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c-Met Initiates Epithelial Scattering through Transient Calcium Influxes and NFAT-Dependent Gene Transcription

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Abstract

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Hepatocyte growth factor (HGF) signaling drives epithelial cells to scatter by breaking cell-cell adhesions and migrating as solitary cells, a process that parallels epithelial-mesenchymal transition. HGF binds and activates the c-Met receptor tyrosine kinase, but downstream signaling required for scattering remains poorly defined. This study addresses this shortcoming in a number of ways.

A high-throughput *in vitro* drug screen was employed to identify proteins necessary in this HGF-induced signaling. Cells were tested for reactivity to HGF stimulation in a Boyden chamber assay. This tactic yielded several small molecules that block HGF-induced scattering, including a calcium channel blocker.

Patch clamping was used to determine the precise effect of HGF stimulation on Ca^{2+} signaling in MDCK II cells. Cell-attached patch clamping was employed to detect Ca^{2+} signaling patterns, and channel blockers were used in various combinations to deduce the identity of Ca^{2+} channels involved in EMT. The results of these experiments show that HGF stimulation results in sudden and transient increases in calcium channel influxes. These increases occur at predictable intervals and rely on proper tubulin polymerization to appear, as determined through the use of a tubulin polymerization inhibitor. Though multiple channels occur in the membranes of MDCK II cells, noticeably TRPV4 and TrpC6, it is TrpC6 that is specifically required for HGF-induced scattering.

These HGF-induced calcium influxes through TrpC6 channels drive a transient increase in NFAT-dependent gene transcription which is required for HGF-induced EMT. This was determined through the use of luciferase-based NFAT reporter assays and confirmed through confocal immunofluorescence.

Using a small-molecule inhibitor of WNK kinase, it was determined that loss of WNK kinase function is sufficient to prevent HGF-induced EMT. Furthermore, patch-clamp analysis demonstrated that WNK kinase significantly increases channel opening at the surface of MDCK cells, indicating a possible mechanism of action for c-Met inhibition, but leaving doubt as to whether WNK kinase is in fact normally involved in c-Met signaling, or whether it is simply permissive.

Keywords: cancer, c-Met, HGF, calcium, NFAT, tubulin, epithelial-mesenchymal transition, WNK kinase

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Chapter 1: Introduction

Epithelial-mesenchymal transition (EMT), where individual cells detach from epithelial tissues and migrate to distant sites, is an essential process in embryonic development. EMT occurs during cancer metastasis, and it is thought that inappropriate activation of signaling that drives EMT in development drives cancer progression in tumors (Thiery, 2003). Scatter factor/hepatocyte growth factor (HGF), which binds and activates the c-Met receptor tyrosine kinase, drives EMT during development (Sonnenberg et al., 1993), scattering of epithelial cells in culture (Weidner et al., 1990), and is implicated in cancer progression (Renzo et al., 1995). Overexpression or activating mutations of c-Met are observed in a number of tumor types and are associated with increased cell proliferation, survival, and cancer metastasis.

Despite the clinical relevance of HGF signaling, it remains poorly defined at the molecular level. One reason is that approaches designed to dissect entire signaling networks have not been applied to HGF signaling. Signaling immediately downstream of the c-Met receptor has received a significant amount of research attention. A number of additional components have also been implicated in HGF signaling. However, their position in any c-Met "pathway" remains unclear and the relationship of specific signaling components with specific signaling outcomes (scattering, proliferation, and survival) also complicates understanding of this signaling network.

This study employs an unbiased chemical screening approach to identify molecular components of HGF signaling. Identification of a neuronal calcium channel blocker as an inhibitor of HGF signaling led us to observe rapid, large, and transient increases in calcium influxes in MDCK cells stimulated with HGF. We show that NFAT-dependent gene transcription is triggered by calcium influxes and that NFAT is required for the HGF-induced cell scattering characteristic of EMT.

A review of c-Met signaling and other receptor tyrosine kinases

Scatter factor or hepatocyte growth factor (HGF) triggers scattering of epithelial cells in culture. Cell treated with HGF undergo dramatic changes in cell morphology, including cell spreading, increased migration, and detachment of cell-cell adhesions. Cells tightly integrated into epithelial tissues instead become solitary, migratory, and invasive. This process mimics the early stages of EMT, a developmental program in which individual epithelial cells detach from tissues and migrate to distant sites as individual cells or groups of cells. In addition to triggering cellular events that strikingly resemble EMT, HGF also triggers increased cell proliferation and survival. In development, HGF triggers EMT in several instances, most notably the complete scattering cells of the dermamyotome (Dietrich et al., 1999). HGF signaling is also linked with cancer progression, driving changes in cell proliferation, survival, and the cellular events that drive metastasis. Cellular events associated with metastasis include breakdown of cell-cell adhesions, initiation of migration, invasion of surrounding tissues, and colonization of distant tissues with tumor cells, a strikingly similar series of events to developmental EMT programs.

HGF is the growth factor ligand for the c-Met receptor tyrosine kinase (Naldini et al., 1991). Receptor tyrosine kinases (RTKs) are typically activated through ligand-induced dimerization, and the c-Met receptor is no exception. In this article we explore the static structure of the c-Met signaling network in an effort to understand how the cell uses the c-Met signaling network to drive EMT. Since the static structure of c-Met signaling is closely related to receptor tyrosine kinase signaling generally, we will also consider how network dynamics and signaling context might allow cells to differentiate c-Met activation from activation of other RTKs.

c-Met as a receptor tyrosine kinase

C-met is one of a diverse array of receptor tyrosine kinases, which are involved both in normal homeostasis and in various disease states. There are 58 receptor tyrosine kinases

belonging to 20 families of between one and six members each. These receptors bear an extracellular ligand binding site and an intracellular tyrosine kinase domain. These two domains are separated by a single transmembrane domain. While each subfamily of receptor tyrosine kinases is activated by a different ligand, the mechanism of ligand-induced activation is largely conserved (Lemmon and Schlessinger, 2010). Receptor dimerization, driven by ligand binding, brings the tyrosine kinase domains of receptor tyrosine kinases into proximity to facilitate *trans* phosphorylation. Receptor phosphorylation is the initial event that then triggers downstream activation of a number of signaling modules. In fact, phosphorylated receptors act as a critical component in RTK signaling, allowing recruitment of a wide variety of SH2 domain-containing downstream effectors. Thus signal branching can occur at the level of the receptor, as multiple signaling modules headed by different SH2 domain containing proteins are activated following receptor phosphorylation.

Proteins recruited to phosphorylated receptor tyrosine kinases have been extensively studied. One such protein is Grb2, which, besides being used by c-Met specifically, is also an important signaling node in networks downstream of most other receptor tyrosine kinases. Grb2 is a critical component of an important signaling module in RTK signaling networks. It stands at the extreme upstream of the MAP/ERK kinase cascade, acting to recruit the Ras GEF SOS1 into an RTK/Grb2/SOS1 signaling complex and thus to the membrane, thereby initiating activation of Ras and of the downstream MAPK/ERK kinase cascade. In fact, SOS1 recruitment to the membrane and the subsequent activation of Ras is a major theme in RTK signaling and can occur through additional mechanisms. SOS1 can be recruited to the membrane by formation of several other signaling complexes, including c-Met/RanBP9/SOS1 (Graziani et al., 1991) and c-Met/Grb2/Shc/SOS1 (Mood et al., 2006b; Wang et al., 2002). In addition to direct recruitment of

SOS1 by activated c-Met receptors, activation of the oncogenic kinase Src by phosphorylated c-Met receptors (Wada et al., 1998) can induce formation of a FAK/Grb2/SOS1 complex (Stefan et al., 2001), which then stimulates Ras nucleotide exchange and activation (Schlaepfer et al., 1994). This indirect mechanism may allow for activation of the SOS1/Ras signaling module at different times following RTK activation.

Other examples of important signaling nodes directly recruited and activated by phosphorylated receptors include phosphatidylinositol-3-kinase (PI3K), PLC, and the namesake of the SH2 domain, Src. Bearing SH2 domains, these proteins are activated downstream of a wide variety of RTKs. Activation of these proteins can be accomplished by direct activation through their SH2 domain-mediated recruitment into c-Met receptor tyrosine kinase complexes (Li et al., 2009). These proteins can also be activated downstream of RTKs indirectly, which can affect the timing of activation following receptor activation. For example, PI3K can also be activated downstream of c-Met indirectly, downstream of GTP-bound Ras (Fan et al., 2001; Graziani et al., 1993). Activation of PI3K downstream of Ras requires c-Met induction of Ras nucleotide exchange by the Ras GEF SOS1, an event that follows SOS1 recruitment to the membrane by the multiple possible mechanisms mentioned in the preceding paragraph.

Activation of the above signaling components is highly conserved within diverse RTK signaling networks, as illustrated by reports where the activity of one RTK is able to compensate for the loss of function of another related RTK. For example, in colorectal cancer cells, expression of c-Met is sufficient to rescue an EMT phenotype when EGFR function is blocked (Liska et al., 2010). Despite similarities in usage of major signaling modules, different RTKs generate different cellular responses in the same cell type. MDCK cells are stimulated to undergo EMT when the c-Met RTK is activated, but not in response to activation of EGFR or VEGFR

receptor tyrosine kinase systems. This is also true in terms of molecular effects following activation of different RTK signaling networks; c-Met and EGFR have opposite effects on Gab1 expression, for example, with c-Met upregulating protein activity and with EGF downregulating it (Maroun et al., 1999a).

The striking conservation of signaling mechanisms between c-Met and other RTK signaling networks begs the question of how the cell differentiates between c-Met activation and activation of other RTK systems in order to generate the molecular and phenotypic responses that drive EMT. Some RTKs employ a greater number of proteins to control signal transduction through the same signaling modules (an example is FGF receptor tyrosine kinase, which has a larger number of regulatory proteins than the closely related EGF receptor tyrosine kinase (Kiyatkin et al., 2006)), which might account for differences in RTK signaling outcomes. This is important since it can affect the relative timing or intensity of shared signaling module activation between otherwise similar RTK signaling network systems, thus generating different cellular signaling outcomes. In fact the timing of signaling seems to alter the outcome of a signaling network significantly. It has been observed that alterations in the timing of MAPK/ERK signaling are associated with distinct signaling outcomes, namely proliferation versus apoptosis, of TGFα signaling in intestinal epithelial cells (Lau et al., 2011). Thus, it is likely more than the static architecture of a signaling network that provides information to the cell in determining cellular responses to network activation.

Feedback loops within signaling modules are also a common feature within receptor tyrosine kinase signaling networks, affecting the intensity of signaling module activation. Generally, negative feedback loops provide stability to signaling networks, while positive feedback loops provide exponential signal amplification or suppression. Examples of feedback

loops in c-Met signaling affect the MAPK/ERK signaling module. In addition to the Ras-dependent activation discussed earlier, the MAPK/ERK signaling module can also be activated by c-Met/Gab1/Shp2 (Lamorte et al., 2003) or c-Met/Grb2/Gab1/Shp2 complexes (Grotegut et al., 2006). These Shp2-containing complexes are affected by positive and negative feedback loops. First, the MAPK/ERK module increases activity of PI3K activity, which increase Gab1 activity, which increases activation of the MAPK/ERK module and yet more PI3K activity. Thus, activation of MAPK/ERK module signaling creates a positive feedback circuit through PI3K and Gab1 to exponentially increase signal output (Maroun et al., 1999b; Schaeper et al., 2007; Weidner et al., 1996). In contrast, a negative feedback loop that reduces late signaling intensity occurs when prolonged activation of c-Met induces degradation of Gab1 through targeted ubiquitylation, though this circuit remains more poorly defined at the molecular level (Yu et al., 2001). Importantly, arrangement of multiple feedback loops within a single signaling module can generate complex effects on overall signaling output, particularly as a function of time (e.g. oscillatory signaling).

Perhaps a more obvious mechanism for generating distinct responses from highly similar RTK systems is to alter the context of signaling by forcing crosstalk of specific RTKs with other signaling networks. Association of RTK with other signaling receptor systems at the cell surface allows for such crosstalk. c-Met signaling has been found to be highly dependent on CD44, as deletion of the CD44 gene renders c-Met haploinsufficiency lethal, demonstrating collaboration between CD44 and c-Met (Matzke et al., 2007), though other receptor systems, most notably ICAM-1, appear to be able to compensate for loss of CD44 in some instances (Olaku et al., 2011). CD44 is a receptor that drives increased migration during wound healing and, like c-Met itself, is tightly associated with cancer progression (Zöller, 2011). CD44, and particularly splice

variant 6, plays a critical role in the c-Met signaling transduction network, where it functions in multiple steps (Orian-Rousseau et al., 2002). Interestingly, in order for CD44 to facilitate Ras signaling downstream of c-Met receptors, it must associate with actin filaments via ezrin/radixin/moesin proteins (Orian-Rousseau et al., 2007), suggesting that c-Met, CD44, and ezrin scaffold the formation of a large signaling particle. Unlike the SH2 interaction with phosphorylated receptors, these lateral associations vary within RTK systems. Since c-Met signaling is dependent on the lateral association of c-Met receptor tyrosine kinases with other plasma membrane receptors, a highly conserved and otherwise undifferentiated RTK signaling network may operate distinctly from other RTK networks simply from alterations of the context in which RTK signaling occurs. In other words, activation of a generic RTK network in combination with different accessory signaling receptors could allow the cell to differentiate its cellular response.

Additional signaling nodes and modules have been implicated in modulating c-Met signaling, also perhaps providing specific context to RTK signaling. Recently an increasing number of studies have recognized the importance of Ca^{2+} fluxes in RTK signaling (Fukumoto et al., 2000; Lewis and Spandau, 2008), particularly in the case of c-Met (Chapuis et al., 2009; Gerber et al., 1998; Hayashi et al., 2000). In EGFR signaling, these Ca^{2+} influxes result from microtubule-dependent vesicular trafficking of Ca^{2+} channels to the plasma membrane (Chen et al., 2007). The potential role of Ca^{2+} influxes in the c-Met signaling network illustrates how activation of signaling within context might also relate to timing of signaling events, as the precise pattern of Ca^{2+} influx periodicity plays a critical role in cellular interpretation of calcium signaling. Perhaps different patterns of Ca^{2+} influxes combined with the same RTK signaling network could elicit different cellular responses.

HGF-induced epithelial-mesenchymal transition

Epithelial-mesenchymal transition is a higher order process that involves many complicated changes in cellular behavior. Activation of EMT by a single cellular signaling network requires the coordinated control of many cell biological processes. The main signaling modules that lead from c-Met receptor activation that initiate EMT drive the cellular processes of actin rearrangement, cell spreading, detachment of cadherin-based cell-cell adhesions, accelerated cell migration, and invasion through extracellular matrices. Spatiotemporally coordinated induction of the correct modules thus drives the larger EMT process. c-Met also activates additional signaling modules not directly tied to EMT and is responsible for EMT-independent cellular behavior changes, including inhibition of apoptosis and increased proliferation that will be discussed in another section. Here, we will address the individual signal transduction modules that lead from the c-Met receptor to specific changes in cellular behavior. It is important to consider where cytosolic signaling alone, without changes in gene transcription, could account for cellular responses to HGF stimulation. When localization and activity of proteins are altered by signaling, as might drive actin dynamics or changes in cadherin internalization, cytosolic signaling may account for the entire specific cellular response. Conversely, events relying on changes in gene transcription and altered levels of protein production, such as in expression of surface proteases required for invasion, gene transcription is clearly fundamental. There are thus likely branch points in the overall c-Met signaling network that depend on whether cellular effects are controlled at the pre- or post-transcriptional levels. Here we will examine the connection of signaling nodes with specific cellular responses to c-Met activation, providing a picture of the overall c-Met signaling network structure.

Induction of actin rearrangement

Morphological changes in cells are driven by actin dynamics and HGF-induced EMT is

no exception. HGF stimulation results in dramatic reorganization of the actin cytoskeleton (Sperry et al., 2010). Actin rearrangements are an essential first step required for nearly every other cell biological process that underlies EMT (Syed et al., 2009). Actin rearrangements are driven by altering the activity or abundance of numerous actin regulatory proteins (Figure 1). Given the large number of proteins that participate in actin dynamics, it is likely that multiple signaling proteins and their regulatory circuits provide the interface between the HGF signaling network and changes in actin organization. Central players in this interface appear to be small GTPases of the Rho family, which act as master regulators of actin dynamics in a number of cellular processes, as well as phosphatidylinositol-3-kinase (PI3K) (Reisinger et al., 2003; Royal et al., 2000).

Induction of cell spreading

Cell spreading is an early event in HGF-induced epithelial-mesenchymal transition. Epithelial cells stimulated with HGF roughly double the area of cell-substrate adhesion, an event that occurs prior to disruption of cell-cell adhesions. Cell spreading results from coordination of actin rearrangements (Bristow et al., 2009) and modulation of integrin-based adhesion with the cell substratum. On certain substrates, cell spreading does not occur effectively (Wang et al., 2010a), perhaps accounting for why the robustness of epithelial-mesenchymal transitions varies greatly depending on the matrix type (Clark, 1994a). Cell spreading, like actin rearrangements generally, relies extensively on Rho GTPases, particularly Rac1. In this pathway, Rac1 activation relies heavily on the Rac1 GEF, βPIX (Cheresh et al., 1999), which is in turn activated by the focal adhesion kinase (FAK)/Src-Yes-Fyn complexes (Lai et al., 2000; Posern et al., 1998; Ruest et al., 2001). FAK/Src-Yes-Fyn complexes assemble in response to Src-dependent phosphorylation of FAK and have been shown to play a critical role in numerous events during

EMT (Klinghoffer et al., 1999) (Figure 2).

Induction of cell-cell detachment

In order for cells to complete scattering during epithelial-mesenchymal transition, epithelial cell-cell adhesions must be disassembled. Detachment of epithelial junctions occurs late in the process of EMT, once cell spreading and initiation of cell migration have been completed. Disassembly of cadherin-based adhesions appears to occur in a series of events. Disruption of cell-cell contacts occurs shortly after spreading and initiation of cell migration. Cadherin switching, meaning altering cadherin family member expression is thought to occur early in EMT. Typically cells switch expression of the epithelial E-cadherin for that of N-cadherin. Interestingly, each cadherin is associated with different actin structures, namely E-cadherin with the actin organization that is observed in epithelial cells and N-cadherin with the actin organization of more mesenchymal cells. It remains unclear whether cadherin switching is a result of changes at the transcriptional levels, or whether this switch is a post-translational event with cells altering the preferential endocytosis of cadherin family members (Christofori, 2006; Wells et al., 2008). Application of tension forces to sites of cell-cell adhesion also appear to play a prominent role in detachment of cell-cell junctions, perhaps allowing detachment of cells from epithelial tissues before cadherin switching has been accomplished. Whatever the combination of mechanisms that reduce cell-cell adhesion, the c-Met signaling network must interface with the cell biological machinery that control the mechanism. Not surprisingly, Rho GTPases occupy a central position here.

Disruption of the tight junction system relies on increased Rac1 activity. Maintenance of the tight junction in the absence of c-Met signaling is thought to rely on Par3 recruitment into aPKC/Par3/Par6 complexes, where it serves to locally depress Rac1 activity. Upon c-Met

activation, Src activity increases and phosphorylates E-cadherin, causing release of Numb. Numb binds phosphorylated aPKC/Par3/Par6 complexes, displacing Par3 and allowing it to translocate to the nucleus (Kallergi et al., 2007). Without Par3 at the tight junction, local Rac1 activity increases and tight junctions are disassembled (Wang et al., 2009). Interestingly, it has been shown that HGF can induce Rac1 disassembly of adherens junctions in a Crk-dependent manner (Kamei et al., 1999), which promotes redistribution of paxillin to focal adhesions. The result is the formation of a Crk/Paxillin/GIT2/βPIX complex, which may then activate Rac1, generating a potential positive feedback loop that exacerbates cell-cell junction disassembly (Kimura et al., 2006).

c-Met also appears to alter the function of cadherin adhesion receptors at cell-cell adhesions, primarily changing the retention of this protein at the cell surface. E-cadherin has been observed to enter endocytic vesicles concurrently with the c-Met receptor (Rooij et al., 2005). Phosphorylation of E-cadherin by Src has been proposed to alter cadherin complex formation and, thus, cadherin function and distribution at the cells surface (Kallergi et al., 2007). c-Met signaling also can affect cadherin trafficking by Ras-dependent activation of Rin2, which stimulated Rab5-dependent vesicle trafficking to endosomes (Chen and Macara, 2005).

Another mechanism for abrogating cell-cell adhesion is by downregulation of E-cadherin transcription. Snail expression is increased in response to the nuclear translocation of transcriptional regulator EGR1, which is activated by the MAPK/ERK signaling module (Lamorte et al., 2002).

Physical rupture of cell-cell adhesion may also play a role in cell-cell detachment, especially during early EMT. This process relies on gaining a sufficiently strong grip on the cell substrate to pull apart cell-cell junctions as the cell contracts (Hiscox and Jiang, 1999). Tyrosine

kinase-induced cell contractility relies on the Rho-ROCK-myosin pathway, which generates actomyosin-based contractile forces on cell-cell contacts (Felici et al., 2010). Here c-Met receptors activate p120-4A, which activates the RhoA signaling module (Goormachtigh et al., 2011) (Figure 3).

