

A hypothesis for a novel role of RIN1-the modulation of telomerase function by the MAPK signaling pathway

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Abstract: Cancerous cells display abnormalities in the signal transduction pathways responsible for responding to extracellular growth factors, or mitogens. Mutations that alter proteins involved in these types of pathways can lead to inappropriate or unregulated cell growth, and therefore predispose the cell to become malignant. The critical role of the Ras/mitogen-activated protein kinase (MAPK) pathway in transducing growth signals to the interior of the cell and subsequently stimulating cell growth and proliferation is underscored by the fact that roughly one quarter of all human tumors contain mutant forms of Ras proteins. A particular focus on the signaling and membrane trafficking adaptor protein known as Ras interference 1 (RIN1) will reveal how this protein can potentially play a significant role in the development of the cancerous phenotype in certain cell types. Of equal interest is the possible connection between the Ras/MAPK pathway, and subsequent expression and enzymatic activity of telomerase—a key enzyme known to be overexpressed in roughly 85% of all cancers. RIN1 is a 783 amino acid (84 kDa) cytosolic protein that is involved in key steps of growth factor receptor-mediated endocytosis and can potentially moderate signaling through the MAPK pathways. RIN1, with its unique ability to compete directly with Raf for activation by Ras, could potentially influence signaling through more than one of the MAPK pathways. If so, RIN1 may then be able to exert a precise and selective effect on the downstream signal(s) of a MAPK target such as telomerase.

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Introduction

Many cancerous cells display abnormalities, at least, in the two major signal transduction pathways (Ras/PI3-K/Akt and Ras/MAPKs) responsible for responding to extracellular growth factors, or mitogens. Hanahan and Weinberg (2000), in a comprehensive review of the molecular hallmarks of cancer cells, list growth factor self-sufficiency as one of the six necessary physiological changes needed to convert a normal cell into a malignant cell. The authors also suggest that malignancy is acquired through a series of successive mutations that fall under two broad categories –dominant gain of function oncogenic mutations and recessive loss of function tumor suppressor mutations.

Unlike normal cells that will only grow and divide in response to external growth signals, many cancer cells have acquired the ability to proliferate in the absence of extracellular mitogenic signals. The apparent autonomy is caused by three key factors: (1) Self-production and

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stimulation by growth factors in an autocrine fashion, (2) Mutant growth factor cell surface receptors, and (3) Mutant intracellular signaling proteins (Hanahan and Weinberg, 2000). Thus, it is this third category of deregulation that appears to be found ubiquitously in most tumors and to which mutants of the Ras signaling pathways fall under.

Specifically, the critical role of the Ras/MAPK pathway in transducing growth signals to the interior of the cell and subsequently stimulating cell growth and proliferation is underscored by the fact that roughly one quarter of all human tumors contain mutant forms of Ras proteins (Hanahan and Weinberg, 2000). Therefore, a more detailed discussion of the significance of the MAPK pathway to cancer development is warranted in an effort to convey a more comprehensive understanding of the tumorigenic process.

MAPK Signaling Pathways in Cancer

At this point, it would be helpful to review the major steps of the Ras/MAPK pathway before proceeding to a more detailed consideration of the roles that downstream mediators of this widespread and critical cell signaling pathway play in cancer cell biology. The Ras/MAPK pathway is one of the principal means by which extracellular and/or mitogenic signals are transduced from the surface of the cell to the cell's interior (Fang and Richardson, 2005). The response often culminates in the nucleus with the transcription and expression of target genes that regulate cellular proliferation, differentiation, and development (Seger and Krebs, 1995; Wang et al., 2002).

The interaction of a particular growth factor with its cell surface transmembrane receptor (also known as a receptor tyrosine kinase-RTK-) sets into motion a series of events that result in the activation of several different cytoplasmic protein kinases. RTK activation by binding of a growth factor ligand induces dimerization followed by auto-transphosphorylation of specific tyrosine residues within the receptor. These phospho-tyrosine residues are then recognized and bound by an adapter protein such as Grb2 through its SH2 domains (Seger and Krebs, 1995). Activation of Ras through the exchange of GDP for GTP by a specific guanine nucleotide exchange factor (GEF) protein known as Sos, then initiates the activation of a cascade of cytoplasmic serine-threonine protein kinases which include RAF1, MEK, and ultimately ERK. Each of these kinases is activated in sequential order by the protein kinase immediately preceding it in the pathway. Once phosphorylated and activated, ERK then proceeds to activate a number of nuclear proteins involved in regulating cell growth and proliferation. The regulation often occurs at the level of transcription.

One of the primary targets of activation by ERK are various types of transcription factors, including the E-twenty-six (Ets) family of transcription factors as well as c-Myc and c-Fos (Fang and Richardson, 2005). These transcription factors are potent stimulators of cellular proliferation (Seger and Krebs, 1995). Mut et al. (2012) provide evidence of the importance of the Ras/MAPK pathway in the activation of the E-twenty-six like transcription factor 1 (Elk-1) in U138 glioblastoma

multiforme cells. The researchers demonstrate that these cells have a high basal proliferative rate that can be reduced in the presence of specific MEK or ERK inhibitors. The authors hypothesize that inhibition of the Ras/MAPK pathway with these types of enzymatic inhibitors prevents the ultimate phosphorylation and activation of Elk-1, which in turn prevents the transcription of specific early cellular proliferation genes such as c-Fos. Activated Elk 1 exerts its effect in the nucleus by binding to the promoters of genes containing a serum response element (SRE) motif. The expression of a number of important cell cycle stimulatory genes, such as Egr1 and c-Fos, can be stimulated by Elk-1. The results also indicate that stimulation with EGF results in a corresponding increase in the proliferative rate, which is most likely mediated by Elk-1 (Mut et al., 2012). For example, the knockdown of Elk-1 through the use of siRNA does not result in an increase in proliferation even after EGF stimulation in U138 cells. The results also suggest that the PI3-K/Akt pathway plays an important role in the regulation of Elk-1 activity. Inhibitors of this pathway do not prevent the phosphorylation of either ERK or Elk-1 in the cytoplasm following EGF stimulation, but rather prevent the movement of these phosphorylated proteins from the cytoplasm into the nucleus (Mut et al., 2012). The step is obviously necessary to produce the full mitogenic response to EGF stimulation.

