–337. <https://doi.org/10.1158/2643-3230.BCD-20-0229>
86. Ratti, S., Lonetti, A., Folio, M. Y., et al. (2020). B-ALL Complexity: Is Targeted Therapy Still A Valuable Approach for Pediatric Patients? *Cancers*, 12(12), 3498. <https://doi.org/10.3390/cancers12123498>
87. Inaba, H., & Pui, C.-H. (2010). Glucocorticoid use in acute lymphoblastic leukemia: Comparison of prednisone and dexamethasone. *The Lancet Oncology*, 11(11), 1096–1106. [https://doi.org/10.1016/S1470-2045\(10\)70114-5](https://doi.org/10.1016/S1470-2045(10)70114-5)
88. Juluri, K. R., Siu, C., & Cassaday, R. D. (2022). Asparaginase in the Treatment of Acute Lymphoblastic Leukemia in Adults: Current Evidence and Place in Therapy. *Blood and Lymphatic Cancer: Targets and Therapy*, 12, 55–79. <https://doi.org/10.2147/BLCTT.S342052>
89. Kothari, A., Hittelman, W. N., & Chambers, T. C. (2016). Cell cycle-dependent mechanisms underlie vincristine-induced death of primary acute lymphoblastic leukemia cells. *Cancer Research*, 76(12), 3553–3561. <https://doi.org/10.1158/0008-5472.CAN-15-2104>
90. Ronghe, M., Burke, G. A., Lowis, S. P., et al. (2001). Remission induction therapy for childhood acute lymphoblastic leukaemia: Clinical and cellular pharmacology of vincristine, corticosteroids, L-asparaginase and anthracyclines. *Cancer Treatment Reviews*, 27(6), 327–337. <https://doi.org/10.1053/ctrv.2001.0243>
91. Cronstein, B. N. (1997). THE MECHANISM OF ACTION OF METHOTREXATE. *Rheumatic Disease Clinics of North America*, 23(4), 739–755. [https://doi.org/10.1016/S0889-857X\(05\)70358-6](https://doi.org/10.1016/S0889-857X(05)70358-6)
92. Lim, S. Z., & Chua, E. W. (2018). Revisiting the Role of Thiopurines in Inflammatory Bowel Disease Through Pharmacogenomics and Use of Novel Methods for Therapeutic Drug Monitoring. *Frontiers in Pharmacology*, 9. <https://doi.org/10.3389/fphar.2018.01107>
93. Peters, C., Schrappe, M., von Stackelberg, A., et al. (2015). Stem-Cell Transplantation in Children With Acute Lymphoblastic Leukemia: A Prospective International Multicenter Trial Comparing Sibling Donors With Matched Unrelated Donors—The ALL-SCT-BFM-2003 Trial. *Journal of Clinical Oncology*, 33(11), 1265–1274. <https://doi.org/10.1200/JCO.2014.58.9747>
94. *Chemotherapy for Acute Lymphocytic Leukemia*. (n.d.). Retrieved October 31, 2025, from <https://www.cancer.org/cancer/types/acute-lymphocytic-leukemia/treating/chemotherapy.html>
95. *Long term side effects of treatment for acute lymphoblastic leukaemia (ALL)*. (n.d.). Retrieved October 31, 2025, from <https://www.cancerresearchuk.org/about-cancer/acute-lymphoblastic-leukaemia-all/treatment/side-effects/long-term>

96. Kantarjian, H., Aldoss, I., & Jabbour, E. (2025). Management of Adult Acute Lymphoblastic Leukemia: A Review. *JAMA Oncology*, 11(7), 771–778. <https://doi.org/10.1001/jamaoncol.2025.0613>
97. *Drugs Approved for Childhood Cancers—NCI*. (2016, August 31). [cgvArticle]. (nciglobal,ncienterprise). <https://www.cancer.gov/about-cancer/treatment/drugs/childhood-cancer-fda-approved-drugs>
98. Yu, G., Chen, F., Yin, C., et al. (2017). Upfront treatment with the first and second-generation tyrosine kinase inhibitors in Ph-positive acute lymphoblastic leukemia. *Oncotarget*, 8(63), 107022–107032. <https://doi.org/10.18632/oncotarget.22206>
99. Hoffman, L., & Gore, L. (2014). Blinatumomab, a Bi-Specific Anti-CD19/CD3 BiTE® Antibody for the Treatment of Acute Lymphoblastic Leukemia: Perspectives and Current Pediatric Applications. *Frontiers in Oncology*, 4. <https://doi.org/10.3389/fonc.2014.00063>
100. Freyer, C. W. (2018). Tisagenlecleucel: The First CAR on the Highway to Remission for Acute Lymphoblastic Leukemia. *Journal of the Advanced Practitioner in Oncology*, 9(5), 537–544.
101. Maude, S. L., Frey, N., Shaw, P. A., et al. (2014). Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia. *New England Journal of Medicine*, 371(16), 1507–1517. <https://doi.org/10.1056/NEJMoa1407222>
102. Cox, A. D., Fesik, S. W., Kimmelman, A. C., et al. (2014). Drugging the undruggable Ras: Mission possible? *Nature Reviews. Drug Discovery*, 13(11), 828–851. <https://doi.org/10.1038/nrd4389>
103. Robinson, D. A., Tanaka, N., Fecce De La Cruz, F., et al. (2024). Sotorasib Is a Pan- RAS G12C Inhibitor Capable of Driving Clinical Response in NRAS G12C Cancers. *Cancer Discovery*, 14(5), 727–736. <https://doi.org/10.1158/2159-8290.CD-23-1138>
104. Bahar, M. E., Kim, H. J., & Kim, D. R. (2023). Targeting the RAS/RAF/MAPK pathway for cancer therapy: From mechanism to clinical studies. *Signal Transduction and Targeted Therapy*, 8(1), 455. <https://doi.org/10.1038/s41392-023-01705-z>
105. Brock, E. J., Ji, K., Reiners, J. J., et al. (2016). How to Target Activated Ras Proteins: Direct Inhibition vs. Induced Mislocalization. *Mini Reviews in Medicinal Chemistry*, 16(5), 358–369. <https://doi.org/10.2174/1389557515666151001154002>
106. Liu, Z., Hu, M., Yang, Y., et al. (2022). An overview of PROTACs: A promising drug discovery paradigm. *Molecular Biomedicine*, 3(1), 46. <https://doi.org/10.1186/s43556-022-00112-0>
107. *MedChemExpress: Master of Bioactive Molecules | Inhibitors, Screening Libraries & Proteins*. (n.d.). MedchemExpress.Com. Retrieved March 12, 2026, from <https://www.medchemexpress.com/>
108. Paulsson, K., Horvat, A., Strömbeck, B., et al. (2008). Mutations of FLT3, NRAS, KRAS, and PTPN11 are frequent and possibly mutually exclusive in high hyperdiploid childhood acute lymphoblastic leukemia. *Genes, Chromosomes and Cancer*, 47(1), 26–33. <https://doi.org/10.1002/gcc.20502>
109. Chen, H., Smaill, J. B., Liu, T., et al. (2020). Small-Molecule Inhibitors Directly Targeting KRAS as Anticancer Therapeutics. *Journal of Medicinal Chemistry*, 63(23), 14404–14424. <https://doi.org/10.1021/acs.jmedchem.0c01312>
110. Welsch, M. E., Kaplan, A., Chambers, J. M., et al. (2017). Multivalent Small-Molecule Pan-RAS Inhibitors. *Cell*, 168(5), 878–889.e29. <https://doi.org/10.1016/j.cell.2017.02.006>
111. Kim, D., Herdeis, L., Rudolph, D., et al. (2023). Pan-KRAS inhibitor disables oncogenic signalling and tumour growth. *Nature*, 619(7968), 160–166. <https://doi.org/10.1038/s41586-023-06123-3>
112. Cregg, J., Edwards, A. V., Chang, S., et al. (2025). Discovery of Daraxonrasib (RMC-6236), a Potent and Orally Bioavailable RAS(ON) Multi-selective, Noncovalent Tri-complex Inhibitor for the Treatment of Patients with Multiple RAS-Addicted Cancers. *Journal of Medicinal Chemistry*, 68(6), 6064–6083. <https://doi.org/10.1021/acs.jmedchem.4c02314>
113. Wolpin, B. M., Park, W., Garrido-Laguna, I., et al. (2026). Daraxonrasib in Previously Treated Advanced RAS-Mutated Pancreatic Cancer. *New England Journal of Medicine*, 394(18), 1790–1802. <https://doi.org/10.1056/NEJMoa2505783>