Induction of increased cell migration

Cells responding to c-Met stimulation increase cell motility, increasing their rate of migration by as much as 2 fold. Migration is driven largely by changes in actin organization and dynamics, further demonstrating the central role of actin reorganization in EMT. Actin drives cellular protrusions at the leading edge that are required for cell translocation across a substrate, while remodeling of cell-substrate adhesions is also required for translocation. Rho GTPases are known to be central players in cell migration, both in regulating actin dynamics at cell protrusions and actin connections to cell-substrate adhesions. Like many essential processes in EMT, there are multiple, partially redundant circuits present in c-Met signaling for inducing cell migration. Like many of the circuits discussed above, c-Met-induced cell migration relies on several parallel circuits, each able to partially compensate for loss of function in its neighboring circuits.

Essential to c-Met-induced cell migration is focal adhesion kinase (FAK), which is required to drive enhanced migration (Zhao et al., 2000). Studies have shown that FAK is activated by phosphorylation by several kinases, including Src, c-Met (Zandy et al., 2007), and the MAPK/ERK module (Yanagisawa et al., 2008). Phosphorylated FAK increases migration by altering membrane protrusion formation at the leading edge. Here FAK acts to facilitate the activation of actin regulatory proteins, including N-WASP (Chen and Chen, 2006). At the trailing edge of the cells, FAK facilitates activation of the RhoA/myosin contractility pathway by

forming a complex with PDZRhoGEF and cooperating in RhoA nucleotide exchange. FAK can also induce migration through activation of Arf6, which stimulates vesicle trafficking pathways that alter Rho GTPase activity, actin dynamics, and cell migration (Wu et al., 2004). FAK/Src-Yes-Fyn complexes, described previously, also facilitate migration, acting via BMX (Iwanicki et al., 2008), Grb7 (Palacios et al., 2001), Rac1 (Chen et al., 2001; Herrera, 1998; Iwanicki et al., 2008; Palacios et al., 2001; Reiske et al., 2000) and focal adhesion disassembly at the trailing edge (Dolfi et al., 1998; Grimsley et al., 2004) (Figure 4).

Induction of cell invasion

Individual, solitary cells resulting from EMT acquire the ability to penetrate connective tissues by remodeling or degrading extracellular matrix. In the context of EMT in development, this allows individual cells to transit through tissues to their final target destination. In the context of metastatic cancer cells from epithelial tumors, this allows cancer cells to invade through surrounding tissues and colonize distant sites. Surface proteases are the primary mediators of cells' ability to invade through connective tissues and cells undergoing EMT are no exception. Tumor invasion relies on the activity of surface matrix metalloproteinase 9 (MMP9) to degrade extracellular matrix proteins, a protein that is also expressed in response to HGF signaling (Graauw et al., 2008). HGF-induced transcription of MMP proteins is mediated E1AF, an Ets family transcription factor (Tague et al., 2004). Ets is activated through nuclear localization caused by signaling through the MAPK/ERK module (Webb et al., 2004). Moreover, Ets-1 has also been shown to take part in an important feedback loop in c-Met signaling, namely driving HGF-induced activation of c-Met transcription (Sridhar and Miranti, 2006) (Figure 5).

c-Met signaling in processes independent of EMT

Though EMT is a major cellular response to c-Met signaling network activation in

epithelial cells, other cellular responses to c-Met signaling are also observed. These are inhibition of apoptosis and increased cell proliferation. HGF-induced inhibition of apoptosis results from inhibition of Bad, a pro-apoptotic member of the Bcl-2 family. This is accomplished by activating PI3K, which in turn generates survival signaling by acting on PDK1 and then AKT (McCawley et al., 1998). Inhibition of apoptosis can also result from upregulation of signaling through the NF-κB module and a downstream increase in Bcl-2 expression (Higashino et al., 2010). Additionally, it has also been shown that HGF induces improved cell survival through phosphorylation of GATA-4 in a MAPK/ERK signaling module-dependent manner (Guo et al., 2011) (Figure 6).

Increased cell proliferation is also mediated by NF-κB signaling module (Guo and Sharrocks, 2009) or alternately, through the MAPK/ERK module and PI3K node. It is interesting to note that cell proliferation negatively correlates with EMT. It has been demonstrated that in proliferating cells, HGF stimulation induces TIMP-2 to inhibit the cell surface protease MMP2, leading to increased matrix deposition and a reduction in invasion. In contrast, HGF stimulation induces quiescent cells to increase matrix degradation through inhibition of TIMP-1 (Esposito et al., 2009) (Figure 7). Clearly proliferative states play a major role in determining the outcome of c-Met signaling, suggesting another instance of crosstalk between cellular processes and the c-Met signaling network that provides context to signaling.

Overall network structure

Regardless of whether the most downstream molecular targets of c-Met signaling are cytosolic or transcriptional, the activation of distinct cellular responses following c-Met activation is branched. Further, that initiation of signaling results in a final phenotypic outcome, transition of cells from epithelial to mesenchymal, suggests at the outset that the overall structure of the c-Met signaling network is linear, rather than cyclical. Activation of the receptor is

transmitted through multiple nodes to a large variety of downstream events, including changes in the regulation of specific genes and proteins. Given the large number of target proteins and genes, it is not surprising that the linear c-Met architecture must be branched, though this does not rule out interconnections between distinct branches. While consideration of the c-Met network as a branched linear network is appealing, there is also evidence that the network is cyclical. c-Met activation does result in a transcriptional increase in production of c-Met receptors (Gambarotta et al., 1996; Paumelle et al., 2002). This could act as a large positive feedback loop system that perpetuates c-Met signaling as a cyclical network.

Summary

Cellular signaling in response to c-Met activation is highly relevant to a number of disease processes, including fibrosis, wound healing, cancer progression, and angiogenesis. A complete map of individual interactions and components in the c-Met signaling network (the static network structure), however, is unlikely to provide a complete understanding of the connection between c-Met signaling and normal cellular processes. Dissection of the c-Met signaling network as an adaptable network system, where temporal dynamics of signaling and cellular context (crosstalk) are considered to be as important as network structure, will be critical to defining how c-Met activation drives normal cellular processes and disease progression.

Established methods for identifying key components of signal transduction pathways in cancer

For many years, one of the challenges in cancer research has been identification of proteins involved in cancer progression. A variety of approaches have been taken to identify these proteins and their respective roles in tumorigenesis. Early approaches involved identification of transcriptional changes in unstimulated versus stimulated tumor cells (Masiakowski et al., 1982), classification of activated cytosolic T lympohcytes in the tumor

environment (Wölfel et al., 1987) and identification of antigens in the serum to which the tumor is exposed (Sahin et al., 1995). More recent approaches have included 2D gel electrophoresis (Zhou et al., 2002), high-throughput retroviral tagging (Mikkers et al., 2002), microarray analysis, which is essentially an improvement on the original technique (Balkovetz et al., 2004) and multidimensional protein identification technology (Mauri et al., 2005). Indeed, high-throughput drug screening is not a new approach and has successfully been used to identify proteins involved in other signaling pathways (Balis, 2002), nor is the concept of using such techniques to block c-Met signaling a new one (Shoemaker et al., 2002).

Nevertheless, to this point, screens for drugs effective at blocking the c-Met pathway have only targeted the regulation and expression of the receptor itself. There is no published evidence of a non-biased drug screen with the intent of identifying essential downstream components of the c-Met pathway. This lack represents a significant gap in the current literature, for, while there is undoubtedly a great benefit to identifying blockers of the c-Met receptor itself, any mutation or dysregulation of the c-Met receptor or, worse yet, a component immediately downstream of the c-Met receptor, can completely negate drugs that target the c-Met receptor specifically. In this study, a transwell migration assay broadens the search for inhibitors to include any compound capable of blocking any essential component of the c-Met signaling pathway. By employing a large chemical library consisting of small, drug-like molecules, the probability of locating several compounds capable of this inhibition is relatively high.

By thereafter identifying the function of the effective drugs, it thus becomes possible to use this technique not only to find potential treatments, but also to identify previously unknown components of the c-Met pathway. This is accomplished in a number of ways, including running functional kinase screens for the compounds that tested effective, structure similarity searches

for analogous compounds with similar function, and bioinformatics-aided identification of drug targets. Compounds of known function are then tested to confirm that drugs with targets similar to those predicted for the unknown compounds are indeed capable of inhibiting HGF-induced transwell migration.

In addition to simply determining whether a specific protein is necessary for HGF-induced EMT-like behavior in general, however, this study goes one step further. By testing the ability of known compounds to inhibit HGF-induced cell proliferation or lateral cell migration, it is then possible to approximate the location of the target protein in the c-Met signal transduction pathway, whether it occurs near the beginning of the pathway and so can inhibit both migration and proliferation, or whether it occurs nearer to the end of the pathway and so affects only cell proliferation or lateral migration, but not both. Thus, through a series of simple high-throughput assays, it is possible to test the ability of a drug to prevent HGF-induced EMT-like scattering in general, as well as migration or proliferation specifically.

Background of calcium signaling

Calcium signaling has long been known to be involved in intracellular signaling. Calcium signaling was first described over 60 years ago as playing a role in muscle contraction (Heilbrunn and Wiercinski, 1947). Over the following decades, calcium was found to interact with a variety of calcium binding proteins, particularly calmodulin, and by 1980 calcium had risen in status to become one of three major cellular regulators, in company with hormones and cyclic nucleotide monophosphates (Cheung, 1980). Expanding on existing knowledge of calcium-induced calcium release mediated by ryanodine receptors in excitable cells, such as muscle, calcium signaling was shown to rely on IP_3 to induce calcium release in inexcitable cells, that is, cells without ryanodine receptors (Putney et al., 1989). By the early 1990s, however, it had become evident that this division between calcium signaling in excitable cells

and inexcitable cells was more perceived than real, as researchers demonstrated the remarkable similarities between ryanodine receptors and IP_3 receptors, which had strikingly similar mechanisms of action, differing only in their methods of activation (Putney, 1993). At about the same time, researchers described how cells, whether excitable or inexcitable, communicated with one another by activating calcium signaling in adjacent cells via gap junctions, IP_3 and ATP signaling, further confirming this unity in calcium signaling (Berridge, 1993).

These first decades of dedicated calcium research provided the foundation for a multitude of recent advances in understanding not only the mechanism of calcium signaling, but identification and classification of a large number of calcium channels, each capable of regulating calcium conductance in diverse and unique ways. In fact, it had become apparent as early as 1975 that there were multiple types of voltage-gated Ca^{2+} channels (Hagiwara et al., 1975). Following this initial discovery, the number of identified voltage-gated Ca^{2+} channels exploded, with each new classification identifying the role of a new subunit, responsiveness to a new drug or inhibitor, or a distinct role in calcium signaling (Catterall, 2000; Clapham and Garbers, 2005).

Concurrent with this identification of voltage-gated Ca^{2+} channels, researchers were also classifying non-voltage-gated channels that, while not necessarily as selective for Ca^{2+} ions as are their voltage-gated counterparts, still play a major role in Ca^{2+} conductance. These non-voltage gated channels fall into a number of categories, including cyclic nucleotide-gated channels, IP_3 receptor channels, and transient receptor potential (TRP) channels. Cyclic nucleotide-gated channels were first described in 1985 in retinal photoreceptor cells (Fesenko et al., 1985), which was followed only two years later by discovery of these cells in the cilia of olfactory neurons and four years later in the pineal gland (Alexander et al., 2008). IP_3 receptor

channels were first hinted at as early as 1981, when it became evident that hydrolysis of phosphatidylinositol was associated with activation of calcium signaling (Berridge, 1981), and in the decades following, knowledge of these channels expanded to encompass several different subtypes, each with distinct characteristics (Alexander et al., 2008). TRP channels are ironic in that they are the most recent addition to the ranks of non-voltage gated channels, and yet TRP channels are more numerous by far than any other channel type discussed to this point. TRP channels fall into several subfamilies, each with a different number of members: canonical (TRPC), melastatin (TRPM), vanilloid (TRPV), polycystic (TRPP), ankyrin (TRPA), and mucolipin (TRPML). TRP channels vary from strongly Ca^{2+} selective to nonselective to selective for some other cation. TRP channels are found throughout the body and play a wide variety of functions, from sensory input to cell cycle regulation, with each of the more than 25 subtypes playing a distinct role in physiological function (Alexander et al., 2008; Nilius et al., 2007).

The role of calcium conductance in cancer progression

Over the past ten years, studies examining the relation of calcium signaling to cancer progression have gained prominence in the scientific literature, and, with few exceptions, the past eighteen years have seen a steady increase in yearly publications relating signaling from Trp channels to cancer. Many of these studies relate intracellular Ca^{2+} concentrations or Ca^{2+}-mediated protein activation to cancer prognosis (Al-Bahlani et al., 2011; Gerhardt et al., 2011; Naik et al., 2011), while a smaller number address the function of a specific calcium channel in cancer progression (Hiani et al., 2009; Lehen'Kyi et al., 2007; Wondergem et al., 2008). From these and other studies, a tapestry of calcium signaling has begun to take shape, with specific channels being revealed essential to specific processes in a multitude of cancer types (Prevarskaya et al., 2010). These studies and many others have contributed greatly to our current

understanding of the roles of Ca^{2+} current in cancer models.

Primarily, however, each of these studies has either taken a single channel and examined its effects on metastasis (Wang et al., 2010b; Wondergem et al., 2008), or else shown that a number of channels cooperate to accomplish a single effect (Rampino et al., 2007). Only a select few studies have addressed the possibility of two different calcium channels functioning in the same pathway with distinct and non-redundant effects (Waning et al., 2007). This study further contributes to this third area of research by demonstrating that not only is inhibition of a single calcium channel sufficient to prevent HGF-induced cell scattering but also that two differentially selective calcium channel blockers capable of preventing transwell migration have distinct effects on other aspects of EMT-like cell scattering.

My experimental strategy combines combinations of inhibitors and patch clamping to determine the identity and activity level of multiple channels and define the function of these channels in the c-Met signal transduction pathway. By testing an inhibitor of unknown specificity in combination with drug concentrations specific to various channels, it becomes possible to identify the target of the novel inhibitor. Following this identification, it is then possible to determine which channels are responsible for specific aspects of HGF-induced scattering.

Tubulin as a mediator of ion channel function

Tubulin, in addition to its cytoskeletal role, is an important mediator of vesicular transport, allowing membrane localization of signaling proteins and channels to be dynamically controlled by the cell. Recent studies have shown that plasma membrane localization of TrpC5 and TrpC6 channels can be included among the many things that tubulin carries via vesicular transport (Greka et al., 2003; Kennedy et al., 2010). This provides an alternate method of regulation for these channels besides the standard diacylglycerol-mediated activation (Hofmann

et al., 1998). Furthermore, recent data confirm a role for vesicular transport in HGF signaling (Kermorgant et al., 2004), and even a role for vesicular transport of ion channels (Steffan et al., 2010). In this study, I use electrophysiological recordings to address the question of whether tubulin is required for vesicular transport of ion channels during HGF signal transduction.

NFAT involvement in cancer signaling

Nuclear factor of activated T-cells (NFAT) is a family of transcription factors first found to function in the immune system (Shaw et al., 1988). In the years since, the members of the NFAT family have been shown to be expressed throughout the body in most cell types and to have a wide variety of functions (Crabtree and Olson, 2002). Among these functions are significant roles in cancer progression, with evidence supporting roles for NFAT in prostate cancer (Lehen'Kyi et al., 2007), breast cancer (Yiu and Toker, 2006), colon cancer (Duque et al., 2005), and glioblastomas (Chigurupati et al., 2010).

NFAT family members NFATc1-c4 are all activated through Ca^{2+}-dependent activation of calcineurin (Clipstone and Crabtree, 1992). This activation is accomplished through nuclear transport of dephosphorylated NFAT proteins when periodic Ca^{2+} influxes occur at sufficient frequencies that the rapid calcium-dependent dephosphorylation of NFAT can no longer be counterbalanced by the slower rephosphorylation of NFAT (Tomida et al., 2003). Thus, in order for NFAT to be activated in cancer pathways, these pathways must first upregulate the rate of Ca^{2+} influx (Thebault et al., 2006).

Numerous studies have tied NFAT activation to cancer progression. Two of the important channels involved in this oncogenic role are TrpV6 in prostate cancer (Lehen'Kyi et al., 2007) and TrpC6 in prostate cancer (Thebault et al., 2006) and glioblastomas (Chigurupati et al., 2010). Remarkably, not only does TrpC6 upregulate NFAT activity, but NFAT has also been shown to increase expression of TrpC6, thus generating a positive feedback circuit (Kuwahara et al.,

2006), which is very characteristic of HGF signaling (Arlt and Stein, 2009; Rasola et al., 2007; Sam et al., 2007).

Based on these data, it is surprising that there appear to be no studies specifically linking HGF signaling to NFAT activation, despite clear suspicions in the scientific community that such might be the case (Gkika and Prevarskaya, 2009). This study also addresses this suspected connection and not only tests the connection to the NFAT protein family in general, but the specific NFAT subtype involved in HGF-induced EMT.

The role of WNK kinase in cancer and cation channel regulation

The with no lysine (WNK) kinase is a unique kinase subfamily in that its members do not contain the characteristic lysine found in subunit II of the catalytic domain, which is replaced with cysteine (Xu et al., 2000). There are four members of this subfamily expressed in humans: WNK1-4. WNK1 is expressed in nearly all tissues, WNK2 in the heart, brain and colon, but not in the kidney (Rinehart et al., 2011), WNK3 primarily in the brain, and WNK4 primarily in the colon (Veríssimo and Jordan, 2001).

One common feature of these kinases is their role in cation transport. WNK1, WNK2 and WNK4 are all implicated in regulation of cation-chloride-coupled cotransporters (Moriguchi et al., 2005; Rinehart et al., 2011), while WNK3 regulates ROMK1, a K^+ channel (Leng et al., 2006). These functions control a variety of important physiological processes. WNK1, WNK3 and WNK4, for example, regulate ion balance in multiple systems, including controlling blood Ca^{2+}, K^+ and hypertension (He et al., 2007; Wilson et al., 2003; Zhang et al., 2008). In addition to the distinct differences among the WNK kinases, there is also evidence of alternative splice variants mediating differential functions. For instance, WNK1 has a kidney-specific splice variant (KS-WNK1) with a distinct and opposing role to that of full length WNK1 (L-WNK1) in K^+ channel regulation (Wade et al., 2006). Furthermore, kidney-specific and brain-specific

isoforms of WNK3 also have opposite effects on regulation of NCCT (Glover et al., 2009).

In addition to these channels, members of the WNK subfamily have been shown to regulate Trp channels, with WNK1 and WNK4 downregulating TrpV4 through endocytosis, WNK1 apparently through kinase activity, and WNK4 more potently than WNK1, though it is unclear whether this effect is a result of kinase activity or an effect of some other domain of the WNK4 protein (Fu et al., 2006). Also, WNK1 and WNK4 activate a pathway that ultimately reduces membrane expression of the NKCC1, TrpC3 and TrpV4 ion channels at the membrane (Fu et al., 2006; Park et al., 2011; Vitari et al., 2005). WNK3, contrariwise, positively regulates TrpV5 and TrpV6 through its kinase domain (Zhang et al., 2008).

In addition to the role of WNK kinases in regulation of cation transport, it is important to note that WNK kinase expression has been linked to cancer prognosis in many cases. WNK1, for example, has been tied to breast, lung and ovarian cancers; WNK2 to colorectal, gastric, lung and ovarian cancers; WNK3 to gliomas and lung and renal cancers; and WNK4 to melanomas and gastric and ovarian cancers (Greenman et al., 2007).

Together, these data indicate a potential role for WNK kinase in the HGF cancer signaling pathway. This study asks whether WNK plays a role in HGF-induced cell scattering and so in the c-Met cancer metastasis pathway. This is important, as no study has previously addressed the connection of HGF signaling and WNK kinase activity. Furthermore, connections between WNK kinase activity and cancer progression have not yet led to the examination of the possibility that specific WNK kinases may regulate cancer progression via their roles as mediators of ion signaling. This is surprising considering the many other ways in which WNK kinase mediation ion balance in tumors has been considered (Moniz and Jordan, 2010).

MDCK as a model system

It was observed over 25 years ago that MDCK cells scatter in response to a factor secreted by fibroblasts (Stoker and Perryman, 1985). In the years since, MDCK has become a well-recognized model system for mimicking the EMT-inducing effects of HGF *in vitro* (Cozzolino et al., 2003; Date et al., 1997; Hellman et al., 2005; Khoury et al., 2005). When MDCK is stimulated by HGF, it undergoes extensive remodeling of the cytoskeleton, flattens, separates from other cells, and increases in motility and invasiveness, which mirrors the behavior of cells undergoing EMT *in vivo*.

