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Cont-RAS-ting the effects hyperactive Ras signaling on cell growth and size control

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MOLECULAR, CELL AND DEVELOPMENTAL BIOLOGY

by

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Abstract

Cont-RAS-ting the effects hyperactive Ras signaling on cell growth and size control in budding yeast

by

Jerry Tyler DeWitt

Severe defects in cell size are a nearly universal feature of cancer cells. Yet, the mechanisms that drive size defects in cancer cells remain unknown, and it is unclear whether they are a direct consequence of primary oncogenic drivers or a secondary consequence of mutations that accumulate during evolution of cancer cells. Hyperactive mutants of Ras are amongst the most prevalent oncogenic drivers. In budding yeast, previous studies have suggested that hyperactive versions of the Ras homolog (*ras2*^{G19V}) cause defects in cell size. However, the mechanisms by which *ras2*^{G19V} drives cell size defects are unknown, and it is unclear whether the size defects are due to accumulation of suppressor mutations. Here, I developed a system for inducible expression of *ras2*^{G19V} that allows for investigation into the immediate consequences of *ras2*^{G19V} expression. I found that *ras2*^{G19V} causes a delay in G1 phase, increased cell size, and aberrant expression of G1 cyclin proteins. Furthermore, *ras2*^{G19V} appears to inhibit a key step in cell cycle entry, in which an early G1 cyclin induces transcription of late G1 cyclins. The data further suggest that Ras does not influence cell size solely via effects in G1 phase. Finally, we found that expression of oncogenic Ras alone is sufficient to cause cell size defects in NIH 3T3 cells, which suggests that defects in cell size in cancer cells could be a direct consequence of primary oncogenic signals. Together, the data suggest that hyperactive forms of Ras influence cell size in both yeast and mammals. Further analysis will determine whether Ras influences cell size via conserved mechanisms.

I dedicate this dissertation to:

My fellow queer community,

who navigate various types of discrimination in everyday life.

First-generation people pursuing a degree in science,

who learn to navigate the educational system with little guidance.

&

My best friend, partner, and husband,

Sergio Alejandro Estrella Ramos

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final rotation and I had listed the Kellogg lab as a back-up, only because I had heard that you were a great mentor. I had no idea, or interest in cell growth and size control at the time. However, during my rotation, I fell in love with the lab. I will never forget when the time came for picking a thesis lab, and after meeting with potential advisors, you were the only mentor (and lab) that truly made me feel wanted. It was a special moment of validation for me. Although you were pretty hands-off during the beginning of my project, it gave me a sense of ownership of my learning and I think that I am better off because of it. Then after my 3rd year candidacy talk, my project took a massive pivot to account for the limitations that COVID presented, and you recognized that I needed more support. You have a way of adjusting your mentorship styles/strategies to fit the needs of the student, and that is something that I aspire to achieve.

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Chapter 1: **Introduction to cell growth & size control, the cell cycle, & Ras.**

"Cells care about how large they are and so should we"[1]
-Paul Jorgensen and Mike Tyers

Cell growth and size control

Cell size is the outcome of the processes that control growth and proper control of cell growth and size is a fundamental requirement across all orders of life. Within the human body, cells range in size across several orders of magnitude **(Figure 1.1)**. However, cells of a particular type maintain tight control of their size around a constant average cell size. Ultimately, cell growth is a fundamental aspect of life and must be properly coordinated with cell division for survival; a failure to undergo adequate growth prior to division would result in increasingly smaller cells over time. Similarly, if growth were to continually occur without cell division, then cells would become abnormally large and impose increased energetic demands.

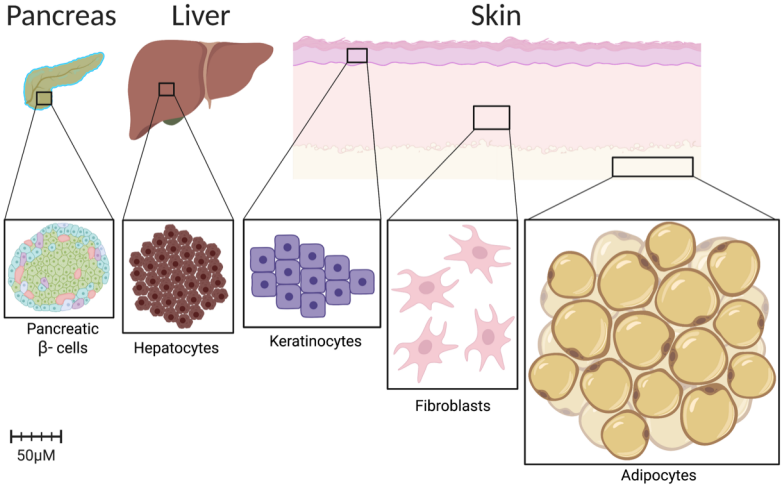

Figure 1.1. Diagram depicting size control of diverse cell within various human tissues.

One of the earliest experiments that sought to test the relationship between cell growth and division came in 1928 when M. Hartmann, a researcher studying amoeba, found that routinely sectioning off parts of cytoplasmic membrane (filopodia and reticulopodia) prevented cell division[2]. These protozoans were observed to resume cell growth following surgical removal of cytoplasmic membrane; however, the amputated cells continually failed to divide for several months of experimentation while the untreated control amoeba had undergone 65 rounds of cell division in the same time-frame. The experiments were later confirmed by an independent researcher in 1956[3]. These critical observations are amongst the first pieces of evidence linking cell growth and size requirements to cell division, a process regulated by the cell cycle.

The coordination between cell growth and cell cycle progression can also be observed by size-checkpoints that control cell cycle entry and exit. Some of the earliest evidence that demonstrates cell growth control came from seminal work conducted by Lee Hartwell using Saccharomyces cerevisiae (budding yeast) as a model organism. Budding yeast undergo asymmetric cell division, which results in the production of a larger mother cell and a smaller daughter cell. The small daughter cell spends more time growing in G1 phase before entering the cell cycle[4].

These observations led to complicated and nuanced questions concerning how cell growth is monitored, controlled, and coordinated with cell cycle progression. Later work in fission yeast found that cells arrested in S phase continue to grow, and therefore cell growth must not require cell cycle progression to occur. Furthermore, upon release from an S phase arrest, cells

rapidly underwent cell division until ultimately converging to an average cell size that was observed prior to the arrest[5,6]. Similar phenomena have been observed when researchers use alternative methods for cell cycle arrests. For example, arresting budding yeast cells in early G1 using a mating pheromone allows cells to still accumulate volume and increase their size. Upon release from this early G1 arrest, cells rapidly advance through the cell cycle, similar to what is observed in fission yeast coming out of an S phase arrest. Together, these observations suggest that cell cycle progression is not a requisite for cell growth.

Cell size control is also observed in bacteria. By conducting single-cell microscopy of two distantly related species of bacteria, *Escherichia coli* and *Caulobacter crescentus*, researchers found that these cells add a constant amount of volume each cell cycle regardless of starting size[7]. These data support a notion where prior cell cycle defects that result in abnormal cell size can be corrected after a few rounds of subsequent division and led to an idea of an "adder" mechanism **(Figure 1.2)**.

