WHITE PAPER

Exploring current and future approaches for targeting KRAS

RAS genes are retrovirus-associated DNA sequences present in all mammalian cells where they express RAS proteins. These proteins are important cytosolic signal transducers for pathways that regulate numerous intracellular functions including cell growth, proliferation, differentiation, and survival.

RAS protein mutations have been found to drive more than 30% of all human cancers across a wide range of cancer types. The KRAS isoform accounts for 85% of all RAS mutations. Thus, intense research initiatives are underway globally to develop effective therapeutic approaches for KRAS-driven cancers. [NCI, Prior].

Key features

- Structure and function of the RAS protein superfamily
- Role and consequences of KRAS gene activation
- KRAS mutations in human cancer
- Targeting KRAS in cancer therapies
- Current challenges in developing KRAS-targeted therapeutics

The RAS superfamily

History

RAS proteins and their encoding genes were first identified in the 1960s – 1970s in retrovirus research in rodents and found to be responsible for the oncogenic properties of RNA tumor viruses. The first human RAS genes/proteins were identified in 1982 and found to be mutationally activated in human cancer cell lines. That marked the first confirmation of RAS oncogenes and led to an explosion of interest in the RAS superfamily that has continued for decades. Today, more than 150 human RAS proteins have been identified. Three RAS genes are responsible for the expression of all RAS proteins: HRAS, KRAS, and NRAS. [Wennerberg et al, Cox & Der, Prior].

Function

The RAS protein superfamily consists of small guanosine triphosphatase enzymes (GTPases) that act as molecular on/off switches for various cellular functions in response to extracellular signals. RAS proteins are responsible for modulating a diverse range of complex cellular processes. The superfamily has five main families that are distinguished by similarities in genetic sequences and cellular functions [Wennerberg et al]. These include:

- Ras: Regulation of signaling networks for cell proliferation, differentiation, and survival.
- Rho: Regulation of signaling networks for actin organization, cell cycle progression, and gene expression.
- Rab: Regulation of intracellular vesicular transport and movement of proteins between different organelles of the endocytic and secretory pathways.

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- Ran: Regulation of nuclear import/export of materials as well as DNA replication, mitotic spindle assembly, and nuclear envelope assembly.
- Arf: Regulation of intracellular vesicular transport, endocytosis, and exocytosis, including vesicle formation and structure.

Structure

RAS proteins are G proteins (membrane-associated glycoproteins) that contain four domains with varying degrees of homology across families. They all share a set of conserved GDP/GTP- binding elements at the N-terminus that make up their G domain. The C-terminus is highly variable within the superfamily which leads to most of their structural and functional differences. RAS proteins also contain two "switch" regions that determine how they react to their substrate and their environment. Conformational changes in the switch regions allow RAS proteins to have many potential configurations [Wennerberg et al].

The diverse functions and mechanisms of RAS superfamily proteins are based on variations in their structures, different post-translational modifications, and the variety of other proteins that regulate RAS protein function.

Guanine nucleotide exchange factors (GEFs) promote formation of the proteins' GTP-bound active form, and GTPase activating proteins (GAPs) promote formation of their inactive GDP-bound form. RAS proteins respond to extracellular signals and relay information to the cell nucleus. They transduce signals relating to cellular differentiation, growth, chemotaxis, and apoptosis. This wide functional influence is possible partially because RAS proteins affect the cytoskeleton, changing the potential shape, migration, and adhesion of cells. Different RAS superfamily proteins gain unique functions through post-transcriptional modifications. These modifications are usually lipids. Lipid modifications help RAS proteins associate with membranes and downstream effectors to regulate cell differentiation, proliferation, and survival [Wennerberg et al].