MDCK as a model system in the high-throughput drug screen

By using MDCK as a model system in the drug screen, this study loses a few benefits that it would have *in vivo*. The primary benefit lost *in vitro* is that drugs discovered *in vitro* may not be viable for use *in vivo*, either because of toxicity or metabolism where an initial animal test would automatically rule out such drugs. However, these losses are minimal when compared with the benefits of an *in vitro* study. First, particularly toxic compounds are easily observed *in vitro*, as there will be no cells left when it comes time to test. Second, it is possible to test several thousands of drugs per week with greatly reduced expense. Third, it avoids unnecessary animal experimentation where less than 0.1% of the animals tested would be more informative than an *in vitro* study. Finally, because I am studying metastasis, a comparable *in vivo* model would also require extensive dissection and close examination in order to determine the extent of metastasis for each drug treatment, which would greatly increase the amount of labor required, the cost and the probability of a false positive result.

MDCK as a model system to determine upstream regulators of Ca^{2+} influx in HGF signaling

In this experimental approach, MDCK is an optimal system. A similar experiment *in vivo*

would be immensely complicated by ubiquitous signaling factors. By limiting the experiment to only one signaling factor, HGF, the situation is simplified. Admittedly, an *in vitro* system does fail to take into consideration a fair number of factors relevant in disease treatment, toxicology, etc. However, the goal of this study is to dissect a specific step from a specific signal transduction pathway, which is greatly facilitated through the increased control permitted by an *in vitro* approach.

MDCK as a model system for calcium channel classification and NFAT testing

An *in vitro* model for calcium channel identification also has a few setbacks. First, when patch clamping cells, the cells are in an artificial environment, and second, past studies show that tumor environment plays an important role in how chemical signals are interpreted (Chung et al., 2011; Clark, 1994b). Thus, *in vitro* studies may not perfectly reflect how the cells would behave *in vivo*. However, as with the drug screen, the advantages bestowed by an *in vitro* setup far outweigh the disadvantages. First, though possible, it is far more difficult and expensive to patch cells in living organisms, particularly mammals. Second, it is more difficult to be certain of patching the same cell type, where a single cell line guarantees minimal variation between individual assays. Finally, because the original drug screen was performed on MDCK cells, patching the same cell type is essential for maintaining consistency.

MDCK as a model for WNK kinase signaling

In this particular aspect, there are significant advantages and equally significant disadvantages to an *in vitro* approach. One main advantage, as mentioned in the reasoning for MDCK in studying regulation of Ca^{2+} signaling, is the reduction of complicating factors. Given the fact that WNK kinase may very easily play a permissive role, it is sufficiently difficult to identify its role in HGF signaling without the complexities of an *in vivo* system. The main inherent disadvantage in an *in vitro* system is that because it may be permissive, the slightest

modification of WNK kinase activity or expression *in vivo* would be nearly impossible to predict and replicate *in vitro*. Therefore, these assays are performed with the understanding that the results will not necessarily translate into an *in vivo* system. However, if the function of WNK kinases is essential *in vitro*, it is very likely that the same is true *in vivo*.

Summary of literature review and my specific aims

In a tumor environment, HGF causes cells to undergo EMT. This reaction is caused by a complex signaling pathway that induces affected cells to flatten, detach, migrate, invade surrounding tissues, and resist apoptosis. Each of these segments of EMT relies on specific signals in the cell. While many of these signals have been identified, it is clear that a large amount still remains unknown.

Specific proteins involved in this signal transduction pathway are gradually being revealed, but frequently it is difficult to identify their precise function and location in the overall sequence, particularly when the experimental setup is competent only to answer the question of its function in the most general sense. This is the case for such techniques as microarray analysis, classification of activated cytosolic T lymphocytes in the tumor environment and 2D electrophoresis. These techniques allow a researcher to determine the expression levels of a large collection of proteins and differentiate between stimulated and unstimulated cells or regions. Though expression levels and phosphorylation states can indicate an important protein in the target pathway, there are many proteins that are upregulated without necessarily playing an essential role in the EMT-inducing pathway.

Over the past few years, it has become increasingly clear that calcium channel regulation is essential for cancer metastasis in multiple tumor types, and that specific calcium channels are indispensible for signal transduction in select cancer types (Hiani et al., 2009; Lehen'Kyi et al., 2007; Wondergem et al., 2008). Moreover, emerging studies have begun to demonstrate not only

the necessity of specific ion channels, but also of specific and consistent patterns of activation (Liu et al., 1998).

Though at first identified as a Ca^{2+}-inducible transcription factor in the immune system, NFAT is widely distributed and has a large number of targets (Crabtree and Olson, 2002). Past studies have tied NFAT function to cancer progression, among other things, and have shown that inhibition of NFAT is sufficient in some cases to restore a more non-malignant phenotype (Duque et al., 2005; Yiu and Toker, 2006).

Specific aim 1: Identify proteins essential to HGF signal transduction and classify them according to function

The high-throughput transwell migration drug screen employed in this study, though unable to identify the specific function of a protein in HGF-induced cell scattering, is superior to most previous methods in that it identifies proteins required for HGF signaling, rather than all the proteins changed during HGF signal transduction. Following this assay and identifying the function of each identified compound as well as possible, these and analogous drugs of known function are employed in assays testing HGF-induced proliferation rate and lateral migration rate. These assays facilitate further classification of proteins as being essential to proliferation, migration, and/or some other aspect of HGF-induced cell scattering. Thus, through a limited number of assays it is possible not only to identify, but to categorize proteins according to their function and placement in the HGF signal transduction pathway.

This study shows that drugs impeding cytoskeletal rearrangement, integrin function, and calcium conductance all effectively prevent HGF-induced cell scattering in MDCK. In addition, further assays with inhibitors of known function show calcium influx, microtubule dynamics and NFAT signaling to be essential for and to have comparable effects on cell proliferation and lateral migration. However, other inhibitors exhibited significant effects only on one of those

two, suggesting that their protein targets may be farther downstream in the pathway.

Specific aim 2: Determine the mechanism of Ca^{2+} signaling in HGF signal transduction

Based on the literature, it is evident that ion conductance plays a role in cell signaling. This conclusion is supported in MDCK cells by the results of the drug screen, as well as the ability of two promiscuous Ca^{2+} channel inhibitors to significantly reduce levels of HGF-induced transwell migration. Interestingly, the two differentially selective promiscuous Ca^{2+} channel inhibitors exhibit different effects on proliferation and lateral migration, though both test positive in inhibition of transwell migration. Together, these data strongly suggest that HGF activates Ca^{2+} signaling in MDCK and that, without this activation, most of the ability of HGF to induce cell scattering is lost, indicating that at least Ca^{2+} influx event is likely near the beginning of the signal transduction pathway. The inability of the second Ca^{2+} channel blocker to significantly inhibit either proliferation or lateral migration suggests the possibility that there may be another instance of Ca^{2+} signaling farther downstream, though this study does not address that possibility in any detail, focusing instead on the initial Ca^{2+} influx.

In order to demonstrate early Ca^{2+} influx, I employ cell-attached patch clamping and examine channel events before and after addition of HGF. Through this, I show that HGF-induced signal transduction upregulates the frequency with which Ca^{2+} channels open at specific times following HGF stimulation. I then determine whether the similar effects shown by the Ca^{2+} channel blocker and the tubulin polymerization inhibitor used in the follow-up drug screen indicate some relation. In order to do this, I perform a patch-clamp assay in which I use HGF to induce cells to scatter, just as before, except that prior to HGF treatment, I administer a functional concentration of the tubulin polymerization inhibitor, which completely eliminates the Ca^{2+} channel surges while leaving the baseline frequency unchanged. This is a strong indication of the importance of tubulin dynamics in HGF-induced Ca^{2+} channel upregulation.

Following this, I use a whole-cell patch to show the ability of La^{3+}, a promiscuous Ca^{2+} channel inhibitor with widely varying IC_{50} values depending on the channel, to block the observed channels one type at a time as concentrations increase. Based on the effective concentrations of La^{3+} and the published sensitivities of various channels to La^{3+}, I identify the channels expressed most abundantly in the plasma membrane.

Finally, I use varying concentrations of La^{3+}, Gd^{3+} (which has similar effects but different IC_{50} values from La^{3+}) and the Ca^{2+} channel blocker identified from our initial drug screen to determine the identity of the Ca^{2+} channel essential for HGF signal transduction. In this assay, I show that TrpC6 is essential for HGF signal transduction in MDCK cells despite the continued function of TrpV4.

Specific aim 3: Characterize the interaction between HGF-induced Ca^{2+} influx and NFAT activation

NFATc1-c4 are transcription factors that are activated through calcineurin in response to Ca^{2+} influx into the cytoplasm (Clipstone and Crabtree, 1992). NFAT-mediated transcription has been proven essential in a wide variety of processes throughout the body (Crabtree and Olson, 2002). It has been implicated in numerous cancer types (Chigurupati et al., 2010; Lehen'Kyi et al., 2007; Thebault et al., 2006; Yiu and Toker, 2006) and has been shown to directly contribute to malignant phenotypes (Duque et al., 2005; Yiu and Toker, 2006). Furthermore, in my own transwell migration experiments, I show that NFAT is essential for HGF signal transduction and that inhibition of NFAT expression shows very similar phenotypes to inhibition of Ca^{2+} influx or tubulin polymerization. This suggests that all three of these proteins may perform a similar function in HGF signaling.

In view of this evidence, I employ reporter assays to determine the pattern of NFAT activation following HGF stimulation. Following this, I combine reverse transcriptase PCR with

immunofluorescent imaging to determine the NFAT subtype influenced by HGF induction. Through the use of reporter assays I demonstrate that the observed large surge of Ca^{2+} influx triggered by HGF signaling is followed by a corresponding surge in NFAT signaling with the precise delay agreeing with a former study on NFAT activation (Tomida et al., 2003), and that in the presence of the Ca^{2+} channel blocker discovered in the drug screen, NFAT activity is reduced to near unstimulated levels. Thereafter, I use immunofluorescence to show that NFATc4 localization is mediated by HGF stimulation, and that low concentrations of Gd^{3+} sufficient only to block TrpC6 are sufficient to significantly reduce this nuclear localization. Thus, I demonstrate the downstream component affected by microtubule-facilitated TrpC6 hyperactivation.

Specific aim 4: Identify the role for WNK kinase in HGF-induced EMT

Members of the WNK kinase subfamily have been shown to be involved in regulation of cation transport through the cell membrane, whether by regulation of cotransporters (Moriguchi et al., 2005; Rinehart et al., 2011) or of ion channels (Fu et al., 2006; Leng et al., 2006; Zhang et al., 2008). Also, mutations in each of these kinases have been shown to be linked to cancer development (Greenman et al., 2007). In addition, initial drug screens show that a small-molecule inhibitor that targets WNK2 and WNK3 is sufficient to prevent HGF-induced cell scattering at concentrations that are not toxic to cells. Also, electrophysiological evidence demonstrates that a WNK kinase inhibitor significantly increases ion flux through the cell membrane, suggesting that the observed effect of WNK kinase inhibitor on EMT may relate to dysregulation of ion signaling. These data suggest that one or more members of the WNK kinase subfamily are either modulated by HGF signaling or else play a permissive role in signal transduction.

Chapter 2: High-Throughput Drug Screen

Design, validation and application of a small-molecule screen for inhibitors of HGF-induced cell scattering

We sought to develop a screening assay that measures HGF-induced EMT. The response of MDCK cells to HGF stimulation has been well documented; treatment of MDCK cells with HGF results in detachment of cell-cell junctions and initiation of cell migration and invasion (Weidner et al., 1990). HGF-induced migration across a transwell filter has been used to monitor EMT-like scattering of MDCK and other cell lines in response to HGF and other stimuli. To validate this assay for screening, MDCK cells were seeded onto transwell filters, allowed to form a monolayer for 24 hours, and then treated with increasing amounts of HGF. A dose-dependent response to HGF is clearly observed (Figure 8A). In control assays, few cells are detected on the underside of the filter. Increasing numbers of cells correlates with HGF addition, but rapidly reaches maximum. To determine the statistical limitations of this screening assay, a series of eight positive (HGF treated) and negative (untreated) control assays were run and quantified, generating a Z prime value of 0.48 for the assay. This indicates that the assay is not quite ideal, but should allow detection of compounds that moderately or strongly inhibit HGF-induced cell scattering.

In validate this assay for small molecule screening, we next tested the assay system against the known c-Met inhibitor SU11274 (Sattler et al., 2003). Importantly, DMSO has no effect on HGF-induced cell scattering in this assay below 2% final concentration (data not shown) and we performed all drug dilutions such that the final DMSO concentration in each assay did not exceed 1%. SU11274 treatment prevented cells from responding to HGF stimulation as measured using the cell scattering assay (Figure 8B). Further, quantitation of the concentration dependence of SU11274 resulted in a calculated EC50 value (17.6nM) that is very

close to the published ID50 for SU11274 (20nM).

Given that our cell scattering assay successfully detects small molecule inhibitors of HGF signaling, library screening was performed on a panel of 50,000 drug-like compounds. To facilitate rapid screening and reduce the number of plates to be analyzed, individual assays were treated with a mixture of 3 distinct compounds, each at 5μM final concentration. Of 16,667 individual assays containing screening compounds, 38 individual assays showed inhibition of HGF-induced scattering. Cytotoxicity, detected when both the upper and lower surfaces of the transwell filter were devoid of crystal violet staining, was observed in only 5 individual assays. Compounds in individual assays demonstrating reduced scattering or cytotoxicity were re-tested as single compounds in subsequent assays. 38 individual compounds that inhibit HGF-induced scattering were validated individually, an initial hit rate of 0.076%, while only 5 individual compounds (0.01%) are cytotoxic. Chemical structure analysis of the 38 compounds identified in the original screen revealed 22 structurally distinct compound families. A single compound that was predicted to be reactive was discarded from analysis at this stage. The average family size was 1.8 compounds, with most families (11 of 22) represented by single chemical structures.

Physicochemical properties of the 37 hit compounds were compared to those of the entire chemical library. While the chemical library contained compounds with a wide molecular weight range (from under 200 to over 500 Daltons), hit compounds were restricted to a narrower molecular weight range, namely 205-413 Daltons (Figure 8C). That the distribution of molecular weights of hit compounds did not appear bell-shaped suggests that there are minimum and maximum size restrictions for compounds in our cell-based assay. Distributions of hydrophobicity, as calculated by clogP, and polar surface area, as calculated by tPSA, properties of hit compounds did not differ significantly from the distribution of the library as a whole

(Figure 8D-E). However, the distribution of solubility properties, as determined by logSw scores, is slightly more negative for hit compounds than for the entire library (Figure 8F). While the reason for this is uncertain it likely reflects solubility and membrane permeability constraints on successful compounds in high content, cell-based assays. Refining criteria used to select compounds for libraries might be expected to increase the hit rate in such screens.

Compounds reveal the cell biology of HGF-induced scattering

Biological activities and molecular mechanisms of hit compounds are expected to reveal molecular componentry required for HGF-induced scattering. Compounds identified in screening can be separated into three groups: 15 compounds work via an unknown molecular target, 4 compounds target microtubules, and 3 work by targeting non-cytoskeletal systems. The first group of compounds shed little light on HGF signaling. Molecular target identification, whether by screening for inhibitory activity in high-throughput biochemical assays or by identifying binding proteins, is required to reveal how HGF signaling is perturbed by the presence of these small molecules.

The second group of small molecules identified in screening share reported activity as microtubule poisons. This includes the largest family of compounds that share significant structural similarity, with 7 piperazine-based derivatives within the cluster (Figure 9A). Three unrelated structure families bearing 1-2 members each are also reported to have activity in preventing microtubule polymerization or in causing depolymerization of existing microtubules (Figure 9B-D) (Gelvan et al., 2003; Kim et al., 2009; Morgan et al., 2008). Microtubules play a fundamental role in a number of cellular processes, including vesicle trafficking and cell polarity. Thus, though it is possible that perturbation of microtubule-based processes exert a direct effect on HGF-induced scattering, it is unsurprising that perturbation of the microtubule cytoskeleton prevents dramatic cellular morphology changes initiated by HGF stimulation.

The third group of small molecules identified in the HGF screen includes those with reported mechanisms of biological activity against specific target proteins, thus revealing the most about molecular events triggered during HGF-induced scattering. One compound affects protein folding by targeting HSP90 (Foley and Ying, 2008) (Figure 9E). Inhibition of protein folding could decrease the levels of components required for HGF signaling, perhaps most importantly the c-Met receptor (Webb et al., 2000). Since drug treatment immediately preceded HGF stimulation, it is unlikely that HSP90 client protein levels would be reduced except in cases of exceptionally high turnover. However, HGF stimulation does require a downstream increase in c-Met expression (Boccaccio et al., 1994). It is our hypothesis that HSP90 inhibitors prevent cells from responding to HGF by reducing the production of available functional c-Met receptors following initial receptor stimulation. A second compound belongs to a class of small molecules that inhibit function of αVβ3integrins (Dayam et al., 2006) (Figure 9F). During EMT, cells alter integrin expression and localization in a manner that is thought to enhance cell migration and reduce cell-cell adhesion (Clark, 1994b). Both of these inhibitors affect targets with obvious connections to HGF signaling. More surprising was the identification of N-allyl-4-(4-morpholinylcarbonyl)benzenesulfonamide, a known neuronal calcium channel blocker (Milutinovic et al., 1999), as an inhibitor of HGF-induced scattering (Figure 9G).

Chapter 3: The Behavior and Control of Calcium Ion Signaling in Hgf-Induced Cell Scattering

HGF stimulation activates microtubule-dependent calcium influxes at the plasma membrane

Since a neuronal calcium channel blocker inhibits HGF-induced cell scattering, we sought to confirm the role of calcium currents in HGF-induced scattering by assessing the effect of two generic calcium channel blockers, econazole and ruthenium red, in our original screening

assay. Both significantly reduce scattering (Figure 10A), confirming that calcium influxes are required for HGF-induced scattering. We then sought to determine whether calcium influxes are required for HGF-induced increases in cell migration and proliferation. Remarkably, only econazole reduced lateral migration and proliferation of HGF-stimulated cells (Figure 10B).

In order to measure changes in calcium influxes following HGF stimulation, we next employed electrophysiological methods to record calcium currents at the plasma membranes of MDCK cells following HGF treatment. Calcium influxes were recorded for 10 minutes prior to HGF addition, revealing a low frequency of channel opening events. These calcium influxes occur as high or low conductance events, allowing us to resolve at least two distinct channels in the plasma membrane of MDCK cells. Though no change in channel opening frequency is observed immediately after HGF treatment, transient and significant increases in channel opening frequency are observed 8.2 (±2.7 = 95% C.I., n=5) and 30.4 (±3.5 = 95% C.I., n=5) minutes after administration of HGF (Figure 10C-F). The first increase is both smaller, at 16 (±9.40 = 95% C.I., n=5) fold above baseline, and shorter in duration, at 2.0 (±0.800 = 95% C.I., n=4) minutes, than the latter increase, which reaches 37 (±26.7 = 95% C.I. n=5) fold above baseline and lasts 7.5 (±3.35 = 95% C.I., n=4) minutes. While the size and duration of these surges are highly variable from experiment to experiment, without exception, the second surge is always higher (2.5 (±0.942 = 95% C.I., n=5) fold above the first peak) and longer in duration (3.7 (±0.611 = 95% C.I. n=4) minutes) than the first. Channel opening frequency returns to near baseline levels between high frequency influx events. Though there was some variability in the amount of time between HGF treatment and the appearance of the first pulse of high frequency calcium influxes (Figure 10C), the amount of time between pulses (23.6 (±3.1 = 95% C.I., n=5) min) was highly reproducible from experiment to experiment (Figure 10D). Channel

conductance and current durations are not significantly affected by HGF treatment, even during high frequency influx events (Figure 10F). This demonstrates that HGF stimulates increased calcium influxes through multiple channel types. Importantly, this result was obtained whether recording were made with cell-attached patches or with whole-cell clamps.

Since HGF alters the frequency of calcium influxes, but not the magnitude or duration of their conductivity, we reasoned that cells are regulating the number of channels, rather than the properties of channels already in the membrane. We propose that the dramatic increase in calcium influx observed following HGF stimulation are a result of mobilization of calcium channels maintained in an internal vesicle population. The requirement of the microtubule cytoskeleton for HGF-induced cell scattering supports this, since previously published data show that Trp channels can be activated via microtubule-dependent vesicle transport (Kennedy et al., 2010). We therefore sought to determine whether perturbation of the microtubule cytoskeleton prevents transient pulses of high frequency calcium influxes in response to HGF stimulation when MDCK cells were treated with a commercial microtubule polymerization inhibitor and subjected to electrophysiological recording, effectively preventing vesicular transport of new ion channels, but not disturbing those already in the membrane. Prior to and immediately after treatment with HGF, cells exhibit a low frequency of calcium influxes through low- and high-conductance channels, as before (Figure 11A). Following HGF stimulation, no increase in frequency of calcium influxes is observed, even after 30 minutes (Figure 11B) and for up to 50 minutes of recording (a 42-minute average of two assays is shown in Figure 11C). Importantly, microtubule polymerization inhibitor did not alter channel conductivity or current duration (data not shown). This result clearly shows that an intact microtubule cytoskeleton is required for HGF-induced pulses of high frequency calcium influxes at the plasma membrane.

