Effects of Oncogenic Ras and p38 Mitogen-activated Protein Kinase on the Adhesion of Normal Human Cells

by

Lynne K. Waldman

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Abstract

Activating mutations in *RAS* oncogenes commonly arise in human cancers. However, in experimental settings, oncogenic *RAS* has most often been studied at supraphysiological levels of expression. Importantly, work by others showed that the response of murine cells to expression of oncogenic *ras* from the endogenous promoter is strikingly different from the response of both human and murine cells to high levels of ectopically expressed oncogenic *RAS*. Thus, to study the outcome of oncogenic Ras signaling in human cells at a more physiological level, I developed a system in which I could activate oncogenic Ras signaling to either low or high extents in normal human fibroblasts. A low level of oncogenic Ras signaling induced cellular hyperproliferation, whereas a high level of signaling induced cellular senescence. A growing body of literature links loss of p38 mitogen-activated protein kinase (MAPK) activity with the promotion of Ras-induced transformation in murine cells. Accordingly, I examined the effect of inhibiting p38 in normal human cells in which I also activated a low level of oncogenic Ras signaling. Interestingly, the inhibition of p38 cooperated with low activation of oncogenic Ras to alter the morphology and adhesive properties of cells. My results suggest that the inhibition of p38 could predispose human cells to partial transformation by oncogenic Ras through alterations in cellular adhesion.

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Chapter 1:

Introduction

I. Cancer as a multistep process

Cell autonomous changes in the genome

The development of cancer is a complex process that consists of genetic alterations within incipient cancer cells as well as the interaction of these cells with their extracellular environment. Regardless of the tissue of origin, developing cancer cells must undergo multiple, cooperating mutational events that together deregulate normal cell proliferation and tissue homeostasis in order to proceed to full-blown malignancy. The genes whose mutation promotes neoplastic development fall within two broad categories: oncogenes and tumor suppressor genes (1).

Oncogenes are genes whose wild-type counterparts, called proto-oncogenes, normally promote pro-growth responses of cells such as cellular proliferation and survival. Normal cells obey signals from their surrounding environment that restrict cell proliferation to those times when it is appropriate and necessary for the well-being of the tissue as a whole. The mutations that convert proto-oncogenes to oncogenes are dominant, gain-of-function mutations, which typically render the resulting oncogenes constitutively active irrespective of a cell's surroundings (1, 2).

In contrast, tumor suppressor genes are genes whose wild-type counterparts normally impede cell growth and proliferation. For example, tumor suppressor genes can induce cells to undergo apoptosis following DNA damage in an effort to eliminate cells that could potentially initiate tumor formation if permitted to survive, since these cells may have incurred pro-cancer lesions in their genes. During cancer development, tumor suppressor genes commonly undergo loss of function through mutations, deletions, or epigenetic changes. This loss of tumor

suppressor activity causes cells to become vulnerable to factors that foster uncontrolled proliferation and cancer development (1, 2).

Multistep tumorigenesis

Tumorigenesis is a multistep process that requires both the activation of proto-oncogenes and the inactivation of tumor suppressor genes. Indeed, analyses of human tumor samples, protocols used to transform normal mammalian cells, experiments using genetically engineered mouse models, and large-scale sequencing of cancer cell genomes have all indicated that tumor cells harbor multiple genetic changes in both proto-oncogenes and tumor suppressor genes, with these genetic changes synergizing to create tumors and drive their progression (3-9). These genetic changes can be as subtle as point mutations or as extensive as chromosomal rearrangements. Importantly, the multiple and distinct genetic changes that are required to convert a normal cell into a cancer cell make mammals resistant to neoplastic development, thus explaining the delayed onset of non-hereditary cancers in the human population (1).

Incipient cancer cells are widely believed to undergo selection as they acquire sequential genetic changes, a process that promotes the expansion of daughter cells having the greatest proliferative advantage. It is this evolution of cells that underpins the metamorphosis of normal cells to cancer cells (1, 10). More specifically, the genetic alterations present within developing cancer cells must work together to confer six crucial abilities upon their host cells if the host cells are to become malignant. These abilities include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (1). Regardless of which proto-oncogenes and tumor suppressor genes become compromised in cells during neoplastic

progression, once cells have gained these six abilities they are thought to be fully malignant and capable of metastasizing to secondary sites, a process resulting in 90% of cancer-related deaths (1, 11).

Transformation and tumorigenicity

The genetic alterations incurred by normal mammalian cells, which enable them to become cancerous, cause these cells to acquire a new set of properties that positively correlate with tumorigenicity. More specifically, in comparison to normal cells, transformed cells generally exhibit differences in their *in vitro* morphology, adhesion, cytoskeletal arrangement, and proliferation as well as their *in vivo* tumorigenicity. The acquisition of these new properties by cells is referred to as transformation (Fig. 1; 12, 13).

When propagated under subconfluent culture conditions *in vitro*, transformed cells commonly exhibit a rounded morphology and refractile appearance, distinguishing them from their flatter, less-refractile normal counterparts (Fig. 1A; 12, 14, 15). Related to these changes in cellular morphology, transformed cells also exhibit reduced adhesion to their substratum and reorganization of their actin cytoskeletons (12, 13, 15). Moreover, when allowed to proliferate in culture to high cell densities, transformed cells usually display an irregular, crisscrossed orientation, which contrasts with the ordered, parallel alignment displayed by normal cells (12, 14-17).

In addition, normal cells undergo density-dependent growth arrest, also known as contact inhibition, when grown to a high density. Normal cells form an ordered monolayer upon reaching confluence, with the possibility of forming an ordered bilayer if maintained at confluence for an extended period of time. Most importantly, normal cells stop proliferating

upon forming a monolayer or bilayer despite the fact that they remain metabolically active. The behavior of transformed cells is strikingly different. Transformed cells fail to undergo contact inhibition and instead pile atop one another, forming multilayered clusters of cells (Fig. 1B; 12, 14, 16-19).

Another important difference between normal and transformed cells is that transformed, but not normal, cells are capable of anchorage-independent growth in either soft agar or suspension cultures (Fig. 1C; 12, 16, 20, 21). Whereas, normal cells rely on proper cell-matrix adhesions to survive and proliferate, transformed cells are able to circumvent the apoptotic and growth arrest programs, which prevent normal cells from surviving and proliferating in the absence of proper anchorage to a solid substrate. This ability permits transformed cells to form viable colonies in soft agar and aggregates in suspension (22, 23).

Although all of the aforementioned transformation-related properties are valuable for predicting whether a population of cells is tumorigenic or not, the tumorigenicity of the cells in question must nonetheless be tested *in vivo* in order to determine whether they are indeed malignantly transformed. For example, a cell population may be able to form colonies in soft agar but not be able to form tumors in an animal host. Cells exhibiting some, but not all of the *in vitro* and *in vivo* properties of transformation are considered to be partially transformed (12, 16, 18).

Importantly, despite that examination of *in vitro* transformation-associated properties is a useful predictor of the *in vivo* tumorigenicity of a population of cells, exceptions exist. For example, a cell line may undergo contact inhibition when grown in culture but prove to be tumorigenic in an animal host. Of note, these exceptions are more frequent in non-fibroblastic cell lines, such as those derived from epithelial tissues (12, 17, 18). However, the vast majority

of tumorigenic cell populations will display some *in vitro* transformation-related properties, causing examination of these properties to remain as a valuable method for scientists to assess the tumorigenic potential of a new cell line.

II. *RAS* oncogenes

Discovery of RAS oncogenes

In a 1964 report, Jennifer Harvey described collecting plasma from a leukemic rat that had been infected with the Moloney leukemia virus (MLV). Injecting the plasma harvested from this rat into animals of different rodent species caused these animals to develop sarcomas prior to their expected development of leukemia (24). The transforming retrovirus responsible for sarcoma formation was later named the Harvey murine sarcoma virus (Ha-MuSV) after the discovering scientist (25).

A similar discovery of another transforming retrovirus was reported in 1967. Serial passage of murine erythroblastosis virus (MEV) led to the identification of a virus capable of inducing the formation of sarcomas in mice. This virus was appropriately named the Kirsten murine sarcoma virus (Ki-MuSV) after its discoverer Werner Kirsten (25-27).

An understanding of the basis for the oncogenic properties inherent in Ha-MuSV and Ki-MuSV emerged in 1973 when Ki-MuSV was shown to contain nucleic acid sequences present in rat cells in addition to sequences derived from MEV (28). Furthermore, a protein product produced from the nucleic acid sequences that are homologous to those found in rat cells was shown to be responsible for the oncogenic properties displayed by Ha-MuSV and Ki-MuSV (29, 30).

By the beginning of 1980s, the genomes of Ha-MuSV and Ki-MuSV had been cloned (31, 32), and it was confirmed that Ha-MuSV and Ki-MuSV were in fact the product of recombination events between the original murine leukemogenic retroviruses and sequences from rat cells, which contain ancestral retroviral sequences called 30S RNA and rat cellular genes (30, 33). The rat cellular genes present in Ha-MuSV and Ki-MuSV were soon discovered to be two distinct but related vertebrate genes (34). These rodent genes were subsequently named with the acronyms *Hras* and *Kras*, respectively, standing for Harvey and Kirsten rat sarcoma (25). The human genes homologous to these rodent genes were identified in 1982 (35) and named *HRAS* and *KRAS* (25). It was later discovered that the *KRAS* gene gives rise to two different protein products, K-Ras4A and K-Ras4B, which result from alternative splicing of the *KRAS* pre-mRNA (36).

While the nature of viral oncogenes was being elucidated, other work was being done to search for mammalian genes capable of inducing transformation and tumorigenesis. In 1979, the genomic DNA from chemically transformed mouse cells was shown to transform NIH-3T3 murine fibroblasts upon transfection, as measured by changes in the morphology of NIH-3T3 cells as well as in their abilities to form foci in monolayer culture, grow in soft agar, and induce tumors in mice (37). Soon after, it was discovered that the genomic DNA from tumorigenic cell lines, which had been derived from different species, could transform NIH-3T3 cells upon transfection (38-40).

In addition, it was shown that Alu sequences, which are found in human but not murine genomes, were present in the murine NIH-3T3 cells that had been transfected with DNA from human tumor cell lines. This experiment, which had not been possible in the initial intraspecies transfection studies, demonstrated that the NIH-3T3 cells had integrated the transfected human

DNA into their genomes (40). Furthermore, in an independent set of experiments, NIH-3T3 cells, which had been transformed with DNA derived from three different human tumor cell lines, were found to harbor the same pattern of Alu-containing sequences, indicating that a singular gene was responsible for the transformation of NIH-3T3 cells (39).

In 1982, the presence of oncogenic genes in human tumor cells was confirmed by the cloning of a transforming gene from the T24 and EJ human bladder carcinoma cell lines (41-43). Furthermore, it was discovered that these human oncogenes were homologous to the *Hras* and *Kras* oncogenes that had been hijacked by the murine leukemia viruses (44-46). Indeed, oncogenic *Kras* was quickly identified as the oncogene present in those chemically transformed mouse cells that had been found to be responsible for transforming NIH-3T3 cells in the initial transfection experiments reported in 1979 (47).

In 1983, the third and final member of the *RAS* family was identified in neuroblastoma and sarcoma human cell lines, being found to share similarity with the *HRAS* and *KRAS* genes (48-50). This gene was named *NRAS* (*Nras* in mice) since it had been identified in neuroblastoma cells (25).

Both prior and subsequent to the discovery of *NRAS*, much additional work was done to understand the oncogenic and normal properties of *RAS* genes and their protein products. Many reports have shed light upon the molecular nature of oncogenic *RAS* and its functions in human tumors. In addition, there is now a wealth of knowledge concerning the molecular players that signal to the Ras protein and receive signals from Ras in both normal and tumor cells (25, 51, 52). The next several sections provide an overview of our current understanding of Ras and its role in tumorigenesis.

Ras proteins as GTPases

It became clear in the early 1980s that oncogenic *RAS* induces transformation as a result of single point mutations in its coding sequence. The first point mutation found to confer oncogenic properties upon Ras was the guanine-to-thymine mutation in codon12, which results in the amino acid valine being substituted for glycine at that position (G12V substitution) (53-56). Subsequently, point mutations at codons 13, 59, 61, 63, 116, and 119 were also found to promote the Ras-induced transformation of cells (57-61).

The functional ramifications of the activating point mutations began to be uncovered as more information was gathered concerning the protein products of *RAS* genes. In the late 1970s, the oncogenic *Ras* genes present in Ha-MSV and Ki-MSV were shown to encode 21,000-Dalton proteins ((29, 30, 62). Soon after their identification, Ras proteins were suspected to be GTPases since they were found to bind guanine nucleotides (63) and to associate with the cellular membranes (64, 65), behaviors which resemble that of GTPases called heterotrimeric G proteins, which had previously been identified in cells (66).

G proteins are intermediaries in transmembrane signaling pathways that consist of three components: upstream receptors, G proteins, and downstream effectors. Extracellular ligands bind to and activate membrane-bound receptors, leading to activation of G proteins. Activated G proteins then activate downstream effector proteins, thus triggering a cellular response to extracellular signals (66).

The activation state of a G protein is contingent upon whether it is bound to GTP or GDP. A G protein's binding of GTP typically activates the protein and enables it to signal to its downstream targets. However, G proteins possess an intrinsic GTPase activity that hydrolyzes

bound GTP to GDP and inorganic phosphate. When bound to GDP, a G protein is usually inactivated and unable to interact with its effectors (66).

Suspicions that Ras is a GTPase were confirmed in 1984 when several reports showed that oncogenic H-Ras proteins harboring point mutations at codon 12 exhibit decreased GTP hydrolyzing activity in comparison to wild-type H-Ras. This observation indicated that GTP hydrolysis is an important regulator of Ras activity, just as it is for the activity of heterotrimeric G proteins (67-69). Likewise, transformation-inducing mutations at codon 61 were also shown to reduce the GTPase activity of Ras (70). This and other research done in the early to mid 1980s indicated that the point mutations able to confer oncogenic activity upon *RAS* do so by compromising the ability of the Ras protein to hydrolyze GTP, thus keeping Ras in the active, GTP-bound state (52).

Post-translational modifications of Ras

Both wild-type and oncogenic Ras proteins must associate with cellular membranes to become biologically active (36, 71, 72). *RAS* gene products are synthesized as cytosolic proteins but undergo two important types of post-translational modifications in order to be targeted to membranes. These modifications are the farnesylation and palmitoylation of amino acid residues at the carboxyl terminus of Ras (36, 52).

H-Ras, K-Ras and N-Ras each have a CAAX amino acid motif at their carboxyl termini, where "C" stands for cysteine, "A" stands for an aliphatic amino acid and "X" stands for any amino acid (36). The enzyme farnesyltransferase covalently modifies the cysteine of the CAAX motif by attaching a farnesyl isoprenoid lipid to this amino acid (73-75). This farnesylation step

is followed by the proteolytic cleavage of the AAX sequence and the carboxymethylation of the now-carboxyl terminal cysteine residue (36, 52).

Interestingly, K-Ras4B, unlike the rest of the Ras family proteins, requires no modification beyond farnesylation to anchor itself to the plasma membrane, due to its series of positively charged lysine residues located within its carboxyl terminus. These lysine residues act as a second signal directing K-Ras4B's localization to the negatively charged membrane (36, 76). The exact mechanism of K-Ras4B's transport to the plasma membrane, however, is unknown (76).

In contrast, H-Ras, K-Ras4A, and N-Ras require the enzyme palmitoyltransferase to attach palmitoyl moieties to one or two cysteines upstream of the farnesylated cysteine in order to localize themselves to cellular membranes (77). These fully modified H-Ras, K-Ras4A, and N-Ras proteins are then shuttled to the plasma membrane through traditional vesicular transport (36). More recently, work has shown that a small amount of H- and N-Ras is ubiquitinated, promoting the redistribution of these proteins from the plasma membrane to endosomes (78, 79).

Activation and regulation of Ras

Ras was first implicated in transmitting extracellular signals in 1984 when epidermal growth factor (EGF) was found to stimulate the binding of Ras to GTP (80). Since then, much has been learned about the proteins that regulate the activation and inactivation of Ras proteins. For example, we now know that the level of GTPase activity exhibited by Ras proteins in a test tube is very low in comparison to that observed in living cells. Similarly, the rate of GDP to GTP exchange that is intrinsic to Ras proteins is too low to account for the rapid nucleotide cycling that occurs upon the stimulation of living cells with extracellular ligands (25, 51). These

observations led to the discovery of two families of proteins that are central regulators of Ras activity: GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (36, 51).

Ras-GAPs negatively regulate Ras signaling by increasing the rate at which Ras hydrolyzes its bound GTP to GDP (81). In contrast, Ras-GEFs positively regulate Ras signaling by promoting the displacement of GDP, thus enabling Ras proteins to bind GTP, which is more abundant than GDP in the cytosol of cells; such GTP-bound Ras molecules are then placed in an actively signaling state (82). As a result, an ongoing tug-of-war between the members of these two large protein families determines whether or not signaling pathways downstream of Ras are activated (36, 51).

Ras-GAPs

Humans and other mammals express several different Ras-GAPs. The first Ras-GAP to be discovered was the ubiquitously expressed p120GAP (83-85). Following p120GAP's discovery, a second Ras-GAP called neurofibromin-1 (NF1) was identified. NF1 displays a more limited expression pattern in the body than p120GAP, with its mutation causing the familial cancer syndrome neurofibromatosis type 1 (82, 86-89).

Subsequent to NF1, a third class of Ras-GAPs was identified, the GAP1 family. The GAP1 family consists of four members: $GAP1^{m}$, $GAP1^{IP4BP}$, Ca^{2+}-promoted Ras inactivator (CAPRI) and RAS-GTPase-activating protein-like (RASAL). However, these Ras-GAPs exhibit a much more restricted pattern of expression than either p120GAP or NF1 (90). Interestingly, the RAS-GAP activity of CAPRI and RASAL, but not that of $GAP1^{m}$ and $GAP1^{IP4BP}$, is

stimulated by an increase in the intracellular concentration of free Ca^{2+}, which causes both proteins to translocate to the plasma membrane (82).

The final Ras-GAP family to be identified is the synaptic GAP (SynGAP) family, of which SynGAP, DAB2-interacting protein (DAB2IP) and nGAP are members. The "n" of nGAP stands for nematode due to its homology to the GAP-2 protein of *C. elegans* (91). SynGAP itself is a Ras-GAP located at the excitatory synapses of neurons and shown to be an important regulator of signaling downstream of Ras in these cells (92, 93). The second SynGAP member, DAB2IP, has been found to act as a tumor suppressor gene and to be inactivated in prostate and breast cancers (94, 95). Little is known about the role of the last SynGAP member, nGAP; however, nGAP was able to rescue loss of Ras-GAP activity in yeast, indicating that it likely functions as a Ras-GAP in human cells (91).

Ras-GEFs

Similar to the Ras-GAPs, the Ras-GEF family of proteins includes many members that fall within different subfamilies. The search for Ras-GEFs originated in efforts that were directed toward understanding how extracellular mitogenic signals promote the binding of Ras to GTP. Because Ras proteins were known to share homology with heterotrimeric G proteins, it was reasoned that Ras family members, like G proteins, would bind GEFs to become activated (25). This thinking led to identification of the first Ras-GEF, a yeast protein called cell-division-cycle-25 (Cdc25), which is not to be confused with the cyclin-dependent kinase phosphatase bearing the same name (96, 97).

The identification of Cdc25 in yeast paved the way for the cloning of the first mammalian Ras-GEF, a protein named son of sevenless (SOS) for its role in eye development in

fruit flies (98). To date, four subfamilies of Ras-GEFs have been identified: SOS, Ras guanine nucleotide-releasing factor (RasGRF), Ras guanine nucleotide-releasing protein (RasGRP) and cyclic nucleotide-dependent Ras GEF (CNrasGEF). In addition, phospholipase C-ε (PLCε) also includes domains that share homology with those of known Ras-GEFs, indicating that it could function as a Ras-GEF. However, whether PLCε performs this function in living cells remains unclear (36).

The ubiquitously expressed SOS family of Ras-GEFs, composed of members SOS1 and SOS2, couples the activation of receptor tyrosine kinases to Ras activation (36, 99). In contrast, RasGRF proteins are principally expressed in the central nervous system (36, 99, 100) and are activated downstream of multiple neuronal receptors in a calcium-dependent manner (101-107). Like RasGRFs, CNrasGEF is expressed predominantly in the brain (108), but appears to operate downstream of G-protein-coupled receptors (109). RasGRPs also display a limited expression profile, but appear to be important in immune cell activation, functioning downstream of non-receptor tyrosine kinases (36, 110-113).

The connection between cell-surface receptors that receive extracellular signals and the activity of Ras-GEFs was elucidated with the identification of the adaptor protein growth factor receptor-bound protein-2 (GRB2) (25). GRB2 is composed of a single SH2 domain flanked by SH3 domains, which participate in protein–protein interactions through amino acid regions rich in proline. GRB2 was found to bind the activated EGF receptor via its single SH2 domain and SOS via its two SH3 domains. Through these interactions, GRB2 forms a bridge that links signals originating from outside of the cell with the intracellular activation of Ras signaling (114-118).

Following the identification of Ras-GEFs and GRB2, much has been learned about the mechanism by which Ras becomes activated in response to extracellular signals. SOS is the Ras-GEF that has been most extensively studied (36), and as such, a rather complete portrait of Ras activation through SOS has been developed:

First, growth factors, like EGF, induce the rapid dimerization and autophosphorylation of their respective tyrosine kinase receptors. Then, GRB2, though its SH2 domain, binds to phosphorylated tyrosine residues in the non-catalytic region of an activated receptor. SOS translocates to the plasma membrane through both its carboxyl-terminal GRB2-binding site and its amino-terminal lipid-binding pleckstrin homology (PH) domain (119-121). In addition to helping SOS anchor itself to the membrane, the binding of SOS to GRB2 causes a conformational change in SOS that frees SOS from self-imposed negative regulation (122).

Furthermore, association of the SOS PH domain with membrane phospholipids also induces the binding of SOS to GDP-bound Ras, permitting a low level of SOS activity. Low-level SOS activity allows Ras to exchange GDP for GTP (122). Once bound to GTP, Ras elicits maximal SOS activity, triggering a positive feedback loop that increases the amplitude and duration of Ras signaling (123).

Ras activation at the structural level

The tertiary structures of H-, K-, and N-Ras proteins are nearly identical, given that they share a very high degree of similarity over the first 87% of their amino acid sequences (124, 125). Each Ras protein is composed of six β-sheets that are surrounded by five α- helices; ten loops link these secondary structures together (52). Magnesium ions present in cells enable Ras

to associate tightly with guanine nucleotides, causing Ras to be continuously bound to either GTP or GDP.

Two regions within Ras proteins, called Switch I (amino acids 30 to 38) and Switch II (amino acids 59 to 67), undergo dramatic structural changes upon the swapping of bound GDP for GTP or vice versa (126). The conformation of Switch I depends heavily upon threonine 35. The exchange of GTP for GDP causes the side chain of threonine 35 to be rotated inward so that it can interact with GTP's γ-phosphate and the bound magnesium ion (52). The γ-phosphate of GTP also causes glycine 60 to reorient itself, inducing changes in the conformational of Switch II (52).

As mentioned previously, oncogenic *RAS* mutations are predominately found at codons 12, 13, and 61. Analysis of crystal structures of mutant Ras proteins has shown that the GTPase activity of Ras is crippled by amino acid substitutions resulting from these oncogenic mutations. More specifically, codons 12 and 13 encode glycine residues in wild-type Ras. Replacing glycine with any other amino acid introduces a side chain at glycine's former position. Codons 12 and 13 are sensitive to mutation, since introducing an amino acid bearing a side chain at either of these positions appears to interfere sterically with the ability of Ras-GAPs to properly stabilize the transition state of the GTP hydrolysis reaction (127-129).

Mutation of codon 61, which normally encodes glutamine, also hinders GTPase activity. Through its interaction with Ras-GAPs, glutamine activates the water molecule of the hydrolysis reaction for attack of GTP's γ-phosphate and contributes to the stabilization of the transition state (127, 129). As a result, oncogenic mutations in Ras stabilize the Ras-GTP complex, leading to constitutive activation of Ras signaling (52, 82).

Ras effector pathways

Active Ras proteins have been shown to communicate with numerous downstream effectors that drive diverse biological outcomes, such as the cancer-relevant processes of cellular proliferation, resistance to apoptosis, invasiveness, and angiogenesis (130). Ras effectors have a high affinity for Ras when it is bound to GTP but not GDP. Although amino acids in both the Switch I and Switch II regions have been implicated in the association of upstream and downstream binding partners with Ras, the Ras effector-binding domain, which is composed of amino acids 32 through 40, is the region that is critical for the binding of Ras to its downstream targets (36, 52, 125).

