Investigation of Oncogenic RAS and Endoplasmic Reticulum-Mitochondria Calcium Flux and their Relationship in the Context of Tumorigenesis

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Abstract

Intracellular calcium as a signaling molecule is a pervasive feature of cellular pathways, especially those that manage internal homeostasis and transitions through the cell cycle, so much so that regulated, responsive calcium flux between the endoplasmic reticulum (ER) and the mitochondria has been suggested to play a major role in cancer development. Another factor commonly implicated in tumorigenesis is RAS, an oncogene that controls signaling for many pathways that are also regulated by calcium. While both calcium and oncogenic RAS signaling are implicated in cancer development, possible links between them have yet to be determined. The identification of these links will provide a further understanding of the mechanisms of cancer development and potential therapeutic targets for cancer treatment.

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Cancer cells hold several characteristics in common, including increased proliferation and decreased sensitivity to apoptosis. Several causes of these changes in oncogenic transformation have been suggested; one commonly implicated cause is the deregulation of calcium signaling, because of its centrality to the control of cell cycle transitions and metabolism. Oncogenes like RAS, a monomeric GTPase that plays an integral role in signal transduction in the cell, are also closely linked to tumorigenesis and the deregulation of these foundational cell pathways. The processes associated with both calcium flux and RAS are complex and nuanced, producing a high likelihood for overlap between the two pathways. Identification of a connection between deregulated calcium flux and oncogenic RAS could provide a therapeutic target for the treatment of cancers with RAS mutations. This paper will explore the existing body of knowledge regarding calcium flux and RAS in both healthy and cancerous cells with the goal of identifying connections between the two processes.

Calcium Flux Between the Endoplasmic Reticulum and the Mitochondria

Cancer research has called special attention to calcium flux in the cell and the various roles it plays, but the impacts of calcium are fundamental to cell life even apart from tumorigenesis. The first part of this paper will describe the current body of literature regarding calcium flux and its role in regulating cellular processes.

Nearly all the organelles in the cell are involved in calcium signaling, but the two most important organelles for calcium homeostasis are the mitochondria and the ER, with the mitochondria being the main site of ATP synthesis in a healthy cell. The tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) are housed by the mitochondria and perform

oxidative phosphorylation for the efficient production of ATP. Functional mitochondria are also central to most major cell events, including cell cycle progression and apoptosis. The mitochondria are not fixed organelles; rather, they make up a network that is continually remodeled by fusion and fission. This dynamic situation is made possible by the cytoskeleton, especially the proteins dynein and kinesin moving with mitochondria on microtubules and actin filaments. The protein phosphatidylinositol 3-kinase (PI_3K) is also implicated in this process (1). This association between mitochondria and the cytoskeleton means that any cytoskeletal construction and deconstruction (especially the remodeling that takes place during cell proliferation) has a dramatic impact not only on the movement of the mitochondria but also on its functions. As the main player in metabolism, the mitochondria adapt to the metabolic needs of the cell by responding to cytoskeletal changes.

The endoplasmic reticulum (ER) is also intimately involved in calcium signaling in the cell. The main roles of the ER are protein and lipid production and sorting, signal regulation, calcium storage, and transport within the cell. Like the mitochondria, the ER is not fixed but a dynamic collection of tubes heavily involved in vesicular transport and associated with the cytoskeleton with bidirectional regulation (2,3). The many functions of the ER provide it with the means to sense and respond to changes in the cell, which it does using calcium signals in a variety of ways.

These two organelles, the mitochondria and the ER, are closely linked. This connection allows for the fluctuation of signals between the two organelles, especially signals mediated by calcium. Calcium, a major second messenger in the cell, is stored in the ER and controls many of the processes in the mitochondria as well as throughout the cell. The mitochondria have a low affinity for the uptake of calcium, too low to allow for uptake from the low cytoplasmic

concentrations. Mitochondrial-associated membranes (MAMs), areas of very close association between the ER and the mitochondria, provide localized increases in cytoplasmic calcium concentration large enough to allow for mitochondrial uptake of the second messenger. Tethers connect the outer mitochondrial membrane to nearby ER tubules at a distance of between 9nm and 30nm depending on the ribosomal content of the ER (4); these distances have also been reported to be as low as or lower than 8.7+/-2.4nm (5). These tethers are clustered together to form very specific pockets of association. Separating the ER and the mitochondria by proteolysis at the MAMs has been demonstrated to completely halt calcium signals between the two organelles, whereas causing even closer association has dramatically improved the calcium signals transmitted to the point of promoting mitochondrial calcium overload (4). Thus, the specific width of the MAMs is integral in determining the quality of the calcium signal, the ratio between the localized concentration of calcium and the total calcium concentration in the cytoplasm (6).

Control of Calcium Flux at MAMs

Specific proteins and signals provide a means of control over calcium flux at MAMs. The number of proteins at one region of association has been estimated to reach one thousand (7), but this estimate likely includes impure and overlapping proteins. 115 unique proteins is a more accurate estimate of MAM protein content (8). This number includes proteins explicitly for use in calcium transfer as well as apoptosis regulators, linking proteins, and regulatory proteins for the mediation of autophagy, inflammation, apoptosis, metabolism, and many other cellular processes. The roles, regulations, and impacts of this collection of proteins have not been fully determined; even though a vast amount of knowledge about the MAMs has been gained in recent years, much remains to be learned. Many of these components are commonly altered in cancer

cells in a manner dependent on the type of cancer (9). The proteins at the MAMs compose a finely tuned mechanism of control, but very small changes can cause it to malfunction with far-reaching effects on the life of the cell.

While MAMs serve several purposes in the cell, their primary function is the mediation of calcium flux. Calcium as a second messenger controls exocrine and endocrine secretion, gluconeogenesis, embryo development and cell differentiation, transcription, nerve growth, migration, muscle contraction, and protein folding in the ER (10), as well as internal homeostatic processes like autophagy (11). Calcium signals specifically directed to the mitochondria act to control the TCA cycle, oxidative phosphorylation, and apoptosis. Dysregulated calcium flux has been linked to Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, ataxias, and autism (12), as well as every hallmark of cancer either directly or indirectly (13,14,15). Calcium can propagate signals within one cell, among a few neighboring cells, or even on a more global level across an entire organ. The regulation and control of calcium flux in the cell, especially between the ER and the mitochondria, is incredibly important for the maintenance of proper cellular function.

IP_3Rs: The Primary Calcium Release Channel

Calcium in the cell is mainly stored in the ER at a concentration of 100-500μM (16). The little calcium that does reside in the cytoplasm exists at a concentration of around 10-100nM (16) and is predominantly bound to buffers or other molecules. Because cytoplasmic calcium is not free, it travels slowly and works more efficiently as a local signal than across the whole cell (12). MAMs provide the perfect setting for calcium to work in this manner. The ER is home to a variety of calcium release channels, some of which work through passive leak while others utilize stimulation by a signal molecule (17). The most important of these channels for the

function of the MAMs is the inositol 1,4,5-trisphosphate receptor (IP_3R). The membrane-spanning section of this channel is near the C-terminus of the protein; the N-terminus of the protein is the site of signal reception (18). Each channel is a tetramer, composed of four identical proteins. IP_3Rs mainly reside in the membrane of the ER, with the majority of their protein mass on the cytoplasmic side of the membrane. The six transmembrane domains of each of the four subunits surround the ion channel and form a filter on the cytoplasmic end to allow for selectivity (12). Many factors influence the selectivity, opening, and closing of the channel, and most of these are still under active investigation. Three isoforms of IP_3R exist and play unique roles in mediating calcium release to regulate apoptosis, secretion, and metabolism with varying sensitivity to stimuli (19,20,21,22,23,24,25). One isoform is usually expressed more highly than the others in a manner dependent on cell type.

IP_3Rs in the ER membrane are localized to areas that require elevated calcium flux, such as the MAMs, but whether they are physically involved in the link between the ER and mitochondria at MAMs is uncertain. Some research demonstrates that the space between the organelles at MAMs is too small for the large cytoplasmic portion of IP_3Rs, so the channels are located on ER tubules very near to MAMs instead (5). This would allow for localized calcium flux, but the tethers that form MAMs would be formed independently of IP_3Rs (4). However, much of the literature points to a tethering system that does involve IP_3Rs (17); GRP75 is generally assumed to physically connect IP_3Rs to mitochondrial proteins in the MAM, as shown in Figure 1 (26). Because the proteins at the MAM are diverse and not fully characterized, further research is required to determine whether IP_3Rs play a role in tethering the ER to the mitochondria. Additionally, IP_3Rs are not fixed within the ER membrane. Rather, like the ER and mitochondria themselves, IP_3Rs move in association with the cytoskeleton through the lipid

bilayer of the ER, forming small clusters of around eight tetrameric channels as they travel. These clusters allow the effective release of calcium in a localized manner (24); calcium released from a cluster of IP_3Rs is called a calcium puff. The localization and mobility of IP_3Rs, in addition to the isoform composition, determine the target of a given release of calcium from the ER (17).

