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Corresponding Author: Se Bok Jang

Authors: Chang Woo Han[1], Mi Suk Jeong[1], Se Bok Jang[1,*]

Institution: [1]Department of Molecular Biology, Pusan National University,

Structure, signaling and the drug discovery of the Ras oncogene protein

Chang Woo Han, Mi Suk Jeong, and Se Bok Jang*

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Jangjeon-dong, Geumjeong-gu, Busan 46241, Korea.

*Corresponding author: Se Bok Jang

Tel: 82-51-510-2523

Fax: 82-51-581-2544

E-mail: sbjang@pusan.ac.kr

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ABSTACT

Mutations in Ras GTPase are among the most common genetic alterations in human cancers. Despite extensive research investigating Ras proteins, their functions still remain a challenge over a long period of time. The currently available data suggests that solving the outstanding issues regarding Ras could lead to development of effective drugs that could have a significant impact on cancer treatment. Developing a better understanding of their biochemical properties or modes of action, along with improvements in their pharmacologic profiles, clinical design and scheduling will enable the development of more effective therapies.

INTRODUCTION

Ras was identified during the extensive study of retroviral oncogenes isolated from the genome of Harvey and Kirsten rat sarcoma viruses. Since the 1980s, the discovery of mutated Ras genes in human tumor cell lines has led to intensive research into the structure and biochemistry of Ras (1). Ras proteins are small GTPases that serve as master regulators of a myriad of signaling cascades involved in highly diverse cellular processes. Activating mutations in Ras are found in about one-third of cancers. Oncogenic mutations in the Ras gene are associated with a single mutation, typically at codons 12, 13 or 61 (2). K-Ras mutations occur frequently in pancreatic, colorectal, endometrial, biliary tract, lung, and cervical cancers. N-Ras and H-Ras mutations predominate in melanoma and bladder cancer, respectively (3). Different isoforms of Ras (H-, K-, and N-Ras) can regulate a variety of cellular processes, including proliferation, differentiation, and apoptosis. Intensive efforts to target these H-, K-, and N-Ras key proteins have been conducted, but no effective pharmacological inhibitors of the Ras proteins have been successfully applied in clinical settings. Recent development of new tools in drug discovery has renewed our hope for development of a Ras inhibitor. However, Ras proteins are highly similar in sequence and structure, particularly in the catalytic domain, although important differences exist. The major driver in most Ras-mutant cancers is K-Ras, but structural, mutational and biochemical data primarily originates from studies conducted using H-Ras (4). For these reasons, some potential binding sites have been identified using computational approaches based on H-Ras structural models; however, they do not appear to have any deep hydrophobic pockets on the surface of K-Ras that would allow tight binding of small molecules (5). While the efforts to

indirectly target Ras through FTIs were rationally designed, this strategy suffered from lack of consideration of the fundamental biology of Ras prenylation. This led to their subsequent failure in large-scale clinical trials targeting K-Ras mediated cancers (6). In previous studies, Ras effector signaling was considered to be a simple process. However, recently studies of various protein kinase cascades have revealed that Ras signaling occurs via a complex and highly dynamic signaling network that can adapt and resist in response to inhibitors. Indiscriminately blocking Ras effectors for both mutant and wild-type Ras may lead to substantial toxicity. Hence, understanding Ras proteins can facilitate investigations of the interaction between development of cancer and cellular signaling pathways. In addition, understanding of the Ras structure has continuously improved since the first crystal structures of Ras were solved, leading to discovery of innovative and exciting venues for targeting inhibitors of Ras development (7). However, most of the inhibitors were ineffective because of low affinity and cellular toxicity. To solve this problem, recent studies have focused on downstream effectors that interact with Ras. These downstream effectors regulate the proliferation, survival, differentiation and motility of cancer cells through complex feedback and cross-talk mechanisms (8).

In this review, we provide an in-depth analysis of the structure, mutational activation, signaling pathway, and inhibitors of Ras. We examine the problems associated with currently available Ras inhibitors and discuss promising avenues for further development.