The three most well-studied families of Ras effectors are Raf serine/threonine kinases, phosphoinositide 3-kinases (PI3Ks), and exchange factors for Ral proteins, known as Ral guanine nucleotide dissociation stimulator (RalGDS) and RalGDS-like (RGL) proteins (130, 131). Although less well-characterized, additional Ras effectors have also been identified, including the following proteins: phospholipase C-ε (PLCε), T-cell lymphoma invasion and metastasis-1 (Tiam1), Ras interaction/interference protein-1 (Rin1), acute lymphoblastic leukemia-1 fused gene on chromosome 6 (AF-6), and Ras association domain-containing family (RASSF) proteins (126).

Raf

The first Ras effector to be discovered was the serine/threonine kinase Raf-1 (132-136), a protein found to be necessary for the Ras-induced transformation of NIH-3T3 cells (137). The related genes *A-RAF* and *B-RAF* were identified subsequently to the discovery of *RAF-1*. The

protein products of this gene family are activated upon binding to GTP-bound Ras, with their activation promoting cell-cycle progression (51, 126).

The association of GTP-bound Ras with Raf localizes Raf to the plasma membrane, a process known to be crucial for its activation (138, 139). Although further details concerning the mechanism of Raf's activation by Ras are not well understood, the binding of active Ras to Raf also stimulates Raf to undergo conformational changes, multiple phosphorylation events, and hetero-oligomerization with other Raf family members, with each of these events contributing to the full activation of Raf (126, 140, 141). Hetero-oligomerization of Raf proteins is believed to occur through the binding of dimers of 14-3-3 adaptor proteins (140). In addition, the assembly of Raf into an active signaling complex appears to require the chaperonin proteins Hsp90 and p50/Cdc37 (142).

Once activated by Ras, and through the assistance of scaffold proteins, Raf phosphorylates and activates MEK1 and MEK2, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinases. MEK in turn phosphorylates and activates the MAPKs, ERK1 and ERK2. Activated ERKs then phosphorylate and activate various cytosolic and nuclear proteins (126, 141).

While an exhaustive list of ERK targets has yet to be defined, the best-characterized targets of ERK are transcription factors. Activated ERK translocates into the nucleus to phosphorylate and activate E26-transcription factor proteins (ETS), like Elk, which interact with serum response factor (SRF) to regulate the expression of many immediate early response genes, such as c-*FOS*. ERK also phosphorylates the c-JUN protein, which binds c-FOS protein to form the activator protein-1 (AP-1) transcription factor (51, 52, 130, 141, 143). The activation of

these transcription factors drives the expression of critical cell-cycle regulators, such as D-type cyclins, thus promoting progression of cells through the G1 phase of the cell cycle (144).

PI3K

After the Raf family of protein kinases, the PI3Ks are the next best characterized Ras effectors (130). Similar to Raf, active PI3K is required for the Ras-induced transformation of NIH-3T3 cells (145). The association of GTP-bound Ras with the catalytic subunit of type I PI3Ks promotes their translocation to the membrane and causes them to undergo conformational changes (145, 146). Once activated, these lipid kinases phosphorylate phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), thereby converting it to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3).

PtdIns(3,4,5)P_3 is a second messenger that binds to a variety of proteins, including 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt/protein kinase B (PKB). The binding of PtdIns(3,4,5)P_3 to PDK1 and Akt localizes these proteins to the plasma membrane, where PDK1 phosphorylates and activates Akt (147, 148). Active Akt then performs an important function downstream of Ras by promoting cell survival through its phosphorylation of multiple proteins that trigger anti-apoptotic signaling pathways (130, 149).

In addition to the production of PtdIns(3,4,5)P_3 leading to activation of Akt, PtdIns(3,4,5)P_3 causes activation of the small GTPase Rac by binding Rac-GEFs; Rac is similar to Ras in that Rac is activated when GTP-bound (130). Rac both regulates the actin cytoskeleton and activates transcription factor pathways, such as the anti-apoptotic protein nuclear factor κB (NF-κB). Importantly, the activity of Rac has been shown to be important for Ras-induced tumorigenesis (150).

RalGDS and RalGDS-like genes

The third most studied class of Ras effectors are the RalGDS and RalGDS-like proteins, which are GEFs for the RalA and RalB small GTPases (130). Initial studies of RalGDS function indicated that activation of this pathway could not itself induce the transformation of NIH-3T3 cells. However, activating the Ral pathway in combination with activation of the Raf pathway was found to enhance the transformation of NIH-3T3 cells beyond that observed for cells in which Raf was activated alone (151, 152).

Experiments performed in human cells also suggest that activation of RalGDS is important in the Ras-induced transformation and tumorigenesis of human cells (153, 154). Furthermore, RalA appears to be necessary for the anchorage-independent proliferation of tumorigenic human cells, and RalB appears to be necessary for their survival (155). In spite of these intriguing data, however, the signaling pathways lying downstream of activated Ral that are critical for promoting Ras-induced transformation remain ambiguous (52).

Additional Ras effectors

Beyond the Raf, PI3K, and RalGDS proteins, various other proteins have been reported to function as Ras effectors. For the most part, the roles of these diverse proteins in Ras signaling and transformation continue to be obscure. However, ongoing work has begun to shed light upon the biology of the following Ras effectors: PLCε, Tiam1, Rin1, AF-6, and RASSF (52, 126).

PLCε catalyzes the hydrolysis of PtdIns(4,5)P_2 to diacylglycerol and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) (51). As mentioned previously, PLCε may be a Ras-GEF given that it shares homology with known Ras-GEFs (36, 156). In addition, PLCε could also regulate Ras-

GAPs and Ras-GEFs whose activity appears to be dependent upon the binding of calcium and/or diacylglycerol. Through its actions on Ras-GEFs and Ras-GAPs, PLCε potentially provides mechanisms of both feed-forward and feedback control of Ras (156).

The identification of Tiam1 as an effector downstream of Ras has provided a second connection between the activation of Ras and the activation of the Rac; the first connection being the activation of Rac through the PI3K pathway as described earlier (157, 158). However, somewhat unexpectedly, Tiam1 appears to play an important role downstream of Ras in its own right. Experiments employing a carcinogen-induced tumor model in which Ras invariably undergoes oncogenic activation showed that knockout of *Tiam1* delayed tumor formation. Also, fibroblasts harvested from *Tiam1*-null mice were resistant to Ras-induced transformation. It is important to note, however, that whereas few tumors formed in the *Tiam1*-null background, a greater percentage of those that did form became invasive and malignant. These intriguing data suggest that Tiam1 may play different roles at different stages of tumor development (159).

The protein AF-6 was found to bind H-Ras *in vitro* (160) and may link Ras signaling with regulation of the actin cytoskeletal and cell-cell junctions. AF-6 was found to bind F-actin (161) and to associate with adherens and tight junctions (161, 162). AF-6 has also been shown to play an active role in regulating cell-cell junctions as it is necessary for the proper formation of epithelial cell-cell junctions and cell polarity during embryogenesis (162, 163). Moreover, Ras competes with the tight junction protein zona occludens 1 (ZO-1) for binding to AF-6, suggesting that Ras may disrupt cell-cell contacts by disrupting the binding of ZO-1 to AF-6 (162).

Unlike the previously described Ras effectors, Ras interaction/interference (Rin) is an example of a Ras effector that impedes Ras-induced transformation and tumorigenesis (164).

There are two potential mechanisms through which Rin could exert its effect as a tumor suppressor. First, Rin has been shown to compete directly with Raf for binding to Ras (165). Alternatively, Rin was found to trigger the endocytosis of growth factor receptors that activate Ras signaling (166).

Another class of Ras effectors is the RASSF family of proteins. Currently, three RASSF proteins, RASSF1, RASSF2, and RASSF5, have been characterized and, like Rin, were found to exhibit tumor suppressive functions. More specifically, RASSF1, 2, and 5 exhibit anti-proliferative and pro-apoptotic functions (167-170). Indeed, multiple human tumor types exhibit loss of *RASSF* gene expression (171-173), and *RASSF1* knockout mice are more susceptible to spontaneous or chemically induced tumorigenesis (174).

Downstream signaling by GDP-bound Ras

Despite the overwhelming attention that has been given to signaling downstream of "active", GTP-bound Ras, there is evidence that GDP-bound Ras can also bind effector proteins and modulate signaling pathways (175). For example, GDP-bound, but not GTP-bound, Ras binds to the transcription factor Aiolos, sequestering Aiolos in the cytoplasm. This sequestration prevents Aiolos from translocating to the nucleus where it promotes expression of the anti-apoptotic protein B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) (176).

Oncogenic RAS and multistep tumorigenesis

Studies of transformation by oncogenic Ras provided crucial experimental evidence of multistep transformation. As mentioned previously, early work illustrated that DNA sequences, later shown to encode *RAS* oncogenes, could transform NIH-3T3 cells (37-40). Around the same

time, the normal *Hras* gene of rats was also found to be able to transform NIH-3T3 cells when overexpressed to high levels by a retrovirus (177). However, oncogenic *ras* could not transform freshly isolated, normal rat cells, indicating that some property of NIH-3T3 cells made them amenable to transformation (3, 4).

The difference in transformation potential between NIH-3T3 cells and normal rat cells turned out to be attributable to differences in the *in vitro* culture histories of these cell populations. NIH-3T3 cells had been established in culture and, as such, possessed an unlimited replicative potential; in other words, they were immortal. In contrast, the normal rat cells had not undergone extensive passage in culture and were not immortalized like the NIH-3T3 cells. It was this unlimited replicative potential of NIH-3T3 cells that rendered them amenable to transformation by *ras* (25, 52).

The property of spontaneous immortalization could be functionally replaced in transformation assays by exposing cells to carcinogens prior to transfecting them with *ras* (178) or by expressing a second oncogene, such *myc*, SV40 large T antigen, or E1A, along with the *ras* oncogene (3, 4). These studies helped lay the foundation for our current understanding of tumor evolution by providing evidence for the concept of multistep tumorigenesis (25). Indeed, subsequent to these pioneering experiments, additional work has shown that normal human cells behave similarly to normal rat cells in that they also require multiple genetic alterations to become fully transformed (6, 21, 154, 179).

RAS mutations in human tumors

The first evidence that point mutations occurred in actual human tumors, and not only in human tumor cell lines that had been extensively propagated in culture, emerged in 1984. Point

mutations were identified in codon 12 of *KRAS* in tumor biopsies from a patient bearing a squamous cell lung carcinoma but not in normal tissue sample from that same patient (180). Following this discovery, a massive effort was undertaken to identify *RAS* mutations in other human cancers (181).

To date, oncogenic *RAS* mutations have been found in many human cancers, with cancers of the pancreas, intestine, biliary tract, and skin displaying the greatest prevalence of *RAS* mutations (Table 1). Furthermore, mutations in different *RAS* genes are found in different types of cancers. For example, mutations in *KRAS* are commonly found in pancreatic, lung, and colon carcinomas (51, 181). Oncogenic *HRAS* mutations are prevalent in bladder carcinomas (51, 182), whereas oncogenic *NRAS* mutations are predominant in leukemias and melanomas (59, 183-186).

In addition, although mutation of codon 12 of *RAS* occurs most frequently, human tumors may also exhibit oncogenic mutations in *RAS* genes at codons 13 and 61. These three mutations together account for nearly all *RAS* mutations found in human neoplasias (51).

ras activation in experimental animal models

Evidence that oncogenic Ras actively contributes to cancer development *in vivo* has been provided though extensive work done using experimental animal models. As a first step, oncogenic *ras* mutations were discovered in rodent models in which tumor formation was induced by carcinogens or γ-irradiation (187-190). The first evidence for the involvement of oncogenic *ras* in the initiation of tumor development was obtained when, in one carcinogen-based tumor model, the second nucleotide in codon 12 of *Hras* was mutated by a guanine-to-

adenine transition, the exact base substitution known to be induced by the carcinogen that had been used (191).

Subsequently, various genetically engineered mouse models of cancer have strongly tied oncogenic *ras* mutations to tumor initiation, development, and maintenance. Oncogenic *ras* induces tumor formation in different organs when aberrantly expressed using tissue-specific promoters (192-194). In addition, experiments performed using genetically engineered mouse models that involve inducible *ras* transgenes indicate that sustained *ras* expression is necessary for continued tumor progression (195, 196).

More recently, mouse models of cancer have become even more adept at recapitulating the events that occur during the development of human cancers by employing the conditional expression of the *Kras* oncogene from the endogenous *Kras* locus. Importantly, experiments utilizing a transgenic mouse model in which oncogenic *Hras* is expressed in mammary tissue in an inducible manner showed that different levels of oncogenic *Hras* dictate different biological outcomes *in vivo* (197). These results argue that expressing oncogenic *ras* genes at physiological levels is necessary to create mouse models of human cancers in the most accurate manner possible.

An increasing number of *RAS*-related studies are incorporating mouse models that express oncogenic *Kras* from the endogenous locus. Importantly, each of these models specifically targets oncogenic *Kras* to a particular organ (198-211). In addition, a subset of these models employs techniques that actively induce the expression of oncogenic *Kras* in mature mice but not younger animals (198, 201, 202, 206, 207, 209, 211). Thus, by using endogenous oncogenic *Kras* mouse models, researchers can mimic the spaciotemporal activation of Ras

signaling in human cancers by expressing oncogenic *Kras* in specific mouse organs and in a delayed manner.

Indeed, the pathologies of several human neoplasias, which frequently harbor *RAS* oncogenes, have been closely imitated in mice by expressing oncogenic *Kras* at physiological levels, either alone or in the presence of another genetic alteration germane to the cancer of interest (198-210). Although there are caveats associated with the use of these endogenous models of oncogenic *Kras* expression, such as concerted activation of oncogenic Kras signaling in a large number of cells and the expression of only one copy of wild-type *Kras* in cells, these models are providing invaluable information concerning the role of K-Ras in the initiation and progression of human neoplasias.

In contrast to the oncogenic *Kras* mouse models described above, there is a lack of studies describing mouse models in which either oncogenic *Hras* or *Nras* is expressed from its corresponding endogenous locus (212). In one colon cancer mouse model, oncogenic *Nras* was expressed from the endogenous *Nras* locus to determine whether oncogenic *Nras* could substitute for oncogenic *Kras* to induce hyperplasia of the colonic epithelium and to and promote high-grade dysplasia in tumors (213). The failure of oncogenic *Nras* to reproduce the tumor-promoting functions of oncogenic *Kras* in this mouse model may shed light upon why human colon cancers are commonly found to harbor oncogenic mutations in *KRAS* but not *NRAS*. As a result, further work is necessary to examine the functions oncogenic *Nras* in mouse models of human cancers that exhibit a high frequency *NRAS* mutation, such as melanoma (51). Similarly, the use of mouse models in which oncogenic *Hras* is expressed at physiological levels in tissue types that give rise to neoplasias known to harbor *HRAS* mutations is likely to provide valuable insight into the role of this oncogene in human cancers.

III. p38 MAPK

Background

Mitogen-activated protein kinases (MAPKs) are members of signaling pathways crucial for the ability of cells to respond to extracellular signals (214, 215). The most well known of the MAPKs are the ERKs (216), which were introduced earlier due to their function downstream of the Ras effector Raf. As expected, given their connection to Ras, ERK1 and 2 are involved in transmitting mitogenic signals within cells and promote cellular proliferation. However, mammalian cells also contain additional MAPK families that respond to disparate extracellular stimuli and regulate cell behavior in response to these stimuli (215). Relevant to the work presented in Chapter 2 is the p38 family of MAPKs.

Unlike ERK, p38 MAPK is a stress-activated protein kinase that becomes highly activated in response to inflammatory cytokines and environmental stresses, such as heat or osmotic shock (214-216). Four different p38 family members (α, β, γ, and δ) have been identified in mammals and are approximately 60% identical at the amino acid level. p38α is highly expressed throughout the body, whereas p38β is ubiquitously expressed but at lower levels, and its contribution to p38 signaling remains ambiguous. In contrast, p38γ and δ exhibit a more restricted expression pattern and may perform more specialized functions (217, 218).

As with other MAPK kinases, p38 is the final tier in a three-tier kinase cascade (Fig. 2). MAPKs are phosphorylated and activated by MAPK kinases (MAP2Ks), and MAP2Ks are phosphorylated and activated by MAPK kinase kinases (MAP3Ks). Indeed, Raf and MEK, which were introduced earlier as being activated downstream of Ras, are the MAP3K and MAP2K, respectively, that are upstream of ERK (216).

p38 MAP2Ks activate each p38 family member by dual phosphorylation of the Thr-Gly-Tyr motif located in the p38 activation loop. Activated p38 proteins then phosphorylate Ser-Pro or Thr-Pro MAPK consensus motifs contained within downstream targets (214, 219). The MAP2Ks and MAP3Ks that are capable of activating p38, in addition to the various substrates activated by p38, will be elaborated upon in the following sections.

Activation of p38

Signaling through the canonical p38 MAPK cascade

p38 proteins are activated by MAP2Ks called MAP kinase kinase 3 (MKK3) and MKK6 (Fig. 2). MKK3 and 6 specifically activate p38, failing to activate proteins of other MAPK families (220-224). p38 can also be activated by MKK4, which was originally discovered as a MAP2K for the JUN N-terminal kinases (JNKs), another family of stress-activated MAPKs. Activation of p38 by MKK4 especially appears to occur in situations where MKK3 and 6 are unavailable (220, 225).

As mentioned above, MKKs that activate p38 are themselves activated through phosphorylation by a MAP3K (Fig. 2). A number of MAP3Ks have been found to activate the p38 pathway, with particular MAP3Ks activating p38 in a manner that is dependent upon both the stimulus and cell type. MAP3Ks found to activate p38 signaling include apoptosis signal-regulating kinase-1 (ASK1), transforming growth factor β-activated kinase-1 (TAK1), thousand and one kinases (TAOs), mixed-lineage kinases (MLKs), and MEK kinases (MEKKs) (226-234).

Importantly, the activated forms of many of these MAP3Ks induce the activation of both p38 and the second stress-activated MAPK, JNK. For example, ASK1, TAK1, MLKs, and MEKKs are all capable of activating both p38 and JNK signaling, indicating that these pathways

often undergo co-regulation in cells in response to stress signals (214, 235). A different dynamic exists with respect to activation of p38 and JNK by TAOs. TAO1 and TAO2 appear to activate p38 specifically, failing to activate JNK in situations of endogenous TAO activation (233). However, the *TAO2* gene also produces a splice variant called prostate derived STE20-like kinase (*PSK*) that specifically activates JNK signaling (236). Thus, *TAO2* gene products also appear to co-regulate the p38 and JNK pathways.

Activation of p38 signaling by GTPases

Although less is known about the upstream molecules that initially trigger activation of the p38 MAPK cascade than is known about those molecules upstream of the better-studied ERK MAPK, a general picture of p38 pathway activation has begun to emerge. Indeed, similar to the activation of ERK signaling by the small GTPase Ras, small GTPases of the Rho family can activate p38. Members of the Rho family capable of activating p38 include Rac, cell division cycle 42 (Cdc42), Rho, and Rit (237-241). In addition, evidence exists that p38 can be activated by heterotrimeric G proteins. However, stimulation of p38 signaling by heterotrimeric G proteins is much less understood than is p38's stimulation by Rho family GTPases (242, 243).

Rho family GTPases are best known for their regulation of the actin cytoskeleton and its related functions, such as cell migration and adhesion, but have also been found to play roles in cell proliferation, transcription, vesicle transport, microtubule dynamics, and neuronal development (244). Despite that further work is also necessary to understand the precise roles that p38 plays in the signaling downstream of Rho family GTPases, some clues have begun to emerge as to which functions downstream of Rho family proteins are mediated by p38. For example, evidence suggests that, in certain cell systems, p38 plays a role downstream of Rac and

Cdc42 in the regulation of the cytoskeleton (245-248). Also, p38 has been found to play a role downstream of Rac in the promotion of bacteria phagocytosis by macrophages (249). Furthermore, p38 has been shown to be necessary for mediating the transcriptional upregulation of several genes downstream of activated Rac or Cdc42 (250-253).

Contributing to the activation of p38 by Rac and Cdc42, the p21-activated kinases (PAKs) are a group of proteins also capable of activating p38 signaling (237, 239). PAKs, which are themselves activated by GTP-bound Rac and Cdc42, are effectors of these GTPases (254-258). Indeed, signaling cascades involving Rac or Cdc42, PAK, MKK3/6 and p38 have been reported to respond to certain stimuli, with each of the proteins in this pathway being necessary for the cytoskeletal remodeling that is induced by Rac or Cdc42 in response to the upstream stimuli (239, 245, 259).

Although the mechanism by which PAKs induce activation of MKKs 3 and 6 is not clear, PAKs may function as MAP3K kinases (MAP4Ks), phosphorylating and activating MAP3Ks upstream of p38. Supporting this idea, the yeast homologue of mammalian PAKs, sterile 20 protein (Ste20p), functions as a MAP4K by phosphorylating and activating the yeast MAP3K Ste11p, thereby activating the yeast alpha-mating factor signaling pathway (260, 261). Further study should provide insight into whether mammalian PAKs behave similarly to their yeast counterparts.

Scaffolding proteins

As alluded to earlier, the p38 and other MAPK pathways regulate numerous cellular responses from a diverse array of extracellular stimuli. In order to manage such complex pathways, cells employ temporal and spatial control of MAPK signaling. One method in which

cells control MAPK signaling is by the use scaffolding proteins. Scaffolding proteins interact with multiple components of a MAPK cascade, thus assembling complexes of MAPK signaling proteins. Scaffolding proteins promote the coordinated, serial phosphorylation of select MAPK cascade constituents, enabling the efficient activation of particular MAPK signaling pathways and the appropriate responses of cells to distinct stimuli (215, 219).

Fewer scaffolding proteins have been identified in mammalian cells to regulate p38 than to regulate either the ERK or JNK MAPK pathways (217). However, one p38-specific scaffolding protein has been identified, which coordinates cellular responses to hyperosmolarity. Osmosensing scaffold for MEKK3 (OSM) binds to actin, the GTPase Rac, the MAP3K MEKK3, and the MAP2K MKK3. Upon osmotic stress, OSM is recruited to membrane ruffles where it binds to Rac and assembles the MEKK3/MKK3/p38 signaling pathway (262).

Other scaffolding proteins found to regulate p38 signaling include JNK-interacting proteins (JIPs). Although, as implied by their name, the JIP family of proteins was originally identified as being scaffolds for JNK MAPKs (263), JIP2 and JIP4 provide scaffolding functions for p38 proteins. In addition to binding proteins of the JNK cascade, JIP2 can bind to MKK3, p38α, and p38δ in addition to upstream signaling molecules, thereby stimulating p38 activity (264-266).

Unlike JIP2, JIP4 appears be a scaffolding protein specific for p38. JIP4 was found to interact with p38α and β, but not with p38γ or p38δ. Moreover, JIP4 enhances p38 signaling through a mechanism that requires either MKK3 or MKK6 (263, 267). In contrast, JIP4 can bind to JNK but does not enhance JNK activation, indicating that JIP4 functions as a scaffolding protein for the p38, but not JNK, pathway (267).

Non-canonical activation of p38

Importantly, p38 can also be activated by non-canonical mechanisms that are independent of its phosphorylation by MKKs 3, 6 and 4. The first mechanism of non-canonical activation involves the autophosphorylation of p38 following its binding to TAK1-binding protein (TAB1) (268). Although there is some debate concerning the physiological relevance of this alternative method of p38 activation (225), accumulating evidence suggests that cells activate p38 through TAB1 binding in certain situations (269-271).

A second MKK-independent mechanism of p38 activation was found to occur downstream of the T-cell receptor (TCR) in antigen-stimulated T cells. This mechanism involves the atypical phosphorylation of a tyrosine residue outside of p38's activation loop by zeta-chain TCR-associated protein kinase 70kDa (ZAP70). ZAP70's atypical phosphorylation of p38 triggers p38 to autophosphorylate the threonine and tyrosine residues present within its activation loop, thereby activating itself (272).

Extracellular signals that activate p38

As already indicated, a multitude of extracellular stimuli lead to the activation of p38 signaling. Environmental stresses that activate p38 signaling include osmotic stress and heat shock (273, 274). In addition, p38 is activated by DNA-damaging agents, such as ultraviolet (UV) radiation, ionizing radiation, and hydroxyurea (275, 276). p38 is also activated by toxic chemicals, such as sodium arsenite and rotenone (274, 277) as well as the biological toxin lipopolysaccharide (273, 275).

As an important mediator of inflammatory responses, p38 activates signaling pathways induced by a variety of cytokines, including tumor necrosis factor α (TNFα), interleukins, and

thrombin (275, 278-283). For example, p38 activity was found to be necessary for TNFα-induced upregulation of vascular cell adhesion molecule-1 (VCAM-1) at the surface of human endothelial cells (280).