114. Frost, A., O'Connor, S., & Ciulli, A. (2026). Allosteric PROTACs: Expanding the Horizon of Targeted Protein Degradation. *Journal of the American Chemical Society*, 148(3), 2823–2837. <https://doi.org/10.1021/jacs.5c14840>
115. Röth, S., Fulcher, L. J., & Sapkota, G. P. (2019). Advances in targeted degradation of endogenous proteins. *Cellular and Molecular Life Sciences*, 76(14), 2761–2777. <https://doi.org/10.1007/s00018-019-03112-6>
116. Kargbo, R. B. (2020). PROTAC-Mediated Degradation of KRAS Protein for Anticancer Therapeutics. *ACS Medicinal Chemistry Letters*, 11(1), 5–6. <https://doi.org/10.1021/acsmchemlett.9b00584>
117. Cieślak, M., & Słowianek, M. (2023). Cereblon-Recruiting PROTACs: Will New Drugs Have to Face Old Challenges? *Pharmaceutics*, 15(3), 812. <https://doi.org/10.3390/pharmaceutics15030812>
118. Luo, X., Archibeque, I., Dellamaggiore, K., et al. (2022). Profiling of diverse tumor types establishes the broad utility of VHL-based ProTaCs and triages candidate ubiquitin ligases. *iScience*, 25(3), 103985. <https://doi.org/10.1016/j.isci.2022.103985>
119. UK, C. org. (n.d.). *Probe ACB13*. Chemical Probes Portal. Retrieved May 11, 2026, from <https://www.chemicalprobes.org/acbi3?>
120. Manabe, T., Bergo, H. C., Li, Q., et al. (2026). Pan-RAF inhibitor exarafenib targets BRAF class II/III NSCLC and reveals ARAF-KSR1 resistance and combination strategies. *Nature Communications*, 17(1), 2484. <https://doi.org/10.1038/s41467-026-69216-3>
121. Feichtenschlager, V., Zheng, Y. J., Qu, T., et al. (2025). Suppression of NRAS-mutant melanoma growth with NRAS-targeting Antisense Oligonucleotide treatment reveals therapeutically relevant kinase co-dependencies. *Communications Medicine*, 5(1), 216. <https://doi.org/10.1038/s43856-025-00932-5>
122. Muñoz-Maldonado, C., Zimmer, Y., & Medová, M. (2019). A Comparative Analysis of Individual RAS Mutations in Cancer Biology. *Frontiers in Oncology*, 9. <https://doi.org/10.3389/fonc.2019.01088>

# Addendum

## Risk analysis

Working in any laboratory requires personal safety measures. In all circumstances, a lab coat, gloves and safety goggles were mandatory to wear, while eating and drinking was prohibited. Since this work involved working with human cell lines and animal derived cells, all work on cells was performed in a cell culture room. Access to this room was only permitted after following a detailed biosafety training and a supervised tour in the room. This biosafety program covered lab safety protocols, emergency exits and handling hazardous waste. Besides following the mandatory protocols, it was important to conduct a detailed assessment of all used chemicals in each experiment, prior to every assay. In both the wet lab and the cell culture rooms, spill kits were present, containing absorbing blankets and cleaning materials.

All cells, including the Ba/F3 cells and all human cells lines, were manipulated under the laminar flow. When entering the cell culture room with a personal badge, new gloves and a personal lab coat were put on. This lab coat never left the cell culture room. To conduct the transductions, immobilized Ecopac viral particles were used. Although this virus should not be able to infect any humans, the transductions were conducted in a separate viral tissue culture room. Additional measures were a green apron on top of your personal lab coat and the use of two pairs of gloves. Everything that was touched during the transductions was put in a plastic bag and never left the laminar flow before the bag was sealed. All waste that came in contact with viral particles was autoclaved. All other materials that came in contact with biological active waste, were put in yellow bins and got disposed, according to the KU Leuven rules. Before and after each use of the laminar flow, everything was disinfected with 70% ethanol.