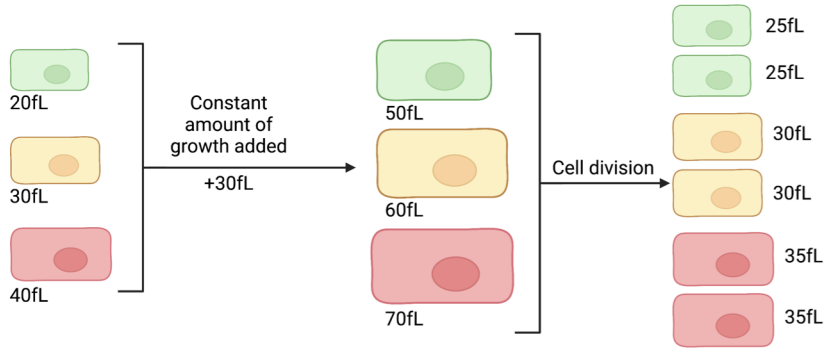

Figure 1.2. A cartoon depicting cells growing under control of an adder growth mechanism. In an adder mechanism, all cells achieve approximately the same amount of growth prior to cell division. In this model, starting size differences within a population will converge upon an average size after a few rounds of division.

In addition to single-cell organisms, evidence for cell size control has been observed in metazoans as well[1,8]. This is particularly evident in developmental biology of multicellular organisms. For example, cell size homeostasis is critical for the proper development and function of tissues, and organs. Normally, cells of a particular type have the ability to coordinate cell growth with division to ultimately generate a population of similarly sized cells as seen in the tissues comprising organs in animals. Ultimately, cell size control is vital for proper cellular function. Cells that fail to properly control growth and size are observed in several human diseases including organ hypertrophy, diabetes, cellular dysplasia, and cancer[9–14].

The importance for size control with regards to proper cellular function can also be observed in various organ transplants between a donor and recipient. For example, following canine liver transplants, surgeons observed that the transplanted liver would grow to fit the body size of the recipient animal[15,16]. Additionally, this phenomenon is not limited to canine livers, because further studies found similar results for rat kidneys[17] and human liver[18]. Although the relationship to cell size control and proliferation in these situations (where there is an adaptive response of organ size) is unknown, it nonetheless requires modulation of cell growth, proliferation, and/or overall regulation of cell size.

Defects in cell size control have important implications for human biology. An example can be observed in cancer. Cancerous cells lack uniformity in terms of cell size, shape, and nuclear-to-cytoplasmic volume ratios. Furthermore, the increasing severity of size defects are associated with poor patient outcomes. Therefore, defects in size control are important for cancer pathology. In fact, in

1966 a cancer pathologist, Dr. Donald Gleason, created a standardized system for grading the severity of prostate adenocarcinoma called the Gleason Score. The Gleason Score accounts for visual cellular abnormalities (atypia, and dysplasia) and modified approaches are still widely in use to date[13,19–21]. Moreover, cytologic atypia and cellular dysplasia are broadly associated with numerous cancer types including, but not limited to, breast, cervical, lung, pancreatic, prostate, and thyroid cancers. Ultimately, the mechanisms underlying control of cell growth and size control are critical, yet poorly understood.

Cytologic atypia and other cellular abnormalities are nearly universal features of cancer cells, and have provided a foundation for cancer pathology for over 100 years. Cytologic atypia is, in part, used to describe the defects in cell size, shape, and nuclear size of a population of cells. Cancer cells show greater heterogeneity of cell size and shape, as well as dramatically altered nuclear to cytoplasmic volume ratios, indicating a loss in cell growth and size control.

Although defects in cell size are closely associated with cancer (among other diseases like diabetes), little is known about the underlying mechanisms

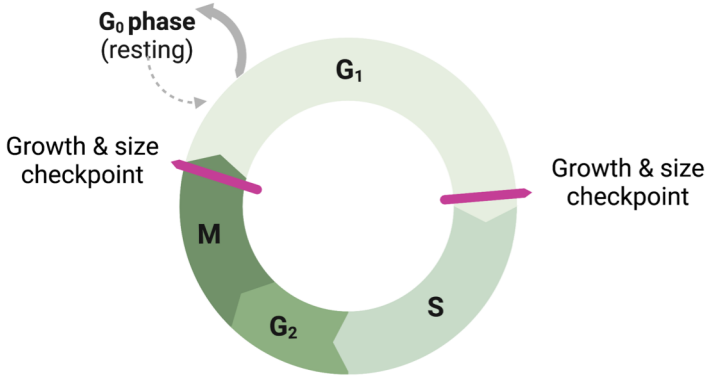

Figure 1.3. A cartoon depicting known growth and size checkpoints of typical cell cycle.

that control cell growth and ultimately cell size. Obtaining a better understanding of how cell size control works in normal cells, and how it is ultimately lost in cancer, can sharpen the collective knowledge surrounding cancer growth and potentially broaden the scope of anti-cancer therapies available.

Sufficient cell growth is required for proper cell cycle progression at several points during the cell cycle; often at the points regulating cell cycle entry/commitment, mitotic entry, and mitotic exit. For example, budding yeast monitor cell growth at cell cycle entry (G1/S), mitotic entry (G2/M), and mitotic exit at the metaphase-anaphase transition. Each growth and size checkpoint of the cell cycle works to ensure that enough cell growth has occurred, prior to progressing through the subsequent phases of the cell cycle **(Figure 1.3)**.

Cell cycle regulation

Overview of the cell cycle

All cells must progress through the cell cycle to divide and proliferate. For successful cell division to occur, cells must grow, duplicate their genome, segregate their genetic material, and ultimately separate into two cells by the process of cytokinesis. Because the appropriate regulation of cell division is vital, cells have evolved mechanisms to control the cell cycle. These processes monitor and regulate 3 critical cell cycle transitions leading to: genome duplication, mitotic entry, and cytokinesis.

The G1/S transition occurs at the end of G1 phase and is a critical commitment-step for the cell cycle entry. Proper progression through the G1/S checkpoint requires a minimum threshold of growth to proceed[1,22]. Failure for a cell to meet the threshold will prevent entry into S-phase. This is the first, and

arguably the most important, checkpoint of the cell cycle because it ensures that all critical requirements for initiating cell division have been met. Moreover, mechanisms that control cell cycle entry in G1 phase are strongly influenced by oncogenic signaling. Upon cell cycle entry, cells enter S-phase of the cell cycle, allowing genome duplication to occur. It is critical that cells perform faithful duplication of their genomic material, because all subsequent daughter cells will contain any mistakes made during DNA replication. To ensure proper duplication of genomic DNA, cells have evolved a G2/M checkpoint that inspects the fidelity of DNA replication and monitors the environmental conditions surrounding the dividing cell. After proofreading and DNA-repair mechanisms, it is thought that ~1 mutation occurs in a single round of division for a human cell containing ~3 billion base-pairs. Therefore, in a healthy cell, genome duplication maintains high fidelity.

After successfully passing the G2/M transition cells enter Mitosis, the process of separating duplicated genetic material prior to cell division. It is crucial that dividing cells properly segregate their genetic material such that each future daughter cell will contain identical information. To monitor this mitotic feat, cells rely on the metaphase-anaphase transition. This checkpoint serves to ensure proper chromosomal segregation and failure to do so will delay cell cycle progression to prevent inappropriate cell division.