RAS STRUCTURE

The Ras is Ras-related protein superfamily of small GTP-binding proteins with structural

similarity (molecular weight 21-25 kDa) (9). Ras-related genes encoding small GTP binding proteins fall into several subfamilies categorized by their amino acid sequences of encoded proteins and their biological functions, Ras, Rho, Rap and Ral (10). The Ras-related protein superfamily of small GTP-binding proteins is characterized by the so-called "G domain," which is unique to this superfamily and plays mostly regulatory functions in many cellular processes. This domain, also called the switch Ⅰ region (amino acids 32-40 in Ras), undergoes conformational changes during conversion of the guanosine diphosphate (GDP)-bound form into a guanosine triphosphate (GTP)-bound form. The Ras constitute a class of phosphate binding loop (P-loop) proteins that work as molecular switches between the GDP-bound inactive and the GTP-bound active state (11). The γ-phosphate interacts with key residues (Tyr32 and Thr35) that hold the switch I region. Conserved Gly60 of the switch Ⅱ region (aa 59-77) makes crucial contacts with the γ-phosphate. The switch Ⅱ region is located between the central β-sheet of Ras and the α2-helix (12).

The Ras genes, which are proto-oncogenes that are mutated in human cancers, are encoded by three expressed genes: H-, K-, and N-Ras (13). Three Ras genes encode 188-89 amino acid proteins that share 82-90% overall sequence identity. Ras proteins are processed in a series of reactions initiated by farnesylation of Ras. Although there are some striking differences in their primary structures, in particular in the variable carboxy terminal region, the enzyme farnesyltransferase recognizes the C-terminal sequence of the Ras gene known as the Cys-A-A-X motif, where A is isoleucine, leucine, or valine, and X is methionine or serine (14). CAAX motif proteins play essential roles in multiple signaling pathways, controlling various processes. These reactions involve prenylation of the cysteine residue, cleavage at the -AAX sequence and methylation of the carboxyl-prenylated cysteine residue

(15). The -AAX sequence is removed by Ras Converting CAAX Endopeptidase 1 (Rce1) and the now C farnesylated terminal cysteine is carboxyl methylated by isoprenylcysteine carboxyl methyltransferase (Icmt) (16). Hence, the CAAX motif comprises plasma membrane anchoring and trafficking of newly synthesized and processed Ras from the cytosolic surface of the endoplasmic reticulum (ER) to the inner surface of the plasma membrane (17). Plasma membrane anchoring and trafficking of Ras proteins cycle between an active, GTP-bound state, and an inactive, GDP-bound state. These exchanges lead to large conformational changes of the switch I and switch II regions in the effector lobe of Ras. Their effector proteins contain Ras association (RA) domains or Ras binding domains (RBDs), which bind specifically to the GTP-bound state. Ras binds numerous effectors, which regulate signals through diverse cellular pathways (18).

SIGNALING PATHWAY

The anchored Ras proteins operate as molecular switches, which in the resting cells are in the GDP-bound inactive state. These Ras proteins become activated in response to extracellular receptors by binding GTP, as catalyzed by guanine nucleotide exchange factors (GEFs) son of sevenless 1 and 2 (SOS1 and SOS2) (19). In the GTP-bound active state, Ras interacts effectively with a set of cytoplasmic target or “effector” proteins (20). The Raf-MEK-ERK cascade is the best characterized Ras effector pathway, leading to deregulated cell growth, inhibition of cell death, invasiveness, and induction of angiogenesis (21). The first mammalian effector of Ras to be characterized was the Rapidly Accelerated Fibrosarcoma (Raf). Activated Ras functions as an adapter that binds to the serine/threonine-protein kinase with high affinity and causes translocation to the cell membrane, where Raf activation takes

place (22). Downstream of this, activated Raf phosphorylates and activates mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2), which can both activate the downstream mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (23). They activate ERK1 and ERK2 via phosphorylation of a -Thr-Glu-Tyr- motif in the activation loop. Notably, ERK1/2 is transcription factors that play a direct role in cell changing gene expression to promote growth, differentiation or mitosis (24). In addition, ERKs can translocate to the nucleus and phosphorylate ETS family transcription factors, i.e. ternary complex factor (TCF) Elk-1, serum response factor accessory protein Sap-1a, Ets1, c-Myc, Tal etc. One of the Ras-induced cellular responses regulates the expression of multiple genes, such as the immediate early gene c-fos, which enables the cell to progress through G0/G1 mitogenic signals of the cell-cycle. As a result, Raf-MEK-ERK pathway activation can promote cell-cycle progression (25).