We then used electrophysiological recording to examine the effect of N-allyl-4-(4-morpholinylcarbonyl)benzenesulfonamide (NA(4MC)BS) on calcium influxes. MDCK cells were treated with HGF and NA(4MC)BS was applied during the second, longer high-frequency calcium influx event. Within two minutes of neuronal calcium channel blocker addition, conductance through a subset of high-conductance channels began to diminish; after an additional 2 minutes, conductance through these channels was nearly undetectable above background noise (Figure 12A). The low conductance channel was resistant to the calcium channel blocker, as were the remaining high-conductance channels. An analysis of high-conductance channel properties reveals that channels that are sensitive to the calcium channel blocker support a slightly higher current than resistant channels (Figure 12B-C). Taken together, these data show that MDCK cells express at least three calcium channels at the plasma membrane, including a low conductance channel and two high-conductance channels. Though HGF stimulation results in transient pulses of high frequency influxes through all three channels types, the neuronal calcium channel blocker identified as an inhibitor of HGF-induced scattering is highly selective for a single high-conductance channel.

Identification of calcium channels required for HGF-induced scattering

Our next goal was to identify the specific channel required for HGF-induced scattering. Our first step was to establish an expression profile for known calcium channels in MDCK cells by reverse-transcriptase PCR. Since c-Met activation triggers dramatic changes in gene expression in MDCK cells (Balkovetz et al., 2004), calcium channel expression was determined before and after HGF treatment. We found detectable levels of expression of a number of Trp calcium channels, including TrpC 1, 4, 6, and 7, TrpV 1-4, TrpM 3-8, and PKD 1-2 (Figure 13A). Importantly, failure to generate a PCR product may indicate a failed reaction, rather than that this channel is not expressed. Several channels showing no expression were tested with

additional primer pairs to confirm negative results. RT-PCR of several channels yielded more or less product in samples derived from HGF-treated MDCK cells, indicating that HGF stimulation could affect expression patterns of calcium channels in MDCK cells. Further, RT-PCR of TrpM3 before and after HGF stimulation yields different sized products that correspond to different splice variants of this transcript, suggesting that HGF stimulation might also alter splicing of calcium channel mRNA.

In order to quickly determine the identity of calcium channels in the plasma membrane, we examined the differential effects of well-characterized calcium channel agonists and antagonists on channel conductivity in MDCK cells. Initial experiments were performed using the trivalent cation La^{3+}, which inhibits a subset of Trp channels with IC_{50} profiles that can vary more than 200-fold (Beech et al., 2003; Campo et al., 2003; Clapham, 2009; Leffler et al., 2007). MDCK cells were subjected to whole-cell patch clamping and calcium influxes recorded for several minutes in the absence of lanthanum ions and then for several minutes in stepwise concentration increases in lanthanum ions (Figure 13B). Analysis of calcium channel currents reveals sudden drops in average conductance at two points, demonstrating that both high-conductance channels are sensitive to lanthanum ions. Before La^{3+} treatment, channels with a conductance greater than 2pA represent over 50% of all channel events, at 1.7mM La^{3+}, these large channels represent less than 3% of detectable channel events. One high-conductance channel is highly sensitive to lanthanum ions, as a subset of high-conductance channel current is blocked when the La^{3+} concentration is raised from 0 to 50μM. The other high-conductance channel is less sensitive, as it is blocked only when the concentration is raised to 500μM. Importantly, the low conductance channel is not affected by lanthanum ions, as low conductance calcium currents are observed even at the highest lanthanum ion concentration tested. Only

TrpC1 and TrpC6 are detected by RT-PCR in MDCK cells and have a La^{3+} sensitivity at or below 50μM, while only TrpV4 has a reported La^{3+} sensitivity near 500μM (Beech et al., 2003; Campo et al., 2003; Clapham, 2009; Leffler et al., 2007). The few lanthanum ion-insensitive high-conductance influxes may be accounted for by TrpM6 channels, which have no published sensitivity to La^{3+}, have a high conductance, and are known to be expressed at the MDCK plasma membrane (Thebault et al., 2009; Topala et al., 2007).

TrpV4-dependent calcium influxes are activated by HGF stimulation of HepG2 cells (Vriens et al., 2004), making it a potential candidate for the NA(4MC)BS-sensitive channel in MDCK cells. Similarly, TrpC6 has been implicated in HGF-induced proliferation in prostate cancer cell lines (Wang et al., 2010b). We next sought to identify whether either channel was the molecular target of NA(4MC)BS. In order to do this, we measured the effect of NA(4MC)BS in MDCK cells recorded in the presence of 10μM La^{3+} or 5μM Gd^{3+}, concentrations sufficient to inhibit TrpC6, but not TrpV4 (Beech et al., 2003; Clapham, 2009). Thus, high-conductance calcium influxes observed under these conditions are accounted for by TrpV4; NA(4MC)BS sensitivity would indicate that TrpV4 is blocked by this drug, while insensitivity would indicate that TrpV4 is resistant to NA(4MC)BS. We recorded baseline channel activity for 5-10 minutes, then added 10μM NA(4MC)BS and continued recording for 10-25 minutes. In five experiments, NA(4MC)BS had little effect on total calcium current compared to MDCK cells recorded without trivalent ion treatment. The average channel current is reduced by only 0.21 (±0.32 = 95% C.I., n=5) pA in the presence of low lanthanum ion concentrations or 0.14 (±0.62 = 95% C.I., n=5) pA in the presence of low gadolinium ion concentrations, compared to 1.49 (±0.92 = 95% C.I., n=5) pA for untreated MDCK cells (Figure 13C). The channel blocked by NA(4MC)BS is clearly sensitive to low concentrations of lanthanum and gadolinium ions,

suggesting that the molecular target of NA(4MC)BS is TrpC6, and not TrpV4. Thus, since NA(4MC)BS blocks HGF-induced scattering in our original screening assay, we conclude that TrpC6 is specifically required for HGF-induced scattering.

To further confirm that TrpV4 and TrpC6 are present in MDCK cell membranes, we measured the effect of channel-specific agonists on calcium currents by electrophysiological recording. Two minutes after the TrpV4-specific agonist 4αPDD (4α-phorbol 12,13-didecanoate) is added to MDCK cells, high-conductance channel opening frequency increases substantially. Analysis revealed that both channel frequency and average channel conductance increases significantly (Figure 13D-E), which is characteristic of the TrpV4 response to 4αPDD (Benfenati et al., 2007). When MDCK cells are treated with the TrpC6-specific agonist hyperforin, the channel opening rate rises from a few events per minute to a nearly uninterrupted stream of channel openings (Figure 13F-G). This confirms the presence of both TrpV4 and TrpC6 at the MDCK plasma membrane.

Chapter 4: The Effect of Calcium Signaling on NFAT Function

HGF stimulation induces NFAT activation through TrpC6-mediated Ca^{2+} influxes

Calcium influxes have been shown to activate NFAT-dependent gene transcription. We reasoned that HGF-induced increases in calcium influxes might increase NFAT-mediated gene transcription and, further, that NFAT is required for HGF-induced EMT. We first tested this by measuring the effect of a commercial inhibitor of NFAT on HGF-induced scattering in our original screening assay. This inhibitor prevents cell scattering following HGF stimulation (reduced to 46.4% (±30.4% = 95% C.I., n=10) of normal level, where 0%=the average untreated control value and 100%=the average HGF-treated control value), demonstrating a role for NFAT. We then sought to determine whether HGF stimulation increases NFAT-dependent gene transcription. We generated a stable MDCK cell line bearing a luciferase-based NFAT reporter

construct and measured the effect of HGF stimulation on luciferase expression. Luciferase activity is transiently and significantly increased 40 minutes after HGF stimulation (Figure 14A), a timing that precisely follows the pulse in high frequency calcium influxes observed 25-30 minutes following HGF stimulation. This suggests a strong connection between calcium influxes and NFAT mediated gene transcription. We sought to further solidify this connection by determining the role of TrpC6 in NFAT activation. We tested whether NA(4MC)BS prevents HGF-induced increases in NFAT-dependent gene transcription. MDCK cells bearing the NFAT reporter system were treated with or without NA(4MC)BS, stimulated with HGF for 40 minutes, and used to generate extracts in which luciferase activity was measured. Luciferase activity in HGF-stimulated cells treated with NA(4MC)BS is reduced compared to untreated control cells, reaching levels comparable to cells that had not received HGF stimulation (Figure 14B). This shows that TrpC6-mediated calcium influxes are required for NFAT-mediated transcription after HGF stimulation, even though calcium influxes through other channels are still increased.

We also examined the effect of HGF stimulation on the nuclear localization of NFAT proteins. In order to first determine the identity of the NFAT protein expressed in MDCK cells, we conducted a series of RT-PCR screens for each of the NFAT subtypes. These experiments revealed that MDCK cells express the c1 and c4 isoforms of NFAT and that HGF stimulation does not significantly affect the expression of these proteins (Figure 14C). Immunolocalization of NFATc4 in MDCK cells treated with HGF reveals that nuclear accumulation of NFATc4 increases significantly 40 minutes after HGF treatment, but this accumulation is blocked by the presence of 5μM Gd^{3+} and 10 μM NA(4MC)BS (Figure 14D-G). This further demonstrates the importance of transient increases in calcium influxes through TrpC6 in NFAT activation. Unlike NFATc4, NFATc1 did not show any significant changes in localization following HGF

stimulation, though this could be a result of antibody incompatibility with canine NFATc1 protein (data not shown).

Chapter 5: The Role of Wnk Kinase in Hgf Signal Transduction

In our initial drug screen, 8-methoxy-2-[2-(3,4,5-trimethoxyphenyl)vinyl]quinolone (8M2[2(345TMP)V]Q), one of the inhibitors that also tested highly effective at preventing HGF-induced cell scattering proved upon closer examination to inhibit WNK2 and WNK3 from the WNK kinase subfamily (Figure 15A-B). It is unknown whether the drug also inhibits WNK1 and WNK4, as it was not tested for inhibition of these proteins. As demonstrated above, HGF signaling relies on very precise Ca^{2+} signaling patterns. Based on this information and on published roles of WNK kinases as mediators of Ca^{2+} influx, we suspected that a member of the WNK subfamily is involved in HGF signaling either in a permissive or an active role, and that it functions by mediating Ca^{2+} signaling.

Inhibition of WNK kinase increases Ca^{2+} influx through the cell membrane

Based on our previous observation of Ca^{2+} signaling at the membrane and the known effect of WNK kinases as regulators of ion signaling (Leng et al., 2006; Moriguchi et al., 2005; Rinehart et al., 2011), we hypothesized that an essential protein for HGF signaling that functions by regulating Ca^{2+} channel function would likely exert a visible effect on Ca^{2+} conductance through the plasma membrane.

In order to test whether calcium signaling at the plasma membrane is mediated by WNK kinase in MDCK cells, we established a series of cell-attached patches on MDCK cells and recorded the basal level of Ca^{2+} channel opening for five minutes each time. Once we had established a baseline, we added our WNK kinase blocker and continued recording a further fifteen minutes to determine its effect on the number, size, and frequency of membrane-bound channel openings.

Repeated assays consistently show that our WNK kinase inhibitor is capable of significantly increasing the frequency of Ca^{2+} channel openings (Figure 16A-B). This is consistent with published data indicating an inhibitory role for WNK1 and WNK4 on TrpV4 and TrpC3 (Fu et al., 2006; Park et al., 2011). Of these, TrpV4 is of particular interest, as MDCK cells express TrpV4 channels in high numbers at the plasma membrane. This suggests that dysregulation of WNK1 or WNK4 could potentially account for the increase in channel opening frequency observed when the inhibitor is added.

Expression patterns of WNK kinase subfamily members in MDCK

Once we had determined the role of WNK kinase in Ca^{2+} channel regulation, we used reverse-transcriptase PCR to determine the expression patterns of each member of the WNK subfamily, including kidney-specific WNK1 and full-length WNK1, which have distinct regulatory properties (Wade et al., 2006), as well as WNK2, WNK3 and WNK4. We tested transcription both in cells treated with HGF for 16 hours and in untreated cells. The results show clear transcription of full length WNK1 and WNK3. WNK2 was not detected for either treatment in either of two PCR assays though it is possible that this denotes a failed experiment rather than the absence of WNK2 mRNA, however, our results agree with previous evidence that WNK2 is not expressed in the kidney (Rinehart et al., 2011) (Figure 17A). There is no observable difference in expression between untreated and HGF-treated cells for any of the members of the WNK subfamily.

Chapter 6: Discussion

HGF signaling plays a critical role in development and in cancer progression, but remains poorly characterized at the molecular level. Application of small molecule screening approaches to dissection of complex signaling networks allows identification of novel signaling components and helps elucidate their role in signaling. Here we show that development of a simple cell-based

assay with a moderate z' score can be successfully used to identify small molecules that target molecular components required for signaling, in this case HGF signaling that drives cell scattering. Our small molecule screening approach specifically implicates microtubule dynamics and calcium signaling in this signaling. We report the occurrence of bursts of high frequency calcium influxes following induction of HGF signaling and demonstrate that such bursts are microtubule dependent, occur through multiple calcium channels, require TrpC6 channels specifically, and affect NFAT-mediated gene transcription in a TrpC6-dependent manner. Finally, we show that an inhibitor of WNK kinase is capable of significantly reducing cell scattering in response to HGF signaling.

Identification of a neuronal calcium channel blocker in our original screen strongly suggests that calcium signaling is required for HGF-induced EMT. Increased calcium signaling downstream of HGF has been observed in a number of systems and occurs through a variety of calcium channels, including TrpC6 (Jin et al., 2002; Wang et al., 2010b; Waning et al., 2007; Wondergem et al., 2008). In HepG2 cells treated with HGF, increased cytosolic calcium results from influxes from the plasma membrane and from intracellular stores, both through TrpV1 and TrpV4 channels (Vriens et al., 2004; Wang et al., 2010b). In glioblastoma, it is TrpM8 that increases cytosolic calcium concentration in response to HGF treatment (Wondergem et al., 2008). In HK2 cells, a renal tubular epithelial cell line, TrpC6 is required for HGF-induced proliferation, migration, and actin rearrangements (Rampino et al., 2007). Further, exogenous overexpression of the calcium channel polycystin-1 in MDCK cells drives scattering (Boca et al., 2007), suggesting that calcium influxes play a role in EMT. Our results confirm that calcium influxes at the plasma membrane occur through multiple channels, including TrpV4 and TrpC6 in MDCK cells. Interestingly, HGF stimulation results in highly reproducible periodic increases

in high frequency calcium influxes. Several lines of evidence suggest that calcium signaling downstream of HGF stimulation occurs as a result of vesicle deposition in the plasma membrane. First, calcium signaling occurs as a result of changes in opening frequency, rather than increases in the duration or magnitude of current. This suggests that the number of channels in the plasma membrane increases, rather than that existing channels alter their conductance properties. Second, high frequency influx events occur through all channel subtypes in the membranes of MDCK cells, consistent with the observation that HGF signaling has been shown to trigger ion channel fluxes generally, including through TrpC6, and a number of ion channel blockers have been shown to block HGF signaling (Jin et al., 2002; Rampino et al., 2007; Wang et al., 2010b). This indicates that regulation of calcium channels is non-specific, as would be expected in the case of vesicle delivery. Third is the observation that microtubule polymerization inhibitors prevent pulses of high frequency calcium influxes in response to HGF. This suggests that microtubule-based vesicle mobilization is required for observed pulses of increased calcium influxes. How HGF might alter vesicle trafficking is unclear, as is how oscillations in frequency of calcium influxes are generated by vesicle trafficking. An alternative hypothesis is that calcium influxes are driven by cell morphology changes that occur in pulses during early EMT. Trp channels are stretch-gated and it is likely that activation of such channels in response to physical forces would impact multiple channel types, though we would expect changes in conductance properties if such was the case.

While an important role for calcium in HGF signaling has been emerging in the literature, less clear has been whether individual channel types make specific, non-redundant contributions to signaling. Our results highlight that TrpC6, among several channels expressed at the plasma membrane, plays a non-redundant and specific role in HGF signaling. In MDCK cells, selective

blockade of TrpC6 prevents EMT-like cell scattering and downstream activation of NFAT-dependent gene transcription, even though calcium influxes through other channels still occur. Importantly, that TrpC6 is required for HGF-induced scattering does not preclude other channels from also being required for EMT, though perhaps in different branches of the HGF signaling network. Though there is evidence that ion channels play a general role in cellular processes downstream of HGF (Jin et al., 2002; Rampino et al., 2007), we propose that calcium influxes through specific channels are connected to specific downstream signaling events. Activation of multiple calcium channels, each tied to distinct downstream signaling events, could facilitate selective and independent activation of multiple downstream effectors. Previously published evidence supports this ideas, as it links TrpV6- to Ca^{2+} signaling and NFAT activation that are required for cell proliferation in prostate cancer (Lehen'Kyi et al., 2007). This idea is further supported by the observation that econazole and ruthenium red have unique effects on HGF signaling; though both reduce HGF-induced cell scattering, their effects on HGF-induced proliferation and migration are distinct. This is likely a result of differential sensitivity of calcium channels to these agents and their coupling with distinct downstream effectors.

A key question is how calcium influxes alter cellular behavior that results in EMT or other responses to HGF signaling. Our identification of NFAT-dependent gene transcription as being required for cell scattering shows that calcium can operate by changing gene expression profiles in HGF-stimulated cells. There is also published evidence that calcium-dependent kinases mediate effects of HGF signaling (Tyndall et al., 2007) . Calcium influxes also impact conductance through other channel types, such as calcium-dependent potassium channels, which have been implicated in mediating HGF signaling, particularly as it affects cell migration (Liu et al., 1998; Rampino et al., 2007). Interestingly, calcium-dependent potassium currents occur with

periodic oscillations that begin minutes after HGF stimulation of SC-M1 cells (Liu et al., 1998). The cell morphology changes of HGF-induced EMT are associated with dramatic rearrangements of the actin cytoskeleton, and it is interesting that many actin regulatory systems are impacted by calcium, either directly or indirectly.

Our results implicating WNK kinase in HGF signaling via modulation of ion transport join together two bodies of evidence: first that members of the WNK kinase subfamily frequently act via regulation of cation transport (Leng et al., 2006; Moriguchi et al., 2005; Rinehart et al., 2011; Wade et al., 2006) and second that mutations in these same proteins are implicated in cancer progression (Greenman et al., 2007). We link these by showing that dysregulation of WNK kinases is sufficient to prevent effective HGF signaling in MDCK. However, it is important to note here that the ability of WNK kinase inhibitors to prevent c-Met-induced EMT does not necessarily mean that c-Met modulates WNK kinase in any way during signaling, only that properly functioning WNK kinase is essential for signal transduction. It is quite possible that this permissive nature of WNK kinase is simply because, in its absence, ion signaling becomes severely dysregulated, and c-Met signaling, which relies on precise Ca^{2+} pulses, is unable to properly regulate these pulses without the normal inhibitory function of WNK kinase.

Our library screen highlights the central role of calcium in HGF signaling and provides molecular details into how calcium influxes are controlled and how calcium influxes exert downstream effects, thus providing a picture of an important biological circuit within the HGF signaling network. Results presented here highlight how an unbiased small molecule screening approach can be applied to dissect complex signaling networks.

Chapter 7: Materials and Methods

Cell culture and assays

MDCK II cells were cultured in DMEM + 10% FBS at 37°C in 5% CO_2. HGF-

conditioned media was generated by MRC-5 cells cultured in DMEM + 10% FBS. Transwell migration assays were performed in Corning plates with an 8μm pore size (Corning). Cells were plated at 75,000 per well on the top of the filter and incubated for 20-24 hours. Where applicable, HGF-conditioned media was added to the lower compartment at the same time as the experimental compound. Cells were incubated a further 20-24 hours and then rinsed with ice-cold PBS, fixed on ice for 15 minutes with 4% paraformaldehyde, and stained with crystal violet. Cells that had not penetrated the filter were swabbed from the top of the filter and plates were scanned for color intensity by a FluorChem photometer (Cell Biosciences). Migration assays were performed using Oris Cell Migration Assays (Platypus Technologies). 75,000 cells were seeded into each well, and plates were incubated 24 hours. Treatments were added to each well and the stoppers were removed. After 18 hours of further incubation, cells were rinsed with ice-cold PBS, fixed on ice for 15 minutes with 4% PFA, and stained with crystal violet. Each well was photographed and cell migration was analyzed using SlideBook software. For proliferation assays, 10,000 cells were seeded into each well of a standard flat-bottomed 96-well tissue culture plate and incubated for 24 hours. Treatments were added and cells were incubated a further 12, 24, 36, or 48 hours before being fixed with 4% PFA and stained with crystal violet. Cell density was then determined using a FluorChem photometer. Unclassified compounds were obtained from ChemBridge.

Gene reporter assay

MDCK cells were infected with a viral vector containing an NFAT Reporter (SABiosciences). Cells were selected with G418 for 14 days to generate a stable polyclonal cell line. Cells from this line were plated at a density of 250,000 cells/well on 24-well plates. Cells were incubated for about 48 hours. The cell media was then treated with 20% HGF-conditioned media with or without drug NA(4MC)BS or left untreated as a control. Cells were harvested

using the Promega luciferase assay kit according to the product instructions. Luciferase intensity was determined with a TD-20/20 luminometer (Turner Designs).