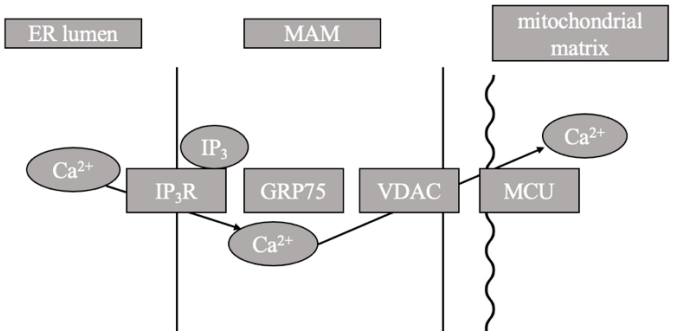

Figure 1. Simplified depiction of proteins at the MAM. *Calcium stored in the ER lumen is released through IP_3Rs into the MAM on stimulus from IP_3 or other signaling molecules. It then travels through the voltage dependent anion channel (VDAC) and finally the mitochondrial calcium uniporter (MCU) into the mitochondrial matrix where it signals for a variety of cell responses. These proteins are closely associated at the MAM to allow for successful uptake of calcium by low-affinity channels on the mitochondrial membrane.*

Regulation of IP_3Rs is mediated by a variety of signal molecules. The primary stimulus molecule is inositol 1,4,5-trisphosphate (IP_3), as implied by the name of the receptor. IP_3 is released by the action of phospholipase C (PLC), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) at the plasma membrane to form diacylglycerol and IP_3. IP_3Rs are also regulated by calcium itself, with low concentrations of calcium stimulating further calcium release and high concentrations inhibiting calcium release. ATP also regulates IP_3Rs (27), along with cAMP, hydrogen ions, NADH, the redox state of the cell (28,29), phosphorylation, ubiquitination, transglutaminase-mediated cross-linking of Gln-Lys residues, nitrosylation (12), proteolysis, and the Bcl-2 family of proteins (27). With the plethora of regulation demonstrated,

IP_3Rs function as a central control hub for every aspect of cell life and situates them at the head of a robust response system that allows the cell to determine its fate in response to any given situation. Because of the delicate position that IP_3Rs hold, however, dysregulation in any of these converging pathways can cause disease in the other pathways. This is commonly the source of the extensive list of pathologies connected to calcium flux.

To initiate a calcium signal, IP_3 binds to the IP_3 binding core on the cytoplasmic side of an IP_3R. Stimulation of the receptor requires four IP_3 molecules, one for each subunit in the tetramer. The phosphate molecules on the fourth and fifth carbons of IP_3 bind to Arg and Lys residues on the receptor (12). Calcium binding sites and sites for regulation by other molecules are less established, but they are known to be present. The binding of IP_3 or any other stimulatory molecule opens the channel, allowing calcium to flow out of the ER and into the cytoplasm.

Other Proteins Involved in ER-Mitochondria Calcium Flux

After calcium is released from the ER into the cytoplasm at a MAM, it travels through a voltage dependent anion channel (VDAC), an uptake channel on the outer mitochondrial membrane (OMM) as depicted in Figure 1 (16). Calcium then passes through the mitochondrial calcium uniporter (MCU) into the mitochondrial matrix, a low affinity process that requires the high localized calcium concentration provided by the MAM (16). Once in the mitochondrial matrix, calcium can carry out its intended purpose, mediating signals to bring about a variety of cellular events. Calcium acts as a main signal to determine cellular life or death; overload of calcium signals can cause the mitochondria to stimulate either metabolism or apoptosis. Other signals also contribute to the decision between life or death, including ATP levels, oxidative stress, and inorganic phosphate levels, as well as signals coming directly from the mitochondria

(16). Any of these signals, especially calcium flux, can be dysregulated in cancerous cells, and could potentially be the target of treatment to restore signaling to normal levels (13).

IP_3Rs are not the only players in cellular calcium homeostasis; proteins like the Bcl-2 family exert control over calcium flux and provide another layer of control over apoptosis versus cell survival. Each molecule in this family of proteins has a unique function, but many of them are suspected to regulate IP_3Rs for the indirect control of apoptosis. For example, Bcl-2 itself can prevent apoptosis by either impacting IP_3Rs to prevent sustained high levels of calcium release from the ER (30,31) or lowering ER calcium concentration by enabling passive leak of ER calcium without inciting a signal (32). Many other proteins in the Bcl-2 family bind to IP_3Rs and impact their stability and ability to allow for calcium flux (17). These proteins are commonly mutated in cancerous cells due to their centrality to processes that control cell fate.

Other Points of Control Over Calcium Flux

Another facet of calcium homeostasis is transport of the electrolyte into and out of the cell. The calcium concentration gradient from the ER to the cytoplasm must be maintained for functional signaling, so transport on this level is integral to calcium signaling. Several pathways allow for this transport to happen. Sodium/calcium exchange and plasma membrane calcium-ATPases (PMCAs) are designed to pump calcium out of the cell to keep cytoplasmic calcium levels low (13). Transient receptor potential (TRP) proteins act as channels to bring calcium into the cell, along with store operated calcium entry (SOCE), the primary method of cellular calcium intake. As calcium is released from the ER via IP_3Rs, luminal concentration decreases, activating stromal interaction molecule 1 (STIM1), which collects at the edge of the ER closest to the plasma membrane to work with ORAI-1, a channel in the plasma membrane that opens on stimulation to allow calcium into the cell (12). SOCE, specifically mediated by these two

molecules, is intimately tied to IP_3R-mediated calcium release (12, 24,33). All of these processes can be dysregulated in cancer at any of these points, as well as at many other protein steps that have been omitted from this summary (13).

After calcium is brought into the cytoplasm, it must be transported into the lumen of the ER for storage. The SERCA pump (sarco-endoplasmic reticulum calcium transport ATP-ase) mediates this transport, using ATP hydrolysis to pump calcium into the more highly concentrated ER lumen and further increase the concentration gradient (10). A number of SERCA pump isoforms exist; like isoforms of IP_3Rs, SERCA pump isoforms have different functions and operate in responses to different stimuli. Because SERCA pumps control levels of ER calcium intake, they also control the amount of calcium available for release from the ER, providing the cell with a point of control over calcium release. SERCA pumps are commonly downregulated in cancer cells (10,34,35), which decreases the likelihood of sustained high levels of calcium release to cause apoptosis while still allowing for metabolic signaling to progress as normal.

When these transport mechanisms are performing properly to maintain cell homeostasis, calcium can fulfill its role as a second messenger in nearly every cellular process. Most importantly for this discussion, calcium is intimately involved in the control of metabolism, mitotic division, and apoptosis. Calcium stimulates TCA cycle dehydrogenases in the mitochondria and works with asparagine/glutamine transporters to allow for healthy metabolism (36). Additionally, it acts as a regulator to allow the cell to progress through checkpoints in the cell cycle or to induce senescence (37). Calcium can also act as a signal for apoptosis at several points. These three pathways are commonly dysregulated in cancer, allowing cancer cells to increase metabolism for sustained high levels of proliferation while simultaneously avoiding apoptosis.

Oncogenic RAS and Cancer Development

Calcium signals are clearly key players in causing cells to become cancerous, but they are not the only factor involved. Oncogenes are another major cause of transformation. One of the most common oncogenes is RAS, a protein that becomes oncogenic on mutation. RAS is related to a vast number of pathways in the cell, and many of these connections are still unclear. However, many of the signaling pathways implicated in dysregulated RAS signaling are the same as those impacted by calcium flux, suggesting a potential connection between the two causes of transformation. This section will explore the basics of oncogenic RAS so that connections between aberrant RAS signals and calcium signaling can be identified.