Ras has been found to interact with and activate other effector pathways that phosphatidylinositol 3-kinase (PI3K)-phosphoinositide-dependent serine/threonine protein kinase (Akt)-mammalian target of rapamycin (mTOR) signaling pathway (26). The PI3K-Akt-mTOR signaling pathway is crucial in signaling downstream of Ras as it regulates cell survival. Ras-PI3K controls the activity of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) (27). PDK1 is a serine/ threonine kinase belonging to protein kinases of the AGC kinase superfamily, including APK/PKB PKA, PKG and PKC (28). Akt/PBK regulates numerous cellular functions, including angiogenesis, metabolism, growth, proliferation, survival, protein synthesis, transcription, and apoptosis by Ras-PI3K-PDK1 (29). In addition, PI3K-dependent activation of Ras-related C3 botulinum toxin substrate (RAC) regulates a wide range of universally important cellular responses, including the actin cytoskeleton, cell

survival, cell/cell contacts and adhesion, transcription and translation. RAC activation also potentiates ERK signaling and increases cellular sensitivity to growth factors (30).

Ras activation has also been shown to stimulate the Ral specific guanine-nucleotide-exchange factors (Ral-GEFs). Ral guanine-nucleotide-exchange factors (RalGDS) are a family of guanine nucleotide exchange factors (GEFs) that promote activation of the Ras family member Ral, resulting in activation on phospholipase D1 (PLD1), an enzyme that regulates vesicle trafficking (31). In addition, Ral stimulation leads to activation of RALBP1 (also known as RLIP1 and RIP1). RALBP1 is a GTPase-activating protein (GAP) for CDC42 and Rac GTPases. One notable feature identified for RalBP1 was GAP activity towards the Rho family GTPases Tac1 and Cdc42, thus giving RALBP1 the potential to impact actin dynamics and the formation of filopodia and membrane ruffling (32).

Phospholipase Cε (PLCε) is a modular protein that incorporates GEF, PKC and Ras-binding domains (33). Moreover, phospholipase Cε could link Ras to calcium (Ca^{2+}) mobilization, which has been known to influence cell proliferation and differentiation (34).

RAS MUTATIONS AND INHIBITORS

Activating Ras mutations occur in ~30% of human cancers, and at even higher frequencies in cancers of the pancreas (90%), lung (35%), thyroid gland (55%), colon (45%), and liver (30%) (35). More than 95% of Ras mutations are found in codons (amino acids) Gly12, Gly13, or Gln61 (36). These mutations make the Ras proteins insensitive to GTP-induced hydrolysis of GTP to GDP and lock them in the activated state (37). Activating mutations in Ras induce constitutive signaling to downstream targets, i.e., Ras effectors. Many factors identified as Ras effectors are PI3K, RalGDS,

RIN1/2, PLCε, and TIAM1 (38).

Oncogenic mutations, such as Q61, are mainly observed in the K-Ras gene. A total of 15-25% of lung adenocarcinoma harbors the K-Ras mutation. This peculiarity suggests that each Ras protein plays a distinct role in a tissue-type dependent manner (39). Hence, there may not be a single Ras-targeted therapy that fits all Ras-mutant cancers (40). Some general strategies for anti-Ras drug development have been suggested, including disruption of regulator/effector interactions, inhibition of membrane associations, downstream effectors, synthetic lethal interactions, and metabolism (41). One of the mutant specific inhibitors reported, SML-8-73-1 (SML), was targeted by the guanine nucleotide binding pocket of K-Ras G12C (42). The G12C mutant form of K-Ras is the high frequency of K-ras mutations and low rates of oncogenic changes in either N-ras or H-ras. The reported rate of K-ras mutations in non-small-cell lung cancer (NSCLC) varies from 16% to 40% (43). Treatment of H358 cells with SML-8-73-1 decreases the downstream phosphorylation levels of ERK and Akt when compared to treatment with negative control, suggesting a compound-dependent effect on K-Ras signaling (44). In another study targeted with guanine nucleotide exchange factors (GEFs), SOS1 converts Ras from a GDP-bound (Ras-GDP) to a GTP-bound (Ras-GTP) state (45). The Ras-SOS1 complex was shown to have an α-helix of SOS1 that binds to a pocket located between the SI and SII regions of K-Ras (46). DCAI and HBS3 peptides are designed to inhibit SOS1-mediated nucleotide exchange by blocking the interaction between Ras and SOS1, which inhibits Ras activation in cells (47, 48). However, as with many other previously reported compounds, the GEF inhibitors reportedly bind only weakly to K-Ras, and the discovery of analogues with large improvements in affinity is likely to be a very challenging task. In another study, farnesyltransferase (FTase) inhibitors (FTIs) and