Cdks are the family of protein kinases that are responsible for all cell cycle progression events. Activation of Cdks requires binding to cyclin protein. Cyclins are expressed during the cell cycle period that they regulate and are rapidly degraded as the cells progress to the next stage of the cell cycle[23]. In

addition to Cdk activation, cyclins are thought to also provide Cdk the substrate specificity that is necessary for properly controlling specific cell cycle events[1,24,25]. Mammalian cells have 20 different Cdks, and several are required for cell cycle progression. However, budding yeast have a single Cdk (Cdk1) that is sufficient for progression throughout the entire cell cycle. Cell cycle-dependent oscillation of cyclin expression and polyubiquitination for subsequent degradation are responsible for the changes in Cdk activity throughout the cell cycle **(Figure 1.4)**.

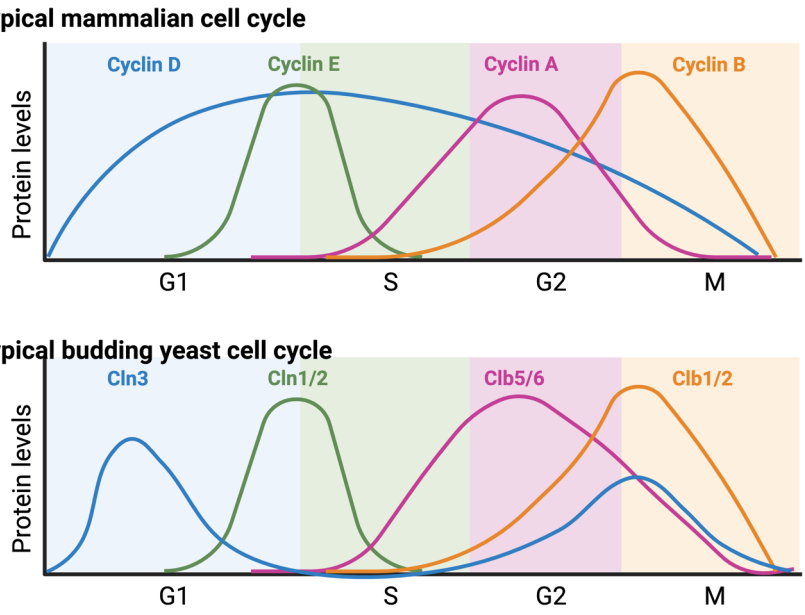

Figure 1.4. Comparative cyclin expression between budding yeast and mammals.
(Top panel) Typical cyclin expression throughout a mammalian cell cycle.
(Bottom panel) Typical cyclin expression throughout a budding yeast cell cycle.

Regulation of cell cycle entry

Cell cycle entry is marked by the transition from late G1 into S-phase. This fateful decision commits the cell to division; cells can only progress in a unidirectional manner after this point. Although cells can slow/pause the cycle,

they must resume with cell cycle progression or undergo programmed cell-death. In budding yeast this transition is referred to as “START” in budding yeast, in animal cells it is referred to as the “restriction point”.

Throughout G1 phase, cells are constantly monitoring growth and outside environmental conditions before committing to cell cycle entry. Once conditions are satisfactory and sufficient growth has occurred, the cell can proceed through the G1/S transition to enter the cell cycle. Although the major components are known, the underlying mechanisms that regulate cell cycle entry are largely unknown. However, it is thought that budding yeast and metazoans control cell cycle entry in a similar manner that is ultimately controlled by G1 cyclins and Cdk.

There are 2 critical types of cyclins that operate in G1 phase: early G1 cyclins and late G1 cyclins. In budding yeast the early G1 cyclin Cln3 accumulates with growth and initiates the expression of the late G1 cyclins Cln1 and Cln2 via inhibition of the transcriptional repressor called Whi5, which binds and inhibits the SBF and MBF transcription factors. SBF (composed of Swi4 and Swi6) and MBF (composed of Mbp1 and Swi6) are heterodimeric transcription factors that control transcription of genes in the G1/S regulon and drive cell cycle entry.

In metazoans, cell cycle entry is thought to operate in a manner similar to that of budding yeast. Cdk, when in complex with the early G1 phase cyclin (cyclin D), functions to relieve the transcriptional repression that retinoblastoma protein (Rb) has on the E2F transcription factor family. This disinhibition of Rb

allows E2F to become active and initiate transcription of cell cycle machinery required for cell cycle entry **(Figure 1.5)**.

In the canonical model of cell cycle entry in budding yeast, inactivation of Whi5 by Cln3-Cdk initiates a positive feedback loop in late G1 whereby Cln1/2, in complex with Cdk, further promote their own expression and additionally facilitate nuclear export of the Whi5 transcriptional repressor to allow seamless transition from G1 to S-phase[26,27] **(Figure 1.5)**. In addition to initiating cell cycle entry, Cln3 is also a dose-dependent regulator of cell size; *cln3*Δ cells are large whereas overexpression of Cln3 results in a small cell size phenotype.

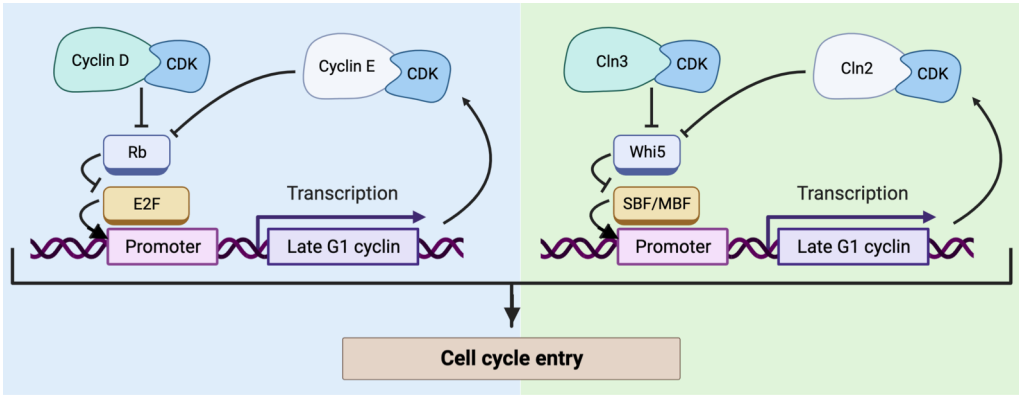

Figure 1.5. Canonical models for cell cycle entry in mammalian cells (blue shading) and budding yeast (green shading).

The overlap between Ras signaling and control of cell cycle progression

The discovery of Ras

The superfamily of Ras proteins was originally identified from rat sarcoma virus which was found to have transduced the Ras gene into its own genome and thereby converting a normal gene into an oncogenic agent. They serve as molecular switches that stimulate vast downstream signaling cascades largely involved in processes that regulate cell growth, survival and cell cycle

progression. Ras-dependent signaling is thought to promote cell cycle entry **(Figure 1.6)**.