RAS was originally discovered in retroviruses in the 1960s and 1970s as one of the first human oncogenes – genes with the power to transform a healthy cell into a cancer cell (38). The RAS gene codes for four proteins: HRAS, NRAS, KRAS-4A, and KRAS-4B. Thirty percent of all human cancers demonstrate a mutation in one of these proteins, and eighty six percent of those mutations are in the KRAS protein (39). Thus, while most initial studies focused on HRAS, studies involving KRAS are more relevant to the current search for a cure for cancer. It has been estimated that over two hundred sixty thousand copies of RAS exist in a single cell, with KRAS being the most abundant form among those copies. With an oncogenic mutation, this copy number increases, magnifying the oncogenic effect (40).

Overview of RAS Function

RAS is a monomeric GTPase, a protein that assists in signal transduction across the plasma membrane (41). To activate RAS, a growth factor must first bind a receptor on the outside of the cell, often a receptor tyrosine kinase (RTK) or a G-protein coupled receptor

(GPCR), although cytokine receptors and extracellular matrix receptors have also been shown to receive growth factors that stimulate RAS (38). As a GTPase, RAS is active when bound to GTP, but inactive when bound to GDP. Receptor activation by an extracellular growth factor triggers the binding of growth factor receptor-bound protein 2 (GRB2) to the receptor itself on one end and on the other end to a guanine nucleotide exchange factor (GEF), giving the GEF protein an affinity for RAS (42). The GEF proteins, most often a protein called son of sevenless (SOS), facilitate the exchange of GDP on an inactive RAS protein for GTP (see Figure 2), activating RAS by a conformational change in the switch I and II domains that gives RAS a greater affinity for its effectors and allows for the activation of pathways downstream of RAS (43). Binding to GRB2 on growth factor reception activates GEFs to perform this function. This is one side of the GTPase cycle that RAS undergoes.

To inactivate RAS and carry out the other side of the cycle, GTPase activating proteins (GAPs) bind to RAS to facilitate the dephosphorylation of bound GTP and form bound GDP (see Figure 2). This reverses the conformational change in RAS, lowering its affinity for its effectors and silencing pathways downstream of this signal. One of the most common GAPs is neurofibromin 1 (NF1) (38). While GEFs and GAPs play a major role in cycling RAS to allow for signal transduction, some intrinsic nucleotide exchange and hydrolysis does occur without the assistance of these enzymes, allowing RAS to slowly cycle between active and inactive states even without another enzyme (44).

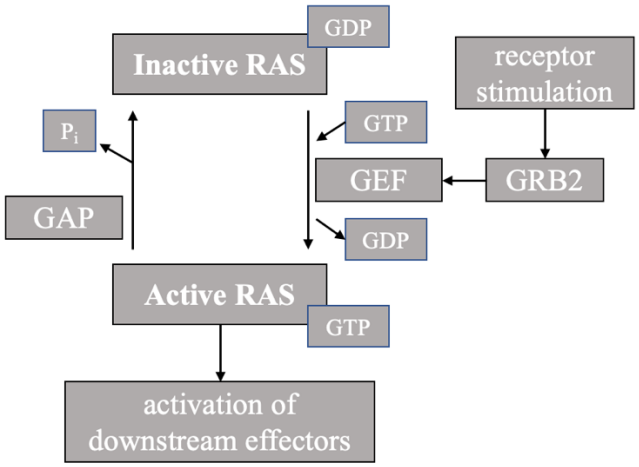

Figure 2. Cycling of RAS through active and inactive states. *Exchange of GDP bound to inactive RAS for GTP by a guanine exchange factor (GEF) activates RAS for the activation of several downstream effectors. This step is reversed by a GTP-ase activating protein (GAP) that provides stabilization for the hydrolysis of GTP to GDP, inactivating RAS to its effector proteins.*

The localization of RAS near the plasma membrane makes possible the signal transduction for which RAS is famous. RAS carries a localization signal at its C terminus that triggers the addition of a lipid chain by farnesyltransferase (45). After an initial lipid chain is added, RAS is moved to the ER and then the Golgi for further lipid modifications and finally an acylation step which sends the protein to the plasma membrane, its final destination (45). While localization to the plasma membrane is the accepted prerequisite for RAS function, some studies have demonstrated functional pools of RAS in the cytoplasm. A farnesylated cytoplasmic pool of NRAS was demonstrated to be permanent, rather than simply a stage in the processing and transport of RAS to the cell periphery (46). That cytoplasmic pool may make a significant contribution to the signal that RAS generates. For this to be possible, several other proteins must be present, a requirement that has been demonstrated by the presence of membrane-less protein granules that allow for the activation of RAS without a lipid membrane (47). The presence of

RAS in the cytoplasm is an unexpected development, and the coordination of the cytoplasmic signal with the signal from the plasma membrane as well as the implications of that coordination have yet to be determined.

Mutations of RAS

When functioning normally, RAS stands as a gatekeeper at the head of a multitude of pathways. A mutation in the RAS gene significantly impacts that role. Most of the RAS mutations are found in loop 4 near the gamma-phosphate of GTP, the location of GAP binding for GTP hydrolysis (38). Ninety-eight percent of oncogenic RAS mutations are found at Gly12, Gly13, and Gln61. These three residues are in the GTP and GAP binding pocket and each includes a single missense point mutation. While these three mutations are the most common, many mutations have been demonstrated in different types of cancers. The specific mutation and its impact depend heavily on the cell and cancer type as well as on a variety of external factors such as the tumor microenvironment (48).

Replacing either Gly12 or Gly13 adds a side chain regardless of the specific mutation and impairs the binding of a GAP by blocking the arginine finger domain commonly found on GAPs. This domain binds next to the gamma-phosphate of GTP, stabilizing the intermediate to allow for efficient hydrolysis. With a side chain at the twelfth or thirteenth residue, GAPs cannot bind, and RAS is effectively locked into the active form bound to GTP (49). Mutations of NF1 (the most common GAP protein) are often found as a co-mutation when Gly13 is mutated in RAS; while some Gly13-mutant RAS molecules retain some level of functionality, the impacts of that activity are dependent on the activity of NF1 (50).

Instead of impacting the binding of a GAP like Gly12 and Gly13 mutations, a mutation at Gln61 directly inhibits the hydrolysis of GTP. For the initiation of this reaction with wild-type

RAS, a catalytic water is situated between Thr35 and Gln61 near the gamma-phosphate of the GTP molecule that is bound to RAS. This water attacks the gamma-phosphate to remove it and form inorganic phosphate. This inorganic phosphate, however, cannot form stably without a proton transfer reaction. The proton transfer reaction is the primary function of Gln61; this residue takes on its imine form with proton transfer, aided in stabilization by Lys16 on RAS and the arginine finger domain from GAP. After the reaction is complete, the amide form of Gln61 is regenerated (51). Any mutation in Gln61 would impede imine formation and thus proton transfer, making GTP hydrolysis virtually impossible (49). Without the ability to perform this reaction, RAS does not revert to its inactive form.

These mutations do not physically lock GTP into RAS, but rather prevent its hydrolysis to GDP, implying that RAS could still release GDP and bind GTP in its stead. The potential for this exchange depends on the relative concentrations of GDP and GTP and on the affinity of RAS for each of them, which usually favors GTP. Additionally, mutated proteins retain some level of intrinsic GAP-like activity, and some mutated proteins still demonstrate low levels of GAP-mediated hydrolysis (39,44). Instead of being statically or constitutively active in the mutated form, mutated RAS is more accurately described as hyperexcitable (52). Additionally, not every tumor with a RAS mutation is dependent on that oncogene. Co-mutations sometimes play an even bigger role in transformation than RAS does; the determination of the mutation with the most weight is heavily reliant on external factors such as the tumor microenvironment (48). A co-mutation could mask the effects of constitutively active RAS, but another mutation could also display the same effects as constitutive RAS activation without any mutation in the RAS gene. Mutations in this category could include increased RTK activation of GEFs like SOS,

mutation or loss of GAPs such as NF1, or any other change that alters the equilibrium between GDP and GTP binding to RAS.

Signal Transduction Downstream of RAS

Once RAS has been activated, it causes a variety of downstream signaling cascades. It is the constitutive activation of these pathways that causes the oncogenic effects of a RAS mutation. The two main effector pathways of RAS are the RAF-MEK-ERK pathway and the PI_3K-Akt pathway; together, these pathways signal for growth, proliferation, differentiation, migration, and apoptosis (43). These are not the only pathways downstream of RAS; the list of effector molecules for RAS continues to grow as further relationships are discovered. All facets of oncogenic RAS signaling combine to cause the characteristic traits of cancer, namely uncontrolled cell growth and proliferation and avoidance of apoptosis, which are the topics of this paper.