Patch clamping

Before patching, cells were rinsed twice in room temperature Ringer's solution. Bath solution: ddH_2O with 129mM NaCl, 11mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 10mM Glucose, brought to a pH of 7.4 with HCl or NaOH. Electrode solution: ddH_2O with 137mM potassium gluconate, 9mM KCl, 1mM $MgCl_2$, 0.1mM EGTA, 10mM HEPES, brought to a pH of 7.4 with HCl or NaOH. $LaCl_3$ and $Gd_2(SO_4)_3$ were dissolved in ddH_2O and other drugs were dissolved in DMSO. For NA(4MC)BS+La^{3+} or Gd^{3+}, $LaCl_3$ or $Gd_2(SO_4)_3$ was added to the electrode solution used for patching. All cell-attached patching was performed at -80mV holding potential and whole-cell patching was performed at -40mV holding potential. All patch clamping was done using an Axopatch 200A amplifier (Molecular Devices), and electrodes were pulled from thin-wall borosilicate glass (GC150TF-10, OD = 1.5 mm with a filament, Warner Instruments).

Reverse-transcriptase PCR

MDCK II cells were cultured as indicated above and were either left untreated or cultured in the presence of 5% HGF-conditioned media for 16 hours. mRNA was harvested and reverse transcribed using the ImProm-IITM Reverse Transcription System (Promega) according to the manufacturer's instructions. Primers are listed in Supplemental Tables 1 and 2.

Immunofluorescence

MDCK cells were cultured on collagen-coated glass coverslips overnight. 10% HGF-conditioned media was then added to each well at 10-minute increments for 70 minutes with an additional well treated with $Gd_2(SO_4)_3$ and 10% HGF simultaneously and allowed to respond for 40 minutes, rinsed in PBS, and then fixed for 15 minutes on ice with 4% paraformaldehyde in

PBS. Cells were blocked in PBS supplemented with 0.2% bovine serum albumin, 50mM NH_4Cl, and 0.5% Triton X overnight. Cells were then stained with primary antibodies against NFATc1 and NFATc4 (Santa Cruz), followed by secondary antibody treatment with goat anti-rabbit or goat anti-mouse antibodies (Invitrogen) conjugated to Alexa-fluor 594 or 488. Coverslips were mounted in VectaShield (Vector Labs) and viewed under an Olympus BX41 phase contrast microscope with a 100x/1.30 oil objective lens. Images were acquired with a Hamamatsu Orca-ER digital camera and analyzed using SlideBook software.

Characterization of nuclear localization of NFATc4

NFATc4 was stained as described above, followed by staining with DAPI. SlideBook was then used to automatically define nuclei by a fixed intensity of DAPI stain. NFATc4 fluorescent intensity was measured throughout the image and within the area identified as the nucleus. Intensity within the nucleus was then divided by total intensity to determine relative percent localization of NFATc4 to the nucleus.

Statistical methods

Unless otherwise noted, all p-values were determined by two-tailed, unpaired Student's t-tests.

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Figure Legends

Figure 1. Induction of actin rearrangement.
Diagram outlining the major events required to induce actin rearrangement in response to HGF. (Al-Awar et al., 2000; Boshans et al., 2000; Bosse et al., 2007; Chen et al., 1998; Egan et al., 1993; Fan et al., 2001; Graziani et al., 1991; Graziani et al., 1993; Kawasaki et al., 2009; Kiyokawa et al., 1998; Li et al., 2009; Mood et al., 2006b; Radhakrishna et al., 1999; Schlaepfer et al., 1994; Stefan et al., 2001; Wada et al., 1998)

Figure 2. Induction of cell spreading.
Diagram outlining the major events required to induce cell spreading in response to HGF. (Cary et al., 2002; Chang et al., 2007; Song et al., 1998; Takino et al., 2005; Wang and Ingber, 1994)

Figure 3. Induction of cell detachment.
Diagram outlining the major events required to induce cell detachment in response to HGF. (Grotegut et al., 2006; Ilic et al., 1995; Lamorte et al., 2003; Maroun et al., 1999a; Maroun et al., 1999b; Schaeper et al., 2000; Schaeper et al., 2007; Stefan et al., 2001; Wang et al., 2009; Weidner et al., 1996; Yu et al., 2001)

Figure 4. Induction of cell migration.
Diagram outlining the major events required to induce cell migration in response to HGF. (Barberis et al., 2000; Carragher et al., 2003; Cuevas et al., 2003; Furuhjelm and Peranen, 2003; Ishibe et al., 2004; Kiyokawa et al., 1998; Klemke et al., 1997; Li et al., 1997; Sakkab et al., 2000; Sawhney et al., 2006; Schlaepfer et al., 1997; Schlaepfer et al., 1994; Schlaepfer and Hunter, 1997; Shen and Guan, 2001; Totsukawa et al., 2004; Westhoff et al., 2004)

Figure 5. Induction of cell invasion
Diagram outlining the major events required to induce cell invasion in response to HGF. (Furlan et al., 2007; Guo and Sharrocks, 2009)

Figure 6. Inhibition of apoptosis.
Diagram outlining the major events required inhibit apoptosis in response to HGF. (Kitta et al., 2003; Liu, 1999; Ma et al., 2005; Müller et al., 2002; Zhou et al., 2006)

Figure 7. Induction of proliferation.
Diagram outlining the major events required to induce cell proliferation in response to HGF. (Finco and Albert S. Baldwin, 1993; Garcia-Guzman et al., 1999; Kermorgant et al., 2004; Lamorte et al., 2000; Mood et al., 2006a; Ramos-Nino et al., 2008)

Figure 8. Design and application of an unbiased chemical screen against HGF-induced EMT.
A. Concentration-dependent effect of HGF in the screening assay. **B.** Effect of SU11274 on HGF-induced cell scattering in the screening assays. **C-E.** Frequency distribution of library and hit compounds by physicochemical properties.

Figure 9. Chemical structures of compounds identified in screening.
Chemical structures of compounds families with reported activity targeting microtubules (**A-D**), HSP90 (**E**), integrins (**F**), or neuronal calcium channels (**G**).

Figure 10. HGF-induced Ca^{2+} influxes are necessary for cell scattering.
A. Effect of econazole and ruthenium red on HGF-induced cell transwell migration. **B.** Effect of econazole and ruthenium red on migration and proliferation, normalized against the HGF-treated controls. **C-D.** Normalized channel opening frequencies of 5 independent experiments aligned by time following HGF stimulation (**C**) or by occurrence of an initial peak in channel opening frequency (**D**). Black circles represent values from each experiment. The gray line represents the average fold increase in channel opening frequency over all 5 trials. **E-F.** Electrophysiology recordings at -80mV beginning (**E**) 10sec and (**F**) 30min35sec after HGF stimulation.

Figure 11. HGF-induced Ca^{2+} surges require microtubules.
A-B. Cell-attached electrophysiology recordings at -80mV holding potential in the presence of

0.5μM tubulin polymerization inhibitor at (**A**) 10sec and (**B**) 30min35sec after HGF stimulation. **C.** Fold change in channel opening frequency after HGF stimulation of cells treated with 0.5μM tubulin polymerization inhibitor (n=2, gray line) or of untreated cells (black line).

Figure 12. NA(4MC)BS selectively inhibits a single high-conductance Ca^{2+} channel.
A. Cell-attached electrophysiology recording at -80mV beginning 45 seconds after 10μM NA(4MC)BS treatment. Some NA(4MC)BS-sensitive (arrows) and -insensitive (arrowheads) high-conductance currents are noted. **B.** Frequency distribution of individual channel conductances by current. Gray vertical lines approximate the conductance range of the two high-conductance channels. Note the reduction in frequency of channels with conductance in the upper range (arrow). **C.** Channel opening events beginning 45 seconds after addition of 10μM NA(4MC)BS. The graph is divided into four quadrants, with the upper and lower halves separating the two high-conductance channels shown in **B**, and the left and right halves representing channel conductance before and after onset of NA(4MC)BS, respectively. Percents represent the portion of total plotted channels located in each quadrant.

Figure 13. Effect of NA(4MC)BS on TrpC6 and TrpV4 channels.
A. RT-PCR amplification of Trp channel mRNA in untreated or HGF-treated MDCK cells. **B.** Average of individual channel currents of a whole-cell patched MDCK cell at a -40mV holding potential in the presence of increasing $[La^{3+}]_o$. **C.** Effect of NA(4MC)BS in the presence of 10μM La^{3+} and 5μM Gd^{3+}, as measured by average channel current. **D.** Electrophysiology recording beginning immediately after addition of 20μM 4αPDD. **E.** Channel currents following addition of 20μM 4αPDD. **F.** Electrophysiology recording beginning immediately after addition of 10μM hyperforin. **G.** Channel currents after addition of 10μM hyperforin. See also Table 1.

Figure 14. HGF-induced NFAT activity requires TrpC6.
A. Luciferase-based gene reporter assay for NFAT activity following administration HGF. **B.**

Effect of NA(4MC)BS on NFAT reporter gene transcription following HGF stimulation. **C.** RT-PCR amplification of NFAT mRNA in untreated or HGF-treated MDCK cells. **D-F.** NFATc4 localization in (**D**) untreated cells, (**E**) cells treated with HGF for 40 min, or (**F**) cells treated with HGF for 40 min in the presence of 5μM Gd^{3+}. Arrowheads indicate cells with strong nuclear localization of NFATc4. **G.** Average percent of GFP intensity localized to the nucleus, where GFP indicates localization of NFATc4; error bars = S.E. among multiple capture fields, n=5 or 6 for each condition. See also Table 2.

Figure 15. 8M2[2(345TMP)V]Q inhibits WNK2 and WNK3 kinase activity.
A. The chemical structure of 8-methoxy-2-[2-(3,4,5-trimethoxyphenyl)vinyl]quinolone (8M2[2(345TMP)V]Q). **B.** The ability of 8M2[2(345TMP)V]Q (B6000) to inhibit kinase activity. Compounds are listed by serial numbers assigned by our lab, and the effect of each drug on each kinase listed is indicated either by a green square to indicate a resultant increase in kinase activity or by a red square to indicate a resultant decrease in kinase activity.

Figure 16. 8M2[2(345TMP)V]Q induces an increased frequency of Ca^{2+} channel openings.
A-B. Electrophysiological readout of a cell-attached patch immediately prior to (**A)** and immediately following (**B**) addition of 10μM 8M2[2(345TMP)V]Q.

Figure 17. Expression patterns of members of the WNK kinase subfamily in MDCK.
A. Results of a reverse-transcriptase PCR series determining the expression levels of members of the WNK kinase subfamily in untreated cells or cells treated with 5% HGF for 16 hours. See also Table 3.

Table 1. Primers used for detection of Trp channels in MDCK.

List of primers used in reverse-transcriptase PCR experiments to detect the expression of Trp channels in MDCK. Forward and reverse primers are listed in order 5' to 3', and Length (bp) refers to the predicted length of the cDNA. In all cases, genomic DNA is significantly longer.

Table 2. Primers used for detection of NFAT in MDCK.
List of primers used in reverse-transcriptase PCR experiments to detect the expression of NFAT subtypes in MDCK. Forward and reverse primers are listed in order 5' to 3', and Length (bp) refers to the predicted length of the cDNA. In all cases, genomic DNA is significantly longer.

Table 3. Primers used for detection of WNK kinase in MDCK.
List of primers used in reverse-transcriptase PCR experiments to detect the expression of WNK kinases in MDCK. Forward and reverse primers are listed in order 5' to 3', and Length (bp) refers to the predicted length of the cDNA. In all cases, genomic DNA is significantly longer.