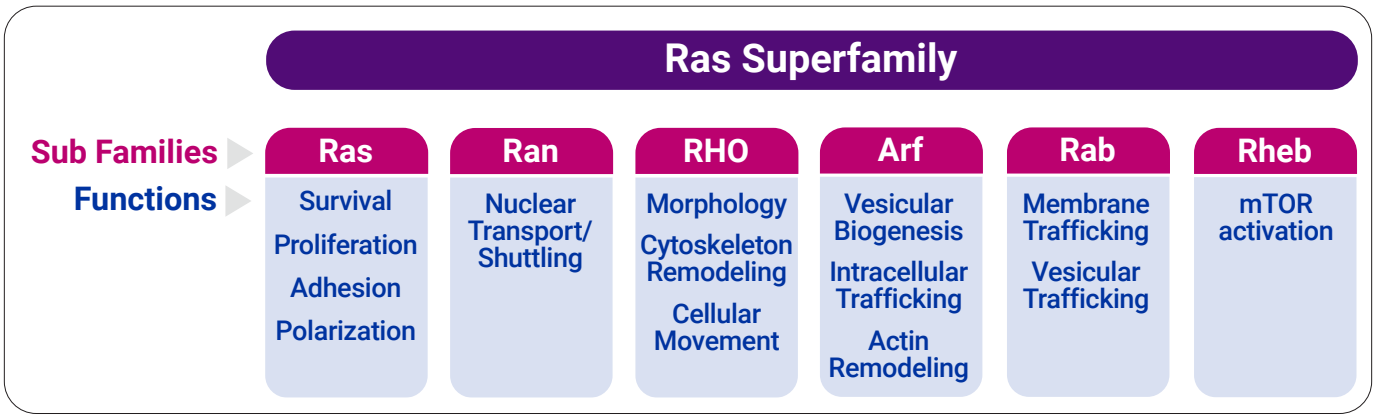

Figure 1: Ras families and main cellular functions

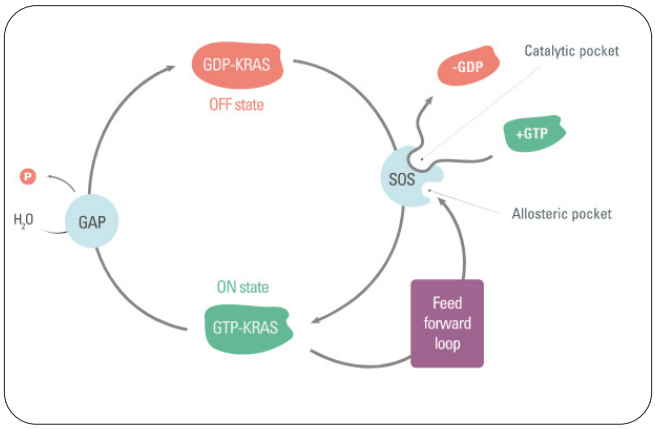

Figure 2: The cycle of KRAS protein from "on state" (KRAS WT-GTP/SOS1) to an "off state" (KRAS WT-GDP/GAP)

In humans, three RAS genes encode the highly homologous RAS proteins HRAS, KRAS, and NRAS. KRAS has become a key therapeutic target in cancer research. This targeting effort is the result of new research on KRAS protein structure, genomics, and its role in human oncogenesis.

KRAS genomics, activation, and function

KRAS genomics

The human KRAS gene is a homolog to the Kirsten rat sarcoma 2 viral oncogene. Genomic analysis of KRAS reveals a complex interplay of genes, isoforms, and proteins. The human genome contains two copies of the KRAS gene: KRAS1 and KRAS2. KRAS2 is a functional proto-oncogene located on chromosome 12 at position 11.1-12.1. KRAS1 is a pseudogene on chromosome 6 that derived from KRAS2.

The KRAS2 gene has six exons. Alternating splicing of exon 4 produces two mRNA forms denoted as 4A and 4B, which in turn give rise to protein isoforms KRAS4A and KRAS4B (also known as isoforms 2A and 2B, respectively). These isoforms differ mainly in their hypervariable region at residues 167-189, with other dissimilarities at residues 151, 153, 165, and 166. The KRAS4B variant predominates in human cells and is commonly known as KRAS. [Pantsar, Liu et al, Jancik et al].

Human KRAS gene expression is regulated at two points:

- During initiation of transcription by the binding of proteins to its promoter
- During transcriptional elongation by microRNAs affecting KRAS mRNA stability

KRAS gene mutations occur most frequently at codons 12, 13, and 61. KRAS mutations are present in many types of human tumors including pancreatic carcinomas (>80%), colon carcinomas (40–50%), lung carcinomas (30–50%), and others. Tumor promotion by the KRAS oncogene can result from overexpression of the mutant KRAS allele or deletion of the wild-type KRAS allele.