The activation of p38 by growth factors is more unpredictable than is its activation by stress stimuli or cytokines. More specifically, growth factors such as fibroblast growth factor (FGF), nerve growth factor (NGF), insulin, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and granulocyte macrophage colony-stimulating factor (GM-CSF) have all been found to activate p38 signaling, but only in specific cell types (284-291). Other cell types either do not respond to these growth factors by activating the p38 pathway or can even downregulate p38 signaling in response to these growth factors. For example, insulin activates p38 signaling in 3T3-L1 adipocytes (291) but inhibits p38 activity in chick forebrain neuron cells (292). Clearly, the activation of p38 by growth factors is highly contextual, whereas the activation of p38 by cellular stressors and cytokines is more universal.

Inactivation of p38

The phosphorylation and activation of p38 occurs quickly in response to most stimuli and is transient (235). The ability of cells to downregulate p38 signaling following stimulation allows them to adapt continuously to changing signals from their surroundings. Just as phosphorylation of p38 plays a crucial role in activating this protein, dephosphorylation plays a crucial role in its inactivation (235, 293).

Mammalian cells contain several types of protein phosphatases capable of inactivating p38 signaling by dephosphorylating one or both of its key activating phosphorylation sites – the

conserved threonine and tyrosine residues present within its activation loop. These phosphatases include serine/threonine phosphatases, tyrosine phosphatases, and dual-specificity phosphatases (DUSPs). Unlike the serine/threonine and tyrosine phosphatases that promote the dephosphorylation of either the threonine or tyrosine residue, respectively, DUSPs dephosphorylate both the threonine and tyrosine residues contained within p38's activation loop (235, 293).

Serine/threonine phosphatases

The phosphatase type 2C (PP2C) family of serine/threonine phosphatases includes three members known to directly inactivate p38, namely PP2Cα splicing variant 2 (PP2Cα-2), PP2Cβ, and PP2Cδ, which is more commonly called p53-induced protein phosphatase 1D (PPM1D) or wild-type p53-induced phosphatase 1 (Wip1) (294). PP2Cα-2 binds to p38 and downregulates its signaling when it is triggered by various stimuli, such as UV radiation and TNFα (295). PPM1D/Wip1, on the other hand, was found to dephosphorylate p38 upon following its induction by UV light. Dephosphorylation of p38 by Wip1 attenuated the p38-induced phosphorylation and stabilization of p53, thereby reducing p53 signaling while cells recovered from UV-induced DNA damage and suppressing UV-induced apoptosis (296).

In addition, PP2C family members can suppress p38 signaling by dephosphorylating and inactivating MAP2Ks and MAP3Ks upstream of p38. For example, PP2Cα-2 was found to inactivate the MAP2Ks MKK6 and MKK4 (295). Furthermore, PP2Cβ and PP2Cε were found to inactive p38 signaling by associating with TAK1 and dephosphorylating this MAP3K (297, 298). PP2Cε was also found to bind and dephosphorylate the MAP3K ASK1 (299).

Tyrosine phosphatases

Some protein tyrosine phosphatases (PTPs) have been shown to dephosphorylate and inactivate p38. More specifically, hematopoietic PTP (HePTP) was found to bind p38 and reduce its activation in T-cells (300). In addition, striatal-enriched protein tyrosine phosphatase (STEP), and STEP-like PTP (PTP-SL) have also been found to bind to p38 and inactivate it through dephosphorylation (301, 302). Further work is necessary to understand the role of PTPs in the regulation of p38 in physiological settings.

DUSPs

Several DUSPs of the MAP kinase phosphatase (MKP) family have been found to dephosphorylate concurrently the threonine and tyrosine residues present in p38's activation loop (293). Importantly, these MKPs can efficiently dephosphorylate p38α and p38β, but not p38γ and p38δ, which are resistant to MKP activity. MKPs identified to target p38 are MKP1/DUSP1, DUSP2, MKP5/DUSP10, DUSP8, MKP7/DUSP16, and MKP8/DUSP26 (219, 293).

The selectivity of these MKPs for their MAPK substrates differs. For example, MKPs like MKP5, MKP7, and DUSP8 selectively inactivate p38 and JNK, whereas MKP1 can also inactivate ERK (303-305). Supporting a role for MKPs in regulating MAPK activity *in vivo*, MKP1-null mice were found to exhibit sustained levels of p38 and JNK activity in response to endotoxic shock and to display increased levels of p38, JNK, and ERK activation in insulin-responsive tissues (306-308).

Based on experiments involving the ERK-specific MKP3, a general model of MKP activation has been suggested: A MAPK specifically and stably binds to the non-catalytic

amino-terminus of a MKP, with this binding stimulating the MKPs' catalytic activity. Indeed, the amino terminus of MKP3 was found to bind tightly with both ERK1 and ERK2, but not to JNK or p38 proteins. Moreover, the binding of purified ERK to MKP3 induced a large increase in the catalytic activity of MKP3 as measured by the dephosphorylation of an artificial substrate. Indeed, when expressed alone, the carboxyl-terminal catalytic domain of MKP3 lacks specificity for dephosphorylation of ERK and instead similarly dephosphorylates ERK, JNK, and p38 with low efficiency (309, 310).

Extending these observations to other MKPs, the catalytic activities of other MKPs such as MKP1, MKP2, MKP4, and DUSP7, were increased upon the binding of these MKPs to their particular substrates (293). MKP4 and MKP1 also bind and undergo catalytic activation by purified MAP kinases to which they exhibit selective binding (311). Interestingly, DUSP2, which can dephosphorylate both ERK and p38, appears only to be catalytically activated by purified ERK, suggesting that regulation of MKPs involves additional complexities (312, 313).

Pathways downstream of p38

p38 has been found to phosphorylate and activate numerous proteins involved in a variety of biological processes, including cell cycle regulation, differentiation, apoptosis, and inflammatory responses. The multitude of substrates downstream of p38 indicates that not only does p38 respond to a plethora of upstream signals, but it also induces a wide array of downstream signaling pathways. In spite of the diversity of signaling pathways activated by p38, however, the vast majority of known p38 substrates fall within one of two broad categories, namely protein kinases and transcription factors (216, 283).

Protein kinases

The first p38 substrate to be identified was MAP kinase-activated protein kinase 2 (MAPKAPK2 or MK2), although it was originally characterized *in vitro* as being phosphorylated and activated by ERK (314). Finding that MK2 phosphorylated the small heat shock proteins 25 and 27 (Hsp25 and 27) in response to stress stimuli led to the discovery that MK2 was not a target of ERK in intact cells, but rather a target for p38 (274, 278, 315, 316). In addition, the closely related kinase MK3 was also found to be phosphorylated and activated by p38 in response to stress stimuli (317).

MK2 and MK3 have been found to phosphorylate and activate different substrates that regulate various biological processes, such as cytoskeletal remodeling, cell migration, cell-cycle regulation, chromatin remodeling, gene transcription and mRNA stabilization. For example, phosphorylated Hsp27 was found to inhibit stress fiber formation induced by the GTPase Rho by competing with phosphorylated cofilin for binding to 14-3-3 proteins. This displacement of cofilin leads to its dephosphorylation and allows its binding to the barbed ends of actin, which blocks actin polymerization (318). Other MK2 and MK3 substrates that are involved in actin remodeling include Hsp25, lymphocyte-specific protein-1 (LSP1), and F-actin-capping protein Z-interacting protein (Cap-ZIP) (319-321).

In addition, MK2 and MK3 phosphorylate and activate various transcription factors, such as serum response factor (SRF), cyclic AMP-dependent transcription factor 1 (ATF1), and cAMP response element-binding protein (CREB) (284, 322). MK2 has also been found to regulate mRNA stability by phosphorylating proteins like tristetraprolin (TTP). The phosphorylation of TTP by MK2 prevents TTP from destabilizing mRNA encoding the cytokine TNFα (323-325). Furthermore, MK2 was found to regulate the cell cycle by inducing the G2/M

checkpoint in UV-irradiated cells by phosphorylating the cell division cycle 25 (Cdc25) phosphatases Cdc25B and Cdc25C, leading to their sequestration by 14-3-3 proteins and inhibiting them from promoting mitotic entry (326).

Another substrate phosphorylated and activated by p38 is the protein MK5, which is also called p38-regulated/activated kinase (PRAK). Like MK2 and MK3, PRAK has been found to phosphorylate and regulate Hsp27 (327). Much remains to be learned about the functions of PRAK downstream of p38. However, PRAK was found to act as a tumor suppressor in an experimental animal model and to activate the p53 tumor suppressor protein by direct phosphorylation, indicating that this kinase may play an important in human cancers (328).

MAPK-interacting protein kinase 1 (MNK1) is also a substrate of p38. MNK1 can phosphorylate eukaryotic initiation factor-4E (eIF-4E), increasing eIL-4E's affinity for capped mRNAs. However, the regulation of eIF-4E is complex as, depending upon the circumstances, phosphorylation of eILF-4E can lead to either a stimulation of translation or a decrease in cap-dependent translation (329-331). Further work must be done to clarify MNK1's role in regulating eIL-4E.

Additionally, p38 was found to phosphorylate and activate p38Mitogen- and stress-activated protein kinases-1 and -2 (MSK1 and MSK2). The activation of MSK1 by p38 may mediate the stress-induced activation of the transcription factor CREB (332-334). MSK1 is also capable of phosphorylating the chromatin proteins histone 2B, histone H3, and high mobility group-14 (HMG-14), indicating that p38 may regulate gene expression through its activation of MSK1 (334-336). Furthermore, both p38 itself and MSK were found to phosphorylate eIF-4E-binding protein 1 (4E-BP1), a negative regulator of eIF-4E activity, in response to UV radiation (337).

Transcription factors

The second major category of p38 substrates is comprised of transcription factors. In addition to regulating gene expression by activating the above-mentioned protein kinases, p38 directly phosphorylates and regulates many transcription factors that modulate the expression of a wide range of genes. The phosphorylation of transcription factors by p38 usually increases their activity; however, p38 has also been found to suppress the activity of select transcription factors through phosphorylation (235, 318, 338). A detailed list of transcription factors directly phosphorylated by p38 is included in Table 2. Importantly, a transcription factor may exhibit varied responses to activated p38 in different cell types, adding further complexity to signaling downstream of p38 (339).

Other substrates

p38 also phosphorylates and regulates a variety of proteins that are neither kinases nor transcription factors. These other substrate proteins include cytosolic phospholipase A2 (cPLA2), peroxisome proliferator-activated receptor (PPARα), Na+/H+ exchanger isoform-1 (NHE-1), stathmin, and keratin 8 (235, 338). Interestingly, p38 family members have been found to exhibit selective binding to select alternative substrates. For example, p38s α, γ, and δ were found to phosphorylate the microtubule-associated protein Tau, whereas p38β was found to phosphorylate glycogen synthase (338). Additional work is needed to elucidate the roles of these alternative substrates in pathways downstream of p38.

p38 MAPKs and cancer

Given that p38 has been found to regulate cellular processes like cell-cycle progression, differentiation, and apoptosis (214, 218), the activities of this kinase were suspected to influence cancer development. Indeed, much evidence has been accumulated that points to p38 playing a substantial role in neoplastic development and progression. This section summarizes our current understanding of p38's role in cancer.

Functions of p38 relevant to cancer

As mentioned at the beginning of this introduction, incipient cancer cells must manipulate or circumvent controls governing normal tissue homeostasis in order to promote neoplastic development. p38 plays important roles in regulating several of the abilities that cells acquire in order to progress toward malignancy. Acquired traits influenced by p38 include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, and tissue invasion and metastasis (1).

Indeed, depending upon the cell type and context, p38 MAPKs have been found to function in several cellular processes that are intimately related to the above-mentioned acquired traits; these processes include proliferation, differentiation, apoptosis, angiogenesis, migration, invasion, and metastasis. Importantly, p38 has been found to act as a tumor suppressor that counteracts neoplastic development much more frequently than to act as an oncogene that promotes neoplastic development. The tumor type, stage, and accompanying genetic alterations most likely determine how p38 activity affects tumorigenesis and tumor progression (214, 216, 218, 340).

Proliferation

In general, p38 activity appears to be anti-proliferative. For example, embryonic fibroblasts, fetal hematopoietic cells, and embryonic stem cells derived from p38-null mice all exhibited hyperproliferation in comparison to their wild-type counterparts (341, 342). In addition, the proliferation of neonatal rat cardiomyocytes was enhanced upon culturing these cells in the presence of a p38 drug inhibitor, and a p38-null background promoted the proliferation of murine cardiomyocytes both *in vitro* and *in vivo* (343). Similarly, p38 was found to inhibit the proliferation of lung progenitor cells in a conditional p38 knockout mouse model (344).

The anti-proliferative effects of p38 activity on cells most likely reflects the regulation of both the G1/S and G2/M cell cycle transitions by p38. For example, p38α can inhibit G1/S progression by downregulating transcription of cyclin D1 (345) as well as by upregulating transcription of the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$ (346). p38α can also regulate G1/S progression through the phosphorylation of D-type cyclins and the CDK inhibitor $p21^{Cip1}$, thereby tagging D-type cyclins for proteasomal degradation and stabilizing $p21^{Cip1}$ (347-349).

In addition, p38α and γ have been found to arrest or delay cell cycle progression at the G2/M checkpoint in response to cellular stressors (350-352). p38-dependent phosphorylation and resulting activation of MK2 appears to be crucial in regulating G2/M arrest (326, 353). As mentioned earlier, MK2 prevents mitotic entry by phosphorylating Cdc25B and Cdc25C, thereby sequestering these proteins and preventing them from activating the Cdc2/Cyclin B complex (326). Furthermore, downregulation of transcription of cyclins A and B may also contribute to p38α's induction of the G2/M checkpoint (343, 354).

Interestingly, p38's activation of the tumor suppressor p53 appears to contribute to p38's ability to halt the cell cycle in both the G1 and G2 phases. p38 can activate p53 through direct phosphorylation as well as by transcriptional upregulation, thereby promoting $p21^{Cip1}$ accumulation and G1 arrest (340). p38 has also been shown to induce the G2/M arrest of thymocytes *in vivo* in response to DNA damage in a p53-dependent manner (355).

Despite the fact that p38 is most often found to inhibit cell cycle progression, there are indications that p38 may positively regulate cell proliferation in certain contexts. For example, one report suggested that p38α positively regulates mitotic progression in HeLa cells independently of its kinase activity (356). Moreover, p38α has been shown to promote the proliferation of particular cancer cell lines as well as cytokine-stimulated hematopoietic cells (340).

Apoptosis

p38 is also a well-established mediator of apoptosis. Many pro-apoptotic stimuli, including chemotherapeutic agents, death receptor signals, and cellular stresses, require p38 activity for the induction of apoptosis (357-362). Some of these pro-apoptotic stimuli may trigger p38-dependent apoptosis by inducing the production of reactive oxygen species (ROS) or DNA damage. Importantly, the ability of p38α to induce apoptosis in response to the production of ROS by select oncogenes, such as Ras, may play a significant role in suppressing tumor initiation (363).

p38 has been found to promote apoptosis through multiple mechanisms. For example, p38 activity has been found to induce the phosphorylation and inactivation of the pro-survival proteins Bcl-2 and basal cell lymphoma-extra large (Bcl-xL). Likewise, p38 can stimulate the

phosphorylation and activation of the pro-apoptotic proteins Bcl-2-antagonist of cell death (Bad), Bcl-2 interacting mediator of cell death (Bim), BCL2-associated X protein (Bax), and Bcl-2 homologous antagonist/killer (Bak) (340). Furthermore, several transcription factors that are activated by p38, such as p53, can upregulate the expression of pro-apoptotic genes like Bax and apoptotic peptidase-activating factor 1 (Apaf-1) (357-359).

Although p38 clearly plays a pro-apoptotic role in many circumstances, several studies have reported pro-survival roles for p38. Depending upon the situation, p38 MAPKs may protect cells from apoptosis by driving the production of anti-apoptotic, inflammation-related proteins like IL-6 or NF-κB or by inducing a cell to undergo cell-cycle arrest rather than apoptosis (340). In addition, p38β has been found to have anti-apoptotic effects in certain cell lines and may buffer against pro-apoptotic effects of p38α (364, 365).

Differentiation

Differentiation blocks the proliferation of cells by entering them into a post-mitotic state. Thus, incipient cancer cells must avoid differentiation in order for a tumor to progress (1). Accumulating *in vitro* and *in vivo* evidence indicates that p38 is an important regulator of differentiation programs responsible for producing cells like adipocytes, neurons, hepatocytes, lung epithelial cells, myocytes, and cardiomyocytes (343, 344, 366-372).

p38's promotion of differentiation contributes to its tumor-suppressive properties. More specifically, activation of p38α in certain cancer cell lines was found to trigger a more differentiated and less transformed phenotype (373-375). In addition, conditional deletion of p38α in mice enhances their development of lung adenocarcinomas due to the failure of lung progenitor cells to undergo differentiation when lacking p38α (344).

p38 can induce cell differentiation in different ways, such as by activating transcription factors that promote tissue-specific differentiation (Table 2) or by targeting chromatin-remodeling enzymes to loci of differentiation-related genes in order to induce their expression (338, 376). p38α has also been found to regulate the proliferative arrest of progenitor cells at the onset of differentiation, an event that is crucial for differentiation to proceed properly (343, 344, 369, 372).

Functions in later stage tumors

In contrast to the tumor-suppressive functions of p38 that are related to its roles in proliferation, apoptosis, and differentiation, p38 may fulfill oncogenic roles in advanced tumors by promoting the processes of cell migration, invasion, angiogenesis, and metastasis. For example, p38 has been found to positively regulate the migration of various normal and tumorigenic cell populations that had been exposed to chemotactic stimuli (259, 377-380). Furthermore, multiple human cancer cell lines exhibit dependence upon p38 activity in order to invade during *in vitro* assays (381-384).

Supporting a role for p38 in invasion, p38α has been found to induce the expression of metalloproteinases (MMPs), including MMP-1, MMP-3, MMP-9, and MMP-13 (283, 384, 385). MMPs facilitate the local invasion of neoplastic cells as well as the extravasation of metastatic cells at distance sites via their ability to degrade proteins of the extracellular matrix (386). Indeed, the inhibition of p38α and p38δ in human squamous cell carcinoma cells was found to reduce their expression of MMP-1 and MMP-13 and to decrease their invasive abilities *in vitro* (382).

Angiogenesis is another process that appears to be affected by p38 activity and is critical for neoplastic progression. In order to progress to a macroscopic size, developing neoplasias must become angiogenic, acquiring the ability to stimulate the growth of new blood vessels (1). In addition to being activated by the potent, pro-angiogenic factor VEGF in certain contexts, p38 has been found to induce expression of VEGF in cells responding to stimulation with particular cytokines (387-389). Moreover, p38α has been shown to activate the transcription factor hypoxia-inducible factor 1 (HIF-1), at least in part through stabilization of its α-subunit (HIF-1α) (390, 391). Through the promotion of pro-angiogenic pathways, HIF-1's regulation of gene expression is central to the adaptive response of cells experiencing a lack of oxygen.

In addition, there exists evidence suggesting that p38 signaling occurring within stromal cells is important for the metastasis of tumor cells to secondary sites. For example, the number of lung metastases formed from intravenously injected B16 or LLC cells was decreased in mice harboring deletion of one copy of p38α in comparison to wild-type mice, with this effect attributed to weaker tumor-platelet aggregates and poorer tumor cell extravasation (392). Furthermore, activation of p38 in myeloid cells by signals originating from tumor cells was implicated in the creation of a premetastatic niche in the lungs of mice injected with B16 or LLC cells (380).

p38 in human cancers

Corresponding with the predominantly tumor-suppressive functions of p38 identified during *in vitro* and *in vivo* experiments, the majority of studies examining p38 activity in human tumor samples point toward the loss of p38 function in cancer cells. For example, p38 activity was lower in hepatocellular carcinomas samples in comparison to nearby normal tissue.

Importantly, among the tumor samples examined, larger tumors exhibited greater reductions in p38 activity (393). Moreover, the sequencing of protein kinase genes in human cancers identified somatic mutations in p38α that are likely to be driver mutations, which positively affect tumor growth and development. However, the effects of these mutations on the biochemical functions of p38α remain to be elucidated (394).

Furthermore, in comparison to their corresponding normal tissues, human cancers exhibit differences in molecules that regulate p38 activity. More specifically, levels of Wip1/PPM1D and DUSP2, phosphatases, which inhibit p38 signaling, were found to be increased in various human cell lines and tumor samples (395-399). Similarly, Gstm1 and 2, which negatively regulate p38 activity by inhibiting the p38 MAP3K ASK1, exhibit elevated levels in several human cancer cell lines (363).

In addition, the p38 MAP2K MKK4 was found to be mutated and inactivated at a frequency of approximately 5% in numerous human cancers, including those of the pancreas, bile duct, breast, colon, lung and testis (400-402). Strikingly, *MKK4* was shown to be downregulated in 75% of ovarian serous carcinomas samples relative to samples of benign ovarian tissues (403). As expected, evidence gathered from model systems also indicates that *MKK4* functions as a product of a tumor suppressor gene (401, 402).

In contrast to the evidence presented above, some studies report increased p38 activity in various tumors. Indeed, increased levels of phosphorylated p38α have been correlated with malignancy in various cancers, including glioma, follicular lymphoma, as well as lung, thyroid, breast, and head and neck squamous cell carcinomas (382, 404-408). The functional ramifications of elevated phosphorylated p38 levels remain to be determined, but appear to include increased invasiveness in gliomas and head and neck carcinomas (382, 408).

When taken together, the experimental evidence and data gathered from human tumor samples suggest that p38 usually inhibits tumor development and progression. However, p38 activity may confer a selective advantage upon tumor cells in certain contexts, depending on coexisting mutations, cancer type, and tumor stage (218, 340). Further study must be conducted in order to understand more precisely when and why p38 displays oncogenic and not tumor suppressive behaviors.

IV. Cell-matrix adhesion

Basics of cell-matrix adhesion

The adhesion of cells to their extracellular environment governs many biological processes that are essential to multicellular organisms, such as cell migration, cell survival, tissue organization, and embryonic development (409). Critical to the formation of cell-matrix adhesions are transmembrane proteins called integrins, which link proteins found in the extracellular matrix (ECM) with a cell's actin cytoskeleton. Integrins create receptors for ECM proteins through the specific heterodimerization of 18 α and 8 β subunits. ECM proteins that bind the large extracellular domains of integrin receptors include molecules like fibronectin, vitronectin, and collagens. The smaller, cytoplasmic domains of integrins interact with proteins that connect them with the actin cytoskeleton (409, 410).

In addition to integrins, many other factors localize to sites of cell-matrix adhesion, causing these structures to be tremendously complex (Fig. 3). Indeed, 90 factors have been found to predominantly reside at cell-matrix adhesions, and another 66 regulatory factors were found to interact with adhesions in a transient manner. Proteins make up 151 of these 156

adhesion-related factors, with the remaining 5 components being 4 lipids and the calcium ion (411). This enormous list of proteins can be broken down into 6 categories based upon the role played by each protein in cell-matrix adhesion; these categories are: integrins, other membrane-bound proteins, integrin-actin linkers, integrin-associated proteins, actin-binding proteins, and adaptor proteins (410).

Although integrins are critical for cell-matrix adhesion, cells also express other membrane-bound proteins that assist in cell-matrix adhesion (red in Fig. 3); these molecules include protein molecules like syndecans (412) and the hyaluronan-binding protein layilin (413). Integrin-actin linkers bind concomitantly to actin filaments and the cytoplasmic domains of integrins, forming critical bridges between integrins and the actin cytoskeleton. Examples of integrin-actin linkers are the proteins talin, α-actinin, and tensin (gold in Fig. 3). In contrast, integrin-associated proteins bind the cytoplasmic tails of integrins, but do not interact directly with actin, interacting with the cytoskeleton indirectly through other adhesion components. A subset of integrin-associated proteins are involved in signaling, such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (410).

Actin-binding proteins do not directly bind integrins, but interact instead with other proteins located at cell-matrix adhesions and play important roles in regulating adhesion dynamics (green in Fig. 3). Actin-binding proteins include vinculin and members of the enabled homolog/vasodilator-stimulated phosphoprotein (Ena/VASP) family of proteins (410, 414, 415). The last group of cell-matrix adhesion proteins consists of a large number of adaptors that interact with actin- and integrin-bound proteins (purple in Fig. 3). Many of these adaptor proteins are enzymes (light purple in Fig. 3), such as Src tyrosine kinases, GEFs and GAPs of

Rho family GTPases, and serine/threonine kinases, notably PAKs, which were introduced earlier as being upstream of p38 (410).