Prior to the start of all experiments, a risk assessment of chemical hazards was conducted. During Western Blot analysis, Methanol was used to activate the membrane. Methanol is toxic when breathing in and can cause organ damage. Methanol was always used under extraction and disposed in a category 3 waste bin (non-halogenated organic fluids). To produce TBS-T to wash the membranes during WB analysis, TWEEN 20 and HCl were used. TWEEN 20 is not categorized as a hazardous fluid, but also needs to be disposed in category 3 waste bins. HCl is a rapid vaporizing fluid, which can irritate the lungs and eyes, and can cause skin burns. It should be disposed in category 1 waste bins (acidic inorganic fluids). Plasmid purification with the CompactPrep Plasmid Midi Kit (Qiagen), required the use of ethanol. Ethanol is non-hazardous in concentrations below 5000 mg/kg, but requires special care since it is highly flammable. Ethanol was disposed in category 3 waste bins. All other used chemicals did not have special requirements regarding their disposal.

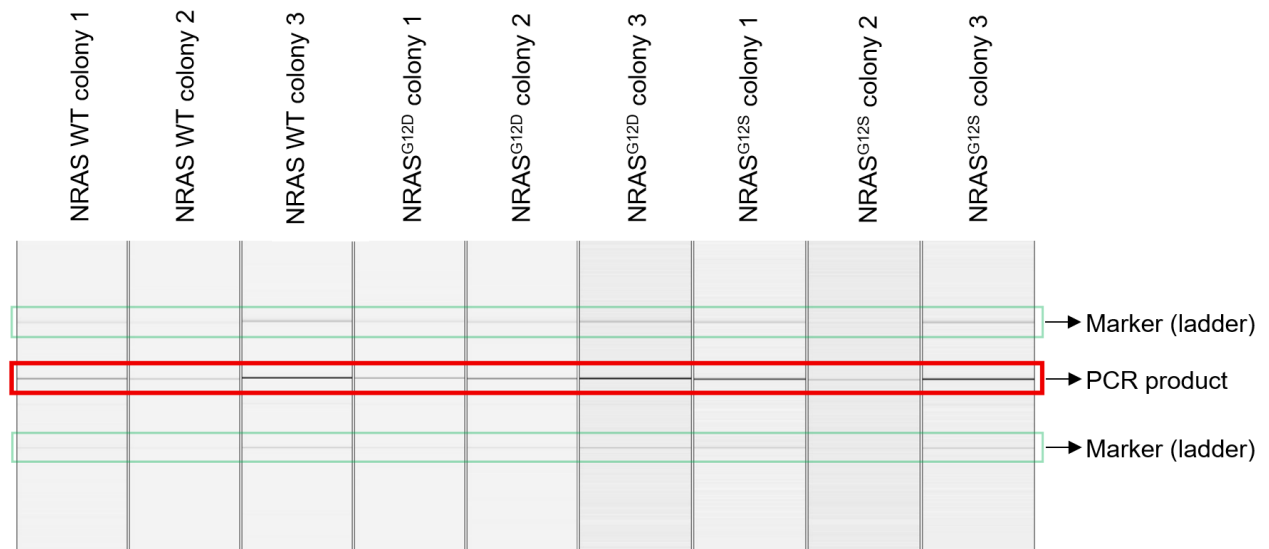
## Supplemental and Extended Data

Table S1: primers used for Q5 site-directed mutagenesis. Red: non-complementary NT compared to original sequence inserted in MSCV plasmid. Bold: mutated codon.  $T_a$  = annealing temperature.  $T_m$  = melting temperature. Primers were designed with NEBaseChanger™ tool.