Wild-type KRAS activation and function

KRAS protein is synthesized in the cytosol in the inactive form and anchored to the cell membrane at the hypervariable region (HVR) of the protein's C-terminus. This localization of KRAS proteins to the cell membrane is influenced by post-translational lipid modifications of the protein, the HVR's electrostatic nature based on the protein isoform, and the composition of the cell membrane based on cell type.

KRAS can be activated by extracellular signals as well as signals from subcellular structures such as organelles. In a normal physiological setting, wild-type KRAS is predominantly in the inactive GDP-bound state. Signals prompt KRAS to disengage from GDP and bind to GTP, transforming KRAS to its active state. This conversion of KRAS from its inactive to active form is mediated by GEFs, and conversion back to the inactive form is mediated by GAPs.

Activated KRAS is then able to bind and activate effector proteins, such as RAF-kinases, PI3K, and RalGDS, that promote downstream cascades of signaling pathways such as the MAPK, PI3K, and Ral-GEFs pathways. These signaling pathways promote cell cycle progression through cell replication, growth, differentiation, and chemotaxis.

Abnormally high levels of wild-type KRAS cause a slowing of cell replication and growth, and an increased pace of apoptosis. Such high KRAS levels can be induced by cellular stress, certain types of radiation, chemical signals, and other prompts. The resulting KRAS reaction is thus considered a defensive mechanism to counteract the effects of over-activation of KRAS. Wild-type KRAS may also protect against mutant KRAS over-activation by dimerizing with mutant KRAS protein.

Oncogenic KRAS activation and function

Oncogenic human KRAS is usually activated due to one of the following:

- Loss of the wild-type gene
- Increased number of copies of the oncogene
- Loss of the p53 tumor suppressor gene

An activated oncogenic KRAS gene results in a mutant KRAS protein that remains in its active GTP-bound state for an abnormally long period of time due to a greatly decreased rate of GTP hydrolysis. When a KRAS protein is stuck in the "ON" position, downstream effector pathways also remain active and cell proliferation rates remain high. Other effector pathways that remain active include those that promote tumor development processes such as mitogenesis, cell migration, tumor invasion, and angiogenesis.

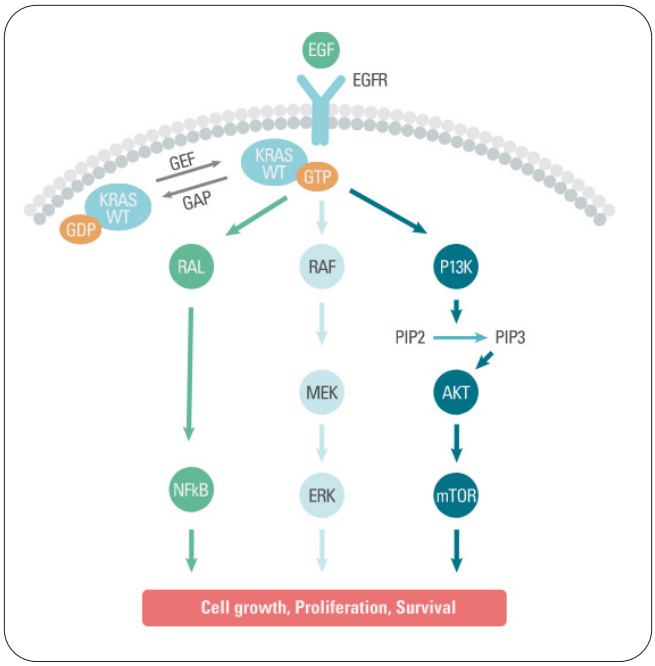

Figure 3: Signaling pathway of KRAS protein activates downstream effector molecules from the PI3K/AKT/mTOR and BRAF/MEK/ERK pathways

Cells that contain mutated KRAS also increase their rate of secretion of chemokines, cytokines, and other compounds that induce remodeling of surrounding cells. The remodeling itself promotes further activation of mutant KRAS and the potential for development of novel mutations in those cells.