Given the central role of the Ras/MAPK pathway in the proliferative response of cells to growth signals, it is logical that abnormalities in any one of its molecular components could lead to the uncontrolled cellular division characteristic of so many different cancers. For example, Fang and Richardson (2005) discuss the significance of the Ras/MAPK pathway in promoting growth, proliferation, and tumorigenesis in intestinal epithelial cells. Indeed, the importance of abnormalities in the MAPK pathway in promoting tumorigenesis is highlighted by the fact that this pathway is deregulated in about 30% of all cancers (Fang and Richardson, 2005). Abnormal MAPK signaling in colorectal cancer typically begins at the surface of the cell with overexpression and activation of EGF receptors. Additionally, protein kinase C (PKC), when activated, can also facilitate the binding of GTP to Ras, which ultimately leads to activation of the MAPK pathway.

Like many other types of cancers, the development of colorectal cancer is a multi-step process involving mutations in specific cell cycle regulatory or signaling genes. Mutations in Ras, specifically the K-Ras isoform, are known to be an early step in colorectal carcinogenesis along with mutations in certain protein kinases such as BRAF (Fang and Richardson, 2005). Hyperactive MAPK signaling through EGF-receptor overexpression or mutant protein kinases in turn leads to the activation of various transcription factors, as mentioned earlier, which promotes cell growth and proliferation. Additionally, unusual MPK signaling can also induce the expression of VEGF, which aids in tumor invasiveness and metastasis by promoting angiogenesis. Invasiveness of colorectal cancer might also be linked to increased synthesis of matrix metalloproteinases such as MMP7, which has been associated with abnormal MAPK signaling (Fang and Richardson, 2005). Given the key

oncogenic role of the Ras/MAPK pathway in a variety of cancers, it is no surprise that a number of MEK and ERK inhibitors are currently being tested as possible chemotherapeutic agents.

RIN1 as a Key Effector in the MAPK Pathway

Besides anomalous cell signaling through the Ras/MAPK pathway, there is a growing body of evidence that suggests that RIN1 might be critical in modulating the cell's response to mitogenic signals (Wang et al., 2002; Smith and Ikura, 2014). The ability of RIN1 to have a moderating effect on signal transduction through the Ras/MAPK pathway lies in its ability to be activated by Ras. In fact, RIN1 has been shown to compete directly with RAF1 for activation by Ras (Wang et al., 2002), and this selective competition could have important implications for cancer biology research. For instance, could RIN1 be a useful target to help dampen or modify excessive signaling through the Ras/MAPK pathway in certain cancer cell lines? If so, could one then reduce the downstream proliferative response induced by the Ras/MAPK pathway as a result of growth factor stimulation? We postulate that RIN1, due to its strong binding affinity for activated Ras, may be able to attenuate signaling through the MAPK pathway and thus potentially regulate the function of a key downstream MAPK effector such as telomerase. Recent experimental findings from our laboratory examining telomerase activity indicate that this as a distinct possibility. Before addressing these possibilities, it is necessary to first review the functional role of RIN1 at the cellular level.

An interesting study by Milstein et al. (2007) indicates that RIN1 behaves as a tumor suppressor in breast cancer cells and that RIN1 expression is reduced in tumorigenic cells when compared to normal breast epithelial cells. The authors report that RIN1 expression is inhibited by overexpression of the repressive transcription factor SNAI1 in ZR75-1 breast cancer cells. Additionally, methylation of the RIN1 promoter in

KPL-1 breast cancer cells is observed to silence RIN1 expression and contribute to the cancerous phenotype. Knockdown of SNAI1 or demethylation of the RIN1 promoter each restored RIN1 expression with the result of inhibiting key tumorigenic processes (Milstein et al., 2007).

Han et al. (1997) and Wang et al. (2002) studied the biochemical properties of RIN1 and reported that RIN1 is an important downstream effector of activated Ras. RIN1 is able to bind to Ras through its Ras binding domain (RBD), which associates with an effector domain within Ras. Furthermore, biochemical analysis reveals that RIN1 has a high binding affinity for activated Ras and that it competes strongly with RAF1 for access to Ras. Molecular studies also indicate that RIN1 binds to 14-3-3 proteins in the cytoplasm and is able to interact with and be phosphorylated by the tyrosine kinase c-ABL.

The ability of RIN1 to bind to Ras lies in its carboxyl-terminal domain, which contains a 433 amino acid sequence (between residues 294 to 727, Fig. 1) necessary for Ras binding (Han et al., 1997). Different Ras effector proteins, such as RIN1 and RAF1, can vary significantly in terms of their overall primary structure but often display a high degree of similarity in the regions required for Ras binding (Ras binding domains). The Ras binding domains of effector proteins typically interact with a short amino acid effector sequence within Ras itself, and this interaction is heavily dependent on Ras being in its activated GTP-bound state. For RIN1, the carboxyl-terminal domain (or Ras Association-RA-Domain) mediates binding to both GTP-Ras and 14-3-3 proteins (Wang et al., 2002).