geranyleranyltransferase I (GGTase I) inhibited the Ras membrane association and subcellular localization (49). These compounds were shown to be an effective therapeutic approach for H-Ras mutant cancers. However, the H-Ras mutant frequency is low and the compounds have been shown to exert serious toxicity in normal tissues.

To date, directly blocking oncogenic Ras activity has been a challenging and unsuccessful endeavor. Therefore, past studies have targeted effector pathways downstream of Ras-mediated oncogenesis (50). The Raf-MEK-ERK and PI3K pathways are the best-characterized Ras effector pathways, initiating cascades of protein-protein interactions that may lead to cell proliferation (51). Sorafenib is the first antitumor of multi-kinase inhibitor that targets Raf kinases to be developed (52). This inhibits the activity of several tyrosine kinases involved in tumor angiogenesis and progression, including the vascular endothelial growth factor receptor (VEGFR) family (53).

As acquired mechanisms of resistance to Raf inhibitors are often due to reactivation of ERK, one obvious approach is to use an ERK inhibitor. The ERK inhibitors targeted that ATP-bound pocket of ERK acts in competition with ATP (54). However, ERK inhibitors block ERK feedback phosphorylation and inactivation of Raf, which leads to enhanced MEK activation. Although effector pathways inhibition seems to be the most promising Ras-targeted strategy, considerable challenges remain.

Other biological studies have pointed that abnormal activation of the Ras-Raf-MEK-ERK signaling pathway frequently results in hepatocellular carcinoma (HCC). The second-generation allosteric non-ATP competitive MEK1/2 inhibitor selumetinib is a benzimidazole derivative that has been shown to contribute to the inhibition of ERK1/2 phosphorylation (55). Selumetinib is well tolerated, but not ideal for treatment of advanced HCC. Recent studies

have shown that salinomycin very specifically interferes with the activity of K-Ras. Salinomycin effectively attenuates effector recruitment to K-Ras, which then compromises at least Ras/MAPK signaling and proliferation (56). Inhibition of effector pathways still seems to be the most promising RAS-targeted strategy, and therefore the drug-discovery group works on the identification and characterization of new Ras effector target drugs.

CONCLUSIONS

Fourth decade research in oncogenic Ras, has generated thus far serves as a rich and instructive backdrop for the questions that lie ahead. Nevertheless, we still have a great deal to learn about these cancers before we can confidently treat them effectively. Ras-mediated changes in cell metabolism have recently been described. In the future, many studies will discuss whether these changes could be exploited for new therapeutic directions. Moreover, in 2014, the United States National Cancer Institute launched the Ras Initiative, which is a US $10 million a year effort to identify new ways to tackle Ras-driven cancers (57). In addition researchers are discovering compounds that could yield the first drugs to target Ras proteins. Targeting Ras in human cancer remains a substantial challenge. A wealth of knowledge acquired through experiments will play a crucial role in facilitating inhibitor development. In summary, critical assessment of past efforts coupled with discussions of biochemical properties will enable the development of more effective cancer therapies.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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FIGURE LEGENDS

Figure 1. Regulation of Ras membrane association. A. Ras protein with a CAAX motif at the carboxyl terminus undergoes three post-translational modifications (PTMs). The first modification step is addition of an isoprenyl group to the cysteine of the CAAX motif by farnesyltransferase (FTase). Next, the isoprenylated CAAX protein becomes a substrate for Ras converting enzyme 1 (RCE1), which removes the last three amino acids (the -AAX of the CAAX motif) by endoproteolysis. Finally, the newly exposed isoprenylated cysteine residue is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT). B. After trafficking and association with the inner face of the plasma membrane, Ras proteins cycle between inactive GDP-bound and active GTP-bound states. Growth factors stimulate transient activation of Ras through activation of GEF. Ras-GTP binds preferentially to downstream effectors. GAP accelerates the intrinsic GTP hydrolysis activity, returning Ras to the inactive state.