The tumor microenvironment is influenced by mutated KRAS in ways that contribute to cancer malignancy. Tumor cells expressing oncogenic KRAS secrete paracrine signals that remodel surrounding stroma cells including fibroblasts, inflammatory cells, innate and adaptive immune cells, and myeloid cells. The paracrine signals secreted into the tumor microenvironment include chemokines, cytokines, and growth factors that direct the stromal cell reprogramming. [Liu et al] Some examples of these paracrine signals and remodeling pathways are:

- Interleukin (IL)-6 and IL-8 maintain stromal inflammatory phenotypes in pancreatic and lung cancer, respectively.
- Granulocyte-macrophage colony stimulating factor (GM-CSF) promotes infiltration of myeloid-derived suppressor cells and inhibits anti-tumor immunity.
- MYC and RAS together program an immune-suppressive stroma involving CCL9-mediated recruitment of macrophages, programmed death-ligand 1 (PD-L1)-dependent expulsion of T and B cells, and IL-23-led exclusion of T, B, and natural killer immune cells.

There are many possible KRAS mutations that cause oncogenic activity, and the same mutation can cause different characteristics in different cancer types. KRAS mutations often make tumors resistant to conventional therapies, including chemotherapy and radiotherapy [Jancik et al].

KRAS mutations in human cancers

RAS mutations occur in 30% of human cancers. Of the three closely related RAS isoforms (HRAS, KRAS, and NRAS), KRAS is the most oncogenic making up 86% of mutated RAS proteins observed in cancer. Mutant KRAS has been identified in specific cancers at the following rates [Liu et al]:

- Pancreas 90%
- Colon 30–50%
- Small intestine 35%
- Biliary tract 26%
- Lung 19%
- Endometrium 17%
- Cervix 8%
- Urinary tract 5%
- Skin (melanoma) 1%

KRAS gene mutations occur most frequently at codons 12, 13, or Q61, with position 12 mutations dominating. The general effects of mutations at these locations are:

- G12 mutations interfere with GAP binding and GTP hydrolysis.
- G13 mutations decrease GAP binding and hydrolysis through steric interference.
- Q61 mutations prevent GTP catalysis by destabilizing catalytic transition states.

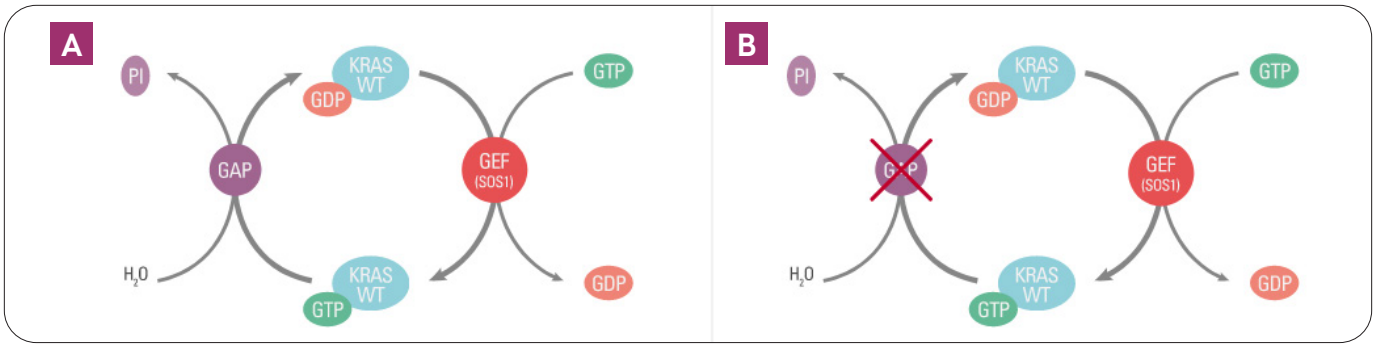

Figure 4: Activity states of KRAS protein. (A) KRAS protein cycle between an active (KRAS WT-GTP/SOS1) and inactive state (KRAS WT-GDP/GAP) (B) Mutational change of KRAS leads to permanent "on" status with KRAS G12C-GTP/SOS1 binding

Specific mutations for pancreatic, colon, and lung cancer are:

- Multiple concurrent KRAS mutations frequently occur in pancreatic cancer. Pancreatic adenocarcinomas predominantly harbor a mutant KRAS gene with a guanine to thymine transversion at codon 12. [Jancik *et al*]
- The mutant KRAS gene associated with colon cancer appears most often at codons 12 and 13. In colorectal cancer, the primary KRAS protein substitution is glycene to aspartic acid. In primary metastatic carcinoma, a substitution of glycene to valine based on a codon 12 mutation is frequently observed. [Jancik et al]
- KRAS mutations occur predominantly at codon 12, occasionally at codon 13, and rarely at codon 61. KRAS mutations predominantly occur in lung adenocarcinomas (the most common histological subtype of non-small cell lung cancer [NSCLC]) at frequencies from 16% to 40%. KRAS mutations have also been observed at a low frequency in squamous cell carcinoma (another subtype of NSCLC), but never in NSCLC). [Westcott & To]

KRAS mutation detection

The determination of a patient's KRAS mutational status using a sample of their tumor is an important clinical tool for physicians treating patients with colorectal or NSCLC. Potential diagnostic techniques must be able to distinguish the range of variants at the appropriate codons and consistently provide the required sensitivity levels for the target alleles. These criteria are important because of the heterogeneity of tumor cell density and genetic makeup.

Most KRAS mutation detection methods use a combination of PCR amplification followed by a detection method to identify wild-type and mutant sequences. Amplification methods can include digital, molecular, or allele-specific PCR. A few commonly used detection methods are:

- Direct sequencing or pyrosequencing of nucleic acids
- Amplification refractory mutation system (ARMS) assay
- Luminex xMap assay
- Single-strand conformational polymorphism analysis
- High-resolution melt curve analysis
- Peptide/nucleic acid probe

Anderson (2011) provides a concise evaluation of several detection techniques including their sensitivity and key features.

Another evaluation (Matsunaga et al) of two sequencing and two assay techniques for detection of KRAS mutations in metastatic colorectal cancer revealed the following capture rates:

- Direct sequencing 93.2%
- Scorpion ARMS assays 97.3%
- Pyrosequencing 95.9%
- Luminex xMap assays 94.5%

Targeting KRAS signaling in cancer therapy

The variety of KRAS mutations that cause human cancers and the persistent difficulty in identifying new mutations are the drivers for multiple types of KRAS therapies being developed or that are already in use.

Immunotherapy is a promising KRAS treatment strategy. Although its application to KRAS mutations is in its infancy, some exciting information has already been uncovered. Immunotherapy has been shown to increase the survival rate of patients who have cancers containing many KRAS variants, including those with poorer prognoses. For example, it has improved the prognoses in lung cancer patients who have multiple KRAS mutations, even though concurrent KRAS mutations are an indicator of poor prognosis. This may indicate that immunotherapies targeting KRAS could have applications in intractable cancers which commonly have multiple mutations, such as pancreatic cancer. Immunotherapy also appears to be an effective choice of treatment for KRAS-positive patients in varying treatment-line settings and has yielded better outcomes than conventional chemotherapy. [Amanam et al].

A specific codon 12 mutation, G12C, has attracted significant interest for mutant KRAS targeting because the mutation creates a unique topographical feature on the KRAS protein. Glycine mutating to cysteine is a common human KRAS mutation. Since that mutation results in the only cysteine residue on the protein's surface, oncogenic KRAS can be targeted while excluding wild-type KRAS. An inhibitor molecule bound to the exposed cysteine residue inhibits the activity of mutant KRAS. This approach may promote tumor regression, although current studies sometimes see varying amounts of success between *in vitro* and *in vivo* tests [Janes et al]. G12D is the most frequent KRAS mutation in cancer and has similar molecular dynamics to wild-type KRAS. Thus, researchers are also looking for inhibitors for the G12D mutation [Liu et al, Pantsar].

Inhibition of KRAS effects through other members of its pathways is also emerging as a promising therapeutic avenue. Antisense nucleotides have shown promise in targeting mutant KRAS mRNA to prevent it from being transcribed into protein. When mutant KRAS proteins have already been generated, interference with post-transcriptional lipid modifications prevents them from binding to their respective cell membranes. Targeting KRAS indirectly can prevent the mutant proteins from causing harm, even if it is not possible to turn off their production. Gene silencing of mutant KRAS has proven difficult due to challenges in accurate transgene delivery [Tomasini et al].