The affinity of RIN1 for Ras can be seen in experiments utilizing antibodies against the GTP bound form of Ras. For example, RIN1 is co-immunoprecipitated with an overly active mutant allele of H-Ras in NIH 3T3 cells when treated with a particular Ras antibody. When the same antibody is pre-attached to Ras, RIN1 is not co-immunoprecipitated. The use of antibodies that bind to the switch II region of Ras also prevents the binding of RIN1. Additionally, the

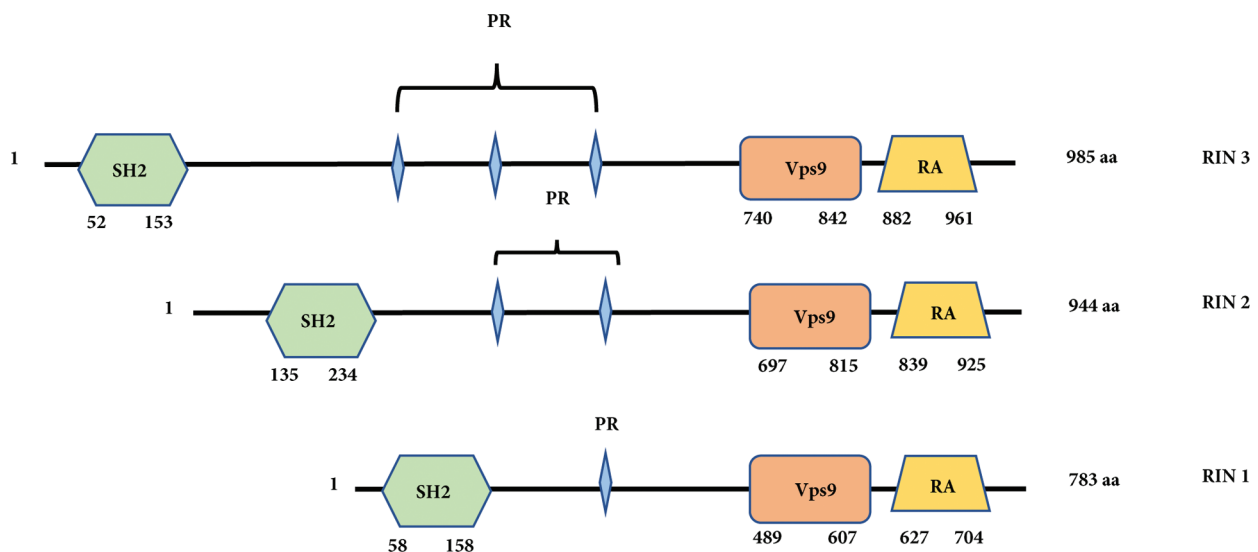


FIGURE 1. These Rin domains are human Rin (1,2,3) based on isoform 1 sequences. SH2 binds phospho-tyrosine residues. Vps9 acts as a guanine nucleotide exchange factor for Rab5. RA binds to activated Ras. Proline-rich (PR) binds to SH3 domains.

effector binding domain within Ras itself is equally important in mediating the proper attachment of certain effector proteins to Ras (Han *et al.*, 1997). The point is illustrated by the fact that mutations in the Ras effector binding domain can selectively inhibit the binding of certain Ras effector proteins without affecting the binding of others. RIN1, for instance, can interact with a constitutively active mutant form of H-Ras (H-Ras^{V12}). However, when this mutant form of H-Ras acquires additional mutations at amino acid positions 35 or 40, RIN1 binding is completely inhibited. The position 35 mutation, on the other hand, does not interfere with RAF1 binding, while a mutation at position 37 permits RIN1 association but prevents RAF1 binding (Han *et al.*, 1997). The ability of full-length RIN1 to interact with Ras is further highlighted by a variant form of RIN1 in which there is a 62 amino acid deletion. The naturally occurring truncated form of RIN1, known as RIN1 delta (D), is the result of alternative splicing of the RIN1 mRNA. This RIN1:D mutant exhibits a much weaker binding affinity for Ras when compared to full-length RIN1 (Han *et al.*, 1997).

Besides interaction with Ras, the carboxyl-terminal domain of RIN1 also mediates binding to 14-3-3 proteins (Han *et al.*, 1997; Wang *et al.*, 2002). These small acidic proteins exist as multiple isoforms in the cytoplasm (epsilon, beta, and zeta) and have been shown to be involved in mitogenesis and malignant transformation of cells through their interactions with signaling proteins. Both RAF1 and RIN1 share the ability to bind to 14-3-3 proteins via their Ras binding domains, and this binding typically results in the activation of RAF1 through a Ras-dependent mechanism that seems to enhance signal transduction functions (Wang *et al.*, 2002). Since RIN1 competes directly with RAF1 for access to Ras, the binding of RIN1 to 14-3-3 proteins would reduce its potential to bind to Ras and thus allow for heightened signaling through the Ras/MPAK pathway. The binding of RIN1 to cytoplasmic 14-3-3 proteins would promote the oncogenic properties of cell growth and proliferation as a result of the increased access of RAF1 over RIN1 for activation by Ras. Interestingly, the deletion of the Ras binding domain within the carboxyl-terminal of RIN1 prevents RIN1 from binding to all isoforms of 14-3-3 proteins, as does the 62 amino acid deletion in the naturally occurring RIN1:D mutant (Wang *et al.*, 2002).