Types of cell-matrix adhesions

Cells can interact with ECM proteins of their substratum to form four types of adhesions: focal complexes, focal adhesions, fibrillar adhesions, and podosomes (Fig. 4). Each of these cell-matrix adhesions is mediated by integrins and consists of similar sets of non-integrin proteins. However, important structural differences exist between these classes of adhesions, with particular proteins preferentially localizing to each kind of adhesion. For example, focal complexes and focal adhesions formed by fibroblasts contain high amounts of the vitronectin receptor $\alpha_v\beta_3$ integrin, whereas fibrillar adhesions preferentially contain the fibronectin receptor $\alpha_5\beta_1$ integrin (409, 410, 416).

Focal adhesions bind cells to their substratum through strong plaques that are roughly elliptical in shape, have a length of 3 to 10 μm, and are typically found near the edges of cells (Fig. 4A; 416, 417). Focal adhesions contain many different proteins, including vinculin and talin as well as tyrosine-phosphorylated proteins, such as focal adhesion kinase (FAK) and paxillin (409, 410). Importantly, cell-matrix contacts displaying similar characteristics to focal adhesions have been found *in vivo* despite that focal adhesions were originally identified as structures created by cells growing on two-dimensional surfaces *in vitro* (418-420).

Fibrillar adhesions tend to assume an elongated shape but can also acquire more rounded forms, exhibiting variable lengths of 1 to 10 μm (Fig. 4B). Unlike focal adhesions, fibrillar adhesions form across the interior of cells, where they direct the formation of extracellular fibronectin fibrils and orient themselves along these fibrils. In addition to containing the

fibronectin receptor $\alpha_5\beta_1$ integrin, fibrillar adhesions also contain high levels the integrin-binding protein tensin and low levels of tyrosine-phosphorylated protein in comparison to focal adhesions (409, 410, 421).

At lengths of only 0.5 to 1 μm, focal complexes are transient, dot-like structures found predominately at the periphery of lamellipodia, the sheet-like extensions at cell edges that contain a branched network of actin filaments (Fig. 4C; 416, 422). Notably, stabilized focal complexes are thought to be the precursors for focal adhesions, which in turn are thought to mature into fibrillar adhesions (409, 423).

The final type of cell-matrix adhesion is the podosome, which is a small ring-shaped structure having a diameter of around 0.5 μm (Fig. 4D). The formation of podosomes by cells is more restricted than is the formation of other cell-matrix adhesions, with podosomes being found in endothelial cells, and smooth muscle cells, and cells of the monocyte lineage as well as in some cancer cells (424, 425). Podosomes contain various proteins that associate with focal adhesions, such as paxillin and vinculin, in addition to the podosome-specific proteins gelsolin and dynamin (410). Unlike the previously described cell-matrix contacts, podosomes actively degrade ECM components, making their activity central to processes like the remodeling of bone by osteoclasts (425).

Steps of cell-matrix adhesion

The adhesion of a cell to its substratum can be broken down into two processes, attachment and spreading. Cells in suspension first attach to their substratum through weak bonds, causing them to retain their spherical shape and display a refractile appearance. Shortly after their initial attachment, however, cells begin extending cytoplasmic protrusions and

forming focal adhesions with their substratum, enabling them to spread outward and assume a flattened shape (426, 427).

Cell attachment

The mechanisms used by cells during their initial attachment to their substratum are not well understood. It is appreciated, however, that the method in which cells first associate with their substratum depends upon both the nature of the substratum as well as other factors present in their environment. For example, human fibroblasts grown in serum-free media were found to attach to their substratum through secreting the ECM protein fibronectin, which adsorbs onto the surface and provides these cells with sites of attachment (426, 428). In contrast, cells grown in serum-containing media attach to their substratum by exploiting soluble ECM components that are present in serum, such as vitronectin and fibronectin. Similar to ECM proteins secreted by cells, these serum ECM components adsorb onto the substratum, providing cells with a matrix upon which to attach. Indeed, polystyrene surfaces specially manufactured to promote cell attachment – a process often performed through oxygenation of the surface – most likely enhances cell attachment by increasing adsorption of serum ECM components onto the substratum (429-431).

The binding of integrins to ECM proteins adsorbed onto a substratum plays a central role in mediating cell attachment. Importantly, though, non-integrin molecules have also been implicated in cell attachment. For example, glycosaminoglycans like hyaluronan appear to play key roles in the initial fastening of cells to their substratum (432). In addition, small peptides found in serum, such as hormones or cytokines, may also adsorb onto the substratum and aid in cell attachment (433). Taken together, the initial attachment of cells to their substratum is an ill-

defined process, depending upon both the chemistry of the substratum and factors present in cell culture media.

Cell spreading and formation of cell-matrix adhesions

As indicated above, cells begin forming integrin-mediated bonds with ECM molecules immediately upon encountering the substratum. The precise composition of the earliest integrin-ECM adhesion complexes is not clear. However, these first adhesion complexes are thought to involve the binding of two dimers of the protein talin to the cytoplasmic domains of two β integrin subunits that are themselves each dimerized with an α integrin subunit. At the same time, talin binds to filamentous actin, bridging the integrins and the actin cytoskeleton (434-437).

Integrins first bind to ECM proteins with low affinity. Binding of integrins to ECM proteins induces the autophosphorylation of focal adhesion kinase (FAK), which enables FAK to bind the non-receptor tyrosine kinase Src. Src then phosphorylates additional residues in FAK, maximizing its activity (438).

The activated FAK-Src complex stimulates the activity of Rac and Cdc42 by recruiting GEFs capable of activating these GTPases. The activation of Rac and Cdc42 at sites of integrin binding enables these GTPases to remodel the actin cytoskeleton and promote the formation of membrane protrusions like lamellipodia and filopodia (439, 440). Not only does the FAK-Src complex facilitate cell spreading by activating Rac and Cdc42, this complex also promotes cell spreading by repressing the activity of the related GTPase RhoA, thus relieving cytoskeletal tension by suppressing actomyosin contractility (441, 442).

Cells spreading along their substratum begin forming focal complexes in response to Rac and Cdc42 activity (443, 444). These complexes are labile structures that, once formed, either

disappear or develop into mature focal adhesions (423). Although the process by which focal complexes mature into focal adhesions remains ambiguous, differences in the types of proteins and their phosphorylation states have been reported to exist between focal complexes and focal adhesions. For example, the protein zyxin was found to reside at focal adhesions but not at focal complexes (445).

In addition, RhoA activity is known to drive maturation of focal complexes into focal adhesions, at least in part through stimulating actomyosin contractility (446, 447). This maturation process tends to occur at the border between lamellipodia and lamellae (448, 449). Lamellae are sheet-like cytoplasmic protrusions that are thicker and more interior to cells than lamellipodia. At later stages of cell spreading, lamellipodia continue to reach forward, whereas mature focal adhesions remain rooted underneath the lamella where they increase in size by recruiting new integrins and other adhesion proteins (423).

The continued application of force to focal adhesions through actomyosin contractility results in the formation of fibrillar adhesions and concomitant remodeling of the ECM (450). Rho activity was found to promote the formation of fibronectin fibrils by cells, indicating that this GTPase may be involved in the formation of fibrillar adhesions (451). The formation of fibrillar adhesions was also found to require Src activity (452). However, additional details of the process by which fibrillar adhesions are formed from focal adhesions remain unclear (450).

Cell-matrix adhesion and cancer

As introduced earlier, uncontrolled cell proliferation, inappropriate cell survival, increased motility, and increased invasiveness are important traits acquired by incipient cancer cells during neoplastic development. These traits are acquired, at least in part, through changes

in how cells communicate with the extracellular matrix (1, 13, 453). Indeed, alterations in the adhesive behavior of cells promote both the transformation of cells in culture as well as tumorigenesis in mice. Likewise, human tumors are regularly found to exhibit differences in the levels and activity of adhesion-related proteins in comparison to corresponding normal tissues (13, 23).

Oncogenes capable of transformation commonly reduce the number of focal adhesions made by cells and reorganize their actin cytoskeletons. For example, oncogenic Src (whose proto-oncogene was introduced above as being activated by FAK at sites of integrin-ECM binding) disassembles actin-microfilament bundles, known as stress fibers, by promoting inactivation of Rho GTPases as well as activation of the actin-destabilizing protein cofilin (454, 455). In addition, the transformation of cells by oncogenic Src induces an increase in the ratio of focal adhesion turnover to focal adhesion assembly, thereby reducing the number of focal adhesions present per cell (456).

Together, many studies have established a causative role for changes in cell-matrix adhesion in the acquisition of additional transformation-related properties, such as anchorage-independent growth and tumorigenicity in mice. Indeed, altering the expression of adhesion proteins in transformed cells has been found to repress their transformation-related properties. For example, ectopic expression of the fibronectin receptor, $\alpha_5\beta_1$ integrin, in transformed hamster cells reduced their density at confluence, inhibited their growth in soft agar, and rendered them non-tumorigenic (457). Similarly, expressing the α_2 integrin subunit in mouse mammary carcinoma cells prevented them from growing in an anchorage-independent manner and reduced their tumorigenicity (458).

In addition to certain integrins, other proteins involved in cell-matrix adhesion, such as α-actinin, gelsolin, and vinculin, have been found to inhibit transformation (459-462). These adhesion proteins were linked with the transformed state when they were found to be downregulated in tumorigenic cell lines in comparison to controls. Importantly, the ectopic expression of these proteins in tumorigenic cells that lacked their expression inhibited the anchorage-independent growth and tumorigenicity of these cells.

The mechanisms by which the ectopic expression of certain adhesion proteins negatively impacts the transformation of mammalian cells are incompletely understood. However, the abovementioned experiments clearly demonstrate the intimate relationship between cell-matrix adhesion and transformation. The sensitivity of anchorage-independence and tumorigenicity to changes in adhesion proteins has stimulated further work aimed toward understanding the mechanisms linking adhesion and transformation.

Examination of anchorage-independence has begun to shed light upon how transformed cells evade the apoptotic and growth arrest programs that normally prevent cell survival and proliferation in the absence of proper cell-matrix adhesions. In order to understand the lack of dependence of transformed cells on cell-matrix adhesions for their proliferation and survival, it is crucial to understand why normal cells stop proliferating or die if not anchored to a substratum. Indeed, integrin-dependent signaling pathways carefully regulate the proliferation and survival of normal cells.

In order to proliferate, normal cells require the collaboration between pro-proliferative signals originating from both growth factors and cell-matrix adhesions. For example, the binding of integrins to ECM proteins synergizes with signaling originating from growth factor receptors to elicit strong or sustained activity of the MAPK ERK (463-467). Importantly, growth

factor-induced activation of ERK can only induce expression of proteins crucial to cell cycle progression, like cyclin D1, when cells are adherent (465, 467, 468). Moreover, these studies suggest that transformed cells are anchorage-independent, at least in part, through the ability of their transforming oncogenes to produce sustained ERK activation in the absence of cell-matrix adhesion.

Additionally, in non-adherent cells, both expression of the CDK inhibitor $p21^{Cip1}$ is increased and degradation of a second CDK inhibitor $p27^{Kip1}$ is decreased. The resulting high levels of these CDK inhibitors prevent the entry of non-adherent cells into S phase by blocking activation of cyclin-CDK complexes (469-471). Cooperation between signaling events downstream of integrins and growth factor receptors is necessary for downregulation of these CDK inhibitors and the resulting transition of cells from the G1 to S phase of the cell cycle (472). Indeed, the activation of RhoA in response to cell spreading was found to induce the degradation of $p27^{Kip1}$ during G1 and to be essential for S phase entry (473). Likewise, active RhoA has also been found to repress the expression of $p21^{Cip1}$ (474, 475).

In addition to cell-matrix adhesion promoting the G1/S transition, the lack of proper adhesion to ECM components can induce apoptosis in normal cells. When bound to ECM proteins, integrins relay cell survival signals; however, when not bound, integrins induce cell death by promoting pro-apoptotic signaling pathways. Thus, integrins preserve tissue homeostasis by preventing the survival of cells not properly adherent to their surroundings (23).

Ligated integrins enhance cell survival by inducing the expression of anti-apoptotic proteins like Bcl-2 (476, 477), by activating pro-survival signaling pathways like the PI3K-Akt pathway (478), or by inactivating pro-apoptotic proteins like p53 (479). In contrast, unligated integrins can induce apoptosis through two different processes, namely anoikis and integrin-

mediated death. Anoikis is triggered by the complete loss of cell adhesion, whereas integrin-mediated death occurs in attached cells that also harbor many unbound integrins (23).

Anoikis may occur through either the intrinsic or extrinsic pathways of apoptosis (480). The extrinsic pathway of apoptosis involves the activation of caspases downstream of death receptors, whereas the intrinsic pathway involves activation of caspases through the release of cytochrome *c* from the mitochondria. The binding of death ligands, such as Fas ligand (FasL), to death receptors, like Fas, induces death receptor oligomerization. Oligomerized death receptors recruit the adaptor protein Fas-associated death domain (FADD) to the cell membrane. Recruitment of FADD to death receptors induces activation of caspases, which degrade cellular proteins, causing cell death (481).

Upon their detachment from their substratum, normal cells may undergo anoikis by upregulating FasL and the Fas receptor or by activating the death receptor pathway downstream of Fas (482, 483). In contrast to normal cells, unattached cancer cells fail to activate the extrinsic pathway apoptosis even though they upregulate FasL and Fas. The resistance of many cancer cells to anoikis lies, at least partly, in their chronic upregulation of FADD-like interleukin-1-converting enzyme-like inhibitory protein (FLIP) (484). FLIP inhibits signaling downstream of death receptors by binding FADD, preventing FADD from triggering caspase activation (485).

Loss of cell-matrix adhesion activates the intrinsic pathway of apoptosis by stimulating the release of factors like cytochrome *c* from the mitochondria into the cytosol. These factors then induce the activation of caspases, ultimately causing apoptosis. The release of the death-promoting proteins from the mitochondria is promoted by pro-apoptotic proteins, such as Bax, Bak, and Bim, and is inhibited by anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (480).

In addition, the heterodimerization of pro- and anti-apoptotic proteins negates the activity

of the dimerized proteins, indicating that the ratio between pro- and anti-apoptotic proteins dictates the vulnerability of a cell to apoptosis (486). Indeed, the downregulation of Bcl-xL was observed upon the detachment of non-transformed intestinal cells that were sensitive to anoikis (487, 488). In contrast, cells transformed with oncogenic Ras and rendered resistant to anoikis exhibited decreased levels of Bak and failed to downregulate Bcl-xL after losing cell-matrix adhesions (487-489).

In contrast to anoikis, the process of integrin-mediated death involves the triggering of apoptosis through the recruitment of caspase 8 to the cell membrane by unligated integrins present on adherent cells, a process that occurs independently of death receptors (490). Overcoming integrin-mediated death may be key for the survival of transformed cells growing in three-dimensional matrices, which are less permissive for formation of cell-matrix adhesions than are two-dimensional substrates commonly used in cell culture. A subset of the integrins on a cell may bind proteins within the surrounding matrix; however, the remaining unligated integrins may promote cell death, resulting in cells proliferating successfully under two-dimensional, but not three-dimensional, conditions.

In an *in vivo* setting, the ability of cancer cells to overcome integrin-mediated death may drive their local invasion and metastasis (491). The primary site of a cancer likely provides cells with an environment rife with opportunities to form adhesions with ECM proteins. Indeed, the primary site hosts a high density of tumor cells that have extensively remodeled of their extracellular matrix. However, if cells invading the surrounding tissue are to avoid integrin-mediated death, they must make many contacts with the unfamiliar matrix that surrounds them. This challenge faced by cells may explain why the cells of aggressively invasive and metastatic tumors commonly express matched integrins and ECM ligands, enabling them to deposit their

own ligands for their own integrins (492).

The strict regulation of cellular proliferation and survival by integrin-mediated adhesion provides mammals with a potent tumor suppressive mechanism counteracting neoplastic development. Only by reducing their reliance on cell-matrix adhesion for pro-growth and pro-survival signals can incipient cancer cells succeed in becoming malignant. Indeed, cancer cells develop ways of manipulating cell-matrix adhesion for their own benefit, co-opting adhesion pathways that promote their invasion into the surrounding tissue or their metastasis to a distant organ.

V. Perspectives

The *RAS* oncogene undeniably plays a role in the development and progression of human cancers, and much has been learned about how the protein product of this oncogene promotes neoplastic development. However, the majority of studies aimed toward understanding the functions of the Ras oncoprotein have involved expressing the *RAS* oncogene in cells to supraphysiological levels. This is particularly true of studies examining the role of oncogenic Ras in human cell lines and cell strains, since the most efficient method of ectopically expressing a gene in human cell populations is through retroviral infection.

Importantly, studies of genetically engineered mouse models in which oncogenic Ras was expressed from the endogenous promoter found that the level of Ras signaling experienced by a mammalian cell determines its response to this signaling (197, 201, 493). More specifically, the supraphysiological expression of oncogenic *RAS* in normal mammalian cells induces them to undergo senescence, a state in which cells are metabolically active but cannot proliferate. In contrast, the endogenous expression of oncogenic *ras* in mouse cells induced their

hyperproliferation and partial transformation, an outcome strikingly different than the terminal growth arrest characteristic of the senescent state.

I hypothesized that, like murine cells, human cells would react differently to high and low levels of oncogenic Ras signaling. To test my hypothesis, I developed a system in which I could activate oncogenic Ras signaling to either a low or high extent. Furthermore, based upon published transformation protocols, I reasoned that inhibition of a tumor suppressor protein would cooperate with low oncogenic Ras signaling in promoting the transformation of normal human cells. Due to increasing evidence that loss of p38 cooperates with oncogenic Ras to transform cells, I examined the effects of inhibiting p38 on the transformation-related phenotypes of cells subjected to a low level of oncogenic Ras signaling.

The results described in the following chapter indicate that, like murine cells, normal human cells respond differently to low and high levels of oncogenic Ras signaling. In addition, evidence is presented arguing that inhibition of p38 cooperates with low activation of oncogenic Ras signaling to reduce the adhesion of normal human cells to their substratum. Taken together, these results suggest that, by promoting proper cell-matrix adhesion, p38 may provide tumor-suppressive functions in early human neoplasias in which oncogenic *RAS* is expressed at a physiological level.

VI. Figures and tables

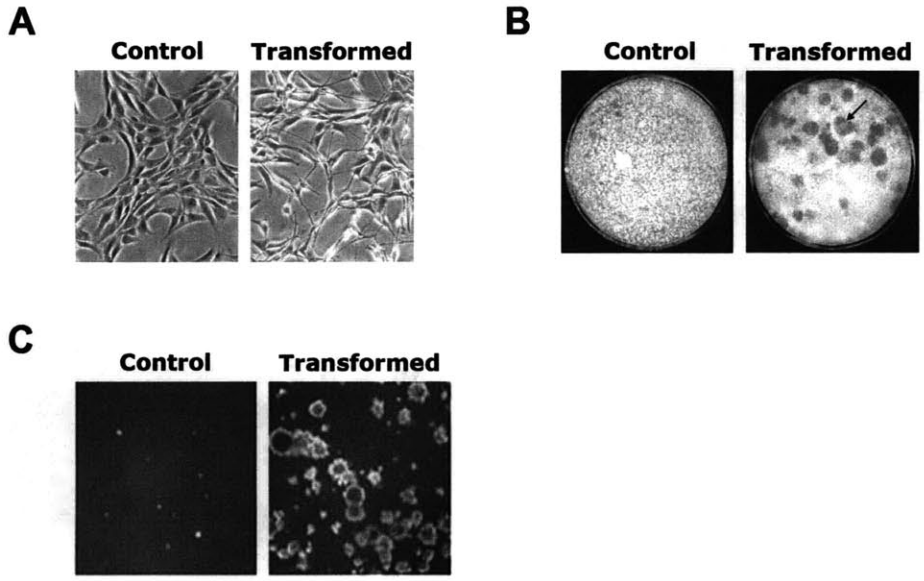

Figure 1. Characteristics of transformed cells. **A.** Morphology of normal NIH-3T3 cells and NIH-3T3 cells transformed with H-Ras^{Q61L}. Photographs reproduced from (Khosravi-Far R, *et al.* Mol Cell Biol 1995; 15:6443-453). **B.** Culture dishes of mouse embryonic fibroblasts (MEFs) that were grown to confluence and then fixed and stained to visualize cells. Control MEFs form monolayers of cells; however, MEFs transformed with endogenous levels of K-Ras^{G12D} pile atop one another, forming clusters of cells visible to the naked eye. Photographs reproduced from (Tuveson DA, *et al.* Cancer Cell 2004; 5:375-387). **C.** Soft agar colony formation by immortalized human embryonic kidney cells (HEKs). Control immortalized HEK cells form few small colonies when seeded in soft agar; however, immortalized HEK cells transformed with H-Ras^{G12V}form many large colonies. Photographs reproduced from (Rangarajan A, *et al.* Cancer Cell 2004; 6:171-183).

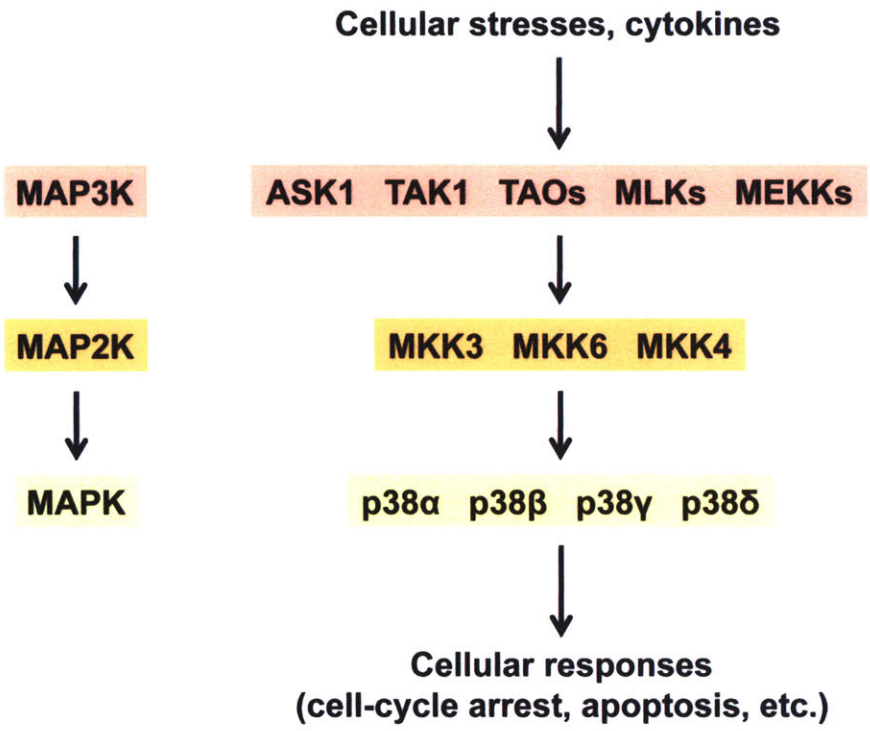

Figure 2. Tiers of the canonical p38 MAPK signaling cascade. p38 family members are MAPKs that are phosphorylated and activated by MAP2Ks. MAP2Ks are phosphorylated and activated by MAP3Ks. MAP3Ks and MAP2Ks known to activate p38 are shown. The MAP3Ks, MAP2Ks, and particular p38 family members activated by a cell in response to an extracellular stimulus depends upon both the nature of the stimulus and the cell type involved.