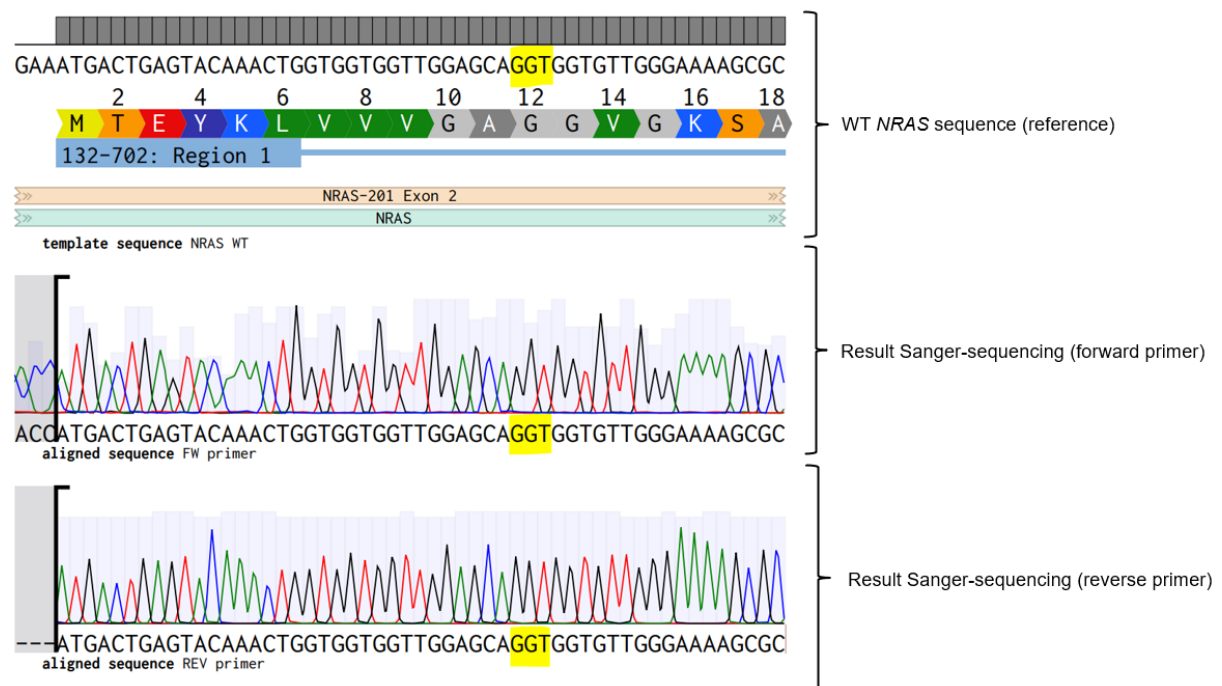
|                                                                    |                                           |                          |                          |
|--------------------------------------------------------------------|-------------------------------------------|--------------------------|--------------------------|
| KRAS WT (based on KRAS <sup>G12V</sup> MSCV plasmid):              |                                           |                          |                          |
| fwd                                                                | AGTTGGAGCT <b>GT</b> TGGCGTAGGCA          | $T_m = 75^\circ\text{C}$ | $T_a = 72^\circ\text{C}$ |
| rev                                                                | ACCACAAGTTTATATTCAGTCATGGTGGCAG           | $T_m = 71^\circ\text{C}$ | $T_a = 72^\circ\text{C}$ |
| KRAS <sup>A146T</sup> (based on WT KRAS MSCV plasmid):             |                                           |                          |                          |
| fwd                                                                | TGAAACATCA <b>CAA</b> AGACAAGACAGGGTG     | $T_m = 68^\circ\text{C}$ | $T_a = 69^\circ\text{C}$ |
| rev                                                                | ATAAAAGGAATTCCATAACTTCTTGCTAAGTCCTGAGCC   | $T_m = 73^\circ\text{C}$ | $T_a = 69^\circ\text{C}$ |
| NRAS WT (based on NRAS <sup>G12C</sup> MSCV plasmid):              |                                           |                          |                          |
| fwd                                                                | GGTTGGAGCA <b>GT</b> TGGTGTGGGA           | $T_m = 71^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| rev                                                                | ACCACCAGTTTGTACTCAGTCATGG                 | $T_m = 69^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| NRAS <sup>G12D</sup> (based on NRAS <sup>G12C</sup> MSCV plasmid): |                                           |                          |                          |
| fwd                                                                | GGTTGGAGCA <b>GAT</b> TGGTGTGGGAAAAGCGCAC | $T_m = 69^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| rev                                                                | ACCACCAGTTTGTACTCAGTCATGGTGG              | $T_m = 73^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| NRAS <sup>G12S</sup> (based on NRAS <sup>G12C</sup> MSCV plasmid): |                                           |                          |                          |
| fwd                                                                | GGTTGGAGCA <b>AGT</b> TGGTGTGGGAAA        | $T_m = 69^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| rev                                                                | ACCACCAGTTTGTACTCAGTCATGG                 | $T_m = 69^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| NRAS <sup>Q61H</sup> (based on WT NRAS MSCV plasmid):              |                                           |                          |                          |
| fwd                                                                | TACAGCTGGAC <b>AT</b> GAAGAGTACAGTGCC     | $T_m = 69^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |
| rev                                                                | TCCAGTATGTCCAACAAACAGGTTTCACCATCTAT       | $T_m = 72^\circ\text{C}$ | $T_a = 70^\circ\text{C}$ |

Table S2: primers used to amplify mutated DNA after the site-directed mutagenesis to check if desired mutations are present.  $T_m$  = melting temperature.

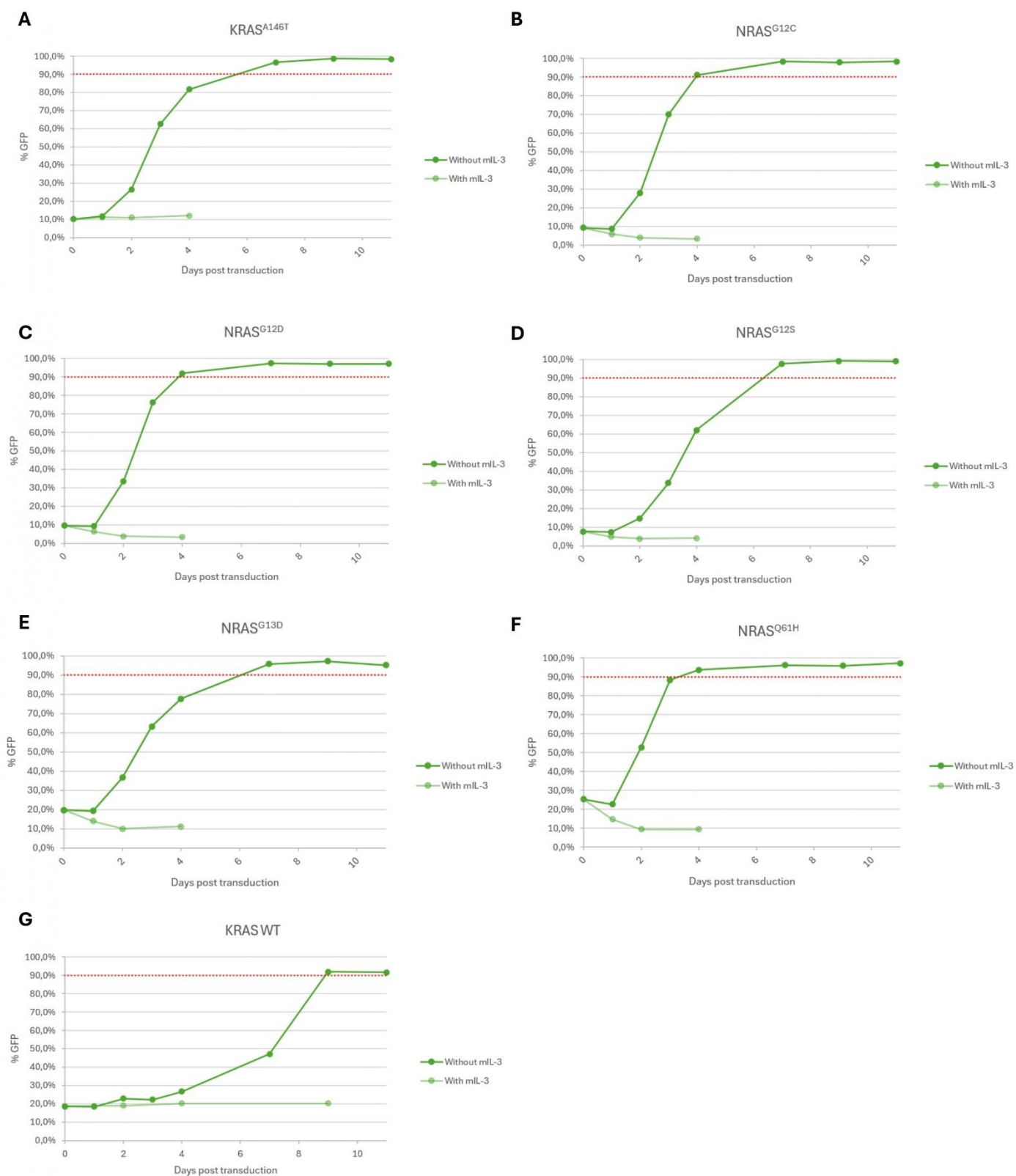
|                            |                       |                          |  |
|----------------------------|-----------------------|--------------------------|--|
| MSCV-GFP universal primer: |                       |                          |  |
| fwd                        | CCTCGATCCTCCCTTTATCC  | $T_m = 54^\circ\text{C}$ |  |
| rev                        | ACGCACACCGGCCTTATTCC  | $T_m = 60^\circ\text{C}$ |  |
| KRAS specific primer:      |                       |                          |  |
| rev                        | TACACAAAGAAAGCCCTCCCC | $T_m = 57^\circ\text{C}$ |  |
| NRAS specific primer:      |                       |                          |  |
| rev                        | TACTCGCTTAATCTGCTCCCT | $T_m = 55^\circ\text{C}$ |  |