New endeavors and challenges

One of the most common KRAS gene mutations is called KRAS G12C. This mutation occurs in most solid tumor types where it is a major driver of tumor growth. Recent years have seen a dramatic spike in the amount of research being conducted into development of G12C inhibitors. Companies leading the way in this important research and development include Amgen, Mirati, and Eli Lilly.

Amgen's LUMAKRAS™ (sotorasib) is a small molecule KRAS G12C inhibitor developed for the treatment of advanced NSCLC, advanced colorectal cancer, and other advanced solid tumors. LUMAKRAS recently received FDA approval for use by adults living with NSCLC that has spread to other parts of the body or cannot be removed by surgery, and whose tumor has an abnormal KRAS G12C gene. Amgen developed its G12C inhibitor to be irreversibly binding in order to provide long-lasting inhibition of G12C tumor promotion. Amgen hopes to also receive FDA approval for use of LUMAKRAS in colon cancer and other solid tumor cancers. [Amgen]

Mirati Therapeutics and the M.D. Anderson Cancer Center have entered into a collaboration that will expand the evaluation of Mirati's investigational small molecules targeting two of the most frequent KRAS mutations in cancer. Their potent, selective KRAS inhibitors are Adagrasib (MRTX849) which is a G12C mutation inhibitor, and MRTX1133 which is a G12D mutation inhibitor. Both molecules are being evaluated for use as monotherapies and in combination with other agents. [BioSpace]

Eli Lilly has developed a new G12C inhibitor that is more potent than their previous investigative molecules but with less toxicity effects. Lilly's LY3537982 has a lower IC50 than both Amgen's and Mirati's inhibitors, and its initial test scores were more potent than the other options. In animal models, Lilly saw outcomes ranging from complete tumor regression to significant tumor growth inhibition. Lilly's inhibitor is scheduled for clinical trials in late 2021. [Taylor]

Revvity facilitates these breakthroughs in mutant KRAS targeting by providing commercially available, no-wash, and ready-to-use homogenous KRAS binding assay kits for KRAS/SOS1 inhibition analysis. Available in both AlphaLISA™ and HTRF™ technologies, these kits streamline workflow by offering fully validated kits to identify novel KRAS inhibitors in a no-wash format with no optimization necessary, and each kit comes with recombinant proteins, detection reagents, and assay buffers.