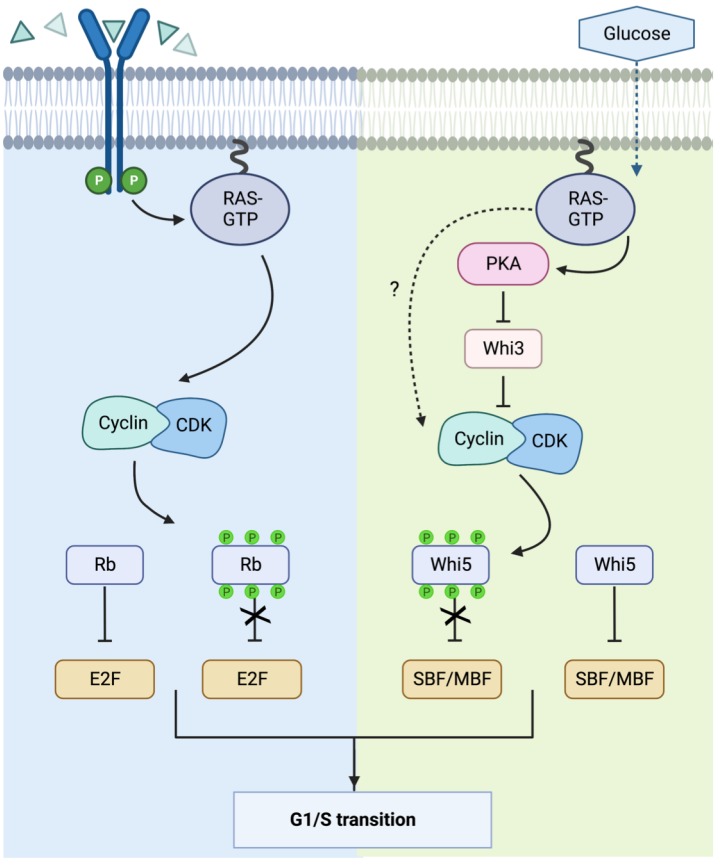

Figure 1.6. Canonical models for Ras-dependent signaling in control of G1/S cell cycle progression

Members of the Ras family comprise a class of G-proteins that are regulated by GTP-binding. Normally, Ras is active when bound to GTP, and can inactivate itself via GTP hydrolysis **(Figure 1.7)**. Ras proteins are also GTPases which allow Ras to hydrolyze its own GTP. However, with weak catalytic activity, Ras relies on accessory proteins to assist with GTP binding and hydrolysis to allow for rapid signaling modulation to occur. For example, guanine exchange factors (GEFs) facilitate the activation of Ras by swapping the bound GDP for

GTP leading to its activation. Additionally, GTPase-activating proteins (GAPs) physically bind Ras-GTP and induce proper conformation of the GTPase domain

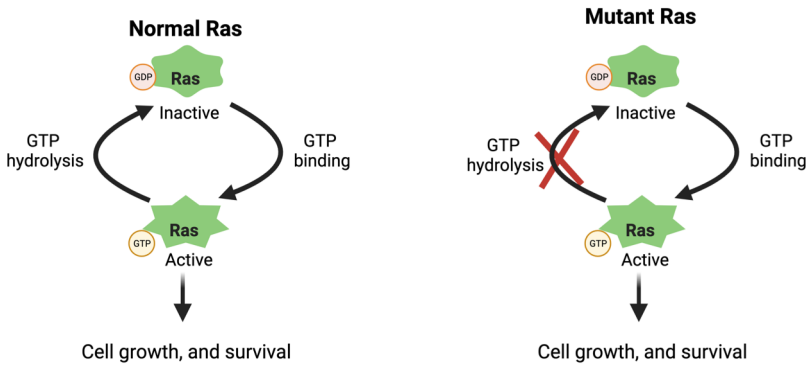

Figure 1.7. A cartoon comparing the normal mechanism of activation for Ras relative to mutant forms that result in hyperactivity.

within Ras to allow optimal positioning for rapid hydrolysis of Ras-GTP (active) to Ras-GDP (inactive).

Currently, there are 5 main subfamilies of proteins encompassed by the Ras superfamily in mammalian cells: Ras, Rho, Ran, Rab and Arf GTPases. Ras was the first to be discovered, in 1964, when the first *ras* oncogene (*Ha-ras* later identified as *HRAS,* in humans) was identified in rat sarcoma virus by Jenny Harvey. Harvey found that *Ha-ras* had transforming capabilities in mice that led to rapid tumor formation, and in 1967 another ras oncogene (*Ki-ras* later named as *KRAS,* in humans) was also identified in viruses and found to contain transforming properties similar to those previously observed in *Ha-ras*[28,29]. In 1979, the *ras* sequences responsible for tumor formation in mice were identified and designated *Ha-ras and Ki-ras*[30]. Soon thereafter, researchers began investigations aimed to identify additional oncogenic drivers, and in 1983, *NRAS* was identified in EJ bladder tumor cells, neuroblastoma, and promyelocytic leukemia cells[31–33]. Because of its clear association with tumorigenesis, Ras has

been heavily studied and much is known about the downstream signaling cascades that are modulated by Ras genes. However, the nuances of Ras signaling between cell types and the complexity of signaling affected by Ras has limited the ability to develop broadly effective drugs against hyperactive forms of Ras; an exception being the development of Sotorasib, a small-molecule drug that specifically targets hyperactive alleles of Ras containing a missense mutation that results in cysteine amino acid. Sotorasib works via formation of a covalent (disulfide) bond with the cysteine mutation allowing it to specifically target hyperactive alleles of Ras that contain this relatively rare mutation. There has been little success in targeting other hyperactive variants of Ras.

Below is a brief overview of the current understanding of Ras signaling in the context of cell growth and size with respect to cell cycle progression for both mammalian and yeast models.

Ras signaling in mammalian cells

The discovery of the Ras protein family, and its association with tumor formation, rapidly led to investigations into the underlying signaling mechanisms that surround Ras GTPases. In mammalian cells, Ras is activated by the epidermal growth factor receptor (EGFR) and other receptor tyrosine kinases (RTKs). This signaling interplay allows Ras to coordinate signals from the extracellular environment to intracellular processes like: cell growth, survival, and cell cycle progression. In general, Ras proteins are master regulators of many canonical signaling axes including, most notably, the MAP kinase cascade and the phosphatidylinositol 3-kinase (PI3K) signaling axis **(Figure 1.8)**.

The MAP kinase cascade is perhaps the most well-known signaling output for Ras and begins with Receptor Tyrosine Kinases (RTKs). Upon growth-factor stimulation of RTKs, cross-phosphorylation of the intracellular domains of the RTKs. This phosphorylation pattern recruits Ras-GEFs that directly activate Ras. This activated form of Ras (Ras-GTP) is able to bind and activate many effectors including the rapidly-accelerated fibrosarcoma (RAF) protein. Once activated, RAF works to stimulate a phosphorylation cascade that ultimately leads to the activation of ERK (extracellular signal-regulated kinase). ERK signaling regulates several transcription factors that control various biological processes including: cell cycle progression, proliferation, and survival.

Additionally, Ras is known to bind and activate phosphoinositide 3-kinase (PI3K). Once activated, PI3K generates PIP3 (phosphatidylinositol-3,4-triphosphate), a signaling lipid that activates several downstream substrates. Among them, include the phosphatidylinositol dependent kinase (PDK1). Once bound to PIP3, PDK1, can then recruit and activate Protein Kinase B (PKB, or more commonly referred to as AKT) to the plasma membrane. When activated, AKT has been shown to interact with the atypical S/T protein kinases, mammalian Target of Rapamycin Complexes (TORC1/2). TORC1 is best understood to promote expression of genes required for cell growth, survival, and metabolism[34–36]. Much less is known about TORC2; however, AKT has been found to be a TORC2 substrate for phosphorylation leading to AKT activation[37–40]. Furthermore, previous works in budding yeast have found that TORC2 signaling is a master regulator for cell growth[41,42].