The RAF-MEK-ERK Pathway

One of the most influential pathways downstream of RAS is the RAF-MEK-ERK pathway, a simplified version of which is depicted in Figure 3. This pathway requires receptor-mediated RAS activation by extracellular growth factors, cytokines, hormones, heat, or oxidative stress mediated across the plasma membrane by a receptor that activates RAS (53). Activated RAS brings Raf to the plasma membrane, where it is phosphorylated and activated by other protein kinases in the vicinity. In addition to Raf being activated by RAS, Raf can also be activated in a number of other ways (54). Activated Raf then phosphorylates and activates MEK (55). Inactive MEK is bound to ERK in the cytoplasm; when MEK is activated, these two proteins dissociate and MEK subsequently phosphorylates and activates ERK (53). This process

is integrated at every step with accessory proteins that form a scaffold and allow for proper docking and anchoring of enzymes and substrates.

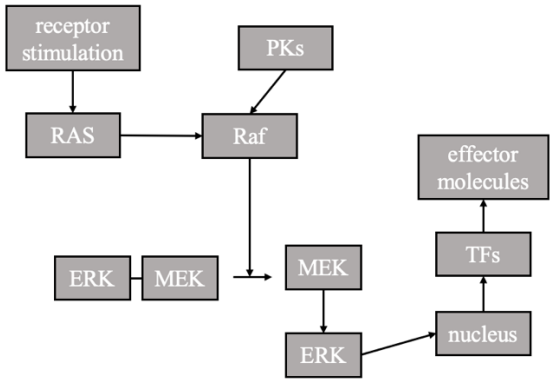

Figure 3. The RAF-MEK-ERK pathway downstream of RAS. *After localization to the plasma membrane by RAS and activation by protein kinases (PKs), Raf activates MEK, breaking its bond with ERK and allowing it to phosphorylate and activate ERK. After activation, ERK moves to the nucleus and acts as a transcription factor for several genes.*

The protein ERK carries out the downstream actions that are connected to the RAF-MEK-ERK pathway. Once activated, ERK can be moved to the nucleus, where it phosphorylates nuclear transcription factors (TFs) such as c-Fos, c-Jun, Elk-1, and c-Myc (54) to stimulate cell cycle entry, angiogenesis, and cell survival (53). ERK can also activate ribosomal S6 kinase (RSK) and MAPK-interacting ser/thr kinase (MNK) (53,56), which work for tumor invasion, metastasis, cell proliferation, survival, migration, and glycolytic flux (57,58,59,60). ERK can negatively regulate SOS, Raf, and MEK (54), and also to regulate the Bcl-2 family of proteins that control the balance of apoptotic signals at the mitochondria (53). ERK usually works for cell survival, growth, and proliferation, but because of its intimate relationship with the Bcl-2 family of proteins it can also cause apoptosis (61). ERK is not necessarily a pro-survival signal, but rather represents the balance of signals that allows the cell to choose between proliferation,

senescence, and death. Inactivation of the RAF-MEK-ERK pathway is mediated by phosphatases, and there are several points of negative feedback built into the system (53). Constitutive activation of this pathway with an oncogenic RAS mutation can cause transformation in cells by allowing increased proliferation; inhibition of this pathway can reverse transformation (54).

The PI_3K-Akt Pathway

The other pathway central to RAS signaling is the PI_3K-Akt pathway, shown in simplified form in Figure 4. Phosphatidylinositol 3-kinases (PI_3Ks) are a family of proteins that work as lipid kinases with a primarily regulatory role. These proteins can be activated by RAS at the plasma membrane in conjunction with allosteric activation by an RTK (43). Many other paths of activation have been demonstrated for PI_3Ks, making it difficult to determine the specific impacts of RAS activation. Regardless of the means of activation, PI_3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), forming phosphatidylinositol 3,4,5-trisphosphate (PIP_3) which then brings Akt, other similar molecules, and 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane. PDK1 acts as an activator of Akt and similar molecules. Akt then requires phosphorylation by a complex of proteins, called mTORC2, to be fully activated, at which point it can cause several downstream effects (43).

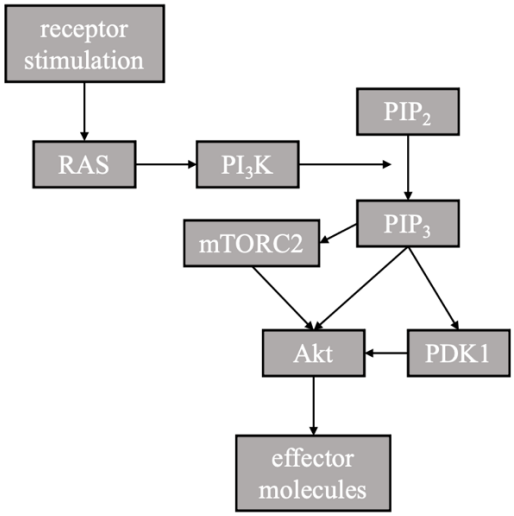

Figure 4. The PI$_3$K-Akt pathway downstream of RAS. *After activation by RAS, PI$_3$K phosphorylates PIP$_2$ to form PIP$_3$, which acts in association with mTORC2 to activate Akt directly and via PDK1. Akt then modifies the various effector molecules of this pathway.*

The targets of Akt generally function to promote cell growth and apoptosis evasion (55). RSK is a target of Akt as well as ERK; activation by either pathway allows for the stimulation of protein synthesis and cell proliferation. Glycogen synthase kinase-3 (GSK-3) is another target of Akt; Akt inhibits this effector to control the expression of cell cycle regulators and thus progression through the cell cycle (43). Akt inhibits several other molecules, including FOXO (forkhead box transcription factors), to inhibit apoptosis and cell cycle arrest, proapoptotic members of the Bcl-2 family such as BAD and BAX, and p53. NFκB is activated by Akt to allow for cancer progression (43). The PI$_3$K-Akt pathway downstream of RAS uses these effectors to make proliferation feasible by allowing progression through cell cycle checkpoints and by allowing the cell to avoid apoptosis. This pathway is most important for cells during development, because of the high levels of cell growth and division during that stage. While this pathway plays a significant role in adult life as well, that role is different in action and in

activation. The PI_3K-Akt pathway also mediates insulin signaling in the cell (62,63) and signal responses to thyroid hormone (64). The endocrine role of this pathway is intimately connected with its oncogenic role; increased glucose intake and metabolism, usually in response to insulin, is easily dysregulated in cells with unchecked PI_3K-Akt activity downstream of RAS. This impact of constitutive RAS activation allows the cell to signal for growth in total disregard of the nutrients available.

Metabolic Remodeling

These two pathways represent the most clearly defined impacts of constitutive RAS activation. Other impacts of RAS are much less clear in their specific connections to aberrant RAS signaling but are experimentally connected to mutant RAS in cancer cells. One of these impacts is metabolic remodeling. In healthy cells, oxidative phosphorylation is the primary means of energy production. This process uses NADH produced in the TCA cycle to produce ATP with the highest efficiency possible. Glycolysis is not usually the first choice for ATP production because it is less efficient than oxidative phosphorylation and because it leads to significant lactate production. Only cells in a hypoxic environment and during times of increased growth and proliferation use glycolysis preferentially over oxidative phosphorylation. A healthy cell will not undergo proliferation without the appropriate signals to confirm that sufficient nutrients are available; when these signals are present, efficient ATP production is not a primary concern for the cell because growth factors confirm that glucose is available in excess to provide energy through glycolysis. A cell undergoing proliferation has an increased need for carbon-skeleton intermediates for the construction of new cell parts, most of which derive from pathways connected to glycolysis. On a signal from growth factors, a cell can begin to proliferate

by remodeling its metabolism to emphasize glycolysis; this provides enough ATP for the cell while also providing the necessary carbon-skeleton intermediates (65).

Healthy cells only make this switch to glycolysis on the signal from growth factors for proliferation. Cancer cells, on the other hand, remodel their metabolism even without growth factor stimulation. This anomaly is called the Warburg effect (65,66). The proliferating cell carefully regulates its internal ATP levels using ATP/ADP and AMP/ATP ratios (65,67,68), sensing how much energy is required and how much is present through a variety of pathways. This allows the cell to maintain its energy supply even when the means of energy production is not the same as usual.