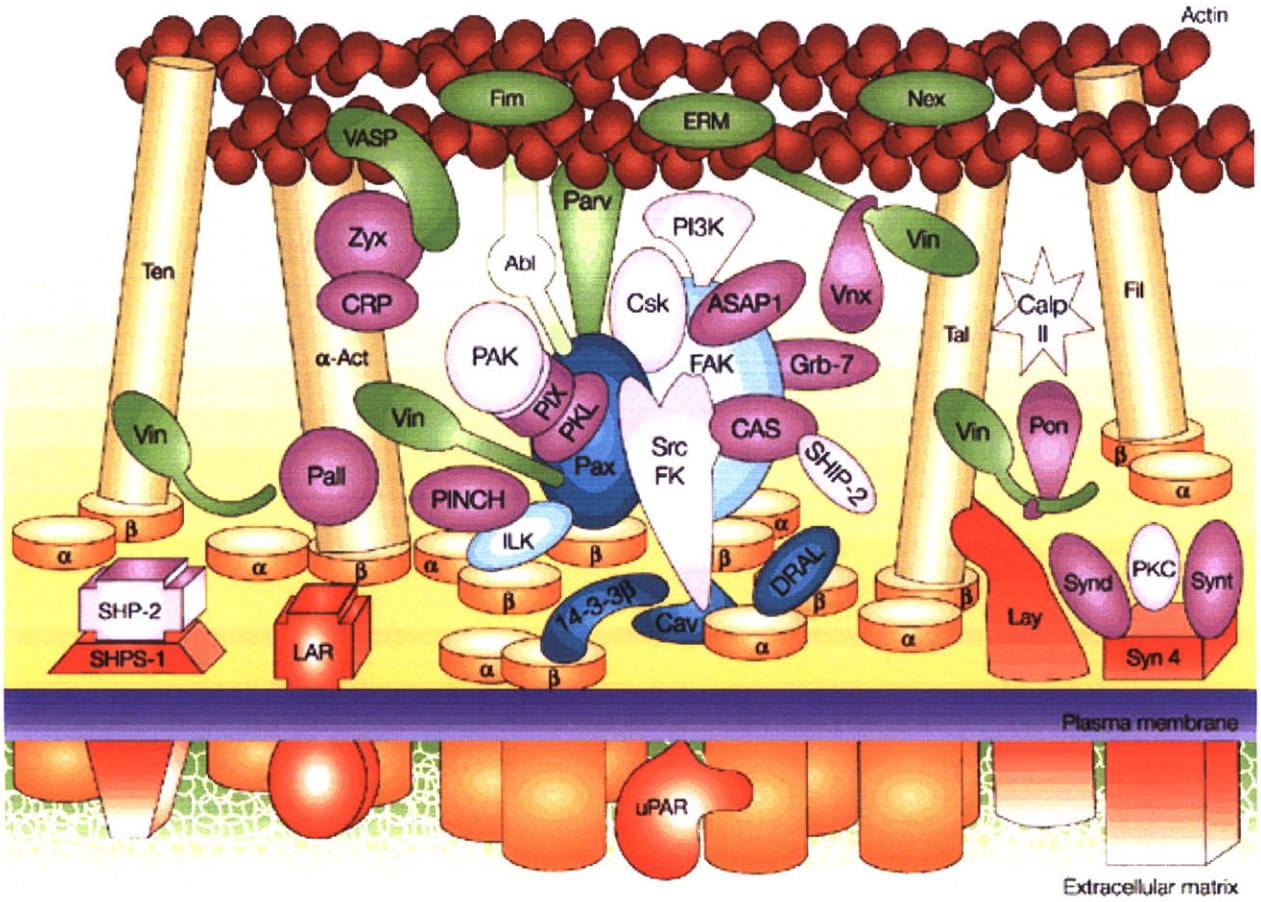

Figure 3. Schematic illustration of proteins associated with cell-matrix adhesions. The actual complexity of cell-matrix adhesions is higher because of the lack of space for including all adhesion-associated proteins and because many of the depicted proteins belong to families having multiple members. Integrins are depicted in orange, other membrane-bound proteins in red, integrin-actin linkers in gold, integrin-associated proteins in blue, actin-binding proteins in green, and adaptor proteins in purple. Adaptor proteins that are enzymes are colored light purple. Abbreviations are as follows: integrin α subunit (α), integrin β subunit (β), syndecan-4 (Syn4), layilin (Lay), phosphatase leukocyte common antigen-related receptor (LAR), SHP-2 substrate-1 (SHPS-1), urokinase plasminogen activator receptor (uPAR), α-actinin (α-Act), talin (Tal), tensin (Ten), filamin (Fil), focal adhesion kinase (FAK), paxillin (Pax), integrin-linked kinase (ILK), down-regulated in rhabdomyosarcoma LIM-protein (DRAL), 14-3-3 and caveolin (Cav), vasodilator-stimulated phosphoprotein (VASP), fimbrin (Fim), ezrin-radixin-moesin proteins (ERM), Abl kinase (Abl), nexillin (Nex), parvin/actopaxin (Parv), vinculin (Vin), zyxin (Zyx), cysteine-rich protein (CRP), palladin (Pall), particularly interesting new Cys-His protein (PINCH), paxillin kinase linker (PKL), p21-activated kinase (PAK), PAK-interacting exchange factor (PIX), vinexin (Vnx), ponsin (Pon), growth factor receptor-bound protein-7 (Grb-7), Arf-GAP protein with SH3 domains ankyrin repeats and pleckstrin homology domains-1 (ASAP1), syntenin (Synt), and syndesmos (Synd), SH2-containing phosphatase-2 (SHP-2), SH2-containing inositol 5-phosphotase-2 (SHIP-2), phosphatidyl inositol 3-kinase (PI3K), Src-family kinases (Src FK), carboxy-terminal src kinase (Csk), calpain II (Calp II), and protein kinase C (PKC). Image reproduced from (Geiger B, *et al.* Nat Rev Mol Cell Biol 2001; 2:793-805).

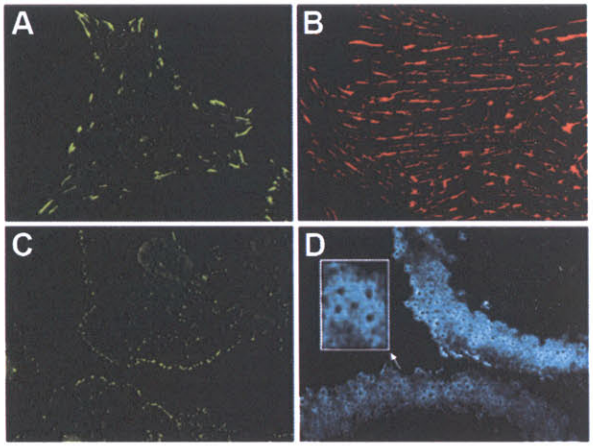

Figure 4. Types of cell-matrix adhesions. **A.** A human fibroblast stained for phosphotyrosine to visualize focal adhesions, structures that are found near the edges of cells. **B.** A human fibroblast stained for tensin to visualize fibrillar adhesions. These adhesions are found more interior to the cell and associate with fibronectin fibrils. **C.** Human fibroblasts treated with a Rho-kinase inhibitor and stained for phosphotyrosine to visualize focal complexes, the dot-like structures at the edges of the lamellipodia. **D.** Rat osteoclasts labeled with paxillin to visualize podosomes. Each podosome consists of a ring of adhesion proteins and an actin-rich center. See insert for higher magnification. Images reproduced from (Geiger B, *et al.* Nat Rev Mol Cell Biol 2001; 2:793-805).

Table 1. *RAS* mutations in human cancers.

Tissue	*HRAS*	*KRAS*	*NRAS*
Adrenal gland	1%	0%	5%
Biliary tract	0%	32%	1%
Bone	2%	1%	0%
Breast	1%	5%	1%
Central nervous system	0%	1%	2%
Cervix	9%	8%	1%
Endometrium	1%	14%	0%
Esophagus	1%	4%	0%
Eye	0%	4%	1%
Gastrointestinal tract (site indeterminate)	0%	19%	0%
Hematopoietic and lymphoid tissue	0%	5%	12%
Kidney	0%	1%	0%
Large intestine	0%	32%	3%
Liver	0%	7%	4%
Lung	1%	17%	1%
Meninges	0%	0%	0%
Ovary	0%	15%	4%
Pancreas	0%	60%	2%
Parathyroid	0%	0%	0%
Peritoneum	0%	6%	Not determined
Pituitary	2%	0%	0%
Placenta	0%	0%	0%
Pleura	0%	0%	0%
Prostate	6%	8%	1%
Salivary gland	16%	4%	0%
Skin	5%	2%	19%
Small intestine	0%	20%	25%
Stomach	4%	6%	2%
Testis	0%	5%	4%
Thymus	0%	15%	0%
Thyroid	4%	3%	7%
Upper aerodigestive tract	9%	4%	3%
Urinary tract	12%	4%	3%

Data derived from the Catalogue of Somatic Mutations in Cancer (COSMIC) of the Wellcome Trust Sanger Institute, Cambridge, UK. Table reproduced from (Karnoub A, Weinberg R. Nat Rev Mol Cell Biol 2008; 9:517-31).

Table 2. Transcription factors directly regulated by p38 MAPKs. (Continued on next page.)

Transcription factor	Complete name	Manner of p38's regulation	Biological process(es) promoted by p38	References
MEF2A, MEF2C, and MEF2D	Myocyte enhancer factors 2A, 2C, and 2D	Activation	Skeletal and cardiac muscle differentiation and cardiac hypertrophy	(494-498)
E47		Activation and heterodimerization	Skeletal muscle differentiation	(499)
MRF4	Muscle-specific regulatory factor 4	Repression	Skeletal muscle differentiation	(500)
BAF60	BRG1-associated factor 60	Activation	Skeletal muscle differentiation	(501)
C/EBPβ	CCAAT/enhancer-binding protein β	Activation	Response to cellular stress	(502)
GADD153/ CHOP	Growth arrest and DNA damage inducible gene 153; C/EBP homologous protein	Activation	Apoptosis	(503, 504)
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1;	Activation	RANKL[b] signaling in osteoclast differentiation	(505)
NFATc4	Nuclear factor of activated T-cells, cytoplasmic 4	Inactivation, nuclear export	Downregulation of adipocyte differentiation[a]	(506)
MITF	Microphthalmia-associated transcription factor	Activation	RANKL[b] signaling in osteoclast differentiation	(507)
MafA	Named after v-*maf* oncogene	Activation	Lens differentiation and response to oxidative stress	(508)
GATA1	GATA binding protein 1	Activation	Interleukin 9 (IL-9) expression in mast cells	(509)
C/EBPε	CCAAT/enhancer binding protein ε	Activation	Neutrophil differentiation	(510)
PGC-1α	Peroxisome proliferator-activated receptor (PPAR) gamma, coactivator 1α	Activation	Metabolism, adaptive thermogenesis in muscle cells, and muscle cell adaptation to exercise	(511-513)
Smad3	SMA- and MAD-related protein 3	Activation	Signaling downstream of TGF-β	(514, 515)

p53		Activation	UV-induced G2/M arrest	(355, 516)
HBP1	HMG box-containing protein 1	Protein stabilization	G1 arrest	(517)
STAT1	Signal transducer and activator of transcription 1	Activation	Signaling downstream of interferons α and γ (IFN-α and IFN-γ), IFN-dependent viral killing, response to stress signaling	(518, 519)
c-FOS	Named after v-*fos* oncogene	Activation	Response to UV radiation	(520)
NFATp	Nuclear factor of activated T-cells, preexisting component	Inhibition via nuclear export	Opposes calcium-promoted activation of NFATp transcription	(521)
ATF2	Activating transcription factor 2	Activation	Activation of ATF2 in response to mitogens via cooperation with ERK, insulin resistance in endothelial cells	(522, 523)
ATF6	Activating transcription factor 6	Activation	Response to cellular stress	(524)
Members of TCF subfamily of Ets transcription factors (Including Elk1, Sap1, SRF, and CREB)	Ternary complex factor subfamily of E26 transcription factors	Activation	Response to interleukin 1 (IL-1) and cellular stress	(339, 525, 526)
Usf-1	Upstream stimulatory factor 1	Activation	Tanning response to UV radiation	(527, 528)

[a] The adipocyte differentiation process is more complicated than simply being downregulated by p38 since pharmacological inhibition of p38 in this system blocked differentiation; other pathways activated by p38 must concurrently promote differentiation.

[b] Receptor activator of NF-κB ligand

Table adapted from (Perdiguero E, Munoz-Canoves P. Top Curr Genet 2008; 20:51-79).

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Chapter 2:

Inhibition of p38 cooperates with low activation of oncogenic Ras signaling to promote loss of normal cellular adhesion

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I. Abstract

Activating mutations in *RAS* oncogenes occur frequently in human cancers. However, in experimental settings, oncogenic *RAS* has most often been studied at supraphysiological levels of expression. To study the outcome of oncogenic Ras signaling in human cells at a more physiological level, we developed a system in which we could activate oncogenic Ras signaling to either low or high extents in normal human fibroblasts. A low level of oncogenic Ras signaling induced cellular hyperproliferation, whereas a high level of signaling induced cellular senescence. A growing body of literature links loss of p38 mitogen-activated protein kinase (MAPK) activity with the promotion of Ras-induced transformation in murine cells. Accordingly, we examined the effect of inhibiting p38 in normal human cells in which we also activated a low level of oncogenic Ras signaling. Interestingly, the inhibition of p38 cooperated with low activation of oncogenic Ras to alter the morphology and adhesive properties of cells. Our results suggest that the inhibition of p38 could predispose human cells to partial transformation by oncogenic Ras through alterations in cellular adhesion.

II. Introduction

Extensive research over the past three decades has yielded numerous insights into the role of oncogenic Ras in tumorigenesis (1-4). However, many of these studies have utilized experimental models in which oncogenic Ras was expressed to supraphysiological levels. Importantly, recent reports have shown that the level of oncogenic Ras signaling experienced by mammalian cells is crucial in determining their response to Ras signaling (5-7). These data

argue that expressing oncogenic Ras to high, supraphysiological extents fails to recapitulate the actions of endogenous oncogenic Ras in the development of human malignancies. Indeed, early in the course of multistep tumorigenesis, when cells contain relatively few genetic abnormalities, this oncoprotein is expressed at levels comparable to those of the normal Ras protein (1, 8, 9).

More specifically, overexpressing oncogenic Ras at a high level in normal mammalian cells induces a state of growth arrest termed senescence, in which cells remain metabolically active but cease to divide (10-14). In contrast, expressing oncogenic Ras in human and murine cells at lower, more physiological levels fails to induce senescence and instead stimulates the proliferation of these cells. Moreover, cells subjected to high and low dosages of oncogenic Ras signaling respond by differentially activating downstream signaling pathways according to their level of exposure to oncogenic Ras (5-7, 15).

The first direct Ras effector to be identified was the Raf serine/threonine kinase. Following its activation by Ras, Raf activates MEK, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase. MEK then in turn activates ERK1 and ERK2 (3). The Raf/MEK/ERK signaling cascade has been intensively studied in the context of neoplastic development and is now known to be a crucial player in the development of tumors initiated by oncogenic Ras (3, 16).

Currently, increasing attention is being focused on the p38 pathway, a second MAPK signaling pathway that appears to be involved in Ras-induced tumorigenesis. Four different isoforms of p38 (α, β, γ, and δ) have been identified in mammals (17). Although the p38 MAPK was first identified through its strong activation in response to cellular stresses (17), the loss of p38α protein has recently been found to induce the hyperproliferation of several murine cell types (18, 19) and to collaborate with oncogenic Ras in the transformation of mouse embryonic

fibroblasts (MEFs) (20). Likewise, genetic ablation of PRAK, a kinase directly phosphorylated by p38, was found to cooperate with oncogenic Ras in the transformation of murine cells, and knockdown of PRAK could substitute for MDM2 in the transformation of human cells when using an E1A/MDM2/Ras transformation protocol (21).

In further support of the idea that p38 negatively regulates transformation-associated phenotypes driven by oncogenic Ras signaling, a p38α null background increased the number and size of tumors induced by oncogenic Ras in a mouse model of lung cancer (22). Similarly, knockout of the Wip1/PPM1D phosphatase and the resulting increased basal level of activated p38 reduced the tumorigenicity of MEFs transformed with H-Ras^{G12V} and E1A (23). In addition, activation of the p38 pathway was found to inhibit the Ras-induced increase in DNA synthesis within NIH3T3 cells and to inhibit DNA synthesis in Ras-dependent, but not Ras-independent, human bladder carcinoma cells (24).

Previous work had shown that human cells respond differently than murine cells to the actions of introduced oncogenes (11). Because very little work has been done to examine the outcomes of low oncogenic Ras signaling with respect to the transformation of normal human cells, we chose to investigate this matter. Furthermore, due to the accumulating evidence that p38 acts as a tumor suppressor by counteracting Ras-induced transformation, we hypothesized that the inhibition of p38 might cooperate with low oncogenic Ras signaling to partially transform cells. Here, we provide evidence that the inhibition of p38 cooperates with the low activation of oncogenic Ras signaling to reduce the adhesion of normal human cells to their substratum. Our results suggest that p38 may provide tumor-suppressive functions in early human neoplasias that express physiological levels of oncogenic *RAS*.

III. Materials and methods

Plasmids and cell strains

A DNA fragment containing ER:H-Ras^{G12V} was subcloned from pBabe-Puro ER:H-Ras^{G12V} (25) into the pBabe-Hygro retroviral vector. Amphotropic retroviruses were generated by using Fugene 6 (Roche Applied Science) to transfect 293T cells with a viral vector and the packaging vectors pUMVC3, and pCMV-VSV. The viral vectors and constructs used are as follows: pBABE-Puro ER:H-Ras^{G12V}, pBabe-Hygro ER:H-Ras^{G12V}, and pBabe-Hygro empty vector. BJ fibroblasts were obtained from the American Type Culture Collection and described previously (26). BJ fibroblasts were infected for 6 hours with viral supernatants that were harvested at 48 and 72 hours post-transfection and that contained 6 μg/ml protamine sulfate. Cells were continuously selected with 100 μg/ml hygromycin (hygro) or 2 μg/ml puromycin (puro) as appropriate.

Cell culture and drug treatment

BJ fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME) containing 10% inactivated fetal bovine serum (IFS), 100 units/ml penicillin, and 100 g/ml streptomycin. SB 203580 (Calbiochem) and BIRB 796 (Axon Medchem) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM. Cells were treated with 1 μM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) to activate ER:H-Ras^{G12V} or EtOH for the vehicle control. SB 203580 was added directly to culture media at final concentrations of 10 or 20 μM. Since BIRB 796 is largely insoluble when directly diluted into culture media at a final concentration of 10 μM, in order to treat cells with 10 μM BIRB 796, we used a previously reported transfection-based

method for delivering an insoluble drug into cells (27). DMSO was transfected as a control. 4-OHT, SB 203580, BIRB 796, and the appropriate vehicle controls were each refreshed every 3 days during experiments. Unless specified, experiments were performed using plastic TC-treated culture dishes or multiple well plates (Corning). Untreated culture dishes (Corning) were used for experiments done under reduced adhesion conditions.

Western blot analysis

Protein expression was measured using standard Western blot techniques and with antibodies specific to H-Ras (sc-520; Santa Cruz Biotechnology), β-actin (sc-47778; Santa Cruz Biotechnology), phosphorylated ERK (#4396; Cell Signaling), ERK (#9102; Cell Signaling), phosphorylated Akt (#4051; Cell Signaling), Akt (#4691; Cell Signaling), phosphorylated p38 (#9211; Cell Signaling), p38 (#9212; Cell Signaling), phosphorylated MK2 (#3007; Cell Signaling), and MK2 (#3042; Cell Signaling). ImageJ software was used to quantify protein levels from Western blots (28).

Senescence-associated β-galactosidase (SA β-gal) assay

SA β-gal activity was detected as previously described (29).

UV treatment

Cells were treated with 200 J/m^2 UV light using a Stratalinker UV crosslinker (Stratagene). The culture media was removed from cells immediately prior to UV treatment and the same media was replaced immediately after UV treatment. Cells were incubated for 1 hr at 37 °C and then harvested for Western blotting.

Immunofluorescence

Cells were cultured in 12-well TC-treated culture plates (Corning) as described above. Cells were fixed for 10 minutes using 3.7% paraformaldehyde in phosphate-buffered saline (PBS). Cells were stained for F-actin using fluorescently labeled Phalloidin (A12381; Cell Signaling) according to the manufacturer's instructions. When staining cells for both F-actin and paxillin, phalloidin staining was completed first. Following phalloidin staining, cells were re-permeabilized for 10 minutes using 0.5% Triton X and incubated for 1 hour with PBG, which contains 0.2% fish gelatin (G-7765; Sigma-Aldrich) and 0.5% bovine serum albumin (BSA) in PBS. Focal adhesions were detected by incubating cells for 2 hours with a 1:100 dilution of anti-paxillin antibody (5H11; Millipore) followed by a 1 hour incubation in a 1:500 dilution of Alexa Fluor 488 goat anti-mouse IgG (Invitrogen); both antibodies were diluted in PBG. Cells were incubated for 30 minutes in 1% BSA in PBS containing 1 μg/ml 4'6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualize cell nuclei and a 1:40 dilution of the phalloidin stock to strengthen actin signal. The number of focal adhesions per cell were quantified by eye.

Cell morphology analysis using CellProfiler

Open-source CellProfiler software (www.cellprofiler.org) was used to identify the nuclei and edges of cells present in microscopy images; cells had been fluorescently labeled using DAPI to visualize nuclei and phalloidin to visualize actin cytoskeletons. Automated algorithms identified each cell's nucleus and edge. For each outlined cell, we then obtained quantitative measurements of cell size and shape. More detailed information is available in the supplementary information.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Student's t test was used for comparisons.

IV. Results

Dose-dependent effects of oncogenic Ras signaling on cellular proliferation

We first developed a system in which we could make normal human cells hyperproliferate when exposed to oncogenic Ras signaling. To this end, we developed a means of activating oncogenic Ras signaling to either a low or high extent in BJ cells, a strain of normal human dermal fibroblasts. Moreover, in order to investigate the initial responses of BJ fibroblasts to the activation of either low or high levels of oncogenic Ras signaling, we exploited the 4-hydroxytamoxifen (4-OHT)-inducible ER:H-Ras^{G12V} construct, in which a modified form of the estrogen receptor ligand-binding domain is fused to the N-terminus of oncogenic H-Ras (25, 30). As previously shown, oncogenic Ras signaling is activated within 10 minutes of the addition of 4-OHT to culture media, and this activation reaches a maximum 24 to 48 hours after addition of 4-OHT (25, 31).

We generated stable populations of BJ fibroblasts in which oncogenic Ras signaling could be activated at a high level by infecting BJ fibroblasts with the pBabe-Puro ER:H-Ras^{G12V} retroviral expression construct. At the same time, we generated stable populations of BJ fibroblasts in which oncogenic Ras signaling could be activated at a low level by infecting BJ fibroblasts with the pBabe-Hygro ER:H-Ras^{G12V} retroviral expression construct. Previous work had shown that the pBabe-Puro and pBabe-Hygro retroviral expression constructs produce

higher and lower amounts of the desired gene product, respectively, making their use ideal for the purposes of this study (32).

In the absence of 4-OHT, minimal amounts of the ER:Ras^{G12V} fusion protein were detected in BJ fibroblasts expressing either pBabe-Hygro ER:Ras^{G12V} (Low ER:Ras) or pBabe-Puro ER:Ras^{G12V} (High ER:Ras) fusion proteins (Fig. 1A), echoing previous results that described the use of the pBabe-Puro ER:Ras^{G12V} construct (25). However, addition of 4-OHT to both Low ER:Ras and High ER:Ras BJ fibroblasts induced the accumulation of the ER:Ras fusion protein to low and high levels, respectively. Once accumulation of ER:Ras protein reached a maximum at 24 hours, the High ER:Ras cells contained 4.5-fold more ER:Ras protein than did the Low ER:Ras cells (Fig. 1A and data not shown).

These levels of activated oncogenic Ras signaling caused, in turn, differential activation of signaling pathways downstream of Ras. For example, as gauged by the phosphorylation status of ERK, both low and high oncogenic Ras signaling activated this kinase following addition of 4-OHT. Importantly, the level of ER:Ras dictated the extent of ERK activation (Fig. 1A). Interestingly, Akt, which is activated by Ras through the PI3K pathway (1), was induced in a non-linear manner as determined by comparing the levels of phosphorylated Akt to the levels of ER:Ras and phosphorylated ERK. Akt was robustly activated by a high level of oncogenic Ras signaling, whereas it was barely activated by low oncogenic Ras signaling (Fig. 1A). These results demonstrate that high and low levels of oncogenic Ras signaling result in the differential activation of signaling pathways downstream of Ras, an observation that is consistent with previous reports (6, 7, 15).

When propagated through repeated serial passages in subconfluent, monolayer cultures and in the presence of 10% serum, Low ER:Ras cells treated with 4-OHT hyperproliferated in

comparison to vehicle control-treated Low ER:Ras cells, generating 13 times more cells over the course of 15 days (Fig. 1B and C). Furthermore, we found that the level of ER:Ras^{G12V} expressed in 4-OHT-treated Low ER:Ras cells was maintained over the prolonged period of culturing these cells (Fig. 1D). This indicated that cells expressing a low level of oncogenic Ras signaling were not counterselected and was consistent with our observation that low oncogenic Ras signaling promoted cell proliferation.

In stark contrast, High ER:Ras cells underwent growth arrest beginning at 8 days of 4-OHT treatment (Fig. 1B and C), with the great majority of cells staining positively for senescence-associated β-galactosidase (SA β-gal) activity (Fig. 1E and F). These data indicated that the cells had entered into a state of senescence in response to a high level of oncogenic Ras signaling. Taken together, the preceding series of experiments demonstrated that by overexpressing ER:Ras to a low extent in BJ fibroblasts we were able to develop a human cell system in which oncogenic Ras induced hyperproliferation rather than senescence, a result that functionally mimics the outcome of expressing oncogenic Ras from the endogenous locus in murine fibroblasts (5, 6).