The interaction of RIN1 with 14-3-3 proteins appears to be largely controlled by a serine residue at position 351 within the Ras binding domain. The serine residue is phosphorylated predominantly by protein kinase D (PKD), and its phosphorylation is required for proper binding to 14-3-3 proteins (Wang *et al.*, 2002). A mutation that substitutes alanine at this position blocks interaction with 14-3-3 proteins and results in an increased ability to suppress signaling through the Ras/MAPK pathway, presumably caused by an increased capacity to compete with RAF1 for access to Ras. The phosphorylation of serine 351 of RIN1 and subsequent attachment to 14-3-3 proteins may act as a suppression control mechanism in cells to unlink RIN1 from activated Ras by sequestering it in the cytoplasm (Wang *et al.*, 2002).

An important point is raised here; for RIN1 to effectively compete with RAF1 for access to Ras, it must be in the proper

subcellular location. Wild type RIN1 in its non-phosphorylated state is weakly associated with the cell membrane and is in close proximity to interact with Ras, which is tightly associated with the plasma membrane. Also, the alanine substitution at position 351 in the mutant form of RIN1 allows for a significant shift to the plasma membrane, which may help to explain its suppressive effect on Ras signaling (Wang *et al.*, 2002). However, when phosphorylated by PKD, wild type RIN1 is confined to the cytoplasm bound to 14-3-3 proteins. The phosphorylation of RIN1 by PKD therefore reduces its capacity to compete with RAF1 for binding to Ras.

The amino-terminal of RIN1, like the carboxyl-terminal, plays an important role in mediating cell signaling through its ability to bind to the tyrosine kinase c-ABL (ABL1). ABL tyrosine kinases are known to be involved in various cellular functions including differentiation, division, migration, and adhesion (Hu *et al.*, 2005). Additionally, the amino-terminal of RIN1 contains an SH2 domain capable of interacting with phospho-tyrosine residues on an activated receptor tyrosine kinase such as the EGF-receptor (Barbieri *et al.*, 2004). RIN1 interacts with c-ABL most likely through a proline-rich sequence in its amino-terminal end and an SH3 domain in c-ABL (Han *et al.*, 1997). Upon binding to c-ABL *in vitro*, RIN1 becomes tyrosine phosphorylated and can then subsequently bind to an SH2 domain within c-ABL. The interaction does not seem to affect the catalytic activity of the enzyme however, and studies have shown that RIN1 has very little interaction with c-ABL *in vivo*. This reduced interaction is caused in part by the different cellular locations of the two proteins, with c-ABL being confined mostly to the nucleus and RIN1 to the cytoplasm (Han *et al.*, 1997).

One interesting exception to this is the oncogenic BCR/ABL fusion protein produced as a result of a translocation between chromosomes 9 and 22. The BCR/ABL is an unregulated tyrosine kinase that is localized primarily to the cytoplasm, where it stimulates cellular proliferation. The BCR/ABL is therefore in the correct location to interact with RIN1 and, indeed, RIN1 is able to bind to BCR/ABL. RIN1 appears to accentuate the tumorigenic, transforming properties of BCR/ABL (Wang *et al.*, 2002; Hu *et al.*, 2005; Afar *et al.*, 1997). Additionally, Hu *et al.* (2005) report that RIN1 is an activator of the ABL2 tyrosine kinase that is involved in the regulation of epithelial cell adhesion and migration. Specifically, RIN1 activation of ABL2 promotes phosphorylation of the adaptor proteins CRK and CRKL. This phosphorylation, in turn, produces conformational changes in CRK and CRKL, which influences cytoskeletal elements to inhibit cell motility. Cells deficient in RIN1 display reduced levels of phosphorylated CRKL and increased cell motility (Hu *et al.*, 2005).

Clearly, the biochemical profile of RIN1 suggests that it potentially plays a key role in modulating signaling through the Ras/MAPK pathway, given its ability to interact with multiple different effector proteins. The greatest capacity for RIN1 to moderate abnormal signaling, as detailed above, appears to lie in its ability to directly compete with RAF1 for access to Ras. The competition, in turn, could be a useful mechanism for dampening or attenuating signaling through the Ras/MAPK pathway by diverting the signal

away from effector proteins downstream of Ras. The exact role that Rin1 plays in tumorigenesis remains unclear. For instance, [Milstein et al. \(2007\)](#) describe a tumor suppressor role for Rin1 in breast cancer cell lines. On the contrary, elevated levels of RIN1 may be associated with increased tumorigenesis and lower survival, as is reported by [Wang et al. \(2012\)](#) for non-small cell lung cancer. It should be noted that although RIN1 is thought to be expressed in most tissues, its expression is highest in brain tissues ([Han et al., 1997](#)). The differential level of RIN1 expression could have important implications for research when investigating RIN1 function in various tissues and/or cell lines ([Han et al., 1997](#)).

RIN1 as an Early Modulator for Leading Telomerase Activity

In addition to its ability to moderate mitogenic signaling through the Ras/MAPK pathway, RIN1 also acts as a guanine nucleotide exchange factor (GEF) for the small monomeric GTPase known as Rab5 ([Tall et al., 2001](#)). The Rab proteins are a diverse group of proteins that belong to the Ras superfamily of small GTPases and play critical roles in regulating the steps of endocytic vesicular transport. Specifically, Rab proteins regulate vesicular traffic from the plasma membrane by controlling cargo selection, vesicle formation, transport along the cytoskeleton, and fusion with intracellular target membranes ([Stein et al., 2003](#); [Hutagalung and Novick, 2011](#); [Stenmark and Olkkonen, 2001](#)). Certain Rab proteins also control the sorting of molecules for return to the plasma membrane or for degradation in lysosomes. The ability of Rab proteins to regulate the many complex steps of vesicular transport lies in their selective activation. There are approximately 70 or 90 different Rab proteins encoded in the human genome, each of which is selectively activated by binding GTP. Once activated, a particular Rab protein serves as a scaffold for the attachment of various effector proteins, which then subsequently direct the completion of a specific step in the endocytic pathway.