Extended Data Figure 1: Desired length of RAS sequence is present in all plasmids. Automated gel electrophoresis performed with QIAxcel, after PCR on the MSCV plasmid that is site-directed mutated. An MSCV universal forward primer was used and a KRAS/NRAS specific reverse primer. Confirmation that the targeted DNA of interest is amplified and present in the *E. coli*. The results are displayed for all three picked colonies per induced mutation. Here an example for NRAS WT, NRAS<sup>G12D</sup> and NRAS<sup>G12S</sup>. The same was done for the other mutations that were induced later.

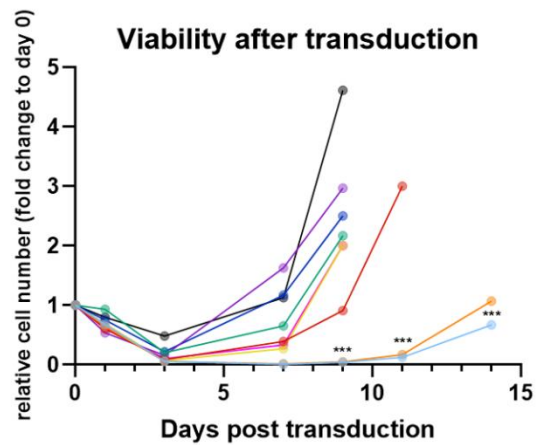


Extended Data Figure 2: Conformation of desired RAS sequence. NRAS WT as an example. Both the result from the forward primer and the reverse primer are aligned against the WT NRAS sequence. Only the results for the first 17 codons are shown. Mutagenesis was based on the NRAS<sup>G12C</sup> plasmid, which has a TGT sequence at codon 12, forming a cysteine. The Sanger-sequencing confirmed a GGT sequence in codon 12, forming a glycine as in WT NRAS. The sequencing results are a 100% match with the reference sequence, indicating the mutagenesis worked (mismatches would have been highlighted red).



Extended Data Figure 3: Evolution of %GFP over time after the transduction, showing that all cell lines, mutant and WT, fully established after 4 – 9 days **A:** KRAS<sup>A146T</sup> **B:** NRAS<sup>G12C</sup> **C:** NRAS<sup>G12D</sup> **D:** NRAS<sup>G12S</sup> **E:** NRAS<sup>G13D</sup> **F:** NRAS<sup>Q61H</sup> **G:** KRAS WT