Unexpectedly, like 4-OHT-treated Low ER:Ras cells, control High ER:Ras cells, which were not exposed to 4-OHT, exhibited hyperproliferation in comparison to control Low ER:Ras cells, which were also not exposed to 4-OHT. This suggested that the ER:Ras system manifests some leakiness, in that there appeared to be a basal level of oncogenic Ras signaling even in the absence of 4-OHT-mediated activation. Indeed, untreated High ER:Ras cells exhibited a higher level of ER:Ras protein and an elevated level of ERK activation in comparison to untreated Low ER:Ras cells (Fig. 1A). More importantly, Low ER:Ras cells that were not treated with 4-OHT proliferated at a rate similar to cells infected with empty vector (Fig. 1G). This latter result

indicated that Low ER:Ras cells not treated with 4-OHT behave like cells that lack the ectopically expressed ER:Ras protein.

p38 inhibition cooperates with oncogenic Ras signaling to affect cellular morphology

Having developed an inducible system in which we could stimulate human cells to undergo Ras-induced hyperproliferation, we next asked whether loss of p38 signaling could cooperate with low oncogenic Ras signaling to promote transformation-related phenotypes. We pursued this line of work, since loss of p38 function in murine cells had been found to promote Ras-induced transformation and tumorigenesis (20-24).

In order to inhibit p38, we used the chemical inhibitors BIRB 796 and SB 203580 to continuously inhibit p38 activity in the 4-OHT-treated Low ER:Ras cells. Importantly, BIRB 796 and SB 203580 are both potent inhibitors of p38α and β, but have different chemical structures, bind to the p38 protein at different sites, and exhibit distinct off-target effects (33). By allowing us to exclude many confounding off-target effects, the use of these two drug inhibitors was ideal for delineating effects specific to the inhibition of p38.

We assessed p38α and β function by monitoring the phosphorylation state of one of their direct substrates, MK2. MK2 is directly phosphorylated in response to UV light exposure in a p38-dependent manner (34). We observed that MK2 was robustly phosphorylated when BJ fibroblasts were exposed to UV light, but that no phosphorylated MK2 was detectable when these cells were first pretreated with either BIRB 796 or SB 203580, indicating that these drugs efficiently inhibit p38 signaling (Fig. 2A).

In addition, we found that BIRB 796, but not SB 203580, inhibited the phosphorylation of p38 itself (Fig. 2A). This result most likely stems from the different mechanisms of p38

inhibition exhibited by these two drugs. The binding of BIRB 796 causes p38 to undergo a large conformational change, preventing p38 from interacting with its upstream kinases (35). In contrast, SB 203580 is a competitive inhibitor of p38 that impedes p38's phosphorylation of downstream targets without perturbing its interaction with upstream kinases (36).

We next asked whether suppressing p38 activity would enhance the proliferation of Low ER:Ras human BJ fibroblasts treated with 4-OHT because of observations recently reported by others who had used murine cell systems (18-20). More specifically, others have shown that p38α-null MEFs infected with a vector specifying oncogenic Ras proliferate faster than corresponding wild-type cells infected with oncogenic Ras (20). Also, multiple p38α-null murine cell types have been shown to proliferate faster than their wild-type counterparts (18, 19).

Unexpectedly, subconfluent Low ER:Ras BJ fibroblasts treated with 4-OHT failed to proliferate more rapidly when exposed to either BIRB 796 or SB 203580 (Fig. 2B and C). Given that BIRB 796 and SB 203580 are both effective inhibitors of p38α, the failure of these inhibitors to enhance the proliferation of Low ER:Ras cells treated with 4-OHT most likely reflects differences in the experimental cell systems used. Whereas we used normal human fibroblasts that possess a limited lifespan in culture, immortalized MEFs were used in the experiments showing that a p38-null background enhances the proliferation of cells containing oncogenic Ras (20).

We did, however, note a distinctive effect of inhibiting p38 signaling in 4-OHT-treated Low ER:Ras cells: cells in which both oncogenic Ras signaling was activated and p38 was inhibited were far more refractile and appeared to be less spread out on their substratum than Low ER:Ras cells treated with 4-OHT alone (Fig. 2D and E). Taken together, these observations indicated that while p38 function had no observable effect on Ras-induced cell proliferation,

inhibition of p38 cooperated with low activation of oncogenic Ras signaling to alter the way in which cells adhere to their substratum.

p38 inhibition and oncogenic Ras signaling affect cell spreading and cell shape

When cells in suspension first attach to a substratum, they retain their spherical shape, display a refractile appearance, and are weakly bound to the substratum. Soon after their initial attachment, however, cells begin extending cytoplasmic protrusions and form strong associations with their substratum known as focal adhesions, enabling them to spread outward and become flattened in shape (37, 38). Importantly, the inhibition of molecules that are crucial to the process of cell spreading, such as Rac and Cdc42, does not prevent the attachment of cells to their substratum but instead causes these attached cells to remain spherical and refractile (39). Accordingly, we hypothesized that the inhibition of p38 signaling in combination with low activation of oncogenic Ras signaling specifically affects cell adhesion by impeding the process of cell spreading.

To assess whether the inhibition of p38 cooperates with the activation of oncogenic Ras signaling to hinder cell spreading, we used image analysis software (40) to quantify the observed changes in cell size and shape (Fig. 3). We found that the activation of low oncogenic Ras signaling significantly decreased the two-dimensional area covered by cells (Fig. 3B and C). Moreover, inhibition of p38 cooperated with Ras signaling to further decrease the area covered by cells, in spite of the fact that inhibiting p38 alone failed to affect cell area or perimeter length.

In addition, low activation of oncogenic Ras signaling and inhibition of p38 had opposite effects on the convexity or roundness of cells (Fig. 3D). Inhibition of p38 alone caused cells to develop more concave areas at their edges. However, low activation of oncogenic Ras signaling

slightly increased the convexity of cells and prevented cells treated with SB 203580 from exhibiting an increase in concavity (Fig 3D).

Similarly, low activation of oncogenic Ras signaling and inhibition of p38 had opposite effects on cell length. The inhibition of p38 caused the elongation of cells in both the presence and absence of oncogenic Ras signaling. In contrast, the activation of Ras signaling slightly decreased the length-to-width ratio of cells relative to that of control cells (Fig. 3E).

These measurements of cell morphology collectively indicated that low activation of oncogenic Ras signaling causes changes in cell shape that are distinct from the changes caused by inhibiting p38. However, the present results also demonstrated that, when acting together, the inhibition of p38 complements the activation of oncogenic Ras signaling to alter the two-dimensional shape of cells, indicating that the perturbations of these signaling pathways cooperate to impede the normal spreading of cells.

The distinct effects on cell shape caused by inhibiting p38 and activating Ras signaling were most likely due to p38 and Ras impinging upon signaling events that modulate cell-matrix adhesion. Nonetheless, we did not observe an increase in the level of phosphorylated p38α – the activated form of this kinase – when we activated oncogenic Ras signaling in our Low ER:Ras cells (Supplementary Fig. S1), agreeing with a previous report published by others that focused upon the dose-dependent activation of p38 by Ras (15). Because low oncogenic Ras signaling was not found to activate p38, we concluded that Ras and p38 both operate in pathways that regulate cell-matrix adhesion but that p38 is not downstream of Ras.

The inhibition of p38 and the activation of Ras work in concert to reduce cell adhesion

Because the inhibition of p38 cooperates with the low activation of oncogenic Ras signaling to alter the spreading of cells, we further investigated changes in cell adhesion, including the first step of cell adhesion, cell attachment. Activation of low oncogenic Ras signaling slightly hampered the attachment of suspended cells to tissue culture-treated plastic at two, but not 12, hours post-plating. In contrast, chemically inhibiting p38 did not affect the attachment of cells at either two hours or 12 hours post-plating and did not cooperate with Ras signaling to affect cell attachment. By 12 hours post-plating, we observed that the cells of all populations were attached to the plastic substratum to similar extents (Fig. 4A and B). These results indicate that although low activation of oncogenic Ras has a modest affect on the kinetics of cell attachment to a substratum, the inactivation of p38 fails to affect the initial attachment of cells to their substratum.

We then examined the second step of cell adhesion, post-attachment cell spreading, at 12 hours post-plating. We found that inhibiting p38 cooperated with activating oncogenic Ras signaling to hinder the spreading of cells on their substratum (Fig. 4C and D). At 12 hours post-plating, cells treated dually with 4-OHT and either BIRB 796 or SB 203580 remained more spherical and refractile than cells in which only one signaling pathway was perturbed or neither signaling pathway was perturbed. Hence, inhibition of p38 appears to cooperate with low activation of oncogenic Ras signaling in constraining the ability of a cell to extend cytoplasmic processes and/or to make focal adhesions with the substratum, both processes being crucial in the spreading of cells.

Given our observation that the p38 and Ras pathways both affect cell spreading, but not the initial cell attachment step of cell adhesion, we wondered whether providing our cell

populations with an alternative substratum would reveal differences in their ability to attach. In fact, the previously described assays were performed using tissue culture-treated dishes whose polystyrene surfaces had been modified using a manufacturing process that caused them to become hydrophilic. Because this hydrophilic treatment promotes cell attachment, we examined the ability of Ras and p38 to affect cell attachment by performing an assay using untreated polystyrene culture dishes.

We found that, as before, cells could attach to untreated plastic if oncogenic Ras were activated alone or if p38 were inhibited alone. However, the attachment of cells to untreated plastic was greatly impaired if both oncogenic Ras signaling were activated and p38 were inhibited. Such cells preferentially formed suspended cellular aggregates rather than contacts with the untreated substratum (Fig. 4E and F). Consequently, when taken together, our measurements of cell adhesion indicate that there exist substratum conditions in which the inhibition of p38 cooperates with the low activation of oncogenic Ras signaling to hinder both the initial cell attachment and subsequent cell spreading processes of cell adhesion.

Because reduced cell adhesion has long been associated with cells that are tumorigenic (41, 42), we next asked whether the inhibition of p38 in combination with the low activation of oncogenic Ras could transform cells. One standard characteristic of transformed cells is their inability to undergo contact inhibition upon reaching high densities (43). Accordingly, we tested whether inhibiting p38 and activating Ras signaling in our Low ER:Ras cells would prevent cells from undergoing contact inhibition upon continuous culture.

While the inhibition of p38 and the low activation of oncogenic Ras signaling each served to increase the density of cells at confluence, as expected from previously reported murine studies (6, 44), perturbing neither signaling pathway on its own enabled cells to

overcome contact inhibition (Supplementary Fig. S2 and data not shown). Furthermore, the inhibition of p38 in combination with low activation of Ras signaling was not sufficient to overcome contact inhibition even after three weeks of continuous culture (Supplementary Fig. S2). These results indicate that although the inhibition of p38 cooperates with the low activation of oncogenic Ras signaling to change the adhesive nature of cells, these perturbations alone are not sufficient to fully transform cells as gauged by their ability to become contact inhibited in monolayer culture.

Inhibition of p38 and activation of oncogenic Ras reduce focal adhesion formation

An intimate and dynamic interplay between the binding of transmembrane integrins to the extracellular matrix and the organization of a cell's actin cytoskeleton is fundamental to the formation of focal adhesions (45). Although cells can begin to extend lamellipodia along a substratum when they are deficient in the ability to form focal adhesions, the formation of focal adhesions is crucial for the continued advancement of lamellipodia and sustained cell spreading. Consequently, focal adhesion formation is crucial for cells to exhibit their normal, spread morphology after attaching to their substratum (46, 47).

Because we had found that the inhibition of p38 cooperates with low activation of oncogenic Ras signaling to alter cell morphology and impede cell spreading, we hypothesized that the Ras and p38 pathways affect the formation of focal adhesions. For this reason, we determined whether the inhibition of p38 acts in concert with the low activation of Ras signaling to reduce the number of focal adhesions formed by a cell with the underlying substrate.

We found that low activation of oncogenic Ras signaling and inhibition of p38 each reduced the number of focal adhesions per cell (Fig 5A and B). The activation of low Ras

signaling resulted in a 63% decrease in the number of detectable focal adhesions. Similarly, inhibiting p38 using 10 and 20 μM concentrations of SB 203580 caused 78% and 86% decreases, respectively, in the number of focal adhesions detected per cell.

Interestingly, when both Ras signaling was activated and p38 was inhibited, we observed a further decrease in the number of focal adhesions per cell (Fig. 5A and 5B). Treatment of cells with both 4-OHT and 10 μM SB 203580 resulted in a 91% decrease in the number of focal adhesions visible per cell. Displaying a dose-dependent response to SB 203580, cells treated with both 4-OHT and 20 μM SB 203580 exhibited a 94% decrease in the number of focal adhesions per cell (Fig. 5B). These results indicate that low activation of oncogenic Ras signaling and inhibition of p38 signaling work together to impede the formation of strong cellular bonds to the substratum by having additive effects on reducing the number of focal adhesions present per cell.

V. Discussion

An interesting hypothesis emerges upon placing our findings in the context of the current understanding of Ras, p38, and cell adhesion: inhibition of the p38 pathway may create an intracellular state in human cells that predisposes them to partial transformation by oncogenic Ras. In contrast to previous studies in which oncogenic Ras was overexpressed to very high extents, we have more closely approximated the physiological levels of oncogenic Ras signaling that are present in human neoplasias by activating this signaling pathway to a low extent. As a result, our experimental system enabled us to study how oncogenic Ras might cooperate with additional genetic alterations in precancerous lesions.

In fact, the aberrant activation of Ras signaling via point mutation often occurs during the early stages of many human cancers at a time when these precancerous lesions are thought to lack many additional genetic alterations. For example, *NRAS* mutations are already present in the early radial growth phase of many melanomas and persist throughout disease progression to the metastatic stage (48). Likewise, the occurrence of oncogenic mutations in *KRAS* is considered to be an important early event in the development of pancreatic cancer. Mutations in *KRAS* are found in approximately 45 percent of papillary pancreatic duct lesions without atypia and undergo positive selection during disease development (49). In addition, the oncogenic mutation of *KRAS* is an important early event that drives the development of colorectal cancer. *KRAS* mutations occur in benign adenomatous polyps that already harbor mutations in the *APC* tumor suppressor gene and precipitate the development of malignant colon carcinomas from these precursor polyps (50-52). Similar patterns of early oncogenic mutation in *KRAS* have also been reported to occur during the development of ovarian (53), ampullary (54), and lung neoplasms (55).

These and other data strongly support the idea that Ras plays a crucial role early in the development of human cancers. However, much research has also demonstrated that in order for benign lesions to progress toward full-blown malignancy, they must harbor mutations in multiple oncogenes and tumor suppressor genes (56). Thus, oncogenic mutation of Ras alone is not sufficient for tumorigenesis. We suspect that the loss of p38 activity may be one of these additional genetic events that promote the transformation and neoplastic development of cells by Ras.

Indeed, evidence exists for the loss of p38 function in human cancers. The negative regulators of p38 signaling, Wip1/PPM1D and DUSP26/MKP8, have been found to be elevated

in various human tumor samples and cancer cell lines (57-61). Although the possibility of additional targets cannot be excluded, both Wip1 and DUSP26 specifically dephosphorylate and inactivate the p38 MAPK and not the ERK or JNK MAPKs, indicating that p38 is the target of their phosphatase activity (61-63). In addition, levels of Gstm1 and 2, which negatively regulate p38 activity by inhibiting ASK1, a kinase upstream of p38 activation, are increased in several human cancer cell lines (20).

Similarly, MKK4, a kinase capable of directly activating p38 (64), is frequently deleted or mutated in a wide variety of human cancers and evidence gathered from model systems indicates that MKK4 is a tumor suppressor gene (65, 66). Furthermore, the sequencing of protein kinase genes in over 200 human cancers identified somatic mutations in p38α within several tumors. While the effects of these mutations on the biochemical functions of p38α remain to be explored, statistical analysis of these mutations indicated that they are likely to be driver mutations that positively affect tumor growth and development (67).

Observations gathered using mouse model systems also indicate that the loss of p38 activity predisposes cells to tumor development by oncogenic Ras. For example, a p38α null background in adult mice was found to promote the development and growth of lung tumors initiated by the expression of endogenous levels of oncogenic K-Ras (22). Likewise, Wip1 null MEFs, which contain high basal levels of p38 activity, exhibit decreased tumorigenicity when transformed with H-Ras^{G12V} and E1A (23). In addition, the liver-specific deletion of p38α increased the burden of hepatocellular carcinomas in mice treated with diethylnitrosamine (DEN) (18). This observation is of interest to us, since oncogenic mutations in H-Ras have been reported to occur in 41 percent of liver tumors induced by DEN (68), indicating that loss of p38α may cooperate with aberrant Ras signaling in this liver cancer model.

The present observations indicate that inhibition of the p38 MAPK pathway cooperates with low activation of oncogenic Ras signaling to cause significant changes in the adhesive behavior of cells, an alteration germane to the process of cell transformation. Altered cell-matrix interactions have long been known to be capable of promoting transformation and tumorigenesis. Although the mechanisms by which cancer cells usurp control of cell adhesion processes and manipulate these programs for their own benefit is undoubtedly complex and incompletely understood, it is clear that modification of cell-matrix adhesions plays a central role in cancer progression (69-75).

In the situation of a developing neoplasia, uncontrolled cell proliferation, inappropriate cell survival, increased motility, and increased invasiveness are important properties of tumor cells. These properties are acquired, at least in part, by changing the way in which cells interact with the extracellular matrix (56, 76, 77). Through various mechanisms, transformed cells commonly circumvent the apoptotic and growth arrest programs that normally prevent the survival and proliferation of cells when lacking proper cell-matrix adhesions. In other words, these transformed cells become anchorage-independent. (78, 79). Importantly, not only are these anchorage-independent cells liberated from their dependence upon cell-matrix proliferation and survival signals, they experience greater autonomy when migrating, which promotes their invasion into the surrounding normal tissue (79). As a result, acquisition of the anchorage-independent phenotype creates a solid foundation for tumor development.

Given the increasing support for p38 as a tumor suppressor gene, we propose that inhibition of the p38 pathway can predispose cells to Ras-dependent neoplastic development, in part though effects on cell adhesion. Further work in human cell systems investigating the

cooperation between low activation of oncogenic Ras and the inhibition of p38 should provide insights into the details of how suppressing p38 complements low levels of oncogenic Ras.

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VII. Figures

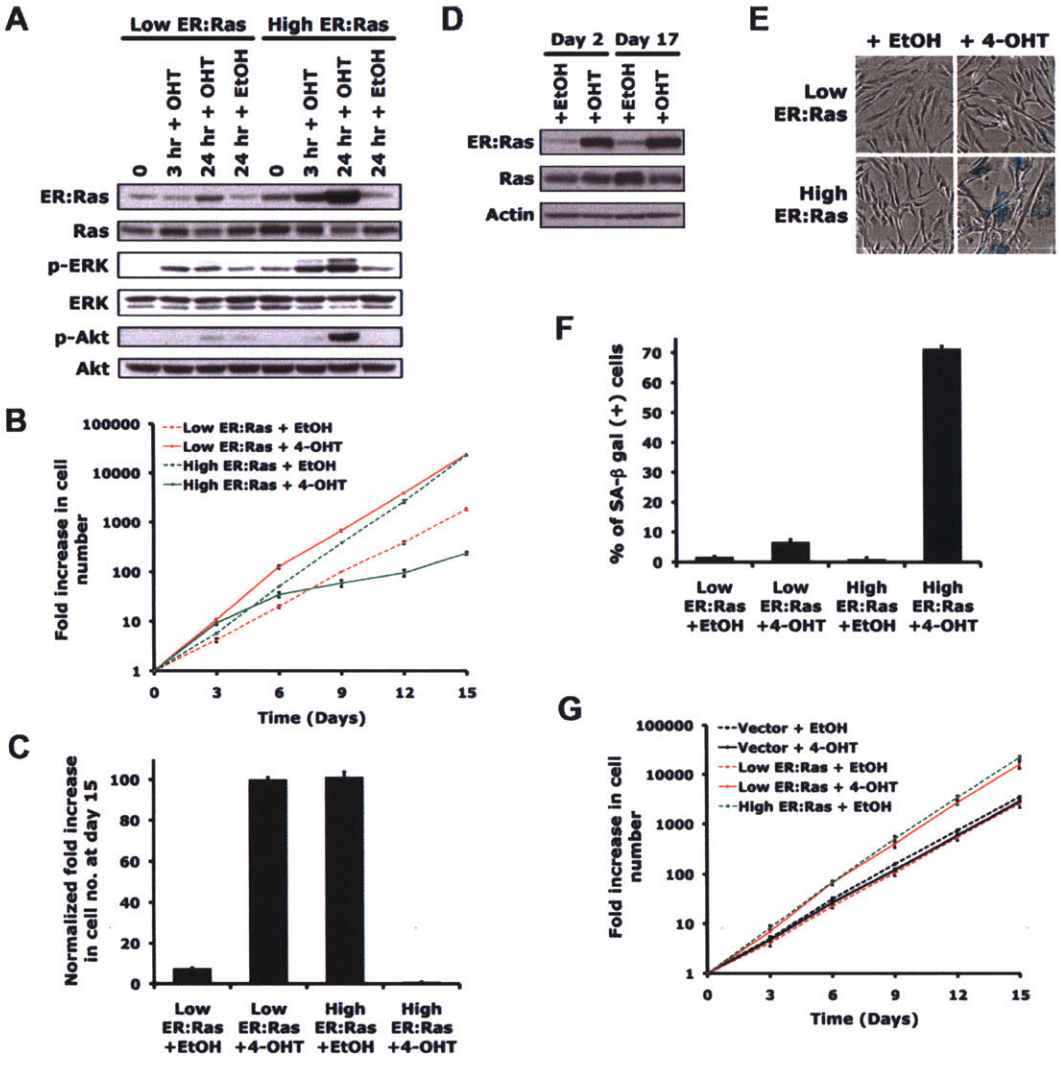


Figure 1. Low activation of oncogenic Ras signaling enhances cellular proliferation and fails to induce senescence. **A**. BJ fibroblasts were infected with pBabe-Hygro ER:Ras^{G12V} (Low ER:Ras) and pBabe-Puro ER:Ras^{G12V} (High ER:Ras) constructs which express inducible oncogenic Ras to low or high extents, respectively. Cells were serum starved for 16 hr and then incubated for the indicated times with the vehicle control (EtOH) or 4-OHT to activate ER:H-Ras^{G12V}. ER:H-Ras^{G12V}, endogenous H-Ras, phosphorylated ERK (p-ERK), total ERK, phosphorylated Akt (p-Akt), and total Akt were detected by Western blotting. **B**. Proliferation of Low ER:Ras and High ER:Ras cells in the presence and absence of 4-OHT. Cells were seeded at a density of 5200 cells/cm^2 and passaged every three days, preventing them from reaching confluence. Treatment with 4-OHT or EtOH began on day 1. Each data point depicts the mean fold increase in the number of cells ± the SEM. The mean and SEM were determined from counting the cells of triplicate culture dishes. **C**. Fold increase in the total number of cells accumulated at day 15 versus the number of cells plated at day 0. Fold increase for each cell population was normalized to the fold increase in cell number experienced by High ER:Ras cells

treated with 4-OHT. **D**. Western blot analysis for ER:H-Ras^{G12V}, endogenous H-Ras, and actin in Low ER:Ras cells at day 2 and day 17. Treatment with EtOH and 4-OHT began on day 1. **E**. Cells were stained for SA β-gal activity at day 15 and visualized at 20X magnification. **F**. The percentage of cells staining positively for SA β-gal activity were quantified. At least 120 cells from each of 3 culture dishes were scored for each cell population. Error bars represent the SEM for each data set. **G**. Proliferation of vector control-infected cells in the presence and absence of 4-OHT in comparison to EtOH and 4-OHT-treated Low ER:Ras cells and EtOH-treated High ER:Ras cells. Growth curve analysis was performed as above.