RAS clearly plays a role in metabolic remodeling in cancer cells, but the nature and specifics of that role are not explicitly clear. One suggested mechanism utilizes the PI_3K-Akt pathway. Because this pathway is so intimately connected to glucose uptake and use (note its connection to insulin signaling), it is logical that the deregulation of this pathway could increase glycolysis by increasing expression of glucose transporters, the access of hexokinase to glucose, and the activation of phosphofructokinase (65). This overemphasis on glucose caused by constitutive activation of Akt results in cancer cells that are addicted to and dependent on glucose, to the point that withdrawal of glucose can cause cell death because the cell can no longer signal for oxidative phosphorylation to fulfill its energy needs (69). This mechanism is by no means the only connection between RAS and the regulation of metabolism; the many effectors of RAS make its impact multifaceted, and our understanding of the impact of RAS is still developing. Regardless, RAS does play some role in the remodeling of metabolism in cancer cells during oncogenic transformation.

Cell Cycle Progression

In addition to metabolic remodeling, RAS has a dramatic impact on cell cycle progression – the root of the connection between RAS and increased proliferation. In healthy cells, growth factor activation causes this increased progression, but constitutive RAS activation in cancer cells allows progression to occur in the absence of growth factors. HRAS specifically has been demonstrated to shorten the cell cycle, cutting the time spent in G1 phase in half. This change is associated with a significant increase in cyclin D and a slight increase in cyclins E and A (70,71). Activation of these cyclins is likely performed by pathways other than RAS; the role of RAS is simply to upregulate their production, and to remove the necessity for platelet-derived growth factor for passage through cell cycle checkpoints (71). RAS is required to make this possible, but other pathways are at work to enhance its effect. ERK has also been demonstrated as necessary to progression to S phase (54), but not sequentially after RAS as would be expected (72), providing further evidence that RAS through the ERK pathway is not the only means of RAS action in cell cycle progression.

The PI_3K pathway is also involved and is likely the primary means of the influence of RAS here. This pathway is known to act at several points in the cell cycle, including during G1 phase and in the mediation of platelet derived growth factor progression to S phase (73). However, the exact steps of the PI_3K pathway and their relationship to cell cycle progression are unclear; this pathway is likely redundant with the ERK pathway and other pathways for the same signaling processes, and the observable changes in protein levels are likely more connected than not. Alternatively, perhaps redundant pathways impact different aspects of G1 phase and allow progression together but in unique ways. The balance among these signals depends heavily on environment and cell type. Regardless of the pathways used, the process of stimulation for cell

cycle progression has been described as wavelike, a system of positive and negative feedback mechanisms working with growth factors and their receptors to create a rhythm that allows the cell to move through the cell cycle (74). Because of its relationship to several of the pathways regulating the cell cycle, an oncogenic RAS mutation does impact on the speed and timing of this rhythm. Its impact can be large enough to be transformative, but likely is not as direct as expected. A shorter cell cycle and increased progression through that cycle, regardless of the specific pathway that brings it about, allows oncogenic RAS to increase cell proliferation to cause the development and progression of cancer.

All of these pathways and the broader impacts of RAS, in addition to others that are even less characterized, contribute to its oncogenic effects. As emphasized in the discussion of every pathway thus far, the impact of RAS is not limited to one clear stepwise process, but rather is nuanced and multifaceted. Despite this, the overall impact of mutated RAS is clear: oncogenic transformation and tumor growth through the dysregulation of normal cellular processes.

Current Therapeutic Strategies for the Treatment of RAS-mutant Cancers

Because of the complexity of RAS signaling, treatment of RAS-mutant cancers has proven quite difficult. RAS has even been dubbed undruggable because no attempt to treat it has proven successful. The key difficulty with a direct treatment of RAS is the lack of allosteric pockets for regulation on the surface of the protein – an inhibitory molecule cannot be designed for a binding pocket that does not exist. Recent advances have been made in developing drugs to directly target RAS, specifically KRAS with a G12C mutation. These small molecules are designed to bind directly to the mutated amino acid; this is ideal because they do not impact the wild-type protein. Binding of these drugs to RAS causes preferential binding to GDP rather than GTP and disrupts the ability of mutant RAS to bind to its effector Raf. These drugs target RAS

while it is bound to GDP, its more flexible state (44). Essentially, this inactivates all mutated RAS molecules, allowing them to avoid the constitutively active state caused by the mutation (75, 52). Several small molecules based on this principle have been developed and are in various stages of trials. While G12C is not the most common RAS mutation, development of these drugs provides hope that others may be developed to target other mutations in the future. Perhaps RAS will not be undruggable for long.

Many other therapeutic strategies exist that do not target RAS directly; this has been the most common method of treatment for RAS-mutated cancers. One strategy is to interfere with localization of the enzyme. Every step involved in localization, from farnesylation to acylation, could be targeted to prevent localization and minimize functionality of mutant RAS simply by removal from proximity to the plasma membrane and the receptors that it interacts with there. Farnesyltransferase inhibitors (FTIs) are the drugs commonly used for this purpose, inhibiting the first step of the process to prevent it from happening at all (45). These drugs, however, have proven to be mostly ineffectual, likely because of the ability of the cell to bypass this step using other enzymes. Another potential reason for the failure of this strategy is the recently discovered cytoplasmic pool of functional, active, and sensitive RAS (46,47); if RAS does not require localization to the plasma membrane for activity, inhibiting the localization process would have no impact on the functionality of RAS.

Targeted degradation has also been explored as a therapeutic strategy for mutant RAS. Proteasomal degradation specific to RAS can effectively downregulate RAS signaling pathways, allowing for growth inhibition and apoptosis initiation to occur in contrast to the commonly demonstrated effects of constitutively activated RAS (39).

Targeting of downstream effectors is another commonly utilized therapeutic approach. Instead of directly inhibiting RAS, drugs have been created to target proteins that are activated or deactivated by RAS or its effectors, such as any protein in the RAF-MEK-ERK pathway or the PI_3K-Akt pathway. This strategy is limited by adaptive resistance; by targeting the pathway so close to its end, this strategy allows the cell to bypass the inhibited steps using other pathways. Many feedback mechanisms exist in the RAS pathways, along with differences in action between the isoforms of RAS. Because so little is known about the intricacies of these pathways, the impacts of effector inhibition can have unexpected and far-reaching effects.

Combination therapy has proven to be the most successful method of treatment thus far. This strategy targets several points in the RAS pathways, and various combinations of drugs have proven effective. For example, PI_3K/mTOR and MEK are commonly inhibited simultaneously; MEK and the anti-apoptotic protein Bcl-xL are another common combination therapy for all KRAS mutations. Either of these combinations (or any other) can be combined with a direct RAS inhibitor like the G12C inhibitors previously mentioned, dramatically increasing the effectiveness of the therapy (76). The benefit of this strategy is its avoidance of feedback inhibition by the utilization of a strategy called vertical inhibition. Combination therapy will grow even more prominent as inhibitors for each point in the known pathways are designed, as more is learned about the details of the pathways downstream of RAS, and as more direct inhibitors for RAS are designed.

Connections Between Calcium Flux and Oncogenic RAS

Calcium flux pathways and RAS pathways are complex and not fully characterized, but because they interact with similar processes in the cell, the potential for connections between them is significant. Identifying a link between these two pathways would allow the exploitation

of that link for therapeutic purposes. On a general level, it has been demonstrated that oncogenic KRAS is associated with remodeled calcium homeostasis (77). But RAS mutations have also been demonstrated to have effects on a more specific level. One of these is modification of ER calcium content. Bcl-2 could be the mediator of these changes, but research disagrees on this topic. Further clarification of the role of Bcl-2 in mediating ER calcium content could provide a point of contact between oncogenic RAS pathways and the regulation of calcium flux. ER calcium content could also be impacted through calcium influx into the cell and then into the ER. SOCE, the process that brings calcium into the cell on depletion of the ER calcium store, has been demonstrated to be downregulated in RAS-mutant cells (78), decreasing the amount of calcium in the cell. RAS has also been demonstrated to effect STIM1 and ORAI-1, which control SOCE and are closely linked to cell cycle transitions. Both of these are localized to the ER and are downstream of ERK, providing a connection between RAS signaling and calcium flux to and from the ER (78). Thus, calcium uptake via SOCE could provide a potential connection between RAS and calcium flux.