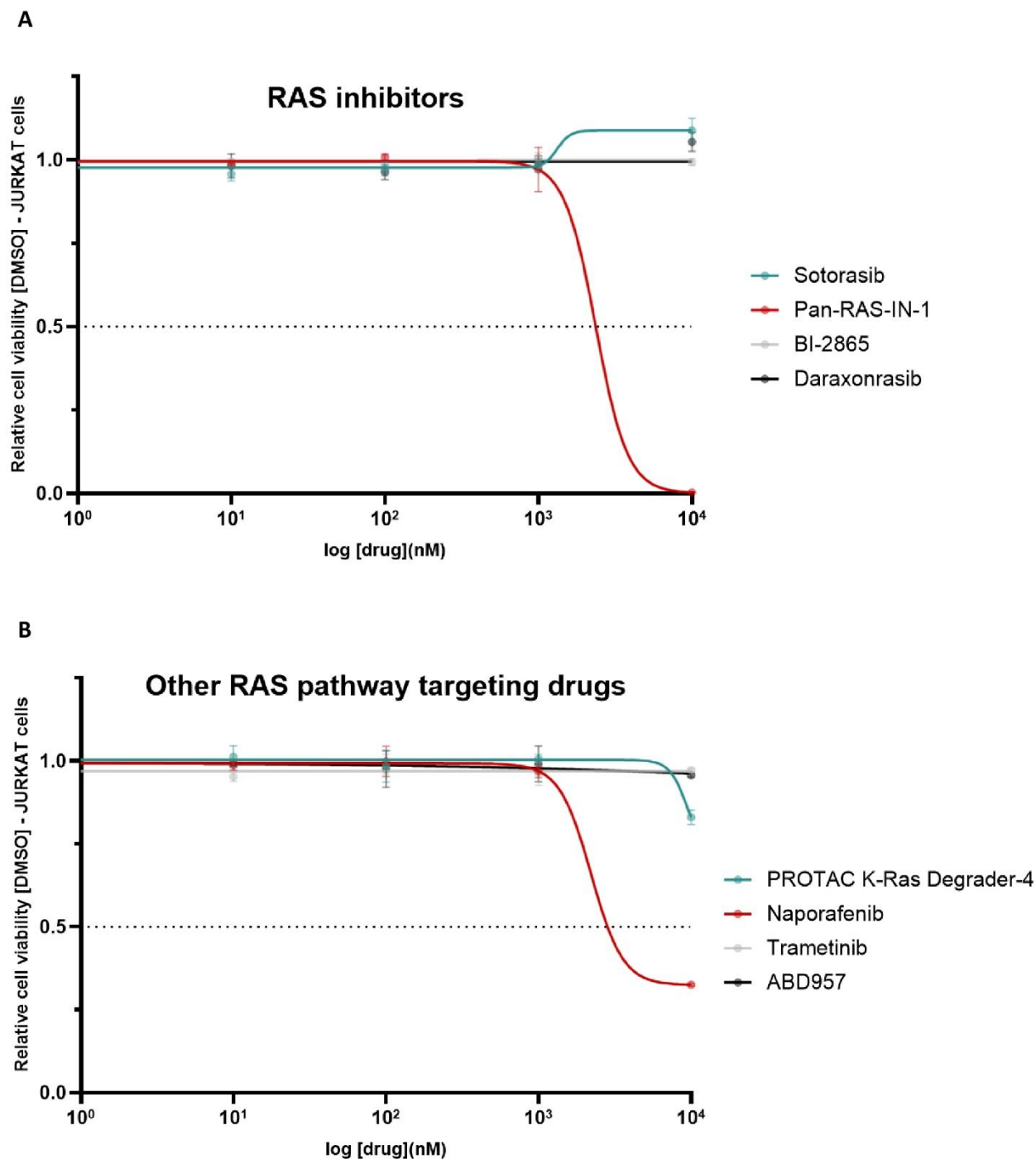
**A**



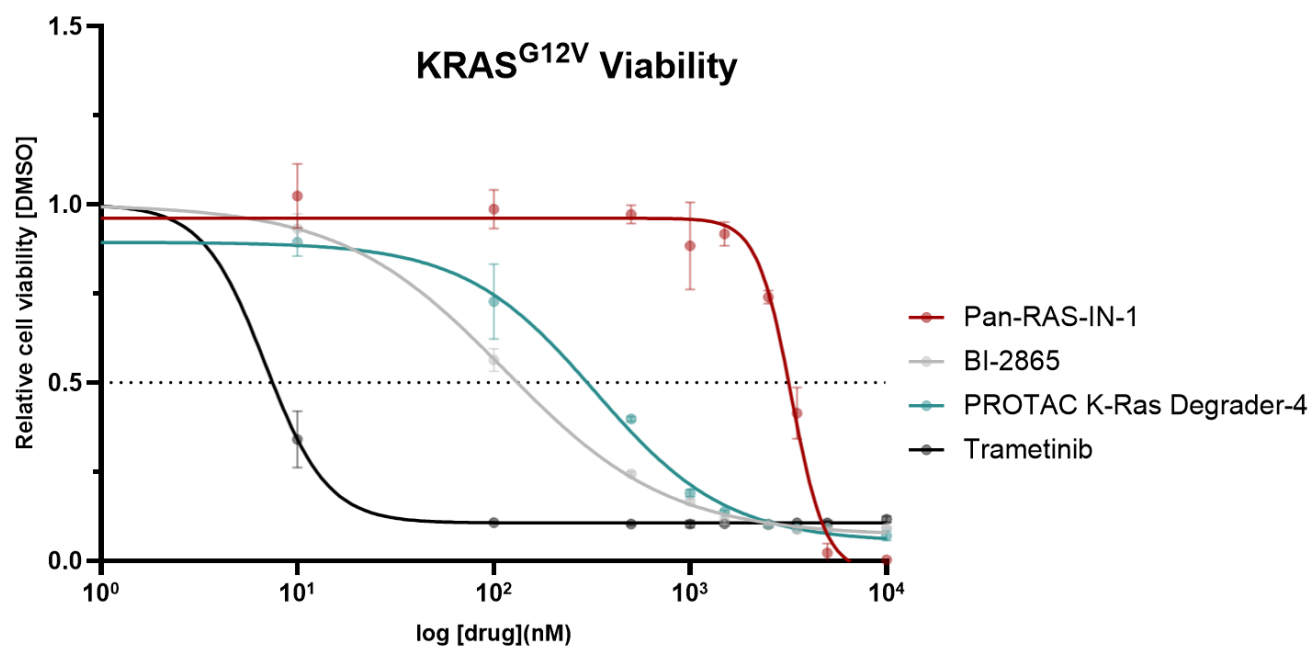
**B**

|             |          |      |      |         |         |
|-------------|----------|------|------|---------|---------|
| day = 0:    |          |      |      |         |         |
| contrast    | estimate | SE   | df   | t.ratio | p.value |
| Mutant - WT | 0.00     | 41.3 | 31.6 | 0.000   | 1.0000  |
| day = 1:    |          |      |      |         |         |
| contrast    | estimate | SE   | df   | t.ratio | p.value |
| Mutant - WT | 4.44     | 41.3 | 31.6 | 0.107   | 0.9151  |
| day = 3:    |          |      |      |         |         |
| contrast    | estimate | SE   | df   | t.ratio | p.value |
| Mutant - WT | 12.87    | 41.3 | 31.6 | 0.312   | 0.7573  |
| day = 7:    |          |      |      |         |         |
| contrast    | estimate | SE   | df   | t.ratio | p.value |
| Mutant - WT | 78.46    | 41.3 | 31.6 | 1.901   | 0.0665  |
| day = 9:    |          |      |      |         |         |
| contrast    | estimate | SE   | df   | t.ratio | p.value |
| Mutant - WT | 240.73   | 41.3 | 31.6 | 5.831   | <0.0001 |
| day = 11:   |          |      |      |         |         |
| contrast    | estimate | SE   | df   | t.ratio | p.value |
| Mutant - WT | 309.31   | 62.0 | 37.0 | 4.993   | <0.0001 |