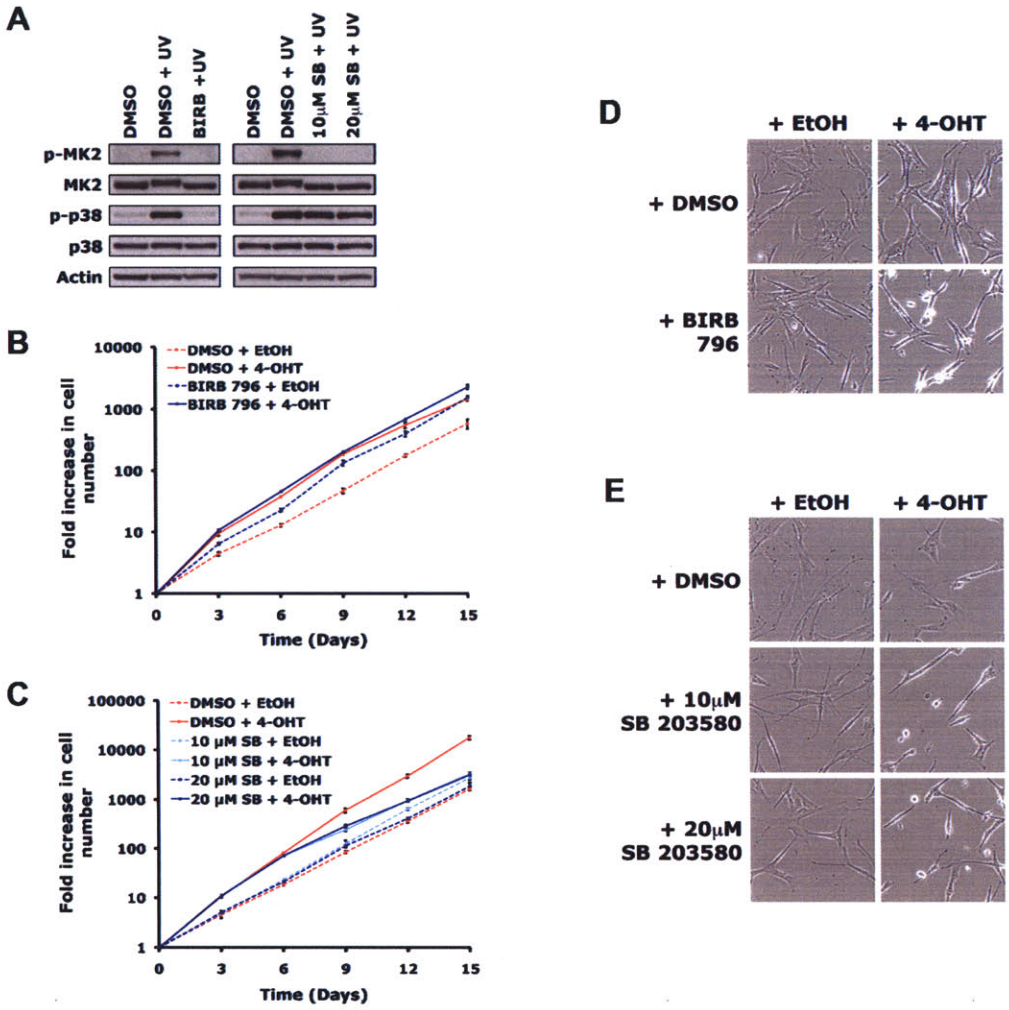


Figure 2. The continuous inhibition of p38 cooperates with low activation of oncogenic Ras signaling to increase the refractiveness of cells. **A**. Low ER:Ras cells were pre-treated for 2 hours with BIRB 796, SB 203580, or the vehicle control (DMSO) as indicated. Cells were then exposed to UV light to activate p38 signaling. Phosphorylated MK2 (p-MK2), total MK2, phosphorylated p38 (p-p38), total p38, and actin were detected by Western blotting. **B**. Proliferation of EtOH and 4-OHT-treated Low ER:Ras cells in the presence or absence of BIRB 796. **C**. Proliferation of EtOH and 4-OHT-treated Low ER:Ras cells in the presence or absence of SB 203580. Growth curve analysis was performed as described in Figure 1B. **D**. Morphology of Low ER:Ras cells after 3 days of treatment with 4-OHT or EtOH (vehicle control) and BIRB 796 or DMSO (vehicle control). **E**. Morphology of Low ER:Ras cells after 3 days of treatment with 4-OHT or EtOH and SB 203580 or DMSO.

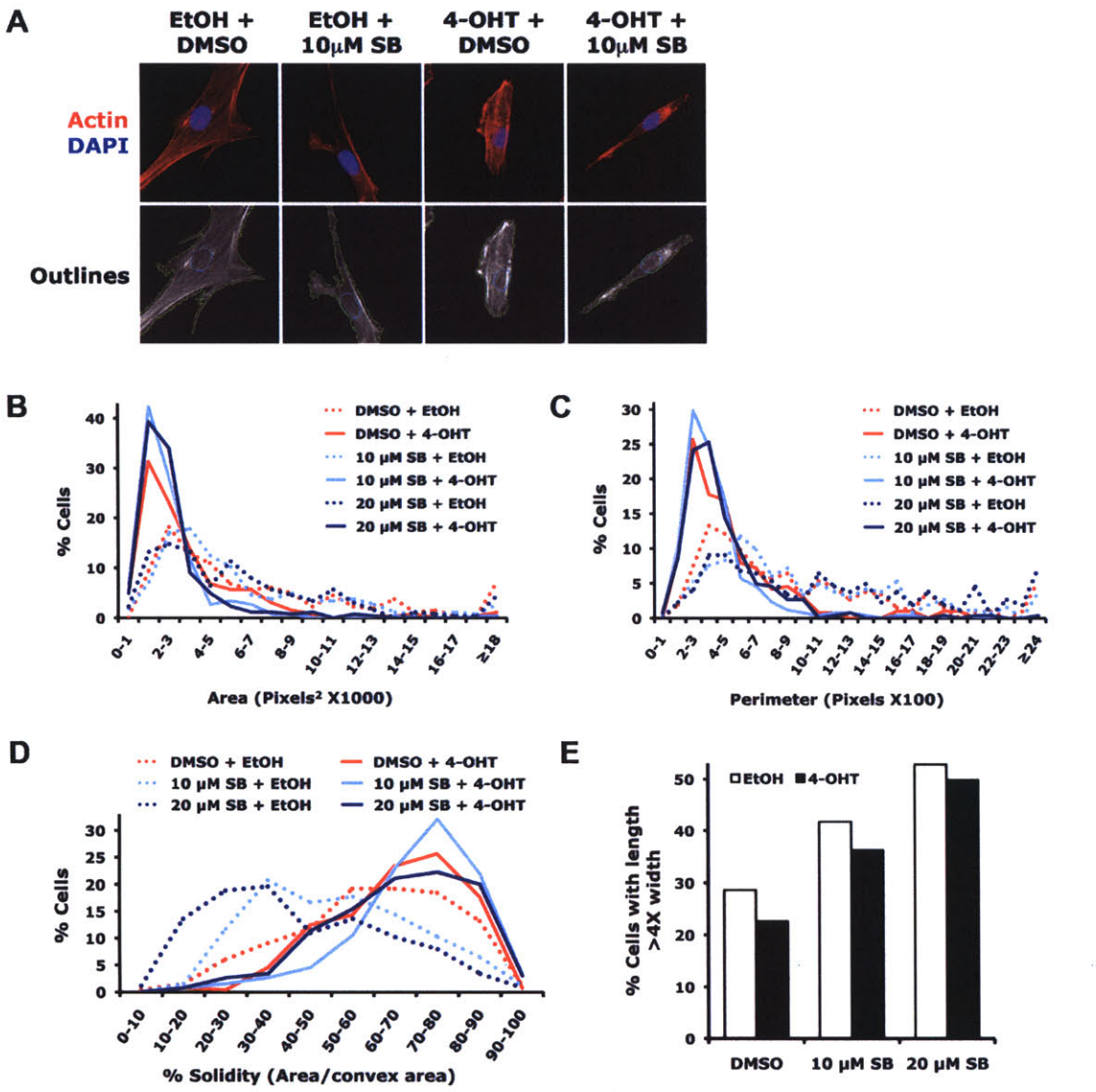


Figure 3. Morphological changes induced by inhibition of p38 and low activation of oncogenic Ras signaling. Low ER:Ras cells were plated at a density of 5200 cells/cm^2 and treated with 4-OHT or EtOH and SB 203580 or DMSO 1 day after plating. After an additional 2 days, cells from each population were trypsinized and replated. Cells of each population were also treated with the appropriate drugs. Cells were fixed and stained for actin (red) and nuclei (blue) at 24 hrs post-plating. **A**. Photographs of cells (10X magnification) and outlines of cell peripheries and nuclei delineated by the image analysis program CellProfiler. **B**. Histogram showing the two-dimensional areas of cells in each population as measured by CellProfiler. **C**. Histogram showing the perimeters of cells in each population as measured by CellProfiler. **D**. Histogram showing the percentages of solidity measured by CellProfiler for cells in each population. A higher percent solidity indicates a more convex cell. **E**. Bar graph showing the fraction of long cells (length is >4X width) in each cell population. Measurements of cell length and width were determined using CellProfiler. 265 cells were analyzed per population to generate graphs in this figure.

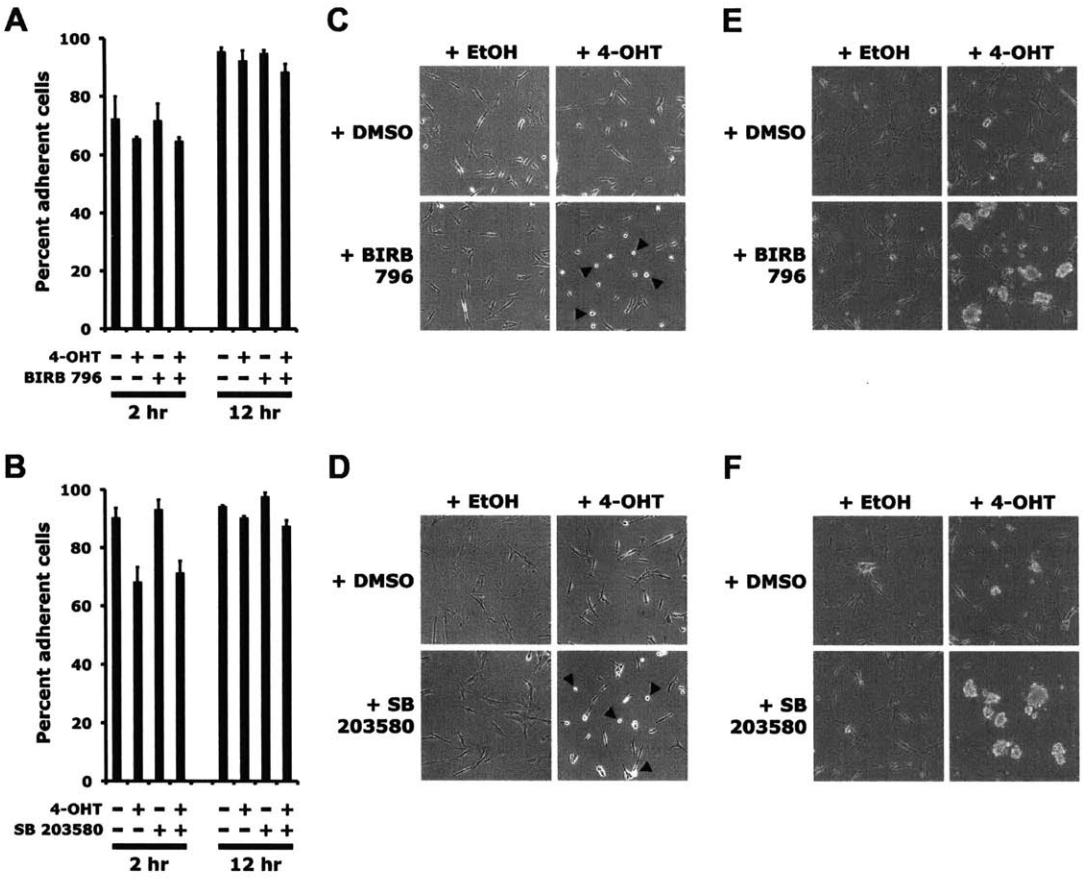


Figure 4. The inhibition of p38 cooperates with low activation of oncogenic Ras signaling to reduce cell adhesion. **A**. Quantification of cell adhesion for Low ER:Ras cells treated with 4-OHT or EtOH and 10 μM BIRB 796 or DMSO. Cells were plated on tissue culture-treated plastic at a density of 5200 cells/cm^2 and treated with 4-OHT or EtOH and BIRB 796 or DMSO 1 day after plating. After 2 additional days, cells of each population were trypsinized and replated in triplicate. Each cell population was also treated with the appropriate drugs. The number of adherent cells was then determined at 2 and 12 hrs post plating for each cell population. Error bars represent the SEM for each data set. **B**. Quantification of cell adhesion for Low ER:Ras cells treated with 4-OHT or EtOH and 10 μM SB 203580 or DMSO. The experiment was performed as described for part A. **C and D.** Photographs of cells described in parts A and B, respectively, at 12 hrs post-plating (10X magnification). Arrows indicate examples of cells that had not begun to spread upon the substratum. **E**. Adhesion of cells treated with 4-OHT or EtOH and 10 μM BIRB 796 or DMSO to cell culture plates not treated for cell attachment. Cells were plated on treated cell culture dishes at a density of 5200 cells/cm^2 and treated with 4-OHT or EtOH and BIRB 796 or DMSO 1 day after plating. After an additional 2 days, cells of each population were trypsinized and replated in triplicate on untreated cell culture dishes. Each cell population was also treated with the appropriate drugs. Photographs of cells were taken after 3 days of growth on untreated culture plates (10X magnification). **F**. Adhesion of cells treated with 4-OHT or EtOH and 10 μM SB 203580 or DMSO to cell culture plates not treated for cell attachment. The experiment was performed as described in part E.

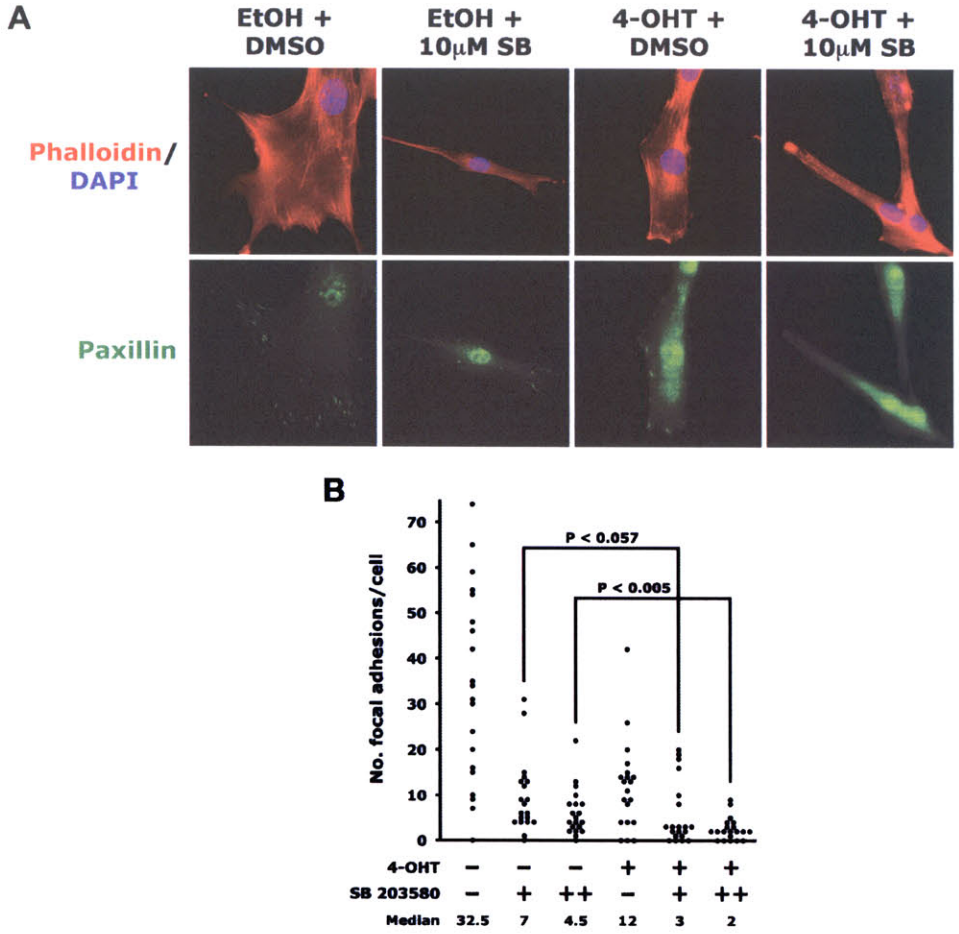


Figure 5. Inhibition of p38 acts in concert with low activation of Ras signaling to reduce the number of focal adhesions. Low ER:Ras cells were plated on tissue culture-treated plastic at a density of 5200 cells/cm^2 and treated with 4-OHT or EtOH and SB 203580 or DMSO 1 day after plating. After an additional 2 days, cells of each population were trypsinized and replated. Each cell population was also treated with the appropriate drugs. Cells were fixed and stained for the focal adhesion-associated protein paxillin (80), (green), actin (red), and nuclei (blue) at 24 hrs post-plating. **A**. Photographs of cells (20X magnification). **B.** The average number of focal adhesions per cell. n = 20 cells per population.

VIII. Supplementary information

Cell morphology analysis using CellProfiler

The image analysis pipeline used to measure cell morphology was created with CellProfiler version 1.0.5811, which can be downloaded under the "Download CellProfiler" tab at www.cellprofiler.org along with the software's instruction manual.

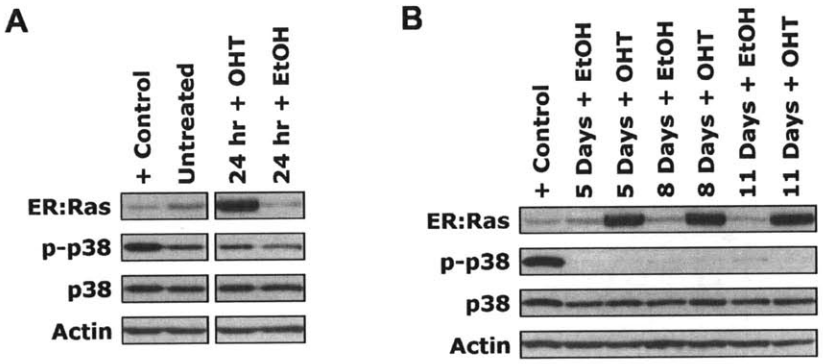
Briefly, the image analysis pipeline operated as follows: Module 1 loaded the images. Modules 2 to 4 performed pre-processing and the identification of nuclei and cell edges. Modules 5 to 12 identified and removed cells (plus their associated nuclei) that touched the image edge to insure accurate measurements of morphology. Modules 13 and 14 performed quantitative measurements. Modules 15 to 19 produced output images and spreadsheets of data. Each module is detailed below:

1. *LoadImages*: On the basis of filename, loaded both the microscopy image that displayed cell nuclei and the corresponding image that displayed actin cytoskeletons.
2. *SmoothOrEnhance*: Top-hat filtered the image of nuclei to reduce background staining.
3. *IdentifyPrimAutomatic*: Identified all nuclei from the filtered image of nuclei.
4. *IdentifySecondary*: Identified cell edges by using both the image of actin cytoskeletons and the nuclei identified by the previous module.
5. *ConvertToImage*: Converted the identified nuclei and cell edges to an image of the cells.
6. *Crop*: Created a new image, identical to that in module 5, but with image border pixels set to 0.

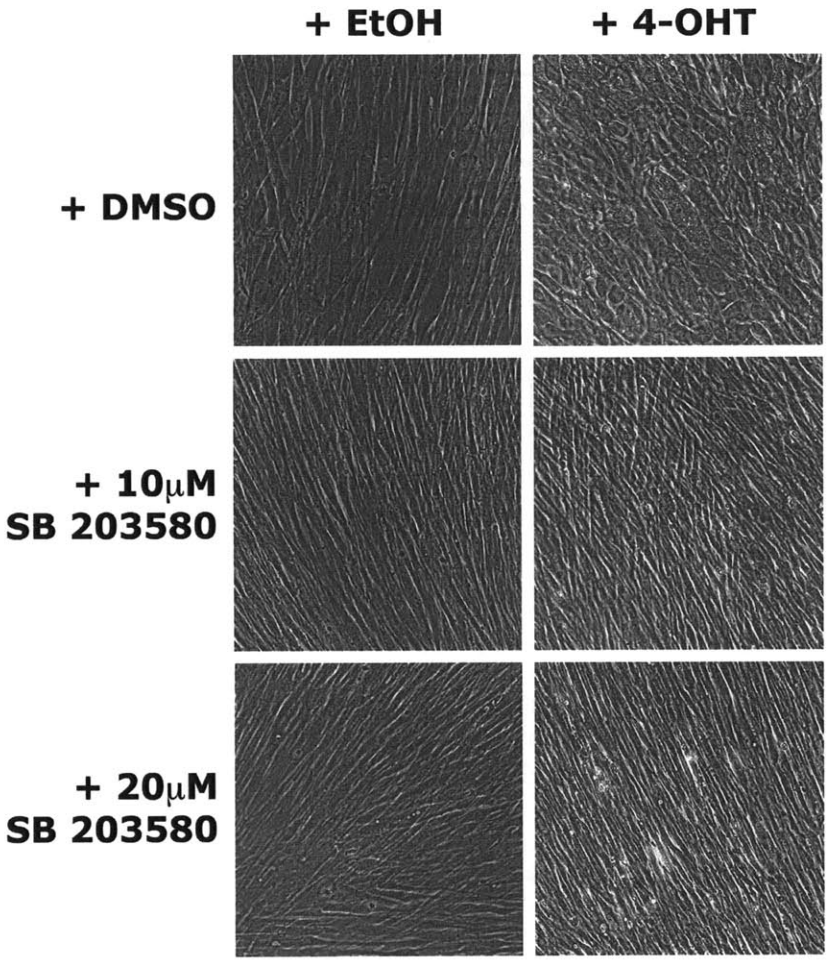
7. *ImageMath*: Subtracted the image created by module 6 from the image created by module 5 in order to obtain an image in which only pixels from cells that touch the image border appear.
8. *MeasureObjectIntensity*: Measured the intensity of each cell found in the image that was produced by module 7.
9. *FilterByObjectMeasurement*: Removed cells in which the measured intensity is non-zero, i.e., the cells that touch the image border.
10. *ConvertToImage*: Converted the remaining cells, which are those cells entirely contained within the borders of the image, to an image of the cells.
11. *MeasureObjectIntensity*: Measured the intensity of each nucleus within the image produced by module 10.
12. *FilterByObjectMeasurement*: Removed nuclei in which the measured intensity is zero, i.e., those nuclei belonging to a cell that was removed since it touched the image border.
13. *MeasureObjectAreaShape*: Measured the morphological parameters of each remaining cell and nucleus.
14. *CalculateRatios*: Obtained the cellular aspect ratio by dividing the major axis length of the cell by the minor axis length.
15. *GrayToColor*: Converted the grayscale image of the actin cytoskeleton to a color image.
16. *OverlayOutlines*: Overlayed the image produced by module 15 with blue oulines of cell nuclei.
17. *OverlayOutlines*: Overlayed the image produced by module 16 with green outlines of cell edges.
18. *SaveImages*: Saved the images produced by module 17.

19. *ExportToExcel*: Exported the morphological measurements from modules 13 and 14 as an Excel spreadsheet.

IX. Supplementary figures



Supplementary Figure 1. Low activation of oncogenic Ras signaling does not activate p38. **A.** Low ER:Ras cells were serum starved for 16 hr and then incubated for the indicated times with EtOH or 4-OHT. Untreated cells were harvested at time 0. **B.** Low ER:Ras cells were treated with 4-OHT or EtOH for the indicated times. ER:H-Ras^{G12V}, phosphorylated p38 (p-p38), total p38, and actin were detected by Western blotting. Lysates from Low ER:Ras cells exposed to UV light were used as a positive control for the detection of phosphorylated p38. Phosphorylated p38 levels were measured at later time points to exclude the possibility that p38 becomes activated by low levels of oncogenic Ras signaling in a delayed manner. Others had shown p38 activation at 8 and 10 days following the infection of BJ fibroblasts with a retroviral construct that overexpressed oncogenic Ras to a high level (15).



Supplementary Figure 2. The inhibition of p38 and low activation of oncogenic Ras signaling are not sufficient to induce cellular transformation. Low ER:Ras cells were plated at a density of 5200 cells/cm^2 and treated with 4-OHT or EtOH and SB 203580 or DMSO 1 day after plating. After an additional 2 days, cells of each population were trypsinized, replated, and treated with the appropriate drugs. Cells were continuously cultured in the same dish with the appropriate drugs for 3 weeks and photographed (10X magnification) at the end of the experiment.

X. References

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Chapter 3:

Overview and future directions

The results presented in chapter 2 indicate that inhibition of p38 and low activation of oncogenic Ras cooperate to reduce the adhesion of normal human fibroblasts. Moreover, the decreased cell adhesion observed upon perturbing these two signaling pathways suggests that inhibition of the p38 pathway predisposes human cells to partial transformation by oncogenic Ras. However, the presented results raise two main questions. First, how does the inhibition of p38 cooperate with low oncogenic Ras signaling to reduce cell adhesion? In other words, what pathways downstream of p38 and Ras regulate the cell-matrix adhesion of human fibroblasts? Second, which other oncogenic or tumor-suppressive pathways must be perturbed in order to transform cells that express near physiological levels of oncogenic Ras? Furthermore, does the inhibition of p38 facilitate the full transformation of cells containing low levels of oncogenic Ras? Potential answers to these questions and their implications will be discussed in this chapter.