Rab5, which is activated by RIN1, has been shown to regulate vesicle budding and cargo selection from clathrin-coated pits as well as early endosome fusion ([Stein et al., 2003](#); [Hutagalung and Novick, 2011](#); [Stenmark and Olkkonen, 2001](#)). [Tall et al. \(2001\)](#) report that RIN1 and Rab5 also play a crucial role in the receptor-mediated endocytosis of epidermal growth factor (EGF)-receptor, following stimulation by EGF. The endocytosis of EGF-receptor occurs through a Ras-mediated mechanism involving upstream activation of RIN1 by GTP-Ras and then subsequent activation of Rab5 by activated RIN1.

The pivotal integrative role of Rab proteins in signal transduction is further illustrated in a study by [Barbieri et al. \(2004\)](#) examining the role of Rab5 in EGF-receptor mediated MAPK signal transduction. RIN1 interacts with Rab5 via its Vps9 domain, which also contains the GEF activation function for Rab5 ([Galvis et al. 2009a](#)). [Galvis et al. \(2009b\)](#) have previously reported the key function of the Vps9 domain of RIN1 in activating Rab5 through a series of mutational studies. [Barbieri et al. \(2004\)](#) demonstrate that a dominant-negative mutant form of Rab5 (Rab5:S34N) is capable of inhibiting activation of the MAPK

pathway in mouse NR6 cells by preventing both the endocytosis and internalization of the EGF-receptor. The inhibition is particular to the MAPK pathway and does not interfere with other EGF induced kinase pathways.

On the other hand, however, expression of wild type Rab5 or the RIN1 delta splice variant leads to increased MAPK activity and increased cyclin D1 expression after EGF stimulation- resulting in heightened cellular proliferation. The authors suggest that Rab5 activation is a key step in linking EGF stimulated endocytosis to signal transduction through the MAPK pathway ([Barbieri et al., 2004](#)). Although the exact biochemical link between Ras association and the Rab5 GEF activity of RIN1 is not entirely clear, the binding of Ras by RIN1 appears to strongly influence EGF-receptor endocytosis.

In a manner similar to EGF-receptor endocytosis, the internalization of insulin receptor following the binding of insulin may involve other steps including Rab5 activation by RIN1 ([Hunker et al., 2006](#)). It is interesting to note that RIN1 sits at the intersection between cell signaling and receptor-mediated endocytosis for mitogens such as EGF and insulin. The increased rate of receptor-mediated endocytosis potentiated by Ras stimulation of the Rab5 GEF activity of RIN1 may be an important negative feedback mechanism by which Ras can divert signaling away from downstream effectors through RIN1. In this model, an increased rate of receptor-mediated endocytosis would favor the quick removal of receptors from the plasma membrane, followed by internalization and degradation ([Hunker et al., 2006](#)). The end result would be an attenuation of mitogenic signal transduction much in the same way as can be achieved by the direct competition of RIN1 with RAF1 for access to Ras. A number of Ras effector proteins have been shown to contain either a Ras association (RA) domain or a Ras binding domain (RBD). Although not identical in terms of sequence homology, these two domains do share a common ubiquitin fold structure that enables interaction with the surface of Ras ([Erijman and Shifman, 2016](#); [Takala and Ylanne, 2012](#)). In a study of integrative Ras signaling, [Smith and Ikura \(2014\)](#) used NMR probes to determine a preferential Ras binding hierarchy among equal concentrations of various Ras effector proteins. The authors report that wild-type Ras binds preferentially to BRAF, followed closely by Rin1 ([Smith and Ikura, 2014](#)).

Having examined the biochemical properties of RIN1 and how it fits into the larger signal transduction machinery of the Ras/MAPK pathway, it would be useful to elaborate on the possible connection between RIN1 and specific downstream effectors of the Ras/MAPK pathway known to induce cellular proliferation. These downstream targets, while varied, include the Ets family of transcription factors mentioned earlier as well as the telomerase reverse transcriptase enzyme. It is intriguing to wonder if RIN1, given its ability to compete directly with RAF1 for access to Ras, could have any influence on the cellular proliferation associated with classical MAPK targets such as the Ets transcription factors and/or telomerase.

The signaling pathways that have been implicated in the stimulation of telomerase expression and activity usually

involve the response to a mitogen such as EGF or IGF-1, and subsequent activation of a number of protein kinases belonging to the Ras/MAPK and PI3-K/Akt pathways (Inui *et al.*, 2002; Seimiya *et al.*, 1999; Zhou *et al.*, 2013). It is feasible then that RIN1 could potentially have an effect on telomerase expression, given its role as a Ras effector molecule. If so, this raises the exciting idea of RIN1 as a potential therapeutic target in specific cancers. To appreciate the role of RIN1 in a therapeutic context, it is necessary to investigate what effect the regulation of the telomerase reverse transcriptase enzyme and the Ets family of transcription factors have on cellular proliferation.

Telomere Structure and Function

Telomeres are the regions of DNA that exist at the very tips or ends of linear chromosomes in eukaryotic cells. Recent evidence suggests that the telomere regions of chromosomes play vital roles in regulating normal cellular processes such as proliferation, aging, and senescence/apoptosis (Ramlee *et al.*, 2016; Shay, 2018). The telomeric regions of chromosomes set a replicative limit on the number of cell divisions a normal cell can undergo before the induction of senescence and/or apoptosis occurs (Chung Low and Tergaonkar, 2013; Maciejowski and de Lange, 2017). The finite replicative capacity, which is approximately 50 to 70 rounds of cell division for most normal somatic cells, is largely due to the progressive loss of telomeric DNA with each round of cell division (Zvereva *et al.*, 2010). The loss of telomeric DNA that accompanies each cycle of cell division is a consequence of incomplete DNA replication at the telomeres (Jafri *et al.*, 2016).