The release of calcium from the ER lumen could likewise connect RAS and calcium signaling. Akt, downstream of RAS via PI_3K, has been demonstrated to phosphorylate IP_3Rs on the C-terminus in the cytoplasm, minimizing apoptosis by preventing normal interaction with caspase-3 that would allow for sustained calcium release to trigger opening of the mitochondrial permeability transition pore and the stimulation of apoptosis (79,80). This phosphorylation does not change normal calcium flux, but rather prevents extreme calcium flux that could initiate apoptosis, stopping the apoptosis cascade before it begins but allowing normal calcium signaling for homeostatic processes like metabolism to continue (79). Akt can also phosphorylate the regulatory unit of the MCU, which acts as a gatekeeper for the entire calcium content of the

mitochondria. This phosphorylation destabilizes the MCU, allowing for ROS production and tumor progression (81). Akt generally is not assumed to be localized to the ER, but its relationship to IP_3R regulation indicates that perhaps its role there is more influential than expected. IP_3R phosphorylation by Akt is just one example of signal integration at IP_3Rs, but it is an important one because it places the regulation of calcium release directly downstream of RAS.

Calcium release from the ER can be impacted in a variety of other ways as well. Increased plasma membrane receptor expression can allow for a greater number of signals to be transmitted, increasing calcium release to allow for heightened metabolism and progression through the cell cycle. Changing IP_3R levels can also dramatically impact calcium release. PLCε, the molecule that releases IP_3 to activate IP_3Rs, is stimulated by the RAF-MEK-ERK pathway downstream of RAS. This connection is part of the way that RAS signals for increased proliferation, increasing IP_3 levels to allow for increased calcium flux (83, 82). Changes in the relative abundance of IP_3R isoforms can also play a major role in regulating calcium release, because each isoform allows for different types of calcium signals (84). The relative abundance of IP_3R isoforms varies by cell type, so a general rule for the impact of RAS on this ratio is difficult to establish although a connection is known to exist. RAS has, however, been demonstrated to play some role in regulating the relationship between the isoforms in cancerous cells. Any alteration of the effect of RAS on these elements of calcium signaling with an oncogenic RAS mutation could act to mediate the oncogenic effects of RAS and be a potential therapeutic target.

Metabolism is another piece of oncogenic transformation that is impacted by both calcium flux and mutant RAS. Metabolic processes are themselves a complex regulatory system,

impacted by several external factors as well as feedback regulation at many points. The TCA cycle dehydrogenases are regulated by calcium; any change in calcium flux will impact their activity. Any impact of mutant RAS on calcium flux between the ER and the mitochondria thus also directly influences the TCA cycle and the balance of metabolism in the cell; this can lead to oncogenic transformation by causing the cell to switch to an emphasis on glycolysis. Of course, this is not the only possible means of influence that RAS has on metabolism. At any point in this complex regulatory system, a pathway downstream of RAS could alter metabolism to bring about oncogenic transformation.

The cytoskeleton provides another potential connection between the balance of calcium in the cell and the oncogenic activities of RAS. The location and orientation of organelles is what allows them to have any meaningful function at all. This is especially true of the ER and the mitochondria. The association between those two organelles and the orientation of the ER primarily allow for functional calcium flux and other signaling cascades. As previously mentioned, the cytoskeleton plays a key role in regulating these things, in addition to the fusion and fission of the mitochondria. Individual proteins also require accurate localization for functionality. IP_3Rs are one example of this. Because they are not uniformly distributed on the surface of the ER, the movement of the cytoskeleton in orienting IP_3Rs is pivotal to their function. Mediating this connection is a protein called KRAS-induced actin binding protein (KRAP), which works with intermediate filaments of the cytoskeleton to allow for localization of IP_3Rs to the ER in general and to some regions in specific in order to fulfill functions unique to each isoform (85,87). This protein is another potential point of connection that allows mutant RAS to play a role in calcium flux.

These are only a few of the potential connections between the calcium and RAS pathways, two of the most nuanced pathways in the cell. Further research will reveal more significant details about the nature of each of these pathways, allowing researchers to identify functional overlaps between the pathways. A point of connection such as this would prove to be an ideal target for drugs that treat RAS-mutant cancers. This field holds much promise for cancer research in the future; the discovery of a practical target for such drugs would provide hope for a large percentage of patients with cancers deemed untreatable by professionals.

References

1. Reynolds IJ, Rintoul GL. Mitochondrial stop and go: Signals that regulate organelle movement. *Science's STKE* 251, 2004.
2. Waterman-Storer CM, Salmon ED. Endoplasmic reticulum membrane tubules are distributed by microtubules in living cells using three distinct mechanisms. *Current Bio* 8: 798-807, 1998.
3. Zheng H. Partnership in action: The endoplasmic reticulum regulates the cytoskeleton. *J Plant Physio* 266: 153540, 2021.
4. Csordás G, Renken C, Várnai P, Walter L, Weaver D, Buttle KF, Balla T, Mannella CA, Hajnóczky G. Structural and functional features and significance of the physical linkage between ER and mitochondria. *J Cell Bio* 174: 915-921, 2006.
5. Iyer R, Franzini-Armstrong C. The location of InsP3 receptors in Purkinje cells of murine cerebellum does not supports a direct interaction in the transfer of calcium ions between ER and mitochondria. *Eur J Transl Myol* 31: 9935, 2021.
6. Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T. Close contancts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* 280: 1763-1766, 1998.
7. Marchi S, Patergnani S, Pinton P. The endoplasmic reticulum-mitochondria connection: One touch, multiple functions. *BBA: Bioenergetics* 1837: 461-469, 2013.
8. Kwak C, Shin S, Park J, Jung M, Nhung TTM, Kang M, Lee C, Kwon T, Park SK, Mun JY, Kim J, Rhee H. Contact-ID, a tool for profiling organelle contact sites, reveals regulatory proteins of mitochondrial-associated membrane formation. *Proc Natl Acad Sci USA* 117: 12109-12120, 2020.
9. Morciano G, Marchi S, Morganti C, Sbano L, Bittremieux M, Kerkhofs M, Corricelli M, Danese A, Karkucinska-Wieckowska A, Wieckowski MR, Bultynck G, Giorgi C, Pinton P. Role of Mitochondria-Associated ER Membranes in Calcium Regulation in Cancer-Specific Settings. *Neoplasia*, 20: 510-523, 2018.
10. Papp B, Brouland J, Arbabian A, Gélébart P, Kovács T, Bobe R, Enouf J, Varin-Blank N, Apáti A. Endoplasmic Reticulum Calcium Pumps and Cancer Cell Differentiation. *Biomolecules* 2: 165-186, 2012.
11. Cárdenas C, Miller RA, Smith I, Bui T, Molgó J, Müller M, Vais H, Cheung K, Yang J, Parker I, Thompson CB, Birnbaum MJ, Hallows KR, Foskett JK. Essential regulation of cell bioenergetics by constitutive $InsP_3$ receptor Ca^{2+} transfer to mitochondria. *Cell* 142: 270-283, 2010.
12. Prole DL, Taylor CW. Structure and function of IP_3 receptors. *Cold Spring Harbor*, 2019.
13. Monteith GR, Davis FM, Roberts-Thomson SJ. Calcium channels and pumps in cancer: changes and consequences. *J Biol Chem* 287: 31666-31673, 2012.
14. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 100 :57-70, 2000.
15. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 144: 646-674, 2011.
16. Grimm S, The ER-mitochondria interface: the social network of cell death. *BBA: Mol Cell Research* 1823: 327-334, 2011.
17. Loncke J, Kerkhofs M, Kaassik A, Bezprozvanny I, Bultynck G. Recent advances in understanding IP_3R function with focus on ER-mitochondrial Ca^{2+} transfers. *Curr Op Physio* 70: 80-88, 2020.

18. Katayama E, Funahashi H, Michikawa T, Shiraishi T, Ikemoto T, Iino M, Hirosawa K, Mikoshiba K. Native structure and arrangement of inositol-1,4,5-trisphosphate receptor molecules in bovine cerebellar Purkinje cells as studied by quick-freeze deep-etch electron microscopy. *EMBO J* 18: 4844-4851, 1996.

19. Mendes CCP, Gomes DA, Thompson M, Souto NC, Goes TS, Goes AM, Rodrigues MA, Gomez MV, Nathanson MH, Leite MF. The type III inositol 1,4,5-triphosphate receptor preferentially transmits apoptotic Ca^{2+} signals into mitochondria. *J Biol Chem* 280: 40892-40900, 2005.