Figure 1: Induction of actin rearrangement

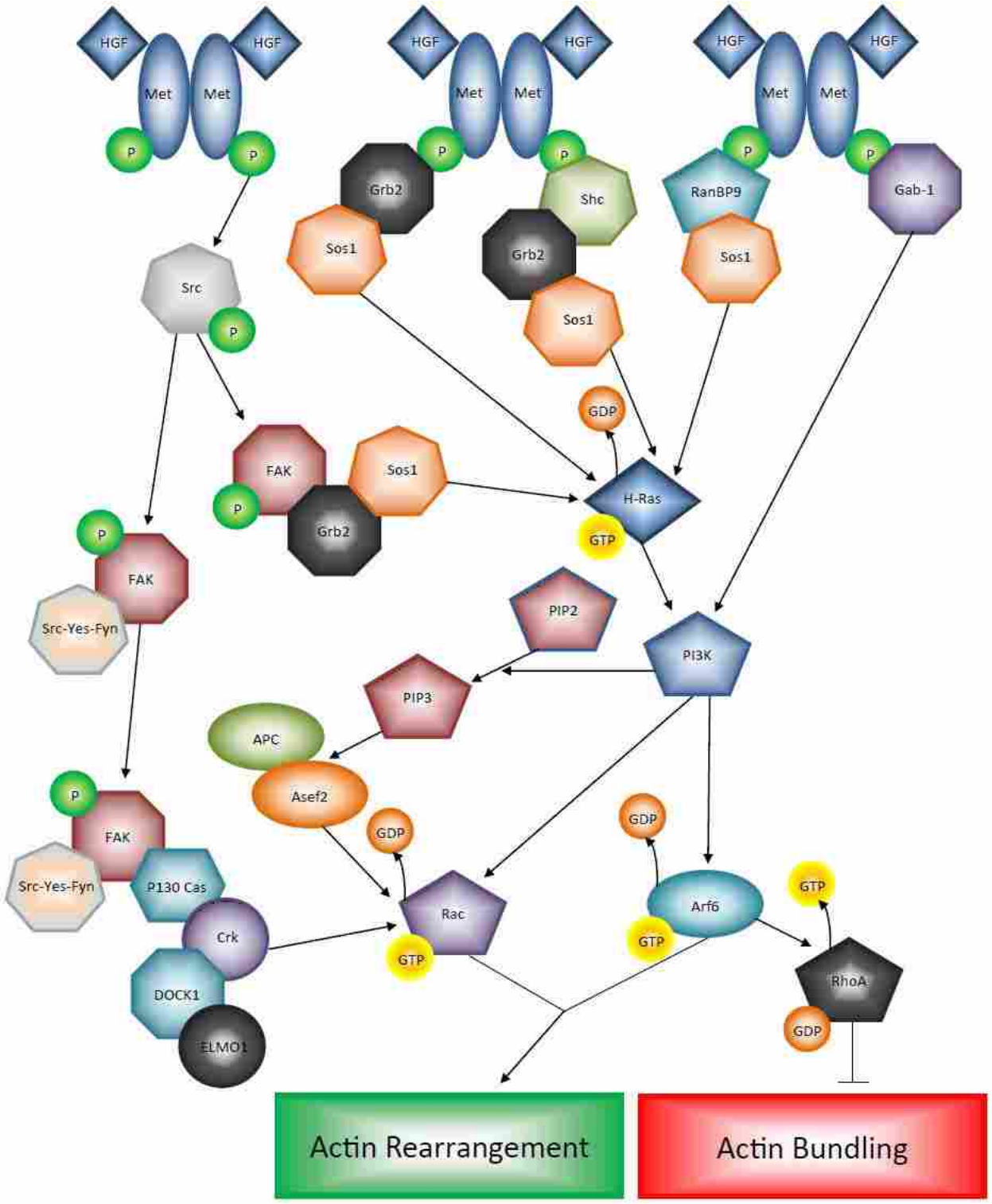

Figure 2: Induction of cell spreading

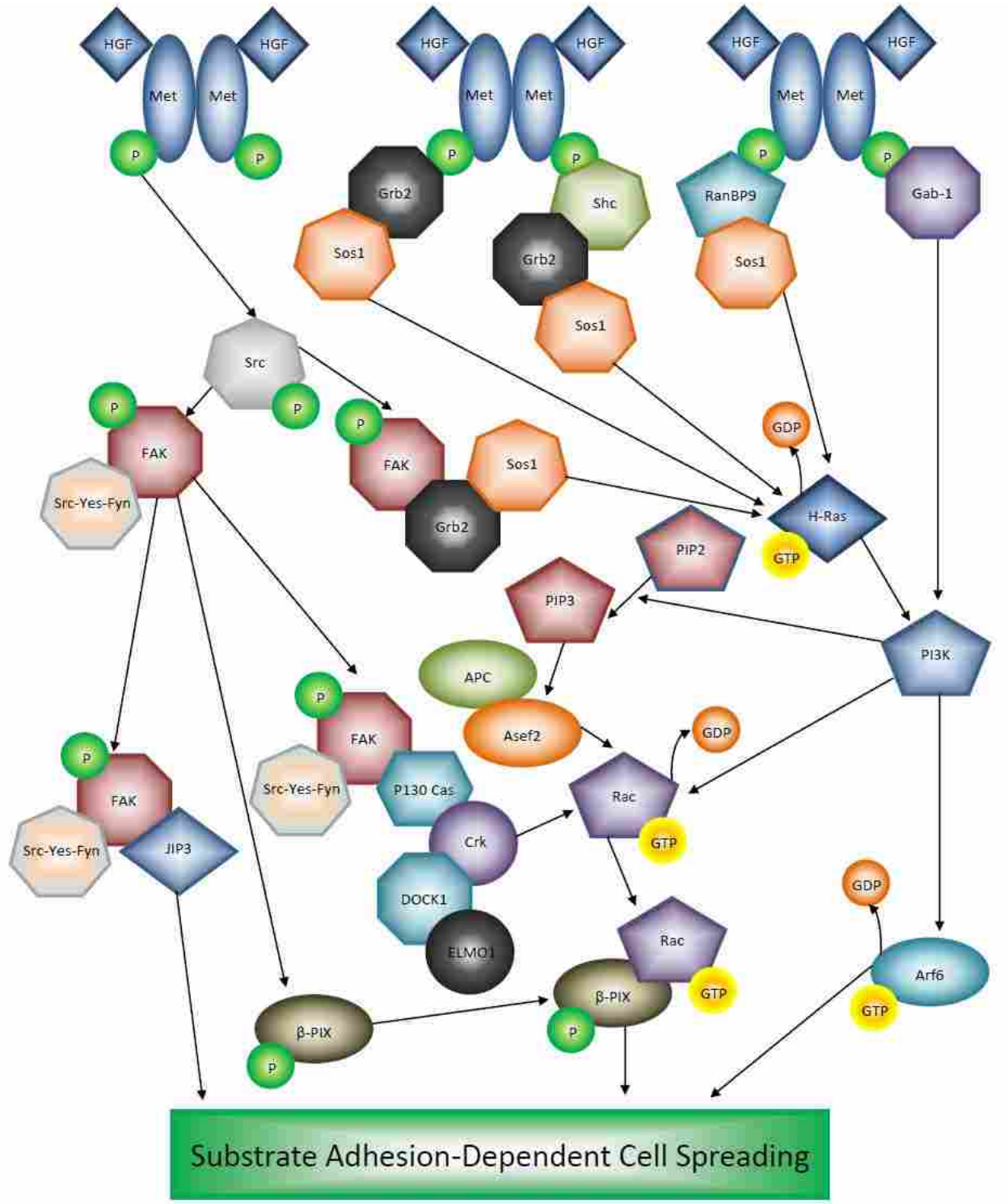

Figure 3: Induction of cell detachment

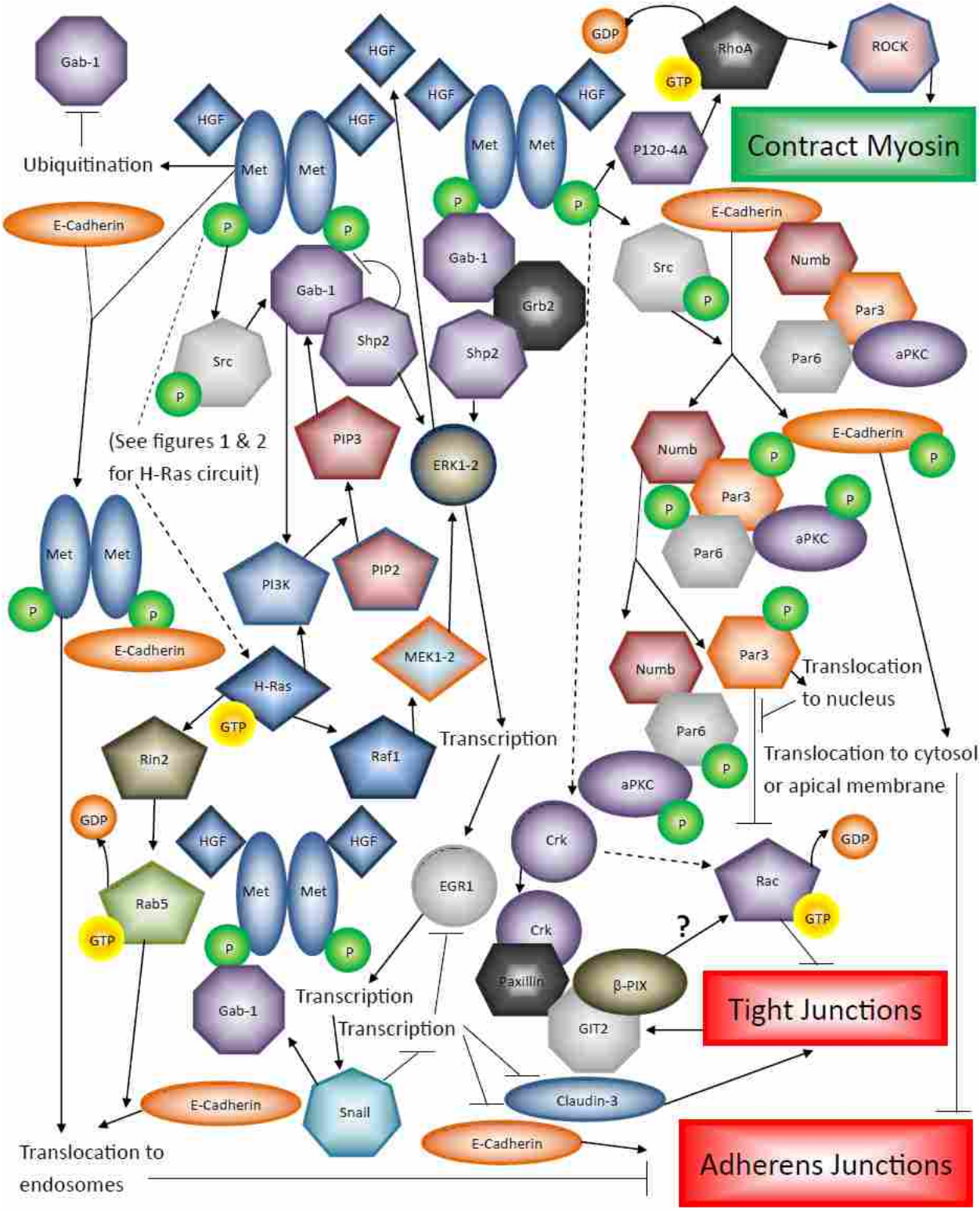

Figure 4: Induction of cell migration

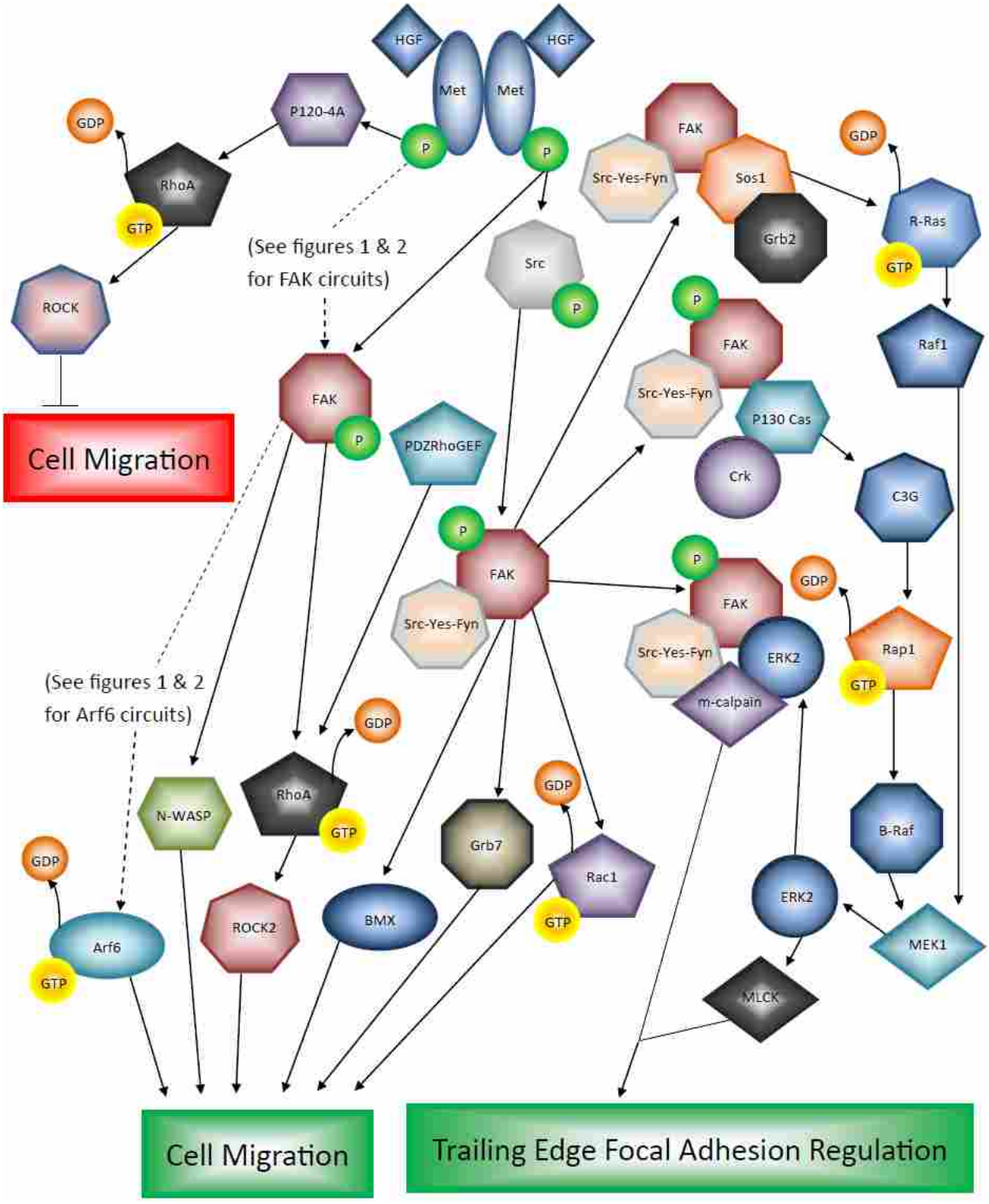

Figure 5: Induction of cell invasion

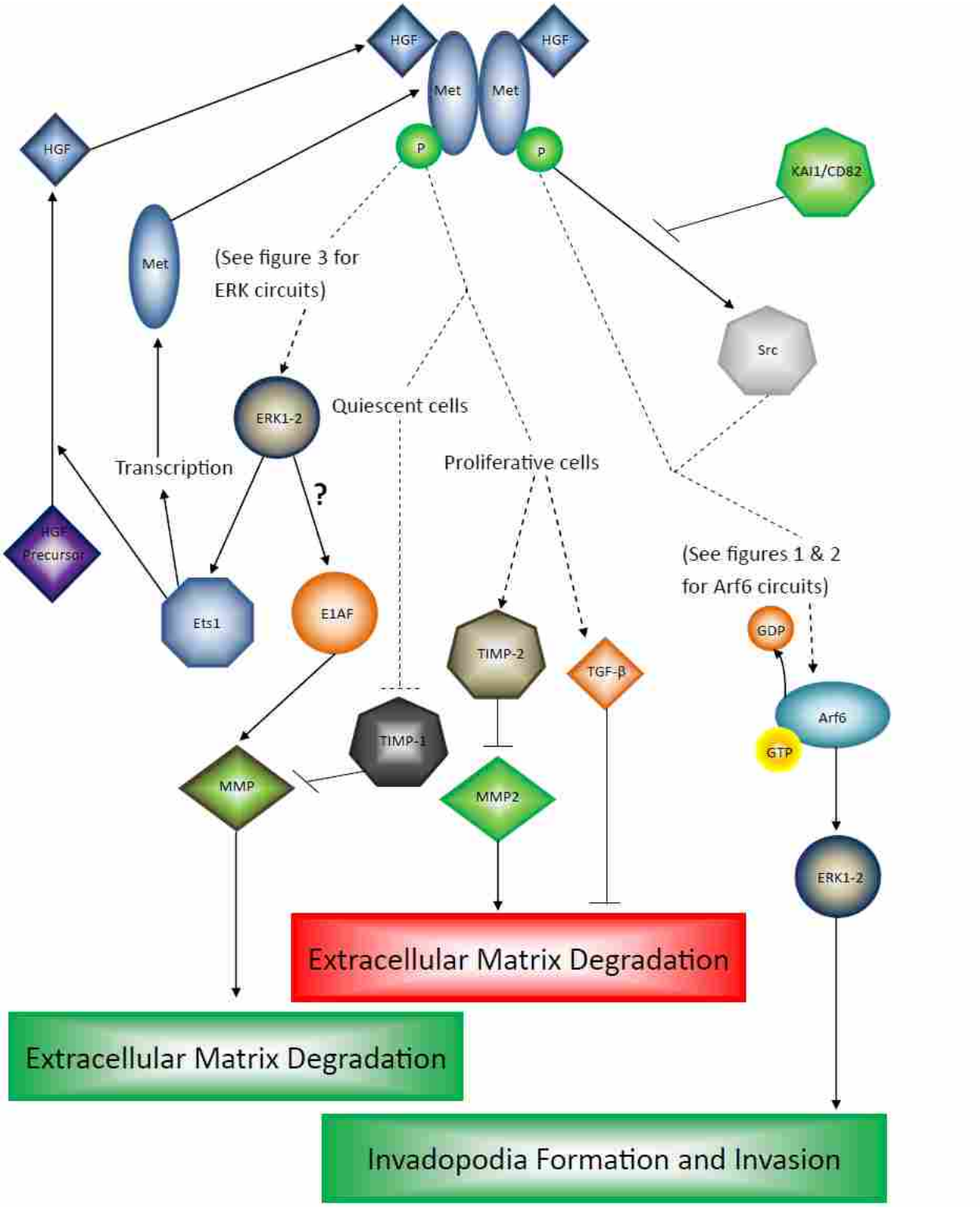

Figure 6: Inhibition of apoptosis

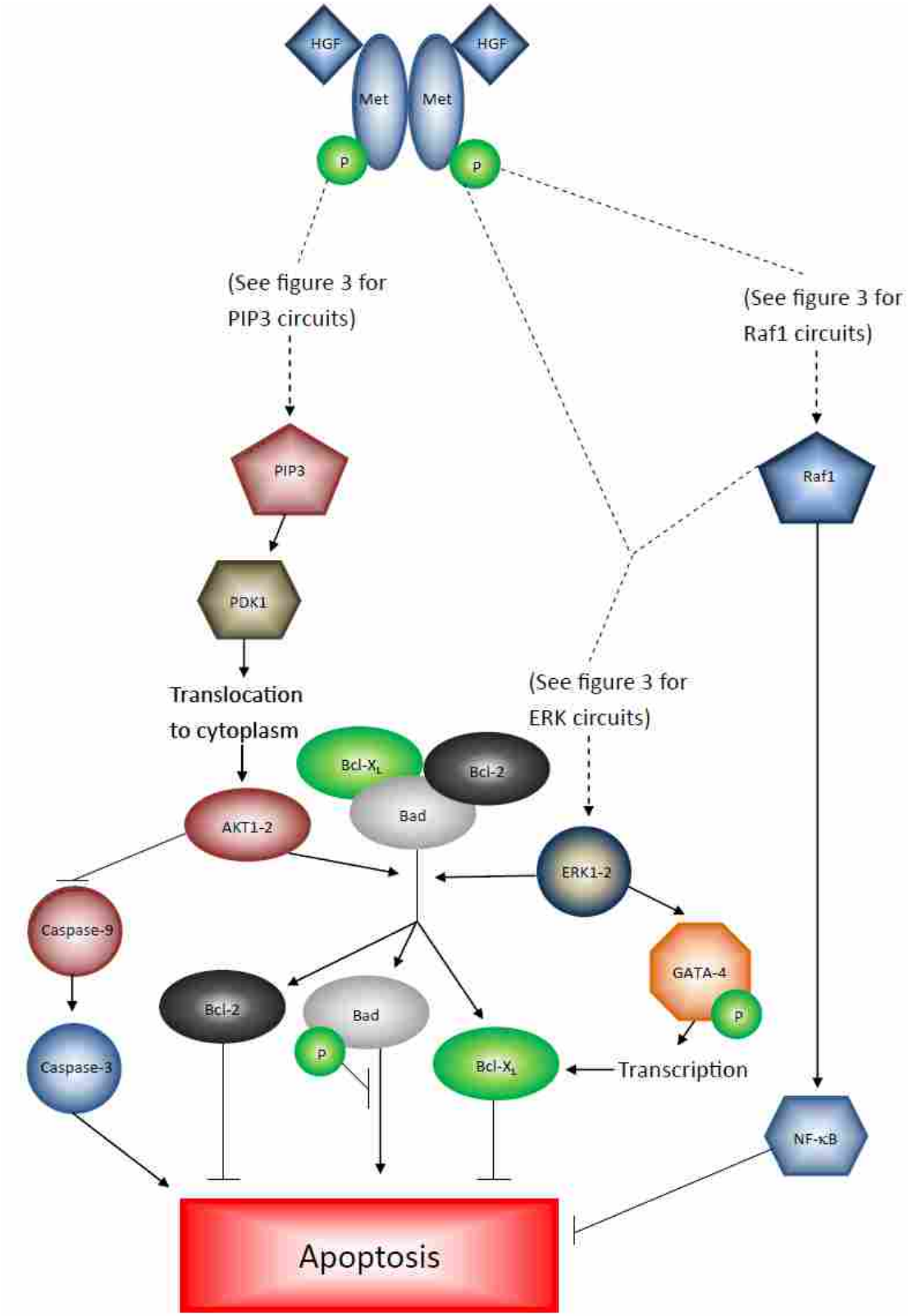

Figure 7: Induction of proliferation

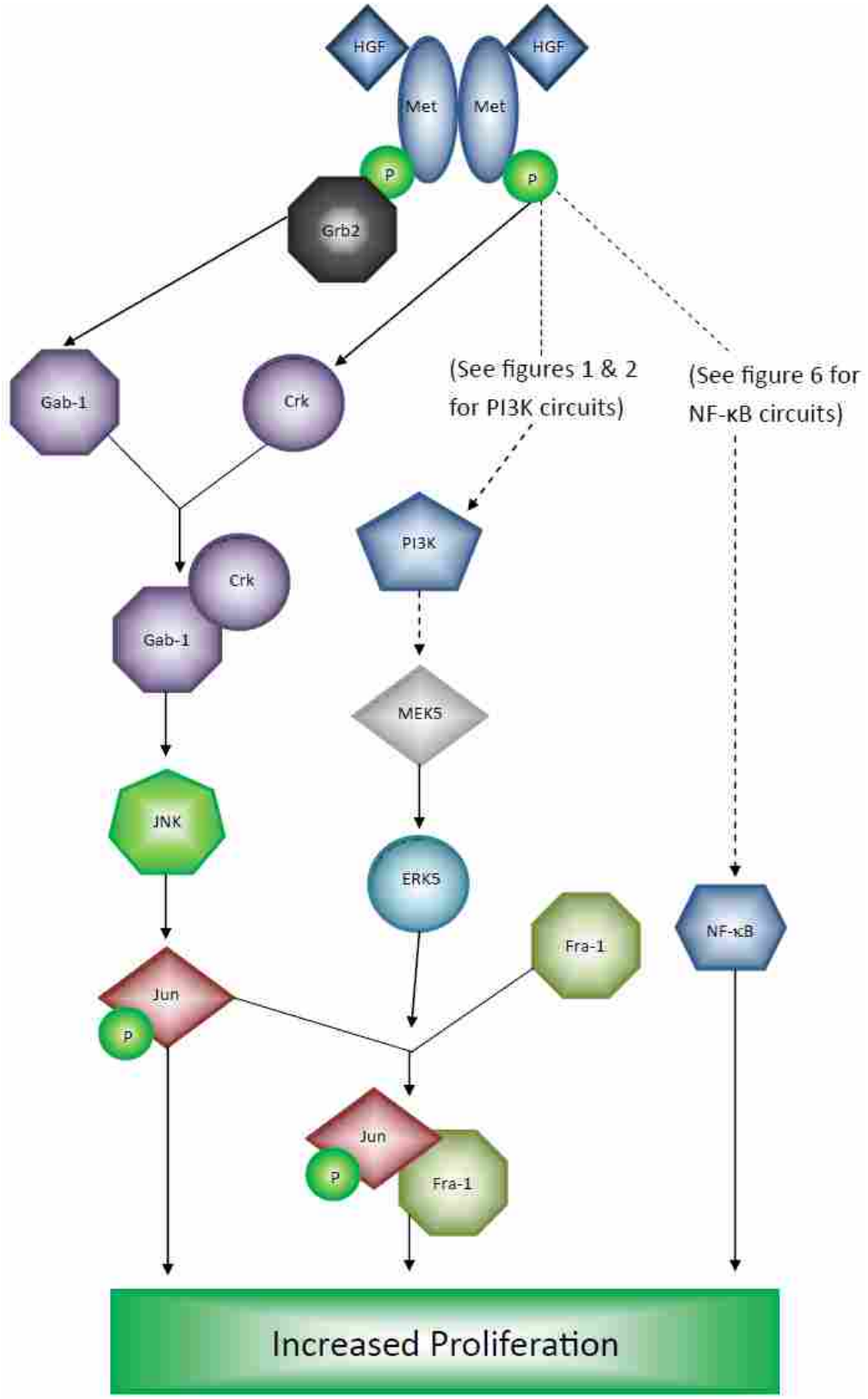

Figure 8: Design and application of an unbiased chemical screen against HGF-induced EMT