While it is clear that normal Ras signaling in mammalian cells is required for cell cycle progression, high aberrant Ras activity promotes accumulation of cyclin kinase inhibitors (CKI's) which are negative regulatory components for cyclin-cdk complexes [43–46]. However, wild type signaling from Ras fails to increase CKI protein expression[43,45,46]. Therefore, Ras signaling in the context of a wild type cell seems to function as pro-growth and pro-cell cycle progression. However, oncogenic mutations in Ras that result in constitutive signaling can produce high levels of Ras signaling. This causes an increase in cyclin D levels in addition to CKI's that work to inhibit cell cycle progression. One model suggests that the increased abundance of cyclin D levels upon hyper-activation of Ras functions as a molecular sink for CKI's to permit functional cyclin E-Cdk2 complexes and allow cell cycle entry. However, the data are mixed. Another study found that hyperactivation of Ras is insufficient to drive an increase in cyclin E levels[47]. Other investigations that tested the biochemical activity of presumably cyclin E-cdk1 complexes found that kinase activity was increased following hyperactive Ras signaling[44]. Although, it is unclear if the same is true in vivo, because the interpretations rely on an increase in histone H1 phosphorylation by cyclin E-cdk. Furthermore, the reported data only show cyclin E-cdk dependent phosphorylation of histone H1 from in vitro kinase assays and not the canonical Rb substrate[44]. Furthermore, there is conflicting evidence between different cell types regarding the effects of hyperactive Ras on cyclin E accumulation and activity. For example, expressing oncogenic Ras in HeLa cells has been shown to modulate cyclin E levels via inhibition of its degradation by the Fbw7 pathway[48]. However, expression of hyperactive Ras failed to modulate

cyclin E levels and activity in primary fibroblasts and REF52 cells[47]. Although these data implicate Ras signaling in the control of G1 cyclin expression and activity, it is difficult to interpret what the functional consequence of hyperactive Ras signaling is throughout the cell cycle, in vivo.

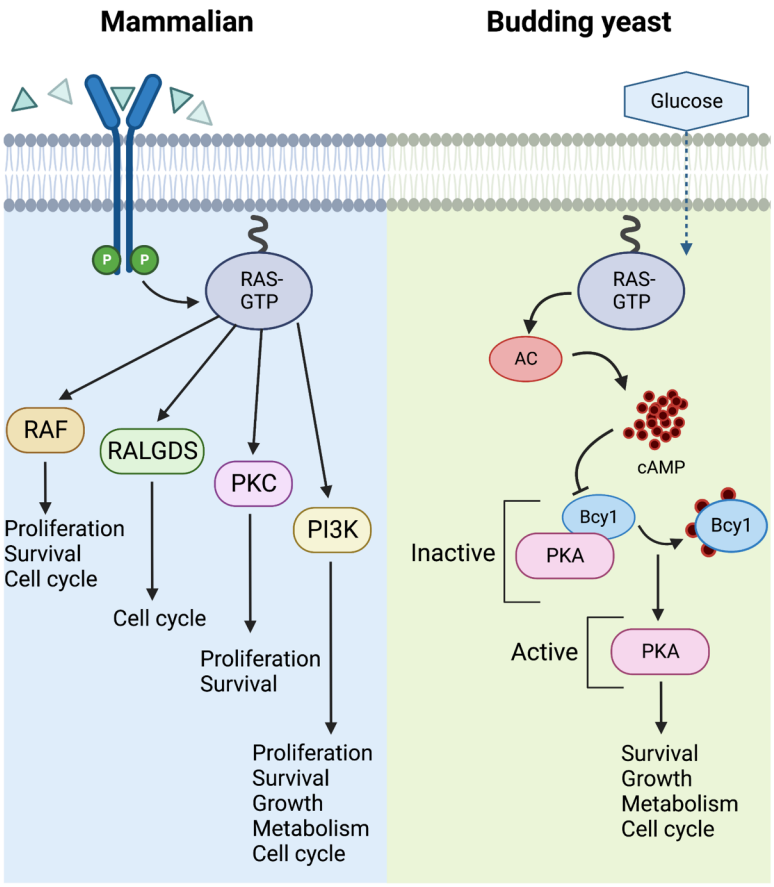

Figure 1.8. Canonical signaling axes stimulated by Ras in mammalian and yeast cells.

Ras signaling in budding yeast

Ras proteins are highly conserved across species and have homologs in budding yeast. This has facilitated work to understand the cellular functions and signaling of Ras, some of which directly translates to mammalian cell biology and

cancer. However, because of the complexity of signaling networks affected by Ras, many of these foundational investigations were limited by the technologies available and relied on indirect manipulation and/or stimulation of Ras. Although limited, these early investigations were nonetheless significant contributions to our current understanding of Ras-dependent signaling and activation.

In the 1980's, investigations carried out in budding yeast identified Ras proteins in the control of cAMP production and ultimately activation of Protein Kinase A[49,50]. Prior to these investigations, it was only known that Ras proteins function as GTPases and are somehow involved in mammalian cell transformation, but very little was understood regarding the molecular mechanisms surrounding Ras. Therefore, these early investigations in budding yeast provided the first glimpses into the underlying biology of Ras. In yeast, Ras proteins, were found to control cAMP production[49–52]. However, prior to discovering that yeast Ras proteins control cAMP production there were conflicting reports that linked cAMP to cell cycle regulation and progression. Some investigators found that addition of exogenous cAMP stimulates cell cycle entry[53,54]. Others found evidence to the contrary – that exogenous cAMP delays the cell cycle[55–58]. Furthermore, researchers also found that yeast cells harboring mutations that result in uncontrolled cAMP production were larger in size[54]. Together, these data provide the first evidence that links Ras and the production of cAMP to the control of cell size, and cell cycle progression; although the underlying mechanisms remained unclear. It is also unclear how aberrant cAMP signaling influences cell size and progression of the cell cycle.

In 1989, researchers examining diploid budding yeast cells found that expression of hyperactive Ras2 (*ras2*^{G19V}) resulted in a large cell size phenotype. Although lacking proper key controls, and not verified in haploids, these data provided the first direct evidence to suggest that Ras-dependent signaling may be involved in cell size control[59]. Yet, under standard growth conditions, the authors failed to detect a change in cell cycle progression. However, in 1994 two groups found that indirect manipulation to stimulate the Ras/cAMP pathway did not affect early G1 cyclin transcription, but did decrease expression of late G1 cyclins, and ultimately delayed cell cycle entry[55,56]. Although, in 1998 it was reported that addition of cAMP produced a substantial increase in expression of the early G1 cyclin, Cln3, and increased levels of subsequent late G1 cyclins as well[60]. Although many of these investigations appear to directly contradict each other, what is clear is that Ras-dependent signaling is somehow important for regulating growth, cell size, and the cell cycle.

In the thesis presented here, I utilize a combination of approaches with modernized techniques to directly investigate the effects of hyperactive Ras signaling on cell growth, size, and cell cycle entry in budding yeast. With this information, I aim to clarify and strengthen the field's current understanding of Ras and provide novel insights into the defects surrounding hyperactive signaling of Ras in the context of mammalian cell biology and cancer.