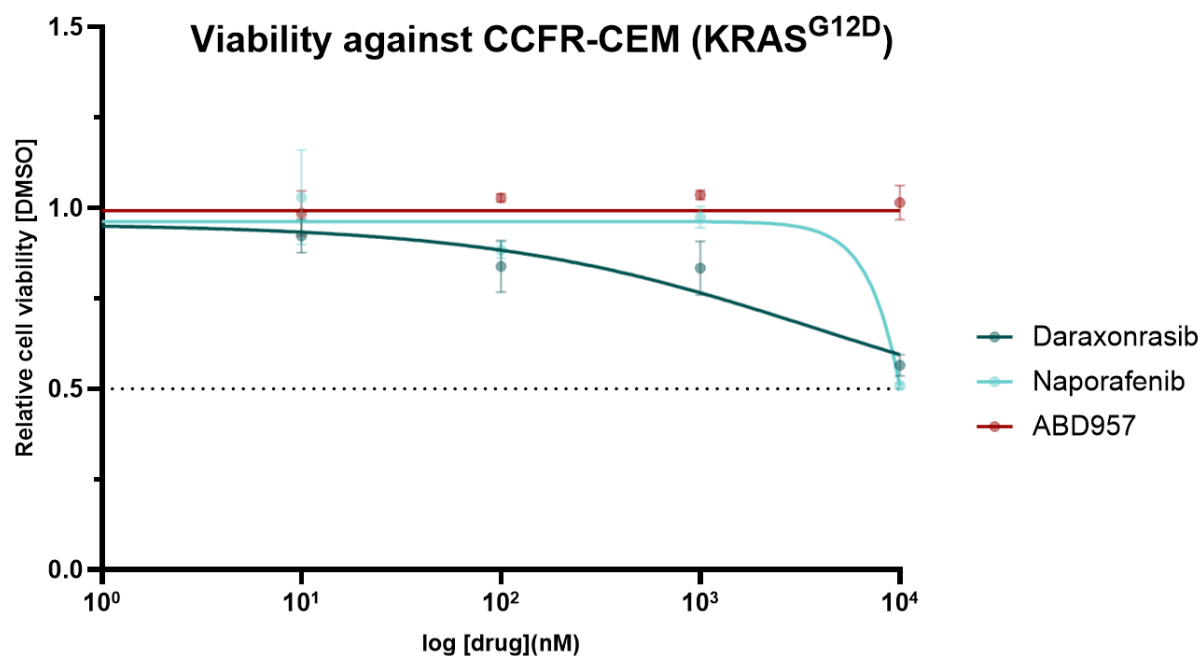
Extended Data Figure 4: **A:** Viability after transduction. Data is normalized for the total number of cells per mutation at day 0. For significance testing, all mutations were grouped and the two WT transductions were grouped. A linear mixed model was produced in R and via Post-hoc comparisons, p-values were obtained. p-value < 0.05 \*, < 0.01 \*\*, < 0.001 \*\*\*. **B:** p-values of a post-hoc comparison (emmeans) between all mutant cell lines and both WT on a linear mixed model.



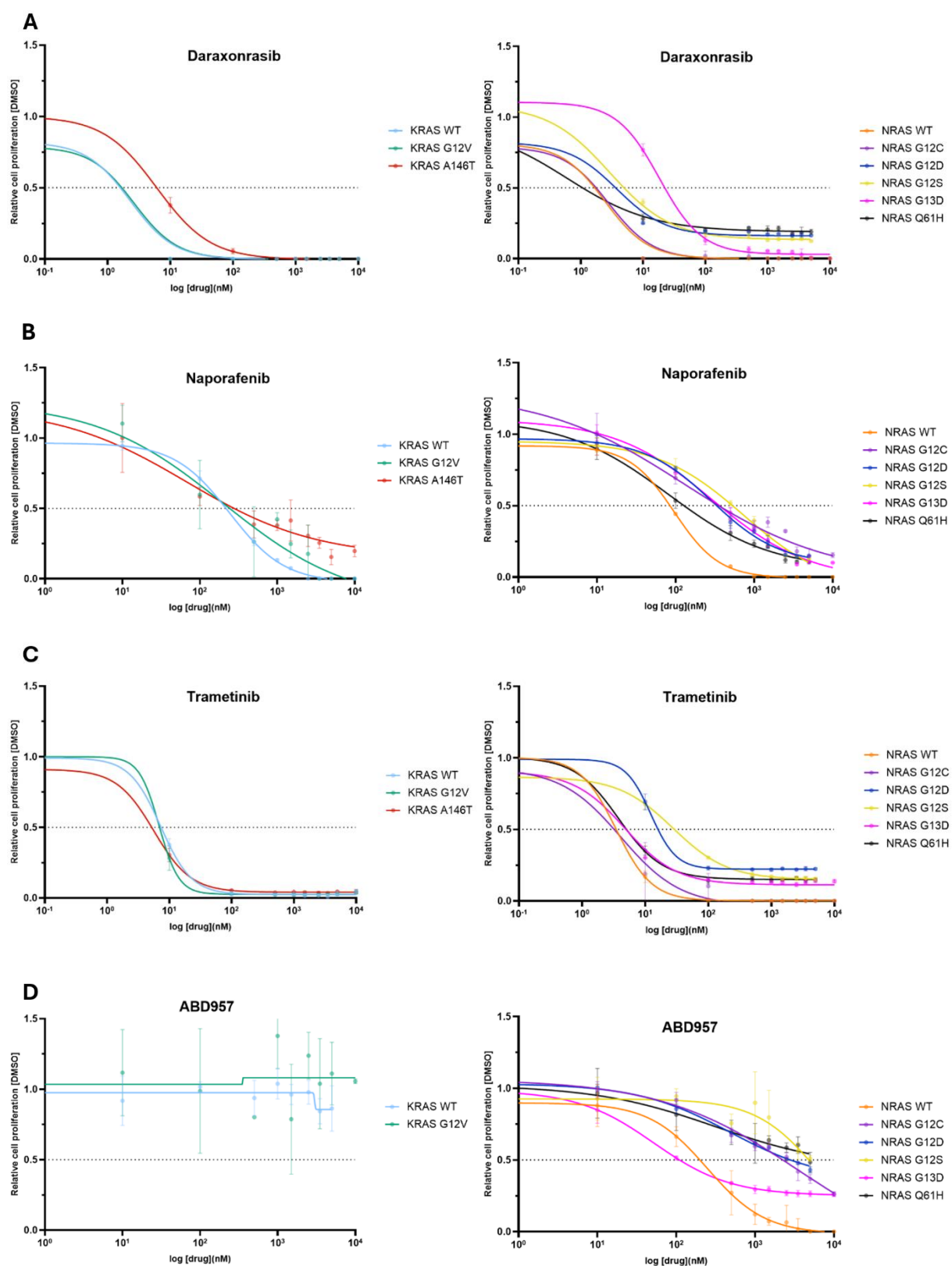
Extended Data Figure 5: Dose-response curve for all drugs against JURKAT cells. Cells were incubated for 24h with different concentrations of all drugs. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. **A:** result for all direct RAS inhibitors. **B:** result for the other drugs.