The telomere regions of vertebrate chromosomes are defined by a distinct structure that distinguishes telomeres from other areas of chromosomal DNA. In humans, a highly repetitive sequence of TTAGGG approximately 5 to 15 kb in length is tightly associated with a complex of six telomeric DNA binding proteins termed a shelterin complex (Stewart *et al.*, 2012). The shelterin protein complex is critical for maintaining genomic stability at the telomeres and consists of the following six proteins: TRF1, TRF2, TIN2, TPP1, POT1, and RAP1 (Stewart *et al.*, 2012). TRF1 and TRF2 (telomeric repeat factor-binding protein) each form separate homodimers that bind to the double-stranded DNA regions of telomeres. Both TRF1 and TRF2 act as negative regulators of telomere length and are linked to each other by the TIN2 linker protein (Walker and Zhu, 2012). The POT1 (protection of telomeres) binds to the 3' single-stranded guanine-rich overhang and mainly functions to inhibit the activation of DNA damage response pathways at the telomeres. The POT1 is connected to the other shelterin complex proteins through the linker protein TPP1 which binds to both POT1 and TIN2 (Heidenreich and Kumar, 2017). The RAP1 is a small protein that binds to TRF2 and aids TRF2 in preventing non-homologous end joining and chromosomal fusions (Stewart *et al.*, 2012).

The shelterin complex, which assists in the formation of T-loops of telomeric DNA, functions as a protective cap that prevents the activation of cellular DNA damage responses that would otherwise recognize the telomeres as double-stranded

DNA breaks (de Lange, 2018; Stewart *et al.*, 2012; Buseman *et al.*, 2012). The major role of telomeres is to maintain genomic stability by acting as a buffer for the gradual erosion of DNA that accompanies each cycle of cell division. Each shelterin complex protein plays a clear role in telomere maintenance, and the loss of any one protein will result in reduced telomere protection (de Lange, 2018; Bandaria *et al.*, 2016; Erdel *et al.*, 2017).

Telomerase is only expressed and active in a relatively small population of somatic stem cells where the depletion of telomeric DNA and the resulting onset of senescence or apoptosis would interfere with the normal functioning of specific tissues (Chung Low and Tergaonkar, 2013). On the other hand, the immortal replicative phenotype that is characteristic of the vast majority of cancer cells is attributed to high levels of telomerase activity. In fact, roughly 85% of all cancers display telomerase activity, and the activation of telomerase activity in these cells is a key step in the tumorigenic process (Zhu *et al.*, 2010; Schmidt *et al.*, 2016). Consequently, the selective inhibition of telomerase activity in cancer cells has emerged as an attractive therapeutic target (Holysz *et al.*, 2013; Buseman *et al.*, 2012).

Telomerase Expression and Regulation

Normal somatic cells lose approximately 50 nucleotides of telomeric DNA with each cycle of cell division as a result of incomplete DNA replication during the S phase. The replication problem is overcome in certain cells by extension of the 3' end of the template DNA strand by the telomerase enzyme. Telomerase is a ribonucleoprotein that functions as a reverse transcriptase by adding sequential repeats of the hexameric sequence TTAGGG to the 3' end of the template DNA strand; and once extended by telomerase, DNA polymerase α -primase (PaP) can then complete replication of the lagging daughter strand (Hockemeyer and Collins, 2015).

The functional telomerase holoenzyme consists of two essential parts: an enzymatic protein component known as TERT that acts as a reverse transcriptase and an RNA template component known as TR (Heidenreich and Kumar, 2017). The TERT mRNA is first synthesized and processed in the nucleus and then exported to the cytoplasm for translation into protein. The TERT is then subsequently moved into the Cajal bodies where it assembles with TR to form the final functional telomerase holoenzyme. The active telomerase enzyme will then be recruited to the telomeres at the appropriate time for telomeric DNA synthesis (Hockemeyer and Collins, 2015; Podlevsky and Chen, 2012).

Interestingly, the regulation of telomerase activity occurs mainly at the level of TERT transcription as TERT mRNA synthesis is highly regulated in most somatic cells (Leao *et al.*, 2018). The synthesis of TERT mRNA appears to be the rate-limiting step in the regulation of telomerase activity as TERT gene transcription is highly repressed in most somatic cells (Zhu *et al.*, 2010; Zhang *et al.*, 2016). The telomerase RNA template (TR), however, is ubiquitously expressed in many cell types (Daniel *et al.*, 2012). The TERT core promoter contains binding sites for several key

transcription factors known to regulate TERT transcription—these include c-Myc, SP1, ER, Ets, AP1, and NF- κ B (Zhu *et al.*, 2010). The c-Myc binds to two E-box consensus sequences within the TERT promoter and is strongly linked to activation of TERT transcription. Similarly, SP1 plays a critical role in activating TERT transcription as the mutation of SP1 binding sites in the TERT promoter significantly reduces TERT expression (Cifuentes-Rojas and Shippen, 2012). Two estrogen response elements are located within the TERT promoter upstream of the transcription start site and enhance TERT expression when bound by ER α (Daniel *et al.*, 2012). On the other hand, TERT expression is negatively regulated by the binding of tumor suppressor proteins such as Wilm's tumor-1 (WT1) protein. Additionally, p53 expression acts to inhibit TERT transcription. The p53 tumor suppressor protein has been shown to interact with both human telomerase-associated protein 1 (hTEP1) and SP1 to inhibit TERT promoter activity (Lu *et al.*, 2013).