Chapter 2 (Published results): Hyperactive Ras disrupts cell size and a key step in cell cycle entry in budding yeast

Introduction

Cells within the human body range in size over several orders of magnitude. However, cells of a particular type maintain a constant average size. Thus, cell growth must be tightly controlled to ensure that cells attain and maintain an appropriate cell size [1,61,62]. At the simplest level, the size and shape of a cell must be the outcome of conserved mechanisms that determine the extent, location, and timing of cell growth. In dividing cells, size is controlled by mechanisms that link cell cycle progression to cell growth. The mechanisms that control cell growth are modulated by nutrients – poor nutrients reduce the threshold amount of growth required for cell cycle progression, leading to a reduction in cell size [63]. The mechanisms that control cell growth and size remain poorly understood.

Previous studies in budding yeast suggested that a Ras signaling network is required for normal control of cell size. Yeast Ras is encoded by a pair of redundant paralogs referred to as *RAS1* and *RAS2*. Cells lacking either paralog are viable but loss of both is lethal [49]. The functions of yeast Ras are best understood in the context of a signaling network that is activated by glucose. High glucose activates Ras1/2, which then activate adenylate cyclase to produce cAMP. The cAMP binds Bcy1, an inhibitory subunit for the yeast homolog of cAMP-dependent protein kinase (PKA) [50,54]. Binding of cAMP to Bcy1 causes it to dissociate from PKA, leading to release of active PKA that initiates a signaling

network with pervasive effects on control of cell growth and metabolism [51,64]. A hyperactive allele of RAS2 (*ras2*^{G19V}) that is analogous to oncogenic alleles of mammalian Ras was found to cause an increase in cell size in diploid cells that also contain a mutant allele of *CDC25*, the budding yeast Ras-GEF [52,59]; however, the effects of hyperactive *ras2*^{G19V} on cell size have not been tested in a wild type background. Deletion of *IRA2*, a budding yeast Ras-GAP that is conserved in mammals, also causes an increase in cell size [65]. Furthermore, a weakly constitutive allele of PKA in a *bcy1*Δ background causes a failure in nutrient modulation of cell size [56]. Together, these observations suggest that a Ras-PKA signaling axis influences cell size and plays a role in nutrient modulation of cell size; however, the mechanisms are poorly understood.

Ras proteins are highly conserved and mammalian Ras homologs serve as master regulators of growth, proliferation, metabolism and survival[66,67]. Ras family members are amongst the most frequently mutated oncogenic drivers; it is thought that between 25-30% of all human cancers have oncogenic mutations in one or more Ras genes[68]. A potential role for Ras family members in controlling cell size is intriguing, since severe defects in cell size homeostasis are broadly linked to cancer[9–14]. For example, most cancers are associated with greater heterogeneity of cell size and shape, as well as dramatically altered nuclear-to-cytoplasmic volume ratios. Defects in cell size and shape have long been used by pathologists to diagnose cancer, and increased heterogeneity of cell size is associated with poor prognosis[9]. The size defects of cancer cells must be caused, either directly or indirectly, by oncogenic signals. However, the mechanisms by which oncogenic signals influence cell growth and size are

largely unknown, and it is unclear whether the size defects of cancer cells are a direct consequence of primary oncogenic signals or a secondary consequence of mutations that accumulate during evolution of cancer cells. The fact that diverse cancers show common defects in cell size suggests the possibility that diverse oncogenic signals converge on common conserved pathways for size control.

Since it is unknown how oncogenic signals influence cell size in human cells, or how hyperactive forms of Ras influence cell size in yeast, it is possible that oncogenic Ras influences cell size via mechanisms that are conserved from yeast to humans. Thus, analysis of how hyperactive Ras influences cell size in yeast could provide new clues to the functions of oncogenic Ras in human cells. Here, we have carried out new analyses of the effects of hyperactive Ras in budding yeast, utilizing modern methods that allow rapid inducible expression of *ras2*^{G19V} at endogenous levels in otherwise wild type cells, which allowed us to discern the immediate consequences of hyperactive Ras during the cell cycle.

Results

Hyperactive Ras increases cell size in budding yeast and mammalian cells

Previous experiments examined the effects of hyperactive Ras expressed from the endogenous promoter on cell size in a mutant background [59]. We therefore started by analyzing the effects of hyperactive Ras on cell size in an otherwise wild type background. A hyperactive version of *RAS2* can be generated by mutating glycine 19 to a valine (*ras2*^{G19V}), which is analogous to oncogenic versions of mammalian Ras. We generated a strain that expresses *ras2*^{G19V} from the endogenous promoter and measured cell size with a Coulter Channelyzer. The *ras2*^{G19V} allele caused an increase in cell size (**Figure 2.1A**). The effects of *ras2*^{G19V} were slightly stronger in a *ras1*Δ background (**Figure 2.1–figure supplement 1A**)

As an independent means of generating hyperactive Ras, we deleted one of the GAPs that contribute to inactivation of Ras. GAPs for Ras are encoded by two partially redundant paralogs referred to as *IRA1* and *IRA2*. Deletion of *IRA2* causes cells to proliferate more slowly, whereas deletion of *IRA1* does not have an obvious phenotype. Deletion of both genes is lethal. We found that *ira2*Δ caused an increase in cell size, which provided further evidence that hyperactive Ras influences cell size (**Figure 2.1B**). A previous genome-wide search for gene deletions that influence cell size also found that *ira2*Δ causes increased cell size [65]. We next tested the effects of loss of function of Ras1 and Ras2. While *ras1*Δ did not affect cell size, *ras2*Δ caused a modest decrease (**Figure 2.1C and Figure 2.1–figure supplement 1B**).

Although many cancer cell lines show defects in cell size[14], it is unclear whether the defects are a direct consequence of oncogenic signals or a secondary consequence of mutations that accumulate as cells adapt to aberrant oncogenic signals. No previous studies have directly tested the effects of a major oncogenic driver on cell size in the absence of the many "passenger" mutations found in cancer cells. We therefore utilized a previously generated NIH 3T3 cell line that expresses $HRas^{G12V}$ to test whether oncogenic Ras alone is sufficient to cause cell size defects[69]. We used a Coulter Channelyzer to compare the size of the $HRas^{G12V}$ cells to an isogenic control. $HRas^{G12V}$ caused a substantial increase in cell size (**Figure 2.1D**).

These results establish that Ras activity influences cell size in both budding yeast and mammalian 3T3 cells.