20. Shibao K, Fiedler MJ, Nagata J, Minagawa N, Hirata K, Nakayama Y, Iwakiri Y, Nathanson MH, Yamaguchi K. The type III inositol 1,4,5-trisphosphate receptor is associated with aggressiveness of colorectal carcinoma. *Cell Calcium* 48: 315-323, 2010.

21. Tu H, Wang Z, Nosyreva E, De Smedt H, Bezprozvanny I. Functional Characterization of Mammalian Inositol 1,4,5-Trisphosphate Receptor Isoforms. *Biophys J* 88: 1046-1055, 2005.

22. Vervloessem T, Yule DI, Bultynck G, Parys JB. The type 2 inositol 1,4,5-trisphosphate receptor, emerging functions for an intriguing Ca^{2+}-release channel. *BBA – Mol Cell Research* 9: 1992-2005, 2015.

23. Furuichi T, Simon-Chazottes D, Fujino I, Yamada N, Hasegawa M, Miyawaki A, Yoshikawa S, Guénet JL, Mikoshiba K. Widespread expression of inositol 1,4,5-trisphosphate receptor type 1 gene (Insp3r1) in the mouse central nervous system. *Recept Channels* 1: 11-24, 1993.

24. Thillaiappan NB, Chavda AP, Tovey SC, Prole DL, Taylor CW. Ca^{2+} signals initiate at immobile IP_3 receptors adjacent to ER-plasma membrane junctions. *Nature Comm* 8, 2017.

25. Newton CL, Mignery GA, Südhof TC. Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP3) receptor with distinct affinities for InsP3. *J Biol Chem* 269: 28613-28619, 1994.

26. Szabadkai G, Bianchi K, Várnai P, De Stefani D, Wieckowski MR, Cavagna D, Nagy AI, Balla T, Rizzuto R. Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca^{2+} channels. *J Cell Bio* 175: 901-911, 2006.

27. Wang L, Yule DI. Differential regulation of ion channels function by proteolysis. *BBA – Mol Cell Research* 11: 1698-1706, 2018.

28. Booth DM, Enyedi B, Geiszt M, Várnai P, Hajnóczky G. Redox Nanodomains Are Induced by and Control Calcium Signaling at the ER-Mitochondrial Interface. *Mol Cell* 63: 240-248, 2016.

29. Beretta M, Santos CX, Molenaar C, Hafstad AD, Miller CC, Revazian A, Betteridge K, Schröder K, Streckfuß-Bömeke K, Doroshow JH, Fleck RA, Su T, Belousov VV, Parsons M, Shah AM. Nox4 regulates $InsP_3$ receptor-dependent Ca^{2+} release into mitochondria to promote cell survival. *EMBO J* 39, 2020.

30. Rong Y, Aromolaran AS, Bultynck G, Zhong F, Li X, McColl K, Matsuyama SS, Herlitze S, Roderick HL, Bootman MD, Mignery GA, Parys JB, De Smedt H, Distelhorst CW. Targeting Bcl-2-IP3 Receptor Interaction to Reverse Bcl-2's Inhibition of Apoptotic Calcium Signals. *Mol Cell* 31: 255-265, 2008.

31. Chen R, Valencia I, Zhong F, McColl KS, Roderick HL, Bootman MD, Berridge MJ, Conway SJ, Holmes AB, Mignery GA, Velez P, Distelhorst CW. Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate. *J Cell Biol* 166: 193-203, 2004.

32. Pinton P, Ferrari D, Magalhães P, Schulze-Osthoff K, Di Virgilio F, Pozzan T, Rizzuto R. Reduced loading of intracellular Ca^{2+} stores and downregulation of capacitative Ca^{2+} influx in Bcl-2-overexpressing cells. *J Cell Bio* 148: 857-862, 2000.

33. Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD. STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. *Nature* 437: 902-905, 2005.

34. Gélébart P, Kovács T, Brouland J, van Gorp R, Grossmann J, Rivard N, Panis Y, Martin V, Bredoux R, Enouf J, Papp B. Expression of endomembrane calcium pumps in colon and gastric cancer cells: Induction of SERCA3 expression during differentiation. *Membrane Transport Structure Function and Biogenesis* 277: 26310-26320, 2002.

35. Prasad V, Boivin GP, Miller ML, Liu LH, Erwin CR, Warner BW, Shull GE. Haploinsufficiency of *Atp2a2*, encoding sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 2 Ca^{2+} pump, predisposes mice to squamous cell tumors via a novel mode of cancer susceptibility. *Cancer Res* 65: 8655-8661, 2005.

36. Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene* 27: 6407-6418, 2008.

37. Humeau J, Bravo-San Pedro JM, Vitale I, Nuñez L, Villalobos C, Kroemer G, Senovilla L. Calcium signaling and cell cycle: Progression or death. *Cell Calcium* 70: 3-15, 2018.

38. Malumbres M, Barbacid M. RAS oncogeense: the first 30 years. *Nature Reviews* 3: 7-13, 2003.

39. Lim S, Khoo R, Juang Y, Gopal P, Zhang H, Yeo C, Peh KM, Teo J, Ng S, Henry B, Partridge AW. Exquisitely Specific anti-KRAS Biodegraders Inform on the Cellular Prevalence of Nucleotide-Loaded States. *ACS Cent Sci* 7, 274-291, 2021.

40. Mageean CJ, Griffiths JR, Smith DL, Clague MJ, Prior IA. Absolute Quantification of Endogenous Ras Isoform Abundance. *PLOS ONE* 10, 2015.

41. Guerra C, Mijimolle N, Dhawahir A, Dubus P, Barradas M, Serrano M, Campuzano V, Barbacid M. Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. *Cancer Cell* 4: 111-120, 2003.

42. Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* 363: 83-85, 1993.

43. Cuesta C, Arévalo-Alameda C, Castellano E. The Importance of Being PI3K in the RAS Signaling Network. *Genes* 12: 1094, 2021.

44. Hobbs GA, Wittinghofer A, Der CJ. Selective Targeting of the KRAS G12C Mutant: Kicking KRAS When It's Down. *Cancer Cell* 29: 251-253, 2016.

45. Cox AD, Der CJ, Philips MR. Targeting RAS Membrane Association: Back to the Future for Anti-RAS Drug Discovery? *Clin Cancer Res* 21: 1819-1827, 2015.

46. Zhou M, Wiener H, Su W, Zhou Y, Liot C, Ahearn I, Hancock JF, Philips MR. VPS35 binds farnesylated N-Ras in the cytosol to regulate N-Ras trafficking. *J Cell Bio* 214: 445-458, 2016.

47. Tulpule A, Guan J, Neel DS, Allegakoen HR, PharLin Y, Brown D, Chou Y, Heslin A, Chatterjee N, Perati S, Menon S, Nguyen TA, Debnanth J, Ramirez AD, Shi X, Yang B, Feng S, Makhija S, Huang B, Bivona TG. Kinase-mediated RAS signaling via membraneless cytoplasmic protein granules. *Cell* 184: 2649-2664, 2017.

48. Huang L, Guo Z, Wang F, Fu L. KRAS mutation: from undruggable to druggable in cancer. *Nature* 6, 2021.

49. Scheffzek K, Ahmadian MR, Kabsch W, Wiesmüller L, Lautwein A, Schmitz F, Wittinghofer A. The Ras-RasGAP Complex: Structural Basis for GTPase Activation and Its Loss in Oncogenic Ras Mutants. *Science* 277: 333-339, 1997.

50. Rabara D, Tran TH, Dharmaiah S, Stephens RM, McCormick F, Simanshu DK, Holderfield M. KRAS G13D sensitivity to neurofibromin-mediated GTP hydrolysis. *Proc Natl Acad Sci USA* 116: 22122-22131, 2019.

51. Khrenova MG, Grigorenko BL, Kolomeisky AB, Nemukhin AV. Hydrolysis of Guanosine Triphosphate (GTP) by the Ras·GAP Protein Complex: Reaction Mechanism and Kinetic Scheme. *J Phys Chem* 119: 12838-12845, 2015.

52. Patricelli MP, Janes MR, Li L, Hansen R, Peters U, Kessler LV, Chen Y, Kucharski JM, Feng, Ely T, Chen JH, Firdaus SJ, Babbar A, Ren P, Liu Y. Selective Inhibition of Oncogenic KRAS Output with Small Molecules Targeting the Inactive State. *Cancer Discov* 6: 316-329, 2016.

53. Sugiura R, Satoh R, Takasaki T. ERK: A Double-Edged Sword in Cancer. ERK-Dependent Apoptosis as a Potential Therapeutic Strategy for Cancer. *Cells* 10: 2509, 2021.