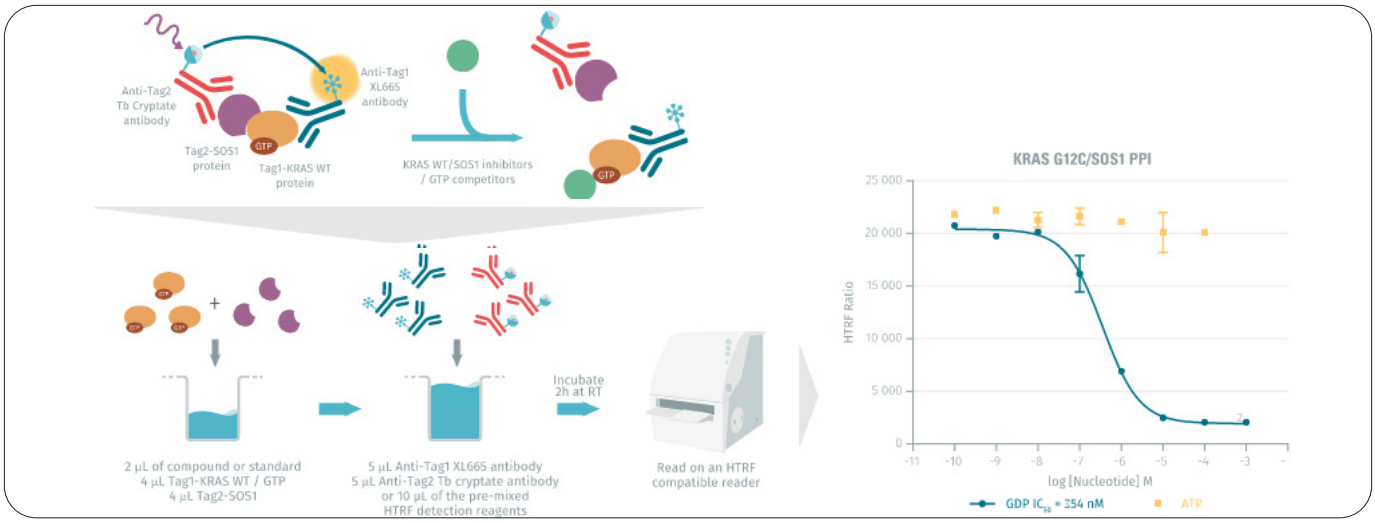

Figure 5: HTRF method used to measure KRAS G12C/SOS1 protein-protein interaction

Revvity also provides a comprehensive portfolio of cell-based assays (Alpha *Surefire Ultra* and HTRF) that help characterize downstream KRAS signaling, including the MAPK and PI3K pathways. These cellular signaling assays enable discrimination between the cellular action of the hit compounds and evaluation of their efficacy in modulating the downstream KRAS pathways. These kits represent efficient tools for the estimation of a therapeutic profile for KRAS-targeting compounds in various cancer cell lines.

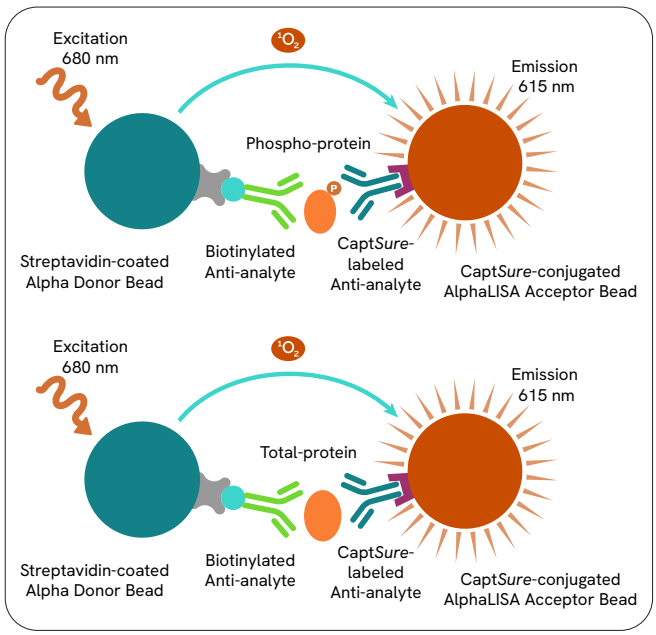

Figure 6: Principle of the Phospho/Total protein AlphaLISA *Surefire Ultra* assays

Sources

National Cancer Institute. https://www.cancer.gov/research/key-initiatives/ras/about

Prior et al. 2021. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3354961/

Wennerberg et al. 2005. https://doi.org/10.1242/jcs.01660

Cox and Der. 2010. https://www.tandfonline.com/doi/full/10.4161/sgtp.1.1.12178

Pantsar 2020. https://doi.org/10.1016/j.csbj.2019.12.004

Liu et al. 2019. https://doi.org/10.1016/j.apsb.2019.03.002

Jancik et al. 2010. https://doi.org/10.1155/2010/150960

Westcott and To. 2013. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3845615/

Anderson. 2011. https://doi.org/10.1586/erm.11.42https://www.medscape.com/viewarticle/746638_1

Matsunaga et al. 2016. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4906624/

Amanam et al. 2020. https://jtd.amegroups.com/article/view/38482/html

Janes et al. 2018. https://pubmed.ncbi.nlm.nih.gov/29373830/

Tomasini et al. 2016. https://theoncologist.onlinelibrary.wiley.com/doi/full/10.1634/theoncologist.2015-0084

Amgen. 2020. https://www.amgen.com/newsroom/press-releases/2021/05/fda-approves-lumakras-sotorasib-the-first-and-only-targeted-treatment-for-patients-with-kras-g12cmutated-locally-advanced-or-metastatic-nonsmall-cell-lung-cancer

BioSpace. 2021. https://www.biospace.com/article/releases/md-anderson-and-mirati-therapeutics-announce-kras-strategic-research-and-development-collaboration-in-solid-tumors/

Taylor. 2021. https://www.fiercebiotech.com/biotech/lilly-rejoins-kras-race-swipe-at-amgen-and-mirati-plans-2021-clinical-trial

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