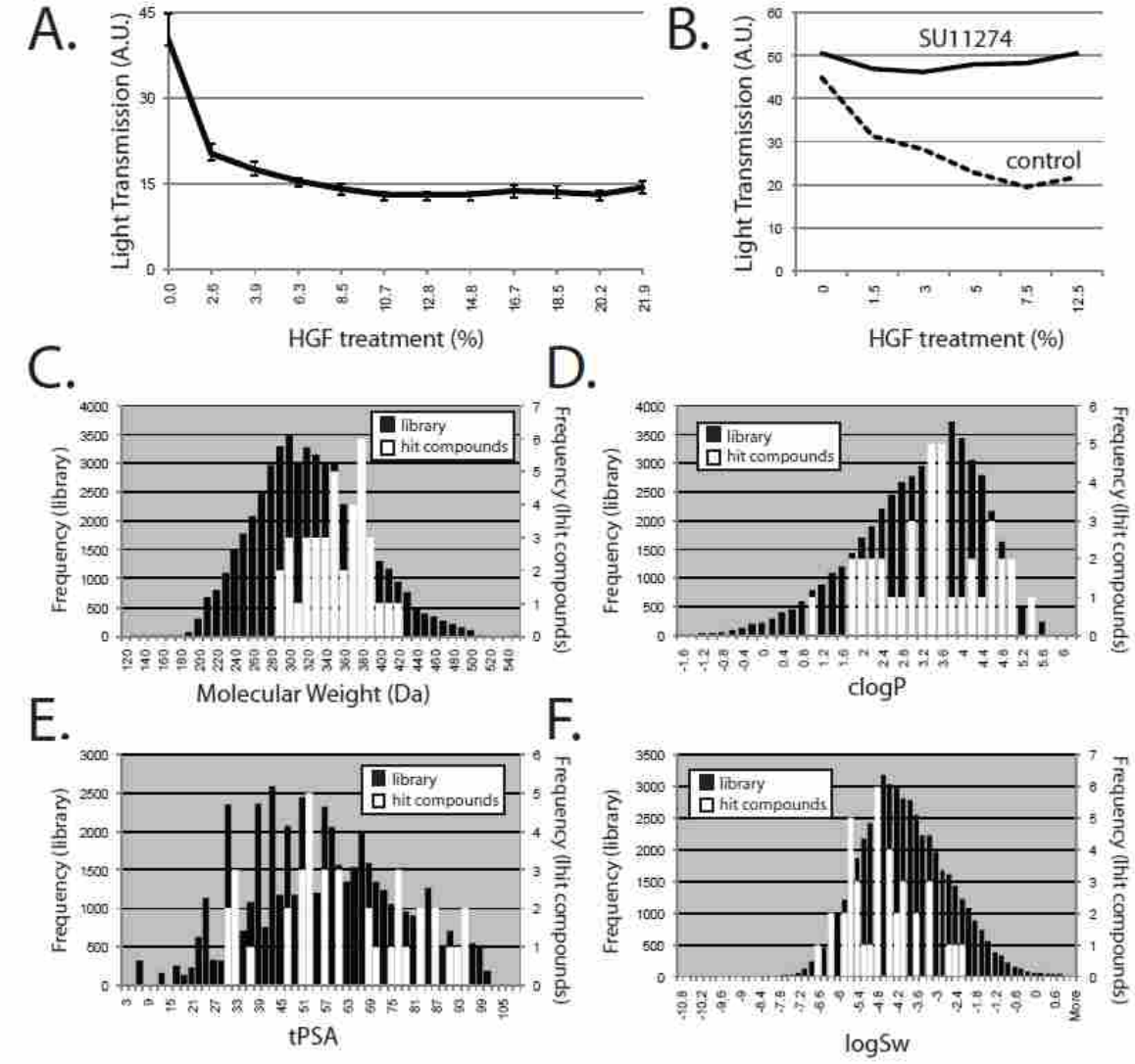

Figure 9: Chemical structures of compounds identified in screening

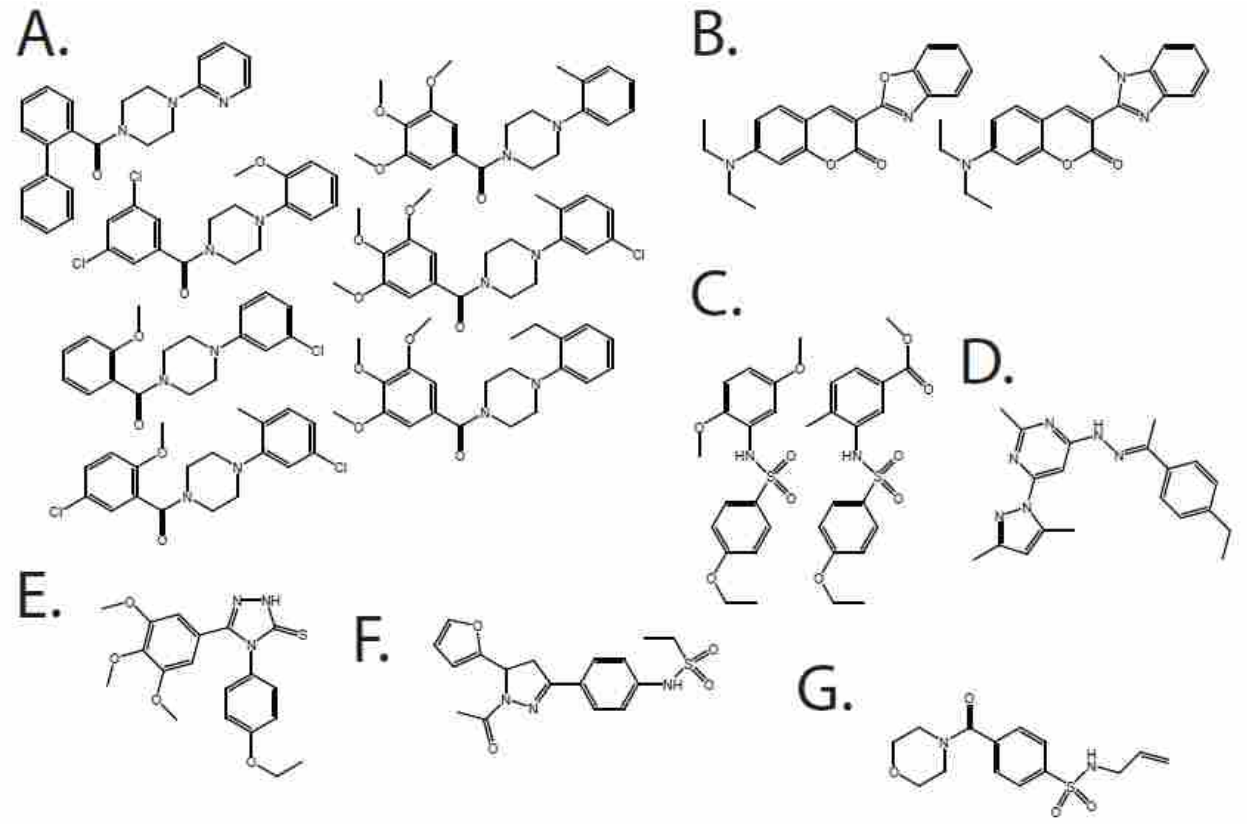

Figure 10: HGF-induced Ca^{2+} influxes are necessary for cell scattering

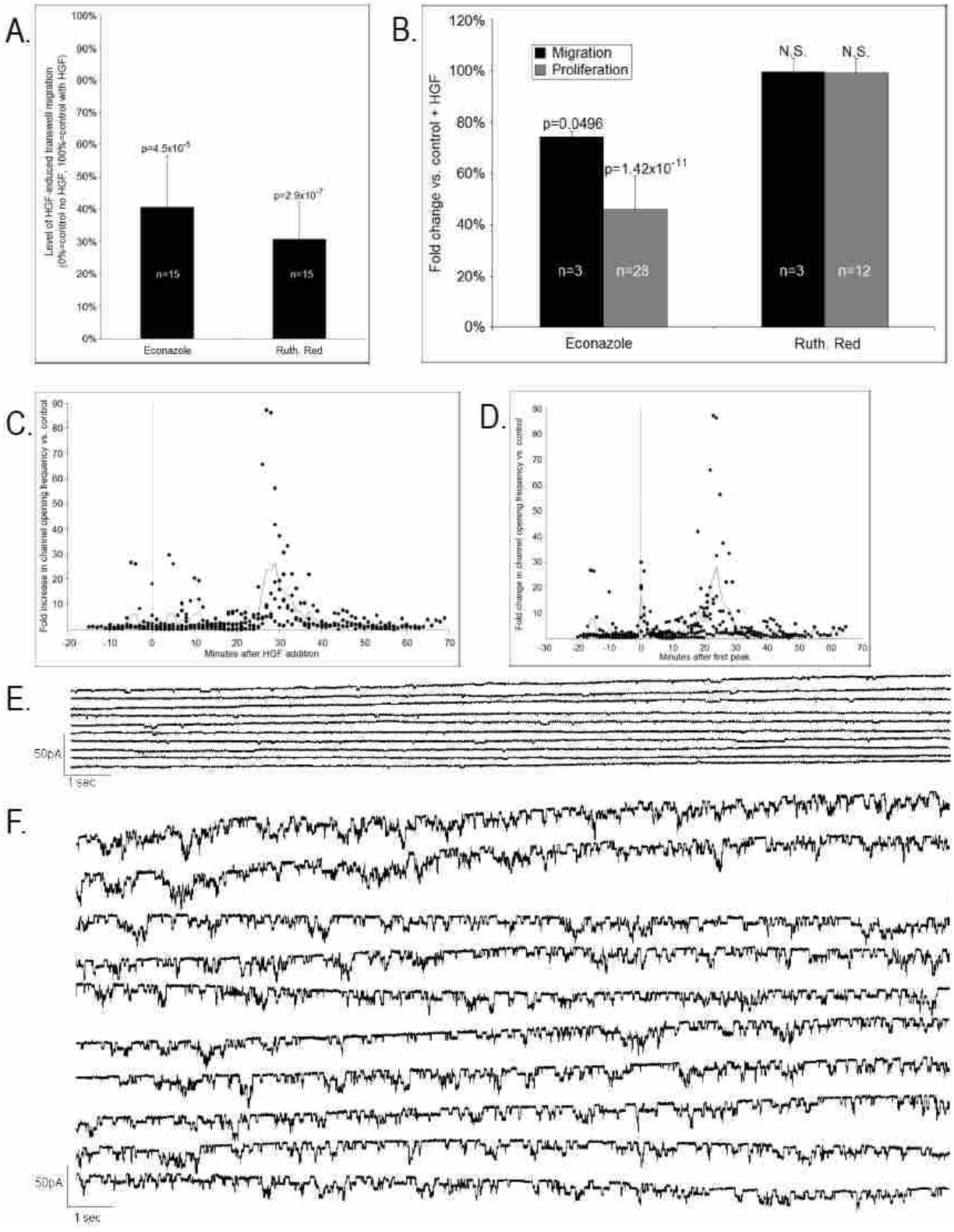

Figure 11: HGF-induced Ca^{2+} surges require microtubules

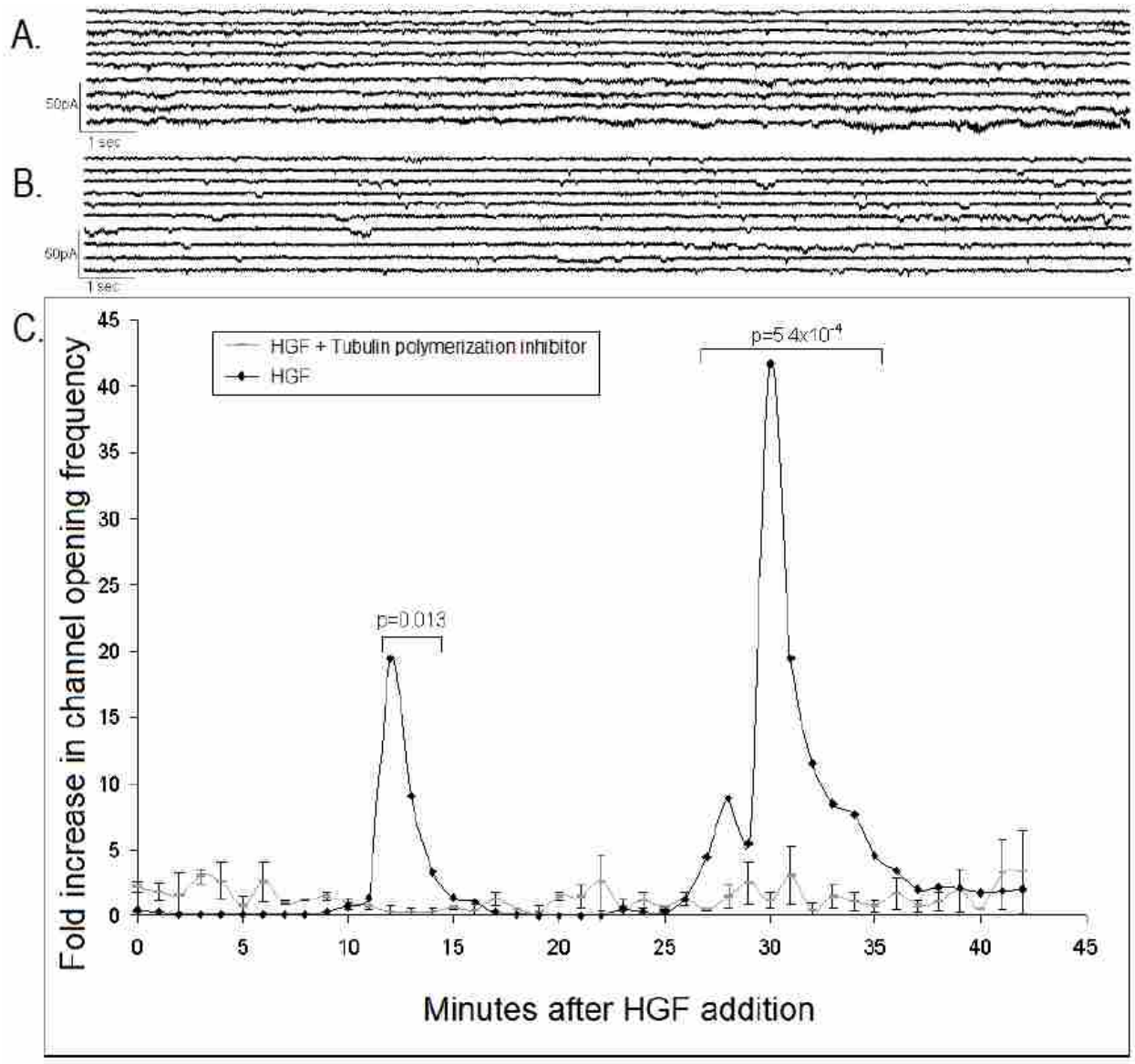

Figure 12: NA(4MC)BS selectively inhibits a single high-conductance Ca^{2+} channel