Figure 2. Regulating signaling downstream of Ras. In the active GTP-bound state, Ras interacts with several families of effector proteins, resulting in stimulation of their catalytic activities. Raf protein kinases activate mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2), which leads to ERK1/2 activation. Phosphoinositide 3-kinases (PI3Ks) generate second-messenger lipids and activate numerous target proteins, including the survival signaling kinase AkT/PDK1. Ras binding activates Ral specific guanine-nucleotide-exchange factors (Ral-GEFs) by targeting them to their substrates, Ral GTPases, which are present in the plasma membrane. Phospholipase Cε (PLCε) catalyses the activation of protein kinase C (PKC) and mobilization of calcium from intracellular stores.

Fig. 1.

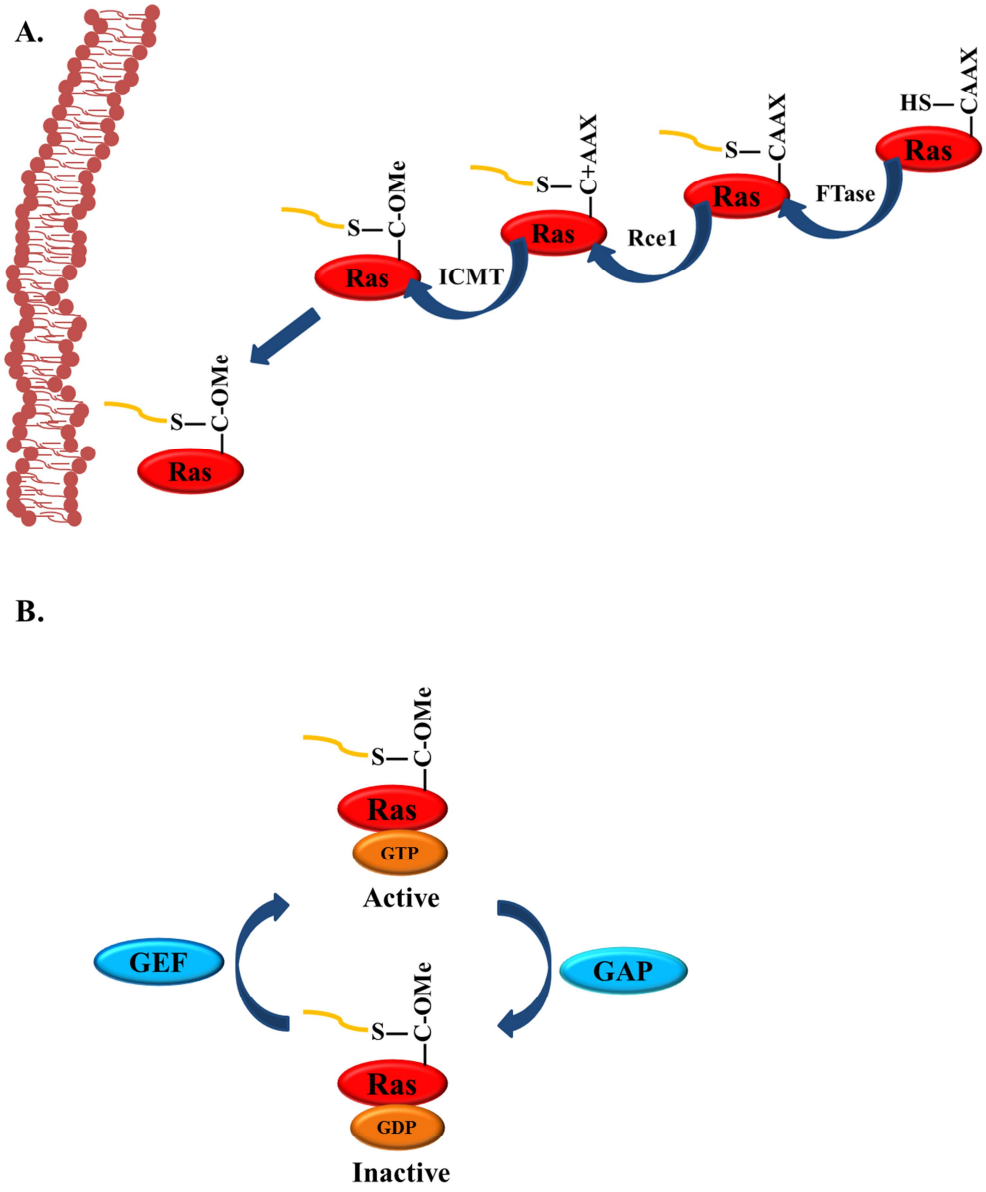
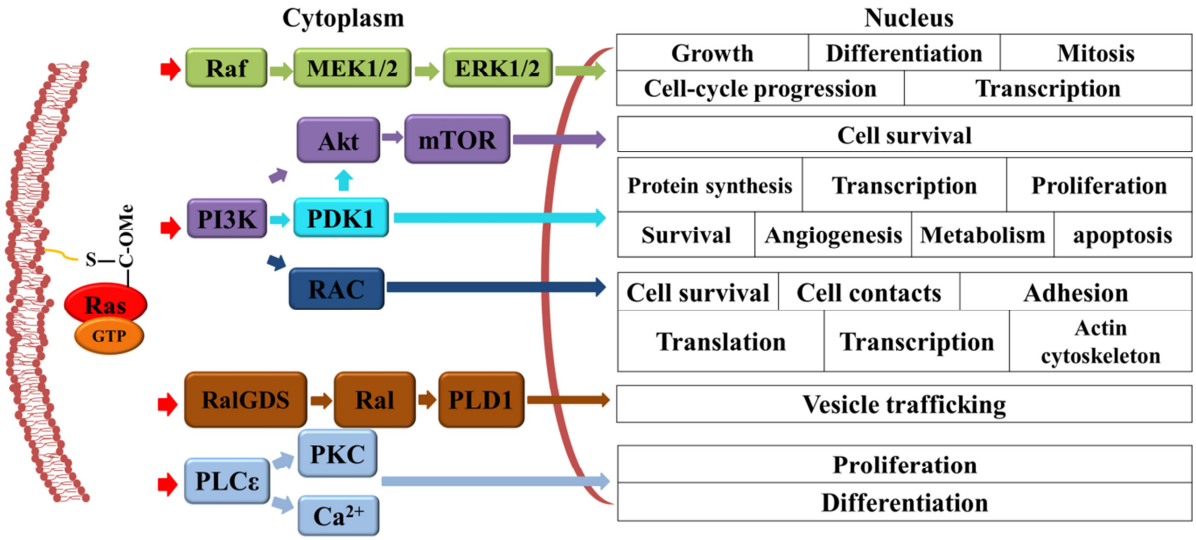

A.
HS—CAAX
Ras
FTase
S—CAAX
Ras
Rce1
S—C+AAX
Ras
ICMT
S—C-OMe
Ras
S—C-OMe
Ras
B.
S—C-OMe
Ras
GTP
Active
GEF
GAP
S—C-OMe
Ras
GDP
Inactive

Fig. 2.


Cytoplasm
Nucleus
Raf
MEK1/2
ERK1/2
Growth
Differentiation
Mitosis
Cell-cycle progression
Transcription
Akt
mTOR
Cell survival
PI3K
PDK1
Protein synthesis
Transcription
Proliferation
Survival
Angiogenesis
Metabolism
apoptosis
RAC
Cell survival
Cell contacts
Adhesion
Translation
Transcription
Actin cytoskeleton
S—C-OMe
Ras
GTP
RalGDS
Ral
PLD1
Vesicle trafficking
PKC
PLCε
Proliferation
Differentiation
Ca2+