When discussing transcriptional control of telomerase expression, it is necessary to examine the hTERT promoter. It is well established that the c-Myc transcription factor is strongly linked to cellular proliferation, and it comes as no surprise that telomerase expression is upregulated by c-Myc. c-Myc is able to interact directly with the hTERT promoter and stimulate hTERT expression. Transcription factors such as c-Myc stimulate gene expression by binding to specific sequences within the promoter region of a gene, and many cancer cells often accumulate mutations within the promoters of key cell cycle regulatory genes. In a large-scale study of 799 tumor samples, Huang *et al.* (2015) identified two specific mutations that were present in the hTERT promoter in a high percentage of the tumor samples. Specifically, these mutations C228T and C250T occur at 124 and 146 bp upstream of the hTERT translation start site and are prevalent in many different types of tumors. The authors conclude that each mutation creates a new binding site for the E-twenty-six (Ets) group of transcription factors which subsequently results in the upregulation of telomerase expression. Upregulation of telomerase activity has also been reported as a consequence of the hypoxia commonly found in solid tumors, where the enzyme may aid in the stabilization of chromosomal damage induced by the low oxygen environment (Seimiya *et al.*, 1999). Increased telomerase expression has also been observed in hepatocytes undergoing cell division in response to partial removal of liver tissue, and this expression is stimulated by both EGF and hepatocyte growth factor (HGF) (Inui *et al.*, 2002). In each instance, the upregulation of telomerase expression was a direct response to signaling through the MAPK pathway.

The central role that the MAPK signaling pathway plays in telomerase activation is illustrated in a study by Maida *et al.* (2002). The effect of EGF stimulation on telomerase expression was investigated by exposing A-431, ME180, MCF-7, and NIH3T3 cells to EGF for various periods of time, and then assessing hTERT mRNA expression. There was a significant increase in hTERT mRNA expression in all the cell types between 6 to 12 h after EGF exposure. The increase in hTERT mRNA expression in response to EGF stimulation was confined to these cell types which

constitutively express telomerase and was not observed in telomerase negative cell types. The authors conclude the MAPK pathway is primarily responsible for EGF induced telomerase expression. A-431 cells stimulated with different concentrations of EGF for either 15 or 30 min showed a marked increase in ERK activity and hTERT expression. Exposure to the MEK inhibitor U0126 abolished this effect but exposure to PI3K or p38 inhibitors did not. Furthermore, the authors demonstrate that EGF exerts its influence on telomerase expression through the MAPK pathway via activation of the Ets family of transcription factors. This group of transcription factors is phosphorylated and activated by Erk, and the hTERT promoter contains two Ets binding motifs within it at the -23 and -18 positions. A-431 cells co-transfected with a wild-type Ets2 expression vector and an hTERT promoter-reporter plasmid revealed high levels of EGF induced transactivation when compared to cells co-transfected with a truncated, dominant-negative Ets2 expression plasmid. A similar result was observed in cells transfected with either a normal hTERT promoter-reporter plasmid or a plasmid containing mutations in the Ets binding motifs (Maida *et al.*, 2002).

The MPAK pathway has also been implicated in the activation of telomerase activity and hTERT mRNA expression in estrogen receptor α (ER α) positive endometrial cells. Zhou *et al.* (2013) report that stimulation of Ishikawa ER α positive endometrial cells with estradiol (E2) results in increased phosphorylation p44/42 MAPK and increased hTERT mRNA expression. The hTERT promoter contains two binding sites for E2-ER α complexes, and the stimulatory effect of E2 on hTERT expression and telomerase activation was greatly reduced by exposure to the MEK inhibitor U0126 or ERK specific siRNA (Zhou *et al.*, 2013). The MAPK pathway therefore appears to play a prominent role in the estrogen-induced regulation of hTERT expression and telomerase activity.

Telomerase activity can also be regulated to a lesser extent by post-translational modifications of the catalytic TERT subunit, such as phosphorylation and ubiquitination. Multiple protein kinases like c-Abl, protein kinase B (PKB) (also known as Akt), and protein kinase C (PKC) are able to influence telomerase activity (Wojtyla *et al.*, 2011). Tyrosine phosphorylation of TERT by c-Abl tends to reduce TERT activity while phosphorylation of serine/threonine residues by Akt tends to stimulate TERT activity. Protein phosphatase 2A has been shown to reduce telomerase activity in specific cell types as TERT phosphorylation is necessary for its import into the nucleus. The TERT stability and half-life in the cytoplasm are controlled by E3 ubiquitin ligases that can target TERT for proteolytic degradation and thus prevent its entry into the nucleus (Cifuentes-Rojas and Shippen, 2012). Several splice variants of human TERT are known to exist each with differing levels of activity.

Epigenetic regulation of the TERT promoter figures prominently for TERT expression as the TERT promoter is located within a region of highly condensed chromatin (Cifuentes-Rojas and Shippen, 2012; Lewis and Tollefsbol, 2016). Both hypoacetylated TERT promoter and CpG island methylation are also commonly associated with the silencing

of TERT expression. Conversely, methylation of lysine 9 of histone 3 (H3K9) increases TERT expression, as does the hyperacetylation of other core histones through the inhibition of histone deacetylase complexes (HDACs) (Zhu *et al.*, 2010). Several transcription factors are known to epigenetically regulate TERT expression through the recruitment of histone acetyltransferases (HATs) or HDACs to the TERT promoter (Lu *et al.*, 2013).