54. Guo Y, Pan W, Liu S, Shen Z, Xu Y, Hu L. ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med* 19: 1997-2007, 2020.

55. Zenonos K, Kyprianou K. RAS signaling pathways, mutations and their role in colorectal cancer. *World J Gastrointest Oncol* 5: 97-101, 2013.

56. Shimamura A, Ballif BA, Richards SA, Blenis J. Rsk1 mediates a MEK-MAP kinase cell survival signal. *Curr Biol* 10: 127-135, 2000.

57. Tanimura S, Hashizume J, Kurosaki Y, Sei K, Gotoh A, Ohtake R, Kawano M, Watanabe K, Kohno M. SH3P2 is a negative regulator of cell motility whose function is inhibited by ribosomal S6 kinase-mediated phosphorylation. *Genes to Cells* 16: 514-526, 2011.

58. Doehn U, Hauge C, Frank SR, Jensen CJ, Duda K, Nielsen JV, Cohen MS, Johansen JV, Winther BR, Lund LR, Winther O, Taunton J, Hansen SH, Frödin M. RSK Is a Principal Effector of the RAS-ERK Pathway for Eliciting a Coordinate Promotile/Invasive Gene Program and Phenotype in Epithelial Cells. *Mol Cell* 35: 511-522, 2009.

59. Houles T, Gravel S, Lavoie G, Shin S, Savall M, Méant A, Grondinn B, Gaboury L, Yoon S, St-Pierre J, Roux PP. RSK Regulates PFK-2 Activity to Promote Metabolic Rewiring in Melanoma. *Cancer Res* 78: 2191-2204, 2018.

60. Cheng DK, Onia TE, Thalappillila JS, Parka Y, Tinga H, Alagesana B, Prasada NV, Addisonnoa K, Riveraa KD, Pappina DJ, Aelsta LV, Tuveson DA. Oncogenic KRAS engages an RSK1/NF1 pathway to inhibit wild-type RAS signaling in pancreatic cancer. *PNAS* 118, 2021.

61. Hong S, Wu P, Park J. A cellular threshold for active ERK1/2 levels determines Raf/MEK/ERK-mediated growth arrest versus death responses. *Cell Signal* 42: 11-20, 2018.

62. Hopkins BD, Goncalves MD, Cantley LC. Insulin–PI3K signalling: an evolutionarily insulated metabolic driver of cancer. *Nature* 16: 276-283, 2020.

63. Szablewski L. *Blood Glucose Levels.* London: InTechOpen, 2020.

64. Cao X, Kambe F, Moeller LC, Refetoff S, Seo H. Thyroid hormone induces rapid activation of Akt/protein kinase B-mammalian target of rapamycin-p70S6K cascade through phosphatidylinositol 3-kinase in human fibroblasts. *Mol Endocrinol* 19: 102-112, 2005.

65. Vander Heiden, MG, Cantley LC, Thompson CB. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* 324: 1029-1033, 2009.

66. Warburg O. On the origin of cancer cells. *Science* 123: 309-314, 1956.

67. Vander Heiden MG, Chandel NS, Schumacker PT, Thompson CB. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol Cell* 3: 159-167, 1999.

68. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantly LC. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA* 101: 3329-3335, 2004.

69. Buzzai M, Bauer DE, Jones RG, Deberardinis RJ, Hatzivassiliou G, Elstrom RL, Thompson CB. The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation. *Oncogene* 24: 4165-4173, 2005.

70. Liu J, Chao J, Jiang M, Ng S, Jong-Young J, Yang-Yen A. Ras Transformation Results in an Elevated Level of Cyclin D1and Acceleration of G1Progression in NIH 3T3 cells. *Mol and Cell Bio* 15: 3654-3663, 1995.

71. Wintson JT, Coats SR, Wang YZ, Pledger WJ. Regulation of the cell cycle machinery by oncogenic ras. *Oncogene* 12: 127-134, 1996.

72. Taylor SJ, Shalloway D. Cell cycle-dependent activation of Ras. *Current Biology* 6:1621-1627, 1996.

73. Jones SM, Klinghoffer R, Prestwich GD, Toker A, Kazlauskas A. PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G1. *Curr Biol* 9: 512-521, 1999.

74. Zhan H, Bhattacharya S, Cai H, Iglesias PA, Huang C, Devreotes PN. An Excitable Ras/PI3K/ERK Signaling Network Controls Migration and Oncogenic Transformation in Epithelial Cells. *Dev Cell* 54: 608-623, 2020.

75. Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM. K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* 503: 548-551, 2013.

76. Kitazawa M, Miyagawa Y, Koyama M, Nakamura S, Hondo N, Miyazaki S, Muranaka F, Tokumaru S, Yamamoto Y, Ehara T, Kuroiwa M, Tanaka H, Komatsu D, Takeoka M, Soejima Y. Drug sensitivity profile of minor KRAS mutations in colorectal cancer using mix culture assay: The effect of AMG-510, a novel KRAS G12C selective inhibitor, on colon cancer cells is markedly enhanced by the combined inhibition of MEK and BCL-XL. *Mol Clin Oncol* 15: 148, 2021.

77. Bittremieux M, Pary JB, Pinton P, Bultynck G. ER functions of oncogenes and tumor suppressors: Modulators of intracellular Ca^{2+} signaling. *BBA – Mol Cell Res* 1863: 1364-1378, 2016.

78. Pierro C, Zhang X, Kankeu C, Treback M, Bootman MD, Rodeereick HL. Oncogenic KRAS suppresses store-operated Ca^{2+} entry and I_{CRAC} through ERK pathway-dependent remodelling of STIM expression in colorectal cancer cell lines. *Cell Calcium* 72: 70-80, 2018.

79. Khan MT, Larry II, W, Yule DI, Bhanumathy C, Joseph SK. Akt Kinase Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors. *Mech Sig Trans* 281: 3731-3737, 2006.

80. Assefa Z, Bultynck G, Szlufcik K, Kasri NN, Vermassen E, Goris J, Missiaen L, Callewaert G, Parys JB, De Smedt H. Caspase-3-induced Truncation of Type 1 Inositol Trisphosphate Receptor Accelerates Apoptotic Cell Death and Induces Inositol Trisphosphate-independent Calcium Release during Apoptosis. *J Biol Chem* 279: 43227-43236, 2004.

81. Marchi S, Corricelli M, Branchini A, Vitto VAM, Missiroli S, Morciano G, Perrone M, Ferrarese M, Giorgi C, Pinotti M, Galluzzi L, Krooemer G, Pinton P. Akt-mediated phosphorylation of MICU1 regulates mitochondrial Ca $^{2+}$ levels and tumor growth. *EMBO J* 38, 2019.

82. Wang X, Fan Y, Du Z, Fan J, Hao Y, Wang J, Wu X, Luo C. Knockdown of Phospholipase Cε (PLCε) Inhibits Cell Proliferation via Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN)/AKT Signaling Pathway in Human Prostate Cancer. *Med Sci Monit* 24: 254-263, 2018.

83. Weber G. Down-regulation of increased signal transduction capacity in human cancer cells. *Adv Enzyme Regul* 45: 37-51, 2005.

84. Pierro C, Cook SJ, Fots TCF, Bootman MD, Roderick HL. Oncogenic K-Ras suppresses IP_3-dependent Ca^{2+} release through remodelling of the isoform composition of IP_3Rs and ER luminal Ca^{2+} levels in colorectal cancer cell lines. *J Cell Sci* 127: 1607-1619, 2014.

85. Dingli F, Parys JB, Loew D, Saule S, Mery L. Vimentin and the K-Ras-induced actin-binding protein control inositol-(1,4,5)-trisphosphate receptor redistribution during MDCK cell differentiation. *J Cell Sci* 125: 5428-5440, 2012.

86. Fujimoto T, Machida T, Tsunoda T, Doi K, Ota T, Kuroki M, Shirasawa S. KRAS-induced actin-interacting protein regulates inositol 1,4,5-trisphosphate-receptor-mediated calcium release. *Biochem Biophys Res Commun* 408: 214-217, 2011.

87. Fujimoto T, Machida T, Tanaka Y, Tsunoda T, Doi K, Ota T, Okamura T, Kuroki M, Shirasawa S. KRAS-induced actin-interacting protein is required for the proper localization of inositol 1,4,5-trisphosphate receptor in the epithelial cells. *Biochem Biophys Res Commun* 407: 438-443, 2011.