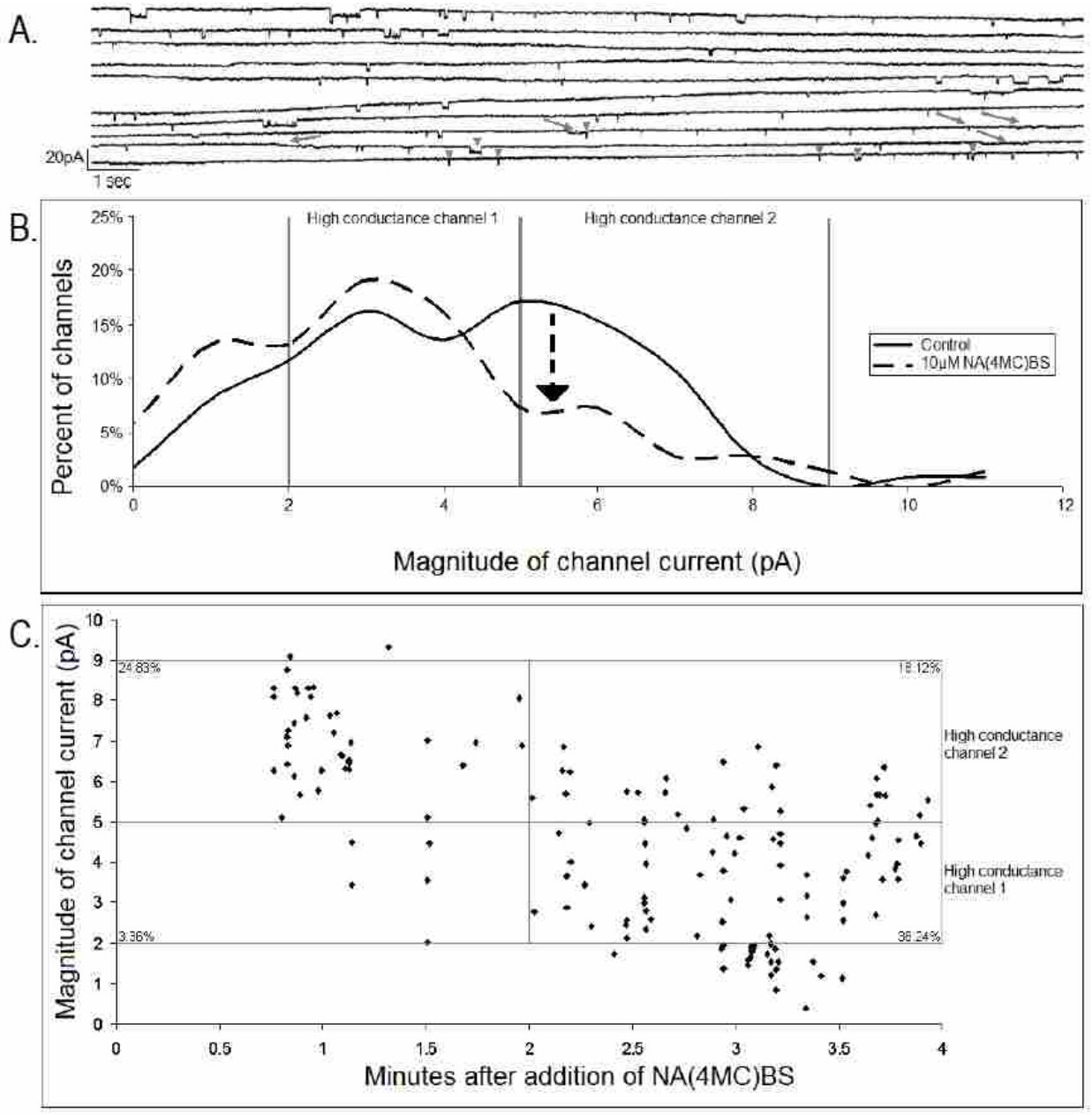

Figure 13: Effect of NA(4MC)BS on TrpC6 and TrpV4 channels

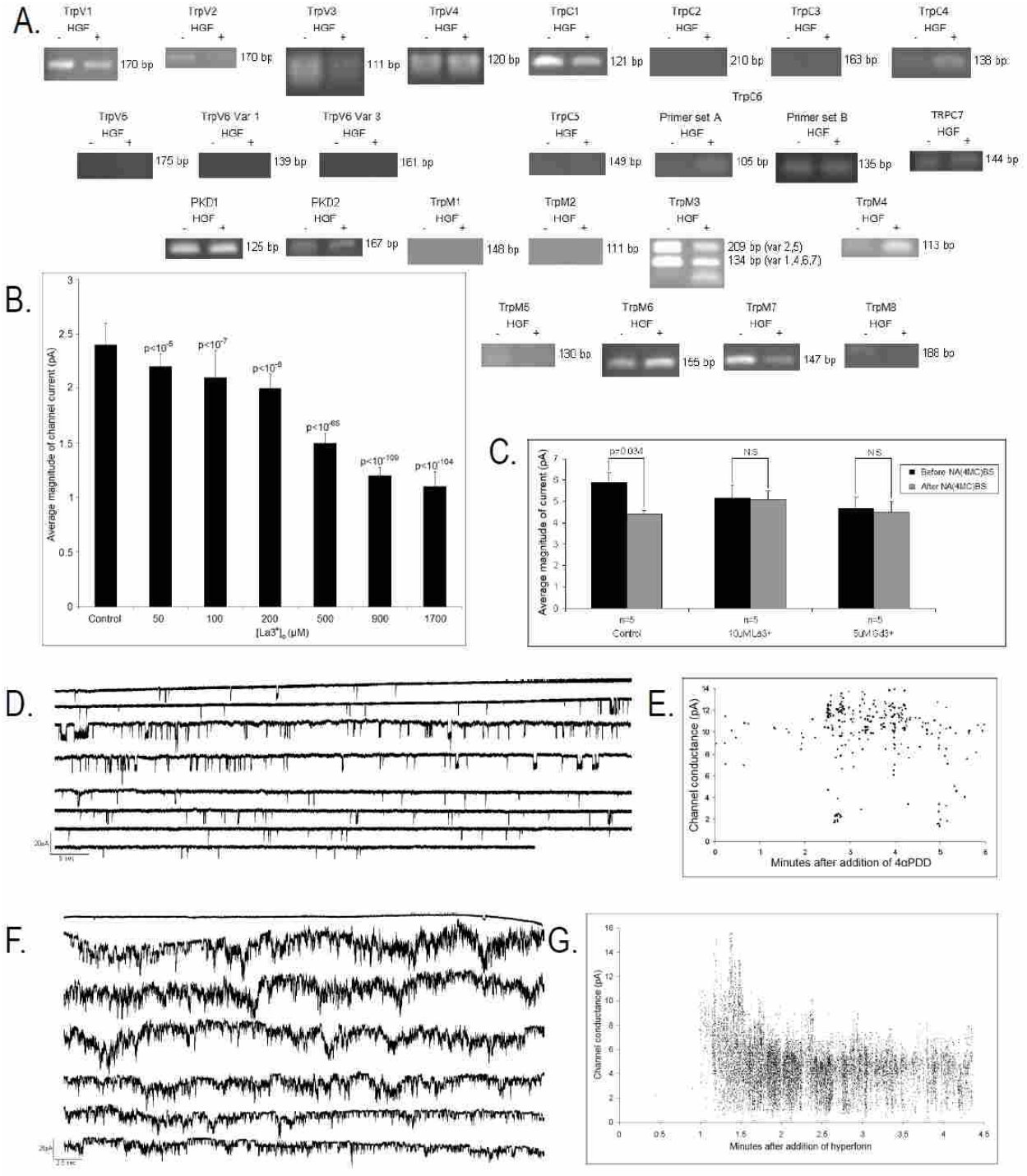

Figure 14: HGF-induced NFAT activity requires TrpC6

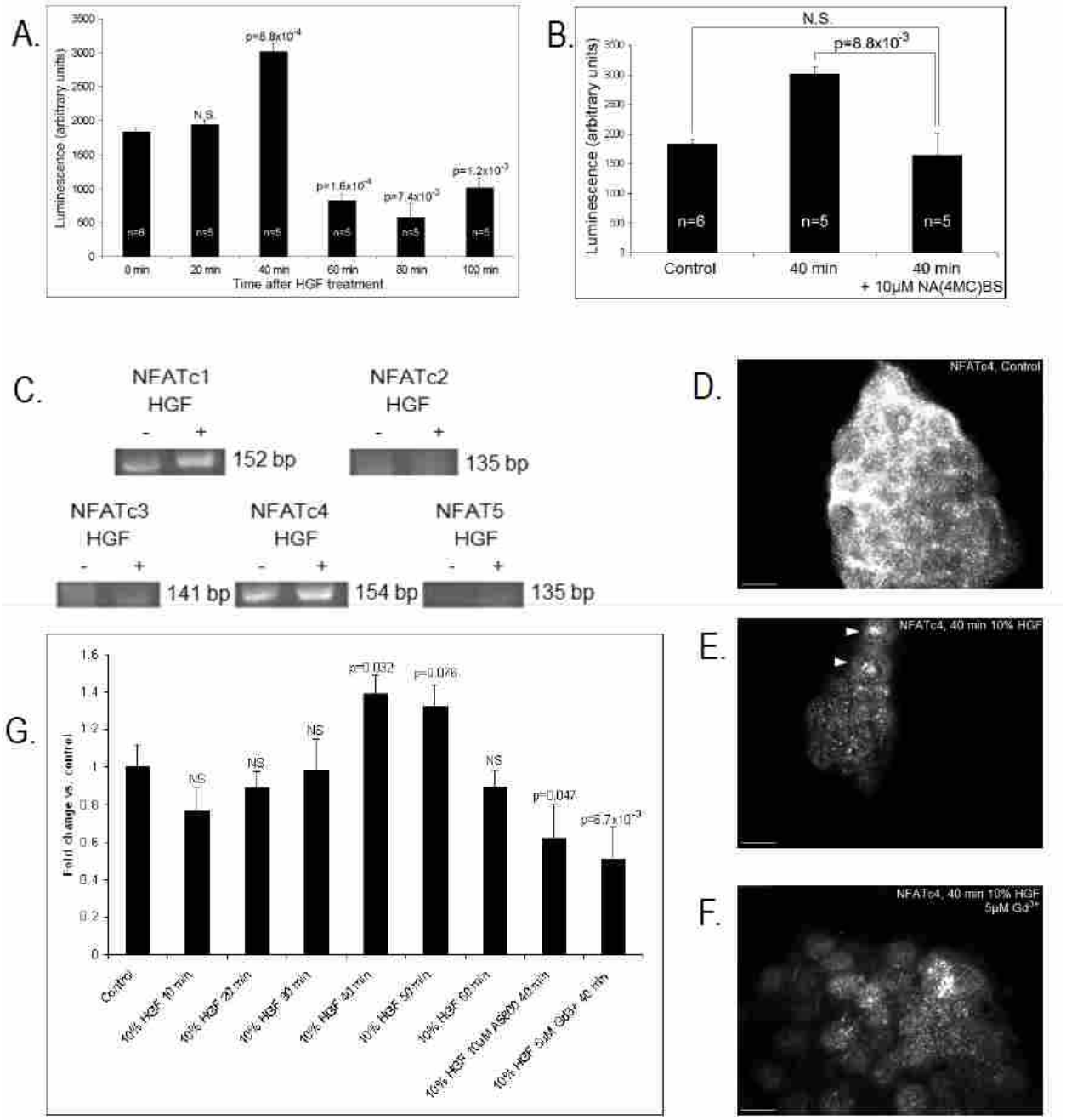

Figure 15: 8M2[2(345TMP)V]Q inhibits WNK2 and WNK3 kinase activity

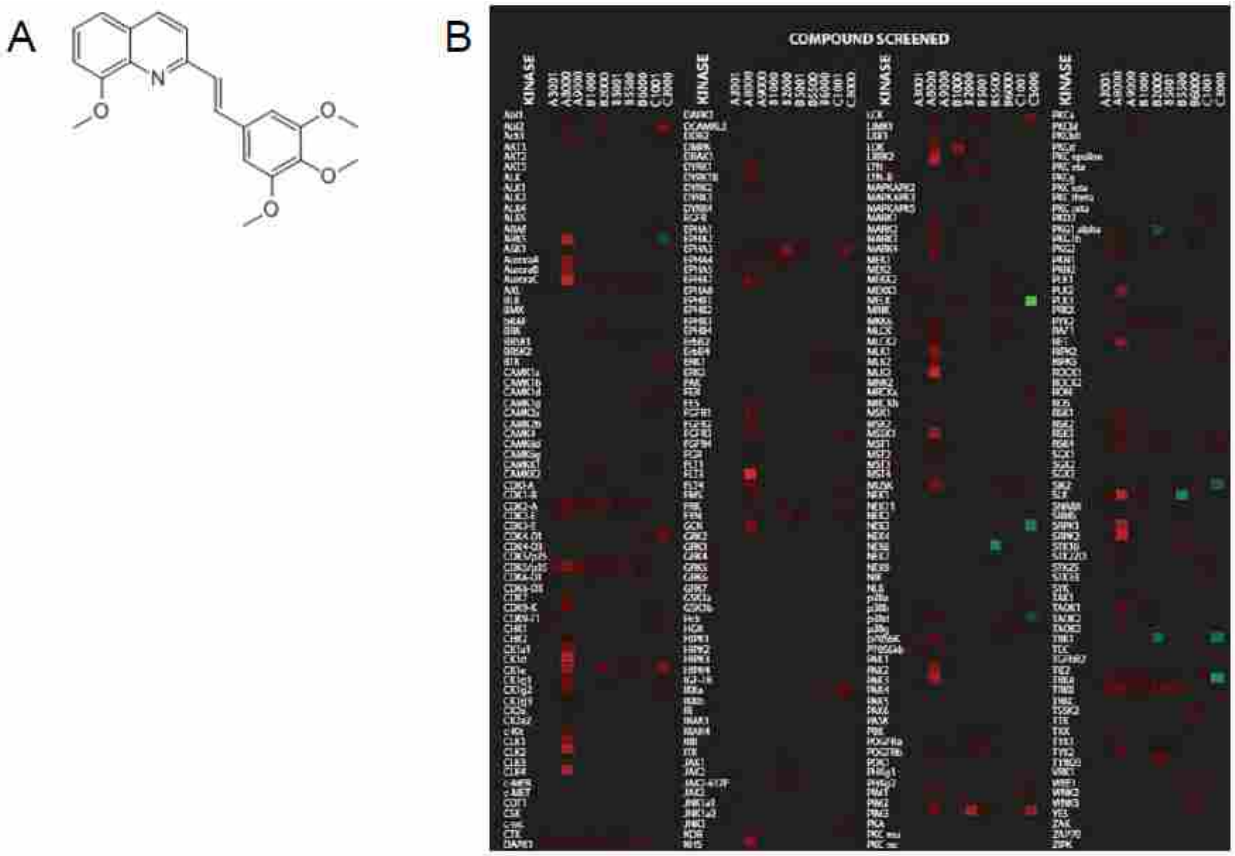

Figure 16: 8M2[2(345TMP)V]Q induces an increased frequency of Ca^{2+} channel openings

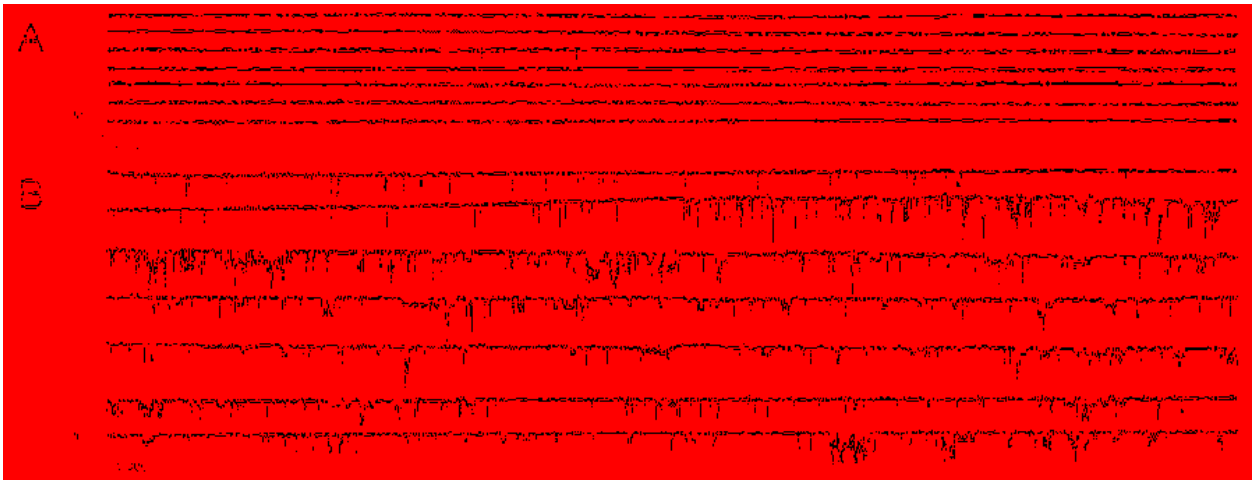

Figure 17: Expression patterns of members of the WNK kinase subfamily in MDCK

Table 1: Primers used for detection of Trp channels in MDCK

TRPC Family Primers			
Primer Target:		**Sequence**	**Length (bp)**
TRPC1	Forward	5'- CTCTACCCAAGCCCCATG -3'	121
	Reverse	5'- CTGGACTGGCCAAACAGC -3'	
TRPC2	Forward	5'- CTGTGAGGGCTCCCTCAG -3'	210
	Reverse	5'- GTTCAGCCCGCATGGTGC -3'	
TRPC3	Forward	5'- CTGGCCAAGCTGGCCAAC -3'	163
	Reverse	5'- CCTCTAGAGGCTCTGCGG -3'	
TRPC4	Forward	5'- GAGGAACCTGGTGAAGCG -3'	138
	Reverse	5'- CCTCGTAGTAAACCCAGG -3'	
TRPC5	Forward	5'- GCCATCCGCAAGGAGGTG -3'	149
	Reverse	5'- GTGTGGGCAGCCAGCATG -3'	
TRPC6	Forward	5'- CTGCTCCTGGACTCGGAG -3'	105
	Reverse	5'- GTGGGCCAGTCTGCTGTC -3'	
	Forward	5'- GTCGGATTGCCCTTCCTG -3'	135
	Reverse	5'- CATGACCAGCAGTCCCAG -3'	
	Forward	5'- CTGGCAGTGCTGGCCAAC -3'	172
	Reverse	5'- GACCGTGATCTCCGCCAC -3'	
	Forward	5'- CAGGAGGATGCAGAGATG -3'	240
	Reverse	5'- CTCCTTATCTACCTGGGC -3'	
TRPC7	Forward	5'- GAAGACCCTGTCCTCACC -3'	144
	Reverse	5'- GTCTCGGCATAGGTCCAG -3'	

TRPM Family Primers			
Primer Target:		**Sequence**	**Length (bp)**
TRPM1	Forward	5’- GACCCCTCAGCTGGACAG -3’	148
	Reverse	5’- GCCCCGAAGTGGTGGAAG -3’	
TRPM2	Forward	5’- CGAGAGCAGAGTGGACTG -3’	111
	Reverse	5’- CACTGGTCCAGGCTGAAG -3’	
TRPM3	Forward	5’- GCTGACAATGGGACCACC -3’	134
	Reverse	5’- CCTTCCACAATGAGCGCC -3’	
TRPM4	Forward	5’- GAACAGGGCTCAGATGGC -3’	113
	Reverse	5’- CAGCTTCAGACTCCAGGC -3’	
TRPM5	Forward	5’- CTCCGAGGAGCTGGACAC -3’	130
	Reverse	5’- CTTGGCGATGTCCACGCG -3’	
TRPM6	Forward	5’- GAATCCCTCCTTGGGGTG -3’	155
	Reverse	5’- GCCCACAGTCCCATCATC -3’	
TRPM7	Forward	5’- GCCCAGCAAATCTAGGTG -3’	147
	Reverse	5’- CTGAAGGCTCATCCTGAG -3’	
TRPM8	Forward	5’- GGAGGAGACCGAGAGTTG -3’	188
	Reverse	5’- GCAGAAGCTTCAGCTGCC -3’	

TRPV Family Primers			
Primer Target:		**Sequence**	**Length (bp)**
TRPV1	Forward	5'- CCACAGCGGTGGTGACGC -3'	107
	Reverse	5'- GGAGCTGTCAGGTGGCCG -3'	
TRPV1 alternate	Forward	5'- CATCAAGCGCACCCTGAG -3'	170
	Reverse	5'- CTTCTGGCTTGAGGGACC -3'	
TRPV2	Forward	5'- GTGTCAGCCAGCCGGACC -3'	113
	Reverse	5'- GCTGGTCCGGGACAGGTACTC -3'	
TRPV2 alternate	Forward	5'- CTGAAGCTGGCTGCCAAG -3'	170
	Reverse	5'- GAGTTCTCCTCCCAGCTG -3'	
TRPV3	Forward	5'- GCCAGCAGAGATCACCCCCAC -3'	111
	Reverse	5'- GGCTTGGAGAAGATGGGG -3'	
TRPV4	Forward	5'- CGGTGGAGGAAGAAGGTC -3'	120
	Reverse	5'- GCCCCGGGACACGATGTC -3'	
TRPV5	Forward	5'- GGCGCGCATCTACAACCC -3'	108
	Reverse	5'- GGGGGTTGGGGCAGTGTGGGG -3'	
TRPV5 alternate	Forward	5'- CCGTCCTCCAGCAGAAAC -3'	175
	Reverse	5'- GGTCCCCCGAGGATAGTC -3'	
TRPV6v1	Forward	5'- CCAGATGTTGGGCCCTTTCAC -3'	109
TRPV6v3	Forward	5'- GGGGCAGCCGCTCAGGGGACC -3'	128
TRPV6v1&3	Reverse	5'- GAAGGCGGAGGCAAAGCCCAG -3'	
TRPV6v1 with TRPV6v1&3 reverse alternate 1	Forward	5'- GGATTCCAGATGTTGGGC -3'	139
TRPV6v1 alternate with TRPV6v1&3 reverse alternate 2			144
TRPV6v3 alternate with TRPV6v1&3 alternate 2	Forward	5'- CAAGGGGCAGCCGCTCAG -3'	161
TRPV6v1&3 alternate 1	Reverse	5'- GTCGGGGTCCTCTGTCTG -3'	
TRPV6v1&3 alternate 2	Reverse	5'- GATGATATAGAAGGCGGAGGC -3'	

PKD Family Primers			
Primer Target:		**Sequence**	**Length (bp)**
PKD1	Forward	5’- CTTCCGGATGTCCCGGAG -3’	125
	Reverse	5’- CCGGGAAGGTGAGGAAGG -3’	
PKD2	Forward	5’- GGATGATGAGCTCCAGTG -3’	167
	Reverse	5’- GATGGGCTGTACTGGAAG -3’	

Table 2: Primers used for detection of NFAT and WNK kinase in MDCK

NFAT Family Primers			
Primer Target:		**Sequence**	**Length (bp)**
NFATc1	Forward	5'- CCTACGAGCTGAGGATTG -3'	152
	Reverse	5'- CTGTAGTGTGAGCGGTTC -3'	
NFATc2	Forward	5'- GACGAGTTCGACTTCTCC -3'	135
	Reverse	5'- CTTGAGGCCATAGTCCAG -3'	
NFATc3	Forward	5'- CACGACGAGCTCGACTTC -3'	141
	Reverse	5'- GGTAGATGGAGGTGGATC -3'	
NFATc4	Forward	5'- GGACACAGCCCCATCTTC -3'	154
	Reverse	5'- CTTTGACAGCTCCTCGGC -3'	
NFATc5	Forward	5'- CCGACAGTGCCAAAGCAC -3'	135
	Reverse	5'- GTCCACACAACATAGGGC -3'	

Table 3: Primers used for detection of WNK kinase in MDCK.

NFAT Family Primers			
Primer Target:		**Sequence**	**Length (bp)**
KS-WNK1	Forward	5'- TTGTCATCATAAATTCTCATTGCTG -3'	???
	Reverse	5'- AGGAATTGCTACTTTGTCAAAACT -3'	
L-WNK1	Forward	5'- CAGATCTACCGTCGAGTG -3'	156
	Reverse	5'- CTCCTGGAAGAAGGCATG -3'	
WNK2	Forward	5'- GGTTCATCATCTGTCCGG -3'	218
	Reverse	5'- GTCTTGAGTCTCTGCCAG -3'	
WNK3	Forward	5'- GGTTGAAGTTGCTTGGTG -3'	247
	Reverse	5'- GCTCCTTAAGACCTTTGG -3'	
WNK4	Forward	5'- GAATGAGCAAGCCATGCAG -3'	172
	Reverse	5'- GTACATATTCTCTGCCTGC -3'	

Curriculum Vitae

Peter R. Langford

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EDUCATION

B.S., Physiology and Developmental Biology with a minor in French Studies, Brigham Young University, Provo, UT, 2008

Ph.D., Physiology and Developmental Biology, Brigham Young University, Provo, UT, December 2011

PROFESSIONAL HISTORY

Graduate Research Assistant, May 2008–present, Brigham Young University, Provo, UT
Focus: Identifying and characterizing previously unknown steps in the c-Met pathway

- Integrated small molecule inhibitors, electrophysiology, gene transfection and genetic expression analyses to identify proteins functioning in the c-Met pathway
- Wrote successful fellowship applications and grant proposals securing over $19,000 across a two-year period, over $5,000 of which was awarded entirely to the lab and over $14,000 which went to the department to pay my summer salaries
- Presented abstracts of my research at the American Society for Cell Biology Conference in 2009 and 2010
- Taught myself cell-attached patch clamping in about three weeks

Teaching Practicum, August–December, 2010, Brigham Young University, Provo, UT
Course taught: Human Physiology (for non-physiology majors)

- Taught basic human physiology to a class of 110 students
- Organized half of the lectures myself based on course curriculum and taught from the professor's slides for the other half
- Received overwhelmingly positive feedback from the students, as well as the professor

Teaching Assistant, August–December 2008, May–June 2009, August-December 2011
Brigham Young University, Provo, UT
Course taught: Advanced Physiology Lab

- Instructed small lab groups of between 8 and 20 students at a time
- Taught the students a variety of experimental protocols, including blood-typing, performing and analyzing an EKG, and interpreting serum hormone and glucose levels

Undergraduate Researcher, May 2007–May 2008, Brigham Young University, Provo, UT
Responsibilities: Management of cell lines

- Maintained multiple cell lines healthy and free of infection during my entire time managing tissue culture
- Set up various types of experiments, including live-cell imaging and immunofluorescence assays
- Trained my successor in tissue culture and sterile technique

FOREIGN LANGUAGE QUALIFICATIONS AND EXPERIENCE

Translator/Interpreter, May–July 2008, Missionary Training Center of the Church of Jesus Christ of Latter-day Saints, Provo, UT
Responsibilities: English-French text translation; English-French/French-English interpretation

- Worked with a team to translate a 100+ page handbook from English to French and to prepare it for publishing
- Provided live interpretations, both in translation booths and in person, for several hours of meetings and diverse professional social interactions

Foreign Language Tutor, May 2007–August 2008, Missionary Training Center of the Church of Jesus Christ of Latter-day Saints, Provo, UT
Languages taught: Tahitian and French
Additional responsibilities: English-Tahitian text translation

- Tutored missionaries, primarily married couples aged 60 and older in both religious and non-religious French and Tahitian
- Translated a packet of language training exercises from English to Tahitian

Missionary, Church of Jesus Christ of Latter-day Saints, August 2004–August 2006
Areas: Kentucky, November 2004–January 2005; French Polynesia, January 2005–August 2006

- Worked as a religious instructor to French, Tahitian, and English speakers for about 11 hours a day, six days a week
- Taught free English classes
- For the final two months, served as the local religious leader for about 20 church members

PUBLICATIONS

Manuscript under revision: Peter R Langford, Lance Keyes and Marc DH Hansen. *c-Met initiates epithelial scattering through transient calcium influxes and NFAT-dependent gene transcription*, returned for revisions by *Journal of Cell Science*.

Article in Press: Peter R Langford and Marc DH Hansen. *Initiation of epithelial-mesenchymal transition by c-Met receptor tyrosine kinase signaling,* submitted for publication in *Current Topics in Biochemical Research,* 31 October 2011.

PRESENTATIONS

Unbiased chemical screening of c-Met signaling reveals the roles of novel kinases and calcium influxes
Poster presented at the ASCB conference in Philadelphia, PA, December 2010

Calcium and Cancer: A Role for TRPV4 in the c-Met Pathway
Research presented at Brigham Young University to graduate students and faculty of the Department of Physiology and Developmental Biology, Provo, UT, 2010

New steps identified in the c-Met pathway
Poster presented at the ASCB conference in San Diego, CA, December 2009

Illuminating the c-Met Pathway
Research presented at Brigham Young University to graduate students and faculty of the Department of Physiology and Developmental Biology, Provo, UT, 2009

AWARDS AND HONORS

Research Assistantship/Teaching Assistantship, Provo, UT, 2008-2011
Brigham Young University Cancer Research Center Summer Research Fellowship, $7,350, Provo, UT, Summer 2011
Women's Research Grant, $5,000, Provo, UT, 2011
Graduate Student Society Travel Grant, $400, Provo, UT, 2010
Brigham Young University Cancer Research Center Summer Research Fellowship, $7,500, Provo, UT, Summer 2010
Graduate Student Society Travel Grant, $400, Provo, UT, 2009
Graduate Student Grant, Provo, UT, Fall 2009, $1,000
Heritage Scholarship (four-year full tuition scholarship), Brigham Young University, Provo, UT, 2003–2004, 2006–2008
Salutatorian, Northview High School, Dothan, AL, 2003
Eagle Scout Award, Dothan, AL, 2000

ACADEMIC ACHIEVEMENTS

Current Graduate GPA: 3.94
GRE Composite Score: 1420, with a 6.0 for Analytical Writing
Undergraduate GPA: 3.79
ACT Score: 33

PROFESSIONAL MEMBERSHIPS

American Society for Cell Biology, 2009–present
American Association for the Advancement of Sciences, 2011-present

RESEARCH SKILLS

Extensive knowledge of tissue culture procedures and sterile technique
Extensive knowledge of cell-based drug-screening methods
Proficient in SlideBook and ClampFit data analysis software
Good statistical background
Competent in western blotting, immunofluorescent staining, and reverse-transcriptase PCR
Proficient in electrophysiology techniques with a focus on cell-attached patch clamping of epithelial cells and some experience with whole-cell patch clamping, as well as electrophysiological data analysis
Some experience with RNAi, as well as both virus- and chemical-mediated gene transfection
Some reporter gene assay experience

OTHER SKILLS AND QUALIFICATIONS

Proficient in Microsoft Office
Proficient in PhotoShop and other image-editing programs
Fluent in French
Fluent in Tahitian

REFERENCES

Dr. Marc Hansen
Research Advisor, 2007–present
574 WIDB
Provo, UT 84602
801-422-4998

Dr. David Busath
Collaborator, 2010–2011 and Former Research Advisor, 2008
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Provo, UT 84602
801-422-8753

Dr. Allan Judd
Graduate Committee Member and Collaborator, 2008–present
585 WIDB
Provo, UT 84602
801-422-3179

Maria Johnson
MTC Supervisor, 2007–2008
4M-121 MTC
Provo UT 84602
801-422-1288

Bill Welsh
Mission President, 2006
D-153 ASB
Provo, UT 84602
801-422-6762