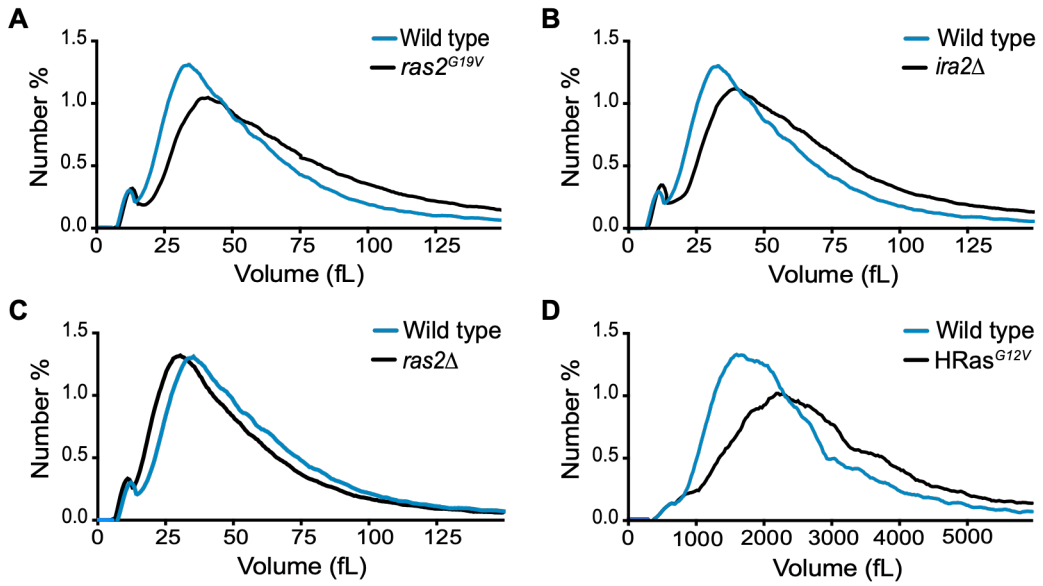

Figure 2.1. Hyperactive Ras increases cell size in budding yeast. (A) Wild type and *ras2*^{G19V} yeast cells were grown to log phase in YPD and cell size was measured using a Coulter counter. (B) Wild type and *ira2*Δ budding yeast cells were grown to log phase in YPD and cell size was measured using a Coulter counter. (C) Wild type and *ras2*Δ budding yeast cells were grown to log phase in YPD and cell size was measured using a Coulter counter.

An inducible and titratable system for expression of *ras2^{G19V}* in yeast

A previous study found that *ras2^{G19V}* causes decreased viability [49], and we found that *ras2^{G19V}* cells show a large reduction in the rate of proliferation and rapidly accumulate suppressor mutations. The effects of hyperactive Ras on cell size could therefore be an indirect consequence of long-term adaptation to constitutive Ras activity. To circumvent this problem, we utilized a previously developed estradiol-inducible promoter to achieve tight temporal and titratable control of *RAS* gene expression in a nutrient independent manner[70]. In brief, cells were engineered to express a fusion protein that includes the bacterial LexA DNA-binding domain, the human estrogen receptor (ER), and a transcriptional activation domain (AD). In addition, a promoter containing two LexA binding sites was integrated in front of the wild type endogenous *RAS2* coding sequence or in front of *ras2^{G19V}*. In this context, addition of ß-estradiol drives transcription of *RAS2* or *ras2^{G19V}* **(Figure 2.2A)**.

To determine the time required to reach peak protein expression, we created strains that express *RAS2-3xHA* and *ras2^{G19V}-3xHA* from the *lexA* promoter (*lexA-RAS2-3xHA* and *lexA-ras2^{G19V}-3xHA)*. Peak protein expression was reached within 60-90 minutes after addition of estradiol (**Figure 2.2B**). Moreover, we found that 200 nM estradiol induced expression of *lexA-RAS2-3xHA* and *lexA-ras2^{G19V}-3xHA* at protein levels similar to *RAS2-3xHA* expressed from the endogenous promoter.

For analysis of *ras2^{G19V}* phenotypes, we utilized untagged versions of *RAS2* (*lexA-RAS2* and *lexA-ras2^{G19V}*). We first tested the effects of inducing expression of *ras2^{G19V}* on cell proliferation. Serial dilutions of cells containing

lexA-ras2^{G19V} and control cells were plated on media containing increasing concentrations of estradiol. Expression of *ras2*^{G19V} at endogenous levels (100-200 nM estradiol) caused a large reduction in the rate of proliferation (**Figure 2.2C**). Expression of higher levels of *ras2*^{G19V} with 400 nM estradiol was nearly lethal.

We next tested whether expression of *ras2*^{G19V} from the *lexA* promoter causes an increase in cell size. Prolonged expression of *ras2*^{G19V} for 12 hours caused a large increase in cell size (**Figure 2.2D**). The increase in cell size was detectable within 3 hours of inducing expression, which suggests that it is a rapid and direct consequence of ras2^{G19V} protein expression (**Figure 2.2E**).