I. Regulation of adhesion and cytoskeletal rearrangement by Ras and p38

As shown in chapter 2, inhibition of p38 and low activation of oncogenic Ras both reduce the number of focal adhesions formed by a fibroblast. However, inhibiting p38 and activating Ras caused dissimilar effects on cell shape. More specifically, low activation of oncogenic Ras signaling increased the roundness of cells and decreased their two-dimensional area, whereas inhibition of p38 elongated cells and failed to reduce cell area. These observations suggest that, although inhibition of p38 and activation of Ras cooperate to reduce the adhesion of cells, manipulation of these two pathways affects the actin cytoskeleton and cell-matrix adhesion in distinct ways. Thus, p38 inhibition and Ras activation most likely induce reorganization of the cytoskeleton and reduced cell-matrix adhesion by modulating different downstream signaling

events that cause independent changes in cell morphology in addition to concomitantly decreasing cell adhesion.

Possible regulation downstream of p38

p38 has been found to phosphorylate several proteins involved in cytoskeletal reorganization and cell-matrix adhesion, for example, Hsp27, paxillin, and ezrin (1-6). Thus, the elongated morphology and reduced number of focal adhesions that I observed in human fibroblasts treated with p38 drug inhibitors are likely to be caused by perturbation of more than one p38 target (Fig. 1A). However, decreased activity of the p38/MK2/Hsp27 pathway, which was introduced in Chapter 1 as being involved in actin remodeling, appears to be the best candidate for the elongated shape and reduced adhesion observed in the presence of p38 inhibitors.

Indeed, the activity of both p38 and Hsp27 was shown to be essential for the formation of lamellipodia in smooth muscle cells (3). These results suggest that p38's activation of Hsp27 may also be necessary for lamellipodia formation in other cell types, such as fibroblasts. Because cell spreading and formation of focal adhesions both require the extension of lamellipodia, reduced signaling through the p38/MK2/Hsp27 pathway could explain the elongated morphology and reduced number of focal adhesions exhibited by fibroblasts treated with p38 drug inhibitors.

Possible regulation downstream of oncogenic Ras

Like p38, Ras has been linked to regulation of the cytoskeleton and cell-matrix adhesion through multiple mechanisms (Fig. 1B). The increased cell roundness, decreased two-

dimensional cell area, and reduced cell adhesion caused by low levels of oncogenic Ras signaling is probably the result of the simultaneous modulation of Ras effectors involved in regulating cytoskeletal reorganization and cell adhesion. Further work is necessary to determine which effectors downstream of Ras are responsible for the alterations in cell morphology and adhesion described in chapter 2; however, several compelling candidates exist, including signaling through the Raf kinase and Rho family GTPases.

For example, oncogenic Ras has been shown to suppress integrin activation in some cell types, such as fibroblasts, in a Raf-dependent manner (7, 8). Integrin activation is central to the continued advancement of lamellipodia, continued cell spreading, and the formation of focal adhesions. Thus, suppression of integrin activation could explain the reduced number of focal adhesions observed in cells in which oncogenic Ras signaling was activated to a low extent. Moreover, the increased roundness of cells induced by low oncogenic Ras signaling may also be due to the suppression of integrin activation, since sustained cell spreading requires the integrin activation and the resulting formation of cell-matrix adhesions (9).

In addition to the possible suppression of integrin activation, low oncogenic Ras signaling may affect cytoskeletal reorganization and cell adhesion through the modulation of the Rho family GTPases RhoA, Cdc42, and Rac. RhoA, RhoB, Cdc42, and Rac are each required for Ras-induced transformation of murine cells, indicating that the activities of these GTPases are crucial mediators of Ras-induced cytoskeletal rearrangements, loss of contact inhibition, and anchorage-independence (10-14).

Indeed, dominant negative RhoA, dominant negative Rac, and dominant negative Cdc42 were each reported to suppress the acquisition of morphological changes typical of cells transformed with Ras, such as increased refractivity and the loss of stress fibers (10, 13, 14).

Furthermore, constitutively active forms of Rac and RhoA enhanced the transformation of cells by oncogenic Ras. Co-expression of active Rac or RhoA with oncogenic Ras made cells more refractile, less adherent, and better at growing in anchorage-independent conditions than when transformed with Ras alone (10).

Proteins downstream of Rho GTPase family members found to be regulated in response to oncogenic Ras include Rho-associated, coiled-coil containing protein kinase (ROCK) and FAK. The activation of ROCK by Rho and activated ROCK's subsequent phosphorylation of the actin-binding protein ezrin were shown to be necessary for transformation of murine cells by oncogenic Ras (15, 16). Thus, the Rho/ROCK/ezrin pathway may contribute to the morphology changes reduction in cell adhesion observed upon the low activation of oncogenic Ras signaling.

In addition, oncogenic Ras was found to inhibit FAK by inducing its dephosphorylation (17). Ras was found to inhibit FAK through a signaling cascade involving activation of faciogenital dysplasia protein 1 (Fgd1), a GEF that specifically activates Cdc42, Cdc42, and ERK. ERK was found to phosphorylate FAK directly, thereby recruiting additional proteins that induce the dephosphorylation of FAK at a separate residue that is critical for its activity. Because FAK activation plays a key role in the early spreading of cells having just attached to their substratum (18), the repression of FAK activation by oncogenic Ras may be responsible for the reduced number of focal adhesions and rounded cell shape observed upon activation of low Ras signaling.

Further work is necessary to determine those pathways downstream of low oncogenic Ras signaling that alter fibroblast morphology and reduce cell-matrix adhesion. Importantly, in the work described above, oncogenic Ras was expressed to supraphysiological levels. Because I observed high and low levels of oncogenic Ras signaling to induce the differential activation of

Ras effector pathways in human fibroblasts, low oncogenic Ras signaling may not activate the same pathways as were activated in the reported experiments.

For example, I failed to see activation of Akt upon low activation of oncogenic Ras signaling but saw robust activation of Akt upon high activation of oncogenic Ras signaling, suggesting that activation of the PI3K pathway is triggered only by high levels of oncogenic Ras signaling. One of the ways in which Ras activates Rac is through the PI3K pathway. Thus, low activation of Ras signaling may not to be sufficient to activate Rac through the PI3K. Due to the differences in signaling stimulated by low and high levels of oncogenic Ras, additional studies using the low ER:Ras fibroblasts must be done to determine which signaling pathways downstream of Ras are activated by low oncogenic Ras signaling.

II. The role of low oncogenic Ras signaling in the transformation of human cells

The results presented in chapter 2 clearly indicate that simultaneous inhibition of p38 and low activation of oncogenic Ras are not sufficient for the full transformation of human fibroblasts. The failure of these two manipulations to transform fibroblasts is expected given what is currently known about the transformation of human cells. Indeed, as discussed in chapter 1, multiple cooperating genetic mutations are required for the complete transformation of normal human cells. Therefore, the question arises as to which other oncogenic or tumor-suppressive pathways must be perturbed in order to transform human cells containing a low level of oncogenic Ras signaling. The answer to this question has important ramifications on how we view the transformation of human cells from normal to malignant.

Impact on current models of transformation and tumorigenesis

Normal human cells have been successfully transformed into cancer cells using several different cocktails of introduced genetic modifications (19). Although the use of these transforming cocktails has provided valuable information in identifying pathways that are important in generating cancer cells from normal human cells, these transformation protocols introduce requirements that may not accurately reflect the acquisition of genetic modifications by incipient cancer cells in actual human tumors. More specifically, two features common among protocols for the transformation of human cells are the need for a strong oncogenic signal, usually provided by highly overexpressing oncogenic Ras, and the disruption of two potent tumor suppressors, namely, p53 and the retinoblastoma protein (Rb).

The high levels of oncogenic Ras required for the experimental transformation of human cells may not accurately model the oncogenic Ras signaling occurring in actual human cancers, where tumor cells most likely never experience such high levels of oncogenic Ras expression. Indeed, studies focused upon one of these frequently used transformation models showed that, unlike high overexpression of oncogenic Ras, moderate overexpression failed to transform human cells robustly, as indicated by decreased anchorage-independent growth and lack of tumor formation in mice (20). Thus, current models of transformation do not account for the promotion of tumorigenesis by near physiological levels of oncogenic Ras.

In addition, the overexpression of oncogenic *RAS* at a high level requires that the genetic modifications disabling p53 and Rb are introduced prior to, or at least at the same time as, introducing oncogenic *RAS* into cells (20-23). Introducing genetic modifications in this order is necessary for the successful transformation of human cells due to the fact that high levels of oncogenic Ras signaling induce the senescence of normal human cells containing intact p53 and

Rb signaling pathways (24). Crippling p53 and Rb functions enables cells expressing high levels of oncogenic Ras to avoid entering into senescence, thus promoting their transformation (21, 25).

Importantly, inhibiting p53 and Rb prior to acquiring oncogenic Ras may not be necessary during the development of actual human tumors. Evidence from human tumor samples and genetically engineered mouse models of cancer indicate that the aberrant activation of Ras signaling is an initiating event in a number of human cancers. For example, oncogenic *RAS* alleles are often detectable in preneoplastic lesions that are believed to lack many additional genetic alterations (26-33). Moreover, expression of oncogenic *Kras* from the endogenous *Kras* promoter in various organs of adult mice, such as the lung, pancreas, ovary, and colon, was found to induce preneoplastic lesions in these targeted organs (34-37).

The relevance of senescence to tumorigenesis

The identification of oncogenic *RAS* mutations in preneoplastic lesions of human cancers and the ability of endogenous oncogenic *Kras* to induce hyperplasias in mouse models, raises the question as to whether models of transforming human cells accurately reflect events occurring in the development of human cancers, due to these transformation models requiring the loss of p53 and Rb functions followed by high overexpression of oncogenic *RAS*. This issue directly relates to whether or not Ras-induced senescence is an important tumor-suppressive mechanism in human cells. Studies focused upon the *in vitro* transformation of human cells indicate that the p53 and Rb pathways must be compromised in order for oncogenic Ras to drive tumorigenesis. In contrast, evidence indicating that oncogenic mutation of Ras is an initiating event in tumor development suggests that disengaging the p53 and Rb pathways prior to the acquisition of oncogenic Ras is not necessary.

Much work has been done to determine whether Ras-induced senescence a biologically relevant tumor-suppressive mechanism. This work has yielded contradictory results. Results obtained from one mouse model of endogenous oncogenic *Kras* activation demonstrate a lack of senescence markers in hyperplasias initiated by oncogenic *Kras* (35). However, results from a second model of endogenous oncogenic *Kras* activation indicate that preneoplastic lesions induced by oncogenic *Kras* do exhibit signs of senescence (38).

Reasons for these conflicting results are not clear, but in combination with other evidence, suggest the possibility that senescence may be a *bona fide* tumor suppressor in some contexts, but not others. For example, markers of senescence have been observed in human melanocytic nevi harboring oncogenic Raf. These nevi are thought to be precursor lesions of melanoma, indicating that some cell types may limit neoplastic development by inducing senescence in the presence of oncogenes (39). On the other hand, multiple studies have shown that cells of some cancers retain the ability to senesce in response to chemotherapeutic agents (40), indicating that transforming a normal cell into a cancer cell does not necessarily involve the ablation of tumor suppressor pathways mediating senescence.

Linking senescence in vivo to the upregulation of Ras

Because high levels of oncogenic Ras are necessary in order to induce the senescence of mammalian cells, it is initially confusing as to why preneoplastic lesions driven by the endogenous expression of oncogenic *Kras* in one mouse model would exhibit senescence, whereas embryonic fibroblasts expressing endogenous oncogenic *Kras* and derived from the same mouse model fail to senesce and instead are immortal (38, 41). A possible explanation for this apparent discrepancy lies in the spontaneous upregulation of oncogenic Ras during tumor

development, for example, through increased copy number of the *RAS* oncogene, increased transcription of the *RAS* oncogene, or loss of negative regulators that inhibit aberrant Ras signaling (42).

Indeed, some mouse models of Ras-induced tumorigenesis have provided evidence for the spontaneous upregulation of Ras during tumor development. Of particular interest, two such studies correlated the upregulation of endogenous Ras with tumor development. The first study described the amplification of wild-type *Kras* (43), whereas the second study reported the spontaneous upregulation of oncogenic *Hras* expressed from the endogenous locus (44). Similarly, a subset of human tumor samples have shown upregulation of oncogenic Ras, with higher Ras levels correlating with later stage tumors (45-50). Thus, there appears to exist a selective pressure for the upregulation of Ras signaling during tumor development.

This upregulation of Ras signaling during tumor development is thought to drive the senescence of cells expressing oncogenic *Kras* from the endogenous promoter. As a proof of principle, the inducible expression of oncogenic *Hras* in mammary epithelia of transgenic mice stimulated the hyperproliferation of mammary cells when oncogenic *Hras* was expressed at a low level. However, increasing the expression level of oncogenic *Hras* in mammary cells triggered their senescence (51). Importantly, the loss of p53 activity prevented the accumulation of senescence cells.

Taken together, these lines of evidence suggest the following model of tumorigenesis: Tumorigenesis is driven by a high, but not low, level of oncogenic Ras signaling. Incipient cancer cells acquire this high level of Ras signaling through the spontaneous upregulation of endogenous Ras oncogenes. Moreover, in order for upregulated levels of oncogenic Ras

signaling to promote tumorigenesis, cells must already have lost the ability to senesce through disruption of the p53 and Rb tumor suppressor pathways.

Implicit in this model of tumorigenesis is the idea that the *in vitro* transformation of normal human cells into cancer cells recapitulates events that occur during tumor development *in vivo*. Indeed, both the model described above and protocols for the transformation of human cells involve the loss of senescence-inducing tumor suppressor pathways and subsequent acquisition of high levels of oncogenic Ras. However, this model is not applicable to genetically engineered mouse models of cancer lacking markers of senescence nor is it applicable to malignant cells capable of senescing in response to chemotherapeutic agents. Therefore, although the model presented above appears to be relevant to certain situations of cancer development, it fails to provide a satisfying explanation for other cases of tumorigenesis.

Tumor-suppressive mechanisms in the absence of senescence

The lack of senescence markers in pre-neoplastic lesions formed in one mouse model of endogenous oncogenic *Kras* activation (35) raises the question as to what other tumor-suppressive mechanisms counteract neoplastic development in these *Kras*-expressing cells. Indeed, the number of functional viral particles administered to the lungs of mice when activating the expression of oncogenic *Kras* in lung cells is many times greater than the number of lesions formed following viral infection (34). Assuming that most infected cells activate their oncogenic *Kras* allele, and assuming each isolated lesion originated from a single infected cell, there is a very high inefficiency of generating lesions upon the endogenous expression of oncogenic *Kras*.

Therefore, the majority of cells exposed to oncogenic Ras signaling fail to form hyperplasias, but why? One possible explanation is that, even though cells expressing endogenous levels of oncogenic *Kras* in this mouse model fail to undergo senescence, aberrant signaling through the Ras pathway triggers the activation of negative regulators of Ras signaling, thus suppressing the ability of oncogenic Ras to promote hyperplastic outgrowths. Supporting this possibility, the *in vivo* activation of oncogenic *Kras* from the endogenous promoter was found to upregulate Sprouty-2, a negative regulator of Ras signaling (52). Moreover, loss of Sprouty-2 promoted the ability of *Kras* to induce the development of lesions in the lungs of mice. These results indicate that cells respond to low levels of oncogenic Ras signaling by engaging negative feedback pathways and that disruption of these pathways promotes *Kras*-induced tumorigenesis.

A second potential tumor-suppressive mechanism operating in the presence of low oncogenic Ras signaling lies in the possibility that most cells in adult tissues fail to be permissive hosts for the initiation of tumor development by oncogenic Ras. In support of this possibility, particular cell types were found to be responsible for initiating tumor development in mouse organs in response to endogenous oncogenic *Kras* expression. More specifically, the expansion of bronchioalveolar stem cells (BASCs) upon oncogenic *Kras* expression was found to initiate the formation of hyperplastic lesions in mouse lungs, lesions which eventually evolved into adenocarcinomas (53).

In addition, the targeted expression of endogenous oncogenic *Kras* to distinct subpopulations of pancreatic cells in mice showed that oncogenic *Kras* was able to induce the hyperplastic growth of a subpopulation of pancreatic and duodenal homeobox 1 (Pdx1)-positive cells, thus initiating the development of Pancreatic ductal adenocarcinoma (PDAC). In contrast,

oncogenic *Kras* was not able to induce the hyperplastic growth of insulin-expressing cells unless the pancreatic tissue was first subjected to chronic injury (37). Taken together, these results indicate that different subtypes of cells in a tissue have innate differences in their susceptibility to forming hyperplastic lesions in response to oncogenic Ras signaling. If true, the number of cells in the mouse or human body that is capable of initiating cancer development is much lower than the overall number of cells in the body.

Take-home messages

Regardless of which tumor-suppressive mechanisms operate to counteract their neoplastic development, cells expressing a single copy of an oncogenic Ras allele appear to undergo an initial period of positive selection, resulting in the formation of hyperplastic lesions. Furthermore, the loss of tumor suppressor functions appears to facilitate Ras-initiated tumor development. Indeed, the loss of tumor suppressor function has consistently been found to expedite tumorigenesis in mouse models employing the expression of oncogenic *Kras* from the endogenous promoter (36, 54-56). Thus, although additional work must be done to understand the role of Ras signaling in the initiation of human cancers, the concept of transforming human cells through multiple genetic alterations certainly relates to human tumor development.

Creating better models of human transformation

There exists a clear need for the development of better models involving the transformation of normal human cells into cancer cells. Much evidence, gathered from human tumor samples and genetically engineered mouse models, indicates that low activation of oncogenic Ras signaling is an initiating event of tumorigenesis. However, current protocols used

to transform human cells fail to recapitulate both the early activation of aberrant Ras signaling and the endogenous levels of this signaling.

Furthermore, differences in the susceptibility of human and murine cells to the process of transformation necessitate the creation of better protocols for transforming human cells. Human cells are more refractory to transformation than mouse cells, indicating that the tumor-suppressive mechanisms operating in human and mouse cells exhibit critical species-specific differences (57, 58). Thus, despite the valuable information gathered using mouse model systems, tumorigenesis in mice cannot fully mimic tumorigenesis in humans. Therefore, improved human transformation models are essential to understand the impact of genetic modifications occurring in the development of human cancers.

A first step in improving current protocols for the transformation of human cells would be to introduce near physiological levels of Ras signaling into cells prior to any other genetic manipulations. Indeed, activating a low, pro-proliferative level of oncogenic Ras signaling in normal human cells is possible as shown in Chapter 2. The Low ER:Ras system could be introduced into a variety of normal human cell types in order to study the transformation of cells originating from different tissues. Furthermore, the Low ER:Ras system would provide a means to identify genetic alterations able to cooperate with low oncogenic Ras signaling in the transformation of human cells.

Importantly, as mentioned earlier, mouse models involving the expression of oncogenic *K*ras from the endogenous locus have shown that successful tumor development relies heavily upon the targeting of a permissive cell type (37, 53). Human tissues are also likely to contain cells that have differential responses to low oncogenic Ras signaling and different susceptibilities

to being transformed. Thus, better models of human cell transformation must begin with the careful selection of parental cell types that innately are more receptive to transformation.

In addition, better models of human cell transformation are likely to rest in experiments involving the transformation of human cells *in vivo*. The robustness with which a potentially tumorigenic cell forms a tumor depends heavily upon the environment in which the cell finds itself (59). For example, human breast carcinoma cell lines were found to form tumors with 100% efficiency when injected in the mammary fad pads of mice, but the efficiency of tumor formation dropped to 40% when these cell populations were injected into the subcutaneous tissue of mice (60).

Despite that *in vitro* transformation assays, such as growth in soft agar, are informative in predicting the *in vivo* tumorigenesis of mammalian cell lines, cells residing in an actual human tumor experience a very different and interactive environment, which is replete with cells, signaling molecules, and extracellular matrix components. Consequently, more accurate methods of studying the transformation of human cells would involve the engineering of human cells with inducible methods of activating oncogenic pathways and repressing tumor suppressor pathways.

Moreover, these engineered cells could be introduced into humanized mouse tissues. For example, the mouse mammary gland could be reconstituted using stromal and epithelial components of human origin (61). Low activation of oncogenic Ras signaling in cells residing in humanized mouse tissues, followed by the inducible manipulation of other oncogenic and tumor suppressor pathways, would advance models of human cell transformation to a position where they closely approximate events occurring during human tumor development.

III. Final Perspective

This study provides evidence that a low level of oncogenic Ras signaling induces the hyperproliferation of human cells, which undergo senescence in response to a high level of oncogenic Ras signaling. This result is directly relevant to data gathered from human tumors and genetically engineered mouse models, which indicate that Ras is an initiating event during tumor development. Thus, studying low oncogenic Ras signaling in human cells is relevant to gaining a better understanding of signaling events occurring in many human cancers.

In addition, I have found that the inhibition of p38 cooperates with low oncogenic Ras signaling to reduce cell-matrix adhesions. By altering cellular adhesion, the inhibition of p38 could predispose human cells to partial transformation by oncogenic Ras. Thus, this finding warrants further study aimed toward elucidating the role of p38 in Ras-induced tumorigenesis.

IV. Figure

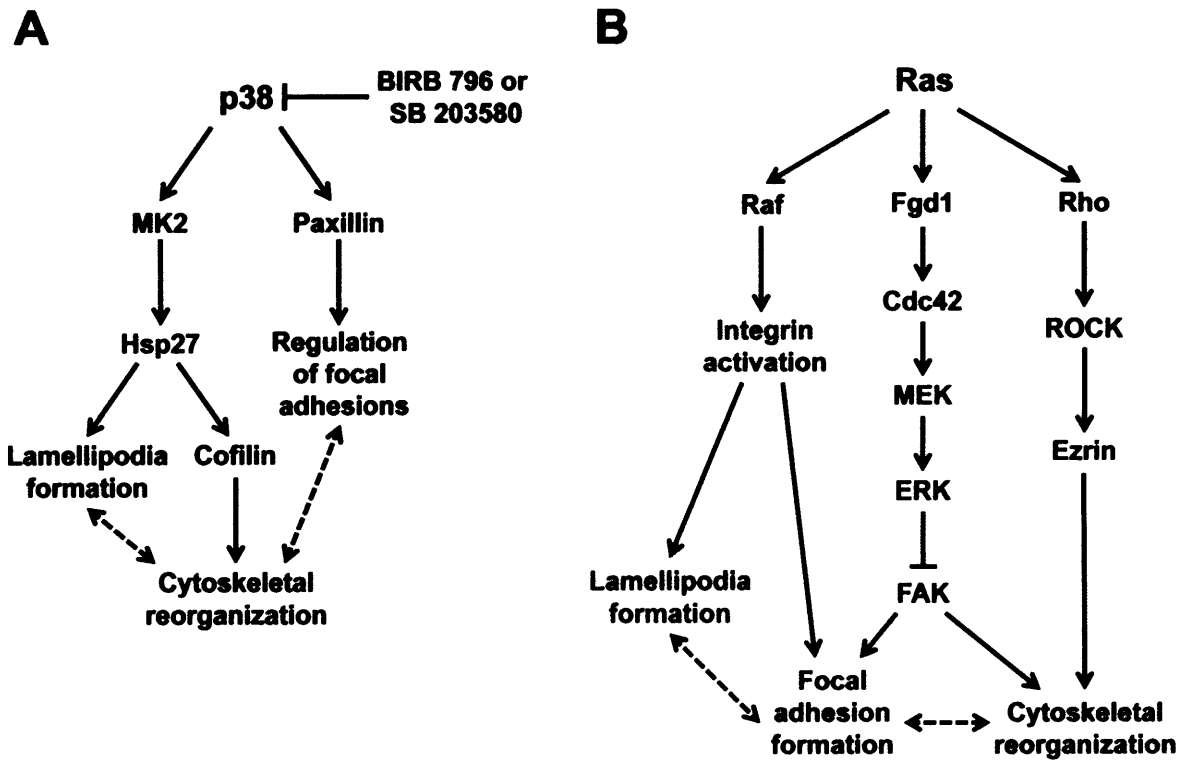

Figure 1. Pathways downstream of p38 and Ras that impact cell-matrix adhesion and/or cytoskeletal reorganization. **A.** Pathways downstream of p38 that have been implicated in controlling lamellipodia formation, cytoskeletal reorganization, and modulation of focal adhesion formation and stability. **B.** Pathways downstream of Ras that have been implicated in these same processes. See the text for details concerning pathways diagramed in this figure. The dotted, double-headed arrows in this figure are present to indicate that lamellipodia formation, cytoskeletal reorganization, and regulation of focal adhesions are intimately related processes, with perturbation of one of these processes often affecting the other two.

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