Telomerase expression and activity can be modulated by various signal transduction pathways. Wnt transcription factor to stimulate expression of both cyclin D and c-Myc. c-Myc is a well-documented activator of TERT expression (Wu *et al.*, 2013). Inflammation is commonly associated with many different cancers, and NF- κ B is a primary regulator of chronic inflammation linked with tumorigenesis and cancer progression. Recent evidence suggests that a reciprocal relationship may exist between NF- κ B activity and TERT expression. NF- κ B can bind upstream of the TERT transcription start site to stimulate TERT expression. The TERT, on the other hand, can reinforce NF- κ B activity by binding to the p53 subunit of NF- κ B to enhance transcription of inflammatory genes such as IL-6 and TNF- α (Ghosh *et al.*, 2012). Additionally, the RAP1 shelterin complex protein is a key regulator of NF- κ B activity (Martínez and Blasco, 2011).

Other signal transduction pathways such as the PI3K/Akt and MAPK pathways also contribute significantly to the regulation of TERT expression and activity. The Akt-mediated phosphorylation of specific effector proteins promotes the proteolytic degradation of p53 and the c-Myc competitor protein MAD1 (Peek and Tollefsbol, 2016). Additionally, the PI3K/Akt pathway has been tied to NF- κ B activation as well as the inhibition of TGF- β signaling through Jab1 activation and SMAD4 degradation (Daniel *et al.*, 2012). The TGF- β signaling pathway is an important inhibitory pathway that mediates cell growth, differentiation,

and proliferation. TGF- β signaling can suppress c-Myc expression but is susceptible to inhibition by estrogen (Peek and Tollefsbol, 2016). Heeg *et al.* (2011) report that EGF-receptor overexpression in OKF6 oral-esophageal cells enhances TERT transcription via the Hif1- α transcription factor and directly stimulates telomerase activity through the Akt-dependent phosphorylation of TERT. Similarly, estradiol (E2) is reported to increase telomerase activity in endometrial cancer cells through MAPK induction of TERT transcription. Inhibition of either MEK or ERK resulted in decreased luciferase activity from a reporter plasmid containing the TERT promoter following treatment with E2 (Zhou *et al.*, 2013).

In both normal and cancerous cells, Ras effector proteins such as RIN1 may be useful targets for modulation of signaling through the MAPK pathway. The detrimental effects of abnormalities in the Ras/MAPK pathway are well documented for a variety of different cancers. Cancer, in the simplest sense, is a disease of the cell cycle, and dissection of the complex molecular interactions which govern cancer cell growth and reproduction will ultimately shed light on instances where moderation of aberrant cell signaling may be possible. RIN1, a known Ras effector molecule, may provide an avenue for attenuation of signaling through the Ras/MAPK pathway. If so, it is intriguing to contemplate what effect molecules such as RIN1 could have on downstream targets of the MAPK pathway known to influence cellular proliferation. Telomerase, for example, is widely expressed in many cancers and has been heavily implicated in tumorigenesis. As a target of the Ras/MAPK pathway, it is interesting to speculate on a possible connection between telomerase expression and the potential MAPK signal modifying ability of a Ras effector such as RIN1 (Fig. 2).

In summary, abnormal telomerase expression and activity is a defining hallmark observed in many different cancers. The immortal replicative phenotype conferred by the enzyme is

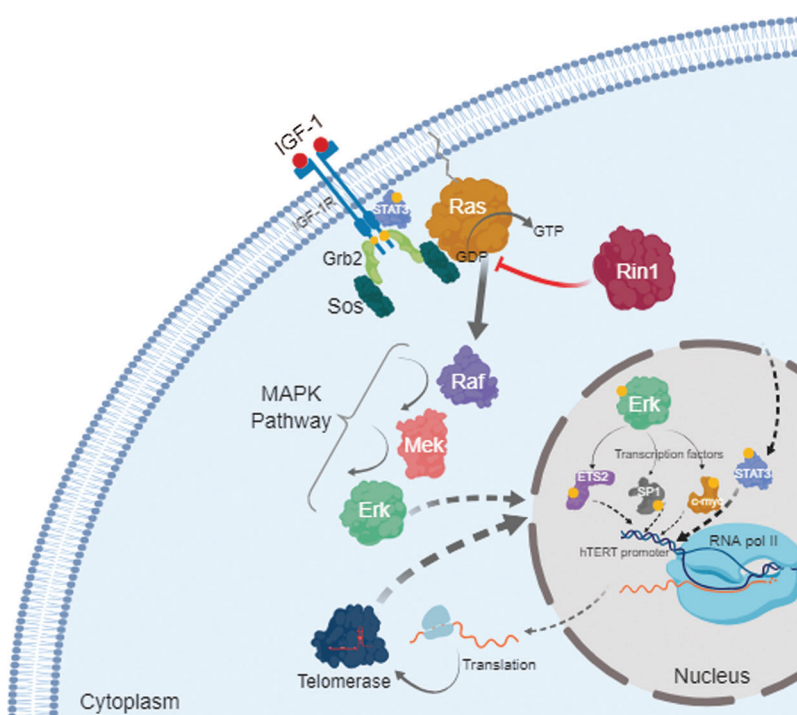


FIGURE 2. Diagram of the proposed model for the moderating influence of RIN1 on signaling through the Ras/MAPK pathway.

The binding of a growth factor such as IGF-1 to its receptor on the plasma membrane activates Ras and subsequently the MAPK pathway. Activation of the MAPK pathway in turn activates various nuclear transcription factors involved in telomerase expression. RIN1 competes with Raf for binding to activated Ras.

critical to the survival and proliferation of malignant cancer cells. The regulation of telomerase activity occurs primarily at the level of transcription and typically becomes abnormal and disordered during the tumorigenic process. The fact that telomerase is not expressed in most normal somatic cells has made the selective inhibition of telomerase activity an attractive chemotherapeutic target. Other strategies that seek to interfere with the signaling pathways responsible for the activation of telomerase expression could also be equally important from a therapeutic standpoint.

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