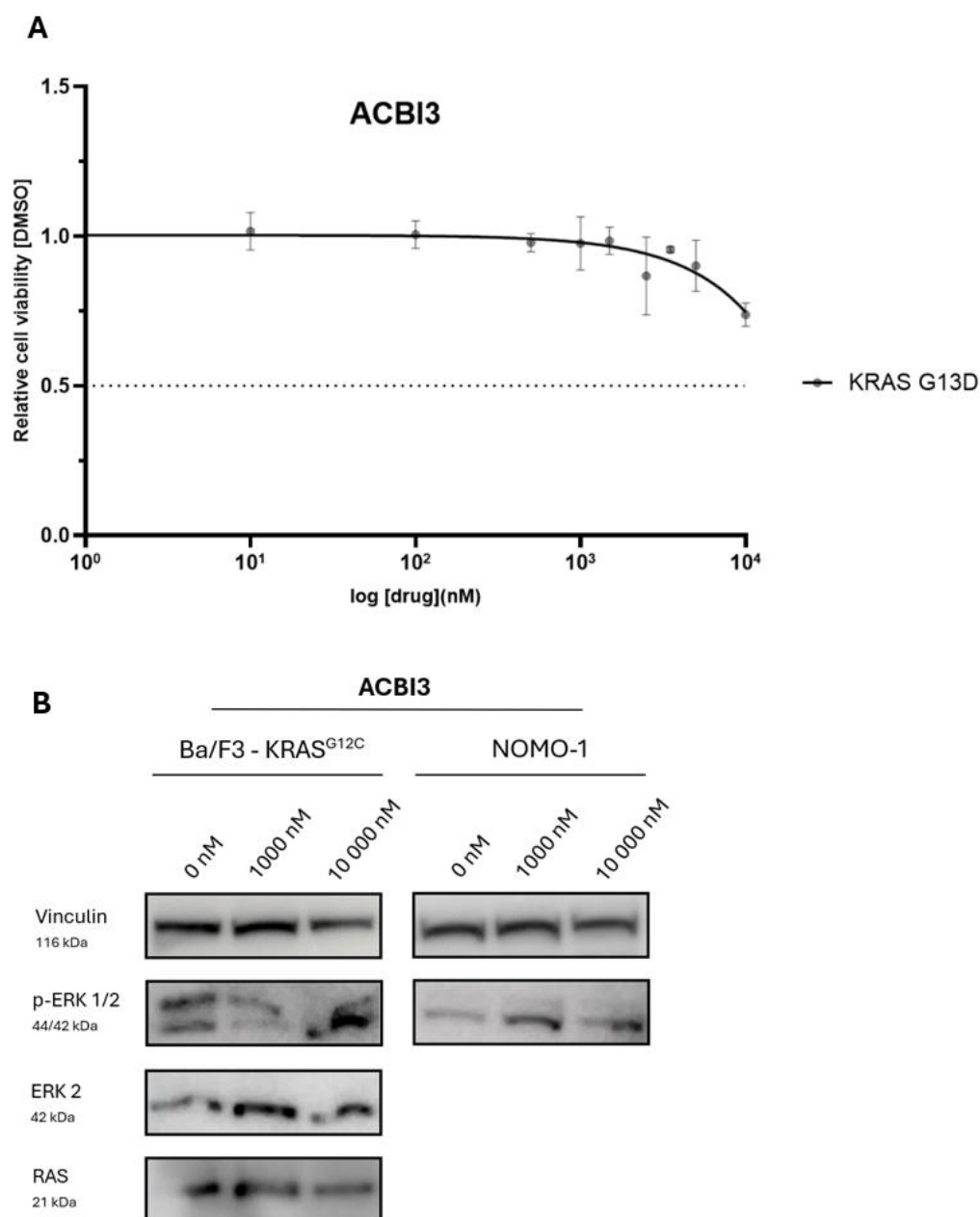
Extended Data Figure 6: Dose response curve of KRAS<sup>G12V</sup> against some of the drugs. Same data as used in the other dose response curves, but without subtracting day 0, leaving here the raw absorbance measurements. Whilst all drugs result in a 10% viability, indicating cytostatic effects, Pan-RAS-IN-1 drops the viability to 0.1%, indicating cytotoxicity.



Extended Data Figure 7: Dose-response curve for Daraxonrasib, Naporafenib and ABD957 against human cell line CCFR-CEM. Cells were incubated for 24h with different concentrations of the drugs. Viability after 24h was calculated by measuring absorbance levels. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO.



*Extended Data Figure 8: Dose-response curve for **A:** Daraxonrasib. **B:** Naporafenib. **C:** Trametinib. **D:** ABD957. Cells were incubated for 48h with different concentrations of the drugs. Proliferation after 48h was measured by subtracting the absorbance at day 0. Since drugs were resolved in DMSO, every well was normalized for the highest total volume of DMSO. Left: results for all KRAS tests. Right: results for all NRAS tests.*



**Extended Data Figure 9: A:** Dose-response curve of KRAS<sup>G13D</sup> overexpressing Ba/F3 cells for after ACBI3 treatment. Note that this is a Cas9 overexpressing Ba/F3 cell system and not a regular Ba/F3 cell (see discussion). **B:** WB analysis results for Cas9 expressing Ba/F3 – KRAS<sup>G12C</sup> cells and NOMO-1 cell lines. ACBI3 is not able to get an effect on proliferation, nor on p-ERK or RAS levels.

**AFDELING**  
Straat nr bus 0000  
3000 LEUVEN, BELGIË  
tel. + 32 16 00 00 00  
fax + 32 16 00 00 00  
[www.kuleuven.be](http://www.kuleuven.be)