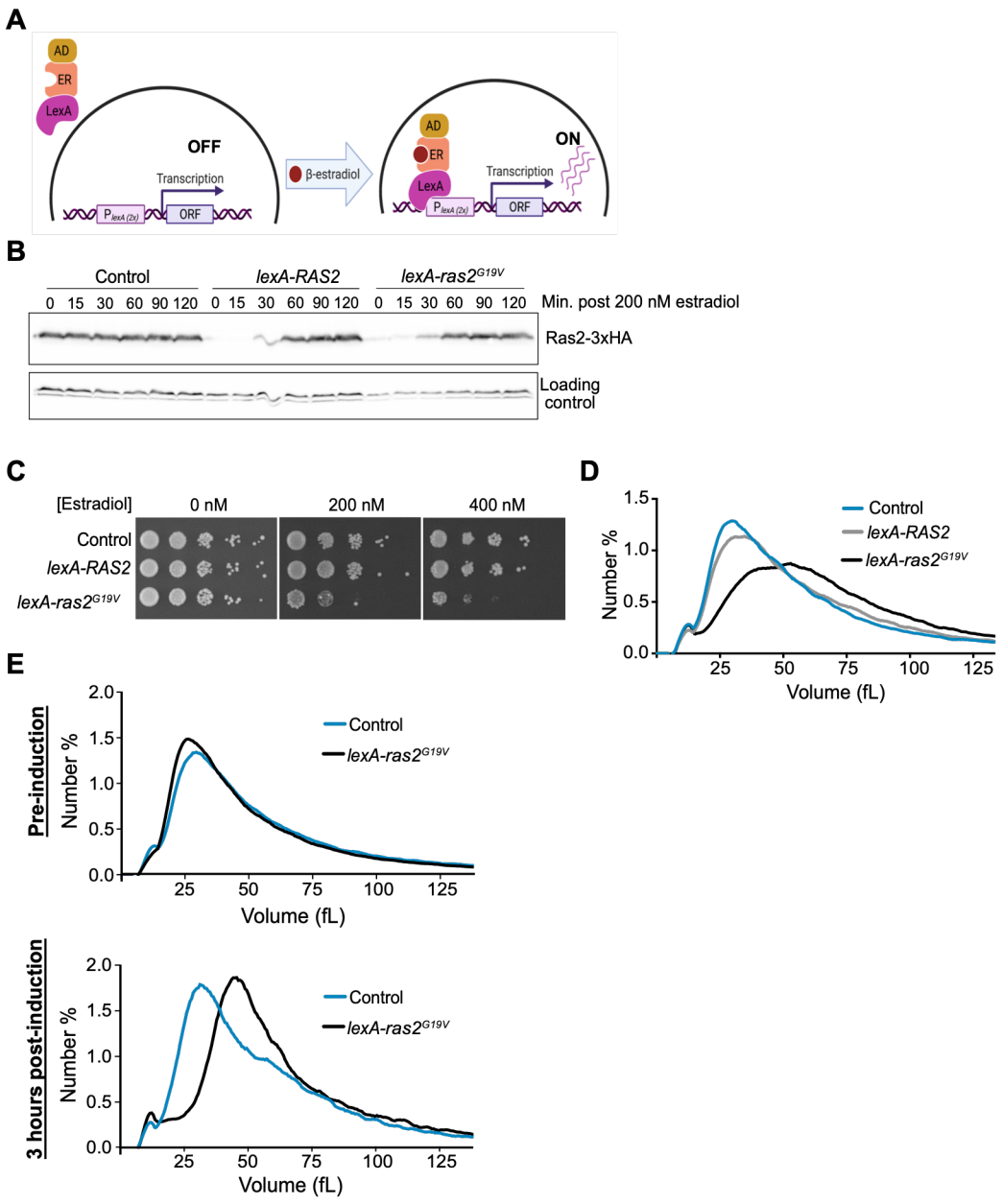

Figure 2.2. An inducible and titratable expression system for expression of ras2^{G19V} in yeast. (A) A diagram of the LexA-ER-AD system for estradiol-dependent gene expression. (B) Estradiol was added to 200 nM and time points were collected at the indicated intervals to measure timing for peak levels of Ras2 or ras2^{G19V} protein expression relative to endogenous levels. (C) Serial dilutions of the indicated strains were spotted onto YPD medium containing the indicated concentration of estradiol. (D) Cells were grown overnight to log phase in YPD + 100 nM estradiol and cell size was measured using a Coulter counter. (E) Cells were grown overnight to log phase in YPD and expression of *ras2^{G19V}* was induced with 100 nM estradiol for 3 hours. Cell size was measured using a Coulter counter.

Expression of *ras2*^{G19V} influences cell cycle progression and cell size

We next set out to learn more about how *ras2*^{G19V} influences cell size. To do this, we first analyzed how *ras2*^{G19V} influences the relationship between cell growth and cell cycle progression. Previous studies analyzed the effects of hyperactive Ras on the cell cycle indirectly by manipulating cAMP levels to mimic Ras-dependent signals that control PKA, or by deleting the BCY1 gene, which leads to a high level of constitutive PKA signaling [50,54–56,60,71]. Together, these studies suggested a link between cAMP-dependent signaling and regulation of G1 cyclin expression; however, in some cases the studies reached conflicting conclusions. For example, two studies found that cAMP-dependent signaling stimulates production of *CLN3* mRNA [56,71] whereas other studies observed no effect [60,72]. Similarly, several studies found that cAMP-dependent signaling increases transcription of *CLN1* and *CLN2* [60,72] whereas two other studies reported a decrease in transcription [55,56]. Differences in results could be due to technical differences in how the experiments were carried out.

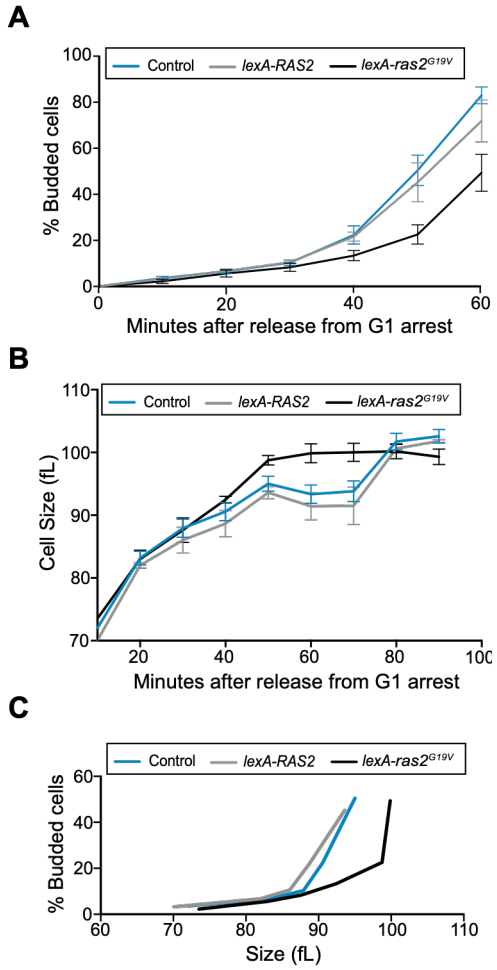

Figure 2.3. Expression of *ras2^{G19V}* influences cell size and delays cell cycle entry.
(A-C) Cells were grown to log-phase overnight in YPD and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC. Cells were treated with 200 nM estradiol beginning 1.5 hours before release from the arrest. (A) The percentage of budded cells as a function of time. (B) Median cell size was measured at 10 min intervals using a Coulter Counter and plotted as a function of time. (C) The percentage of budded cells as a function of cell size. Error bars represent SEM of three biological

Previous investigations were limited by the tools available at the time and were unable to analyze the immediate effects of Ras-dependent signaling on the cell cycle. Moreover, although production of cAMP is a well-established output of Ras-dependent signaling in budding yeast, it is possible that Ras has targets beyond cAMP production. An additional limitation of previous studies is that they did not analyze how cAMP-dependent signals influence G1 cyclin protein expression during the cell cycle.

Here, we directly examined how expression of *lexA-ras2^{G19V}* influences cell cycle progression, cell growth, and cyclin expression in synchronized cells. Cells were arrested in G1 phase with mating pheromone and expression of *RAS2* or *ras2^{G19V}* from the *lexA* promoter was induced prior to release from the arrest. Wild type cells that express *RAS2* from the endogenous

promoter were included as a control. Since bud emergence marks cell cycle entry, we first analyzed the percentage of cells undergoing bud emergence as a function of time. Expression of *lexA-ras2*^{G19V} caused a prolonged delay in bud emergence (**Figure 2.3A**). We also analyzed cell size as a function of time with a Coulter Channelyzer (**Figure 2.3B**) and plotted bud emergence as a function of cell size (**Figure 2.3C**). *ras2*^{G19V} caused cells to undergo bud emergence at a larger cell size. These data are consistent with a previous report that increased levels of cAMP inhibit cell cycle entry and cause increased cell size at the G1/S transition (Tokiwa et al., 1994).

Expression of *ras2*^{G19V} blocks a key step in cell cycle entry

We next analyzed the effects of *ras2*^{G19V} on expression of G1 phase cyclins. In budding yeast, a cyclin called *CLN3* is expressed in early G1 phase and accumulates gradually [41,73,74]. Accumulation of Cln3 protein is correlated with growth and appears to be dependent upon cell growth, which suggests that it could be a readout of the extent of growth [41,73,75]. Cln3 eventually triggers transcription of a redundant pair of late G1 cyclin paralogs called *CLN1* and *CLN2* [76–78]. Expression of late G1 cyclins is the key molecular event that drives cell cycle entry. To assay accumulation of both early and late G1 cyclins, we used a strain that contains Cln3-6xHA and Cln2-13xMyc. Wild type, *lexA-RAS2* and *lexA-ras2*^{G19V} cells were released from a G1 arrest and the behavior of Cln3-6xHA and Cln2-13xMyc were assayed by western blot during the cell cycle. *ras2*^{G19V} caused a nearly 3-fold increase in the expression of Cln3-6xHA protein in G1 phase, as well as delayed and decreased expression of Cln2-13xMyc

(**Figure 2.4 and Figure 2.4–figure supplement 1A**). Expression of *lexA-ras2*^{G19V} also caused a decrease in Cln2-13Myc levels in asynchronously growing cells (**Figure 2.4–figure supplement 1B**).

Previous studies suggested that increased expression of *CLN3* drives premature cell cycle entry [79–81]. Thus, the finding that *ras2*^{G19V} strongly promotes expression of Cln3, yet inhibits expression of Cln2, was unexpected.

We next used northern blotting to test whether *ras2*^{G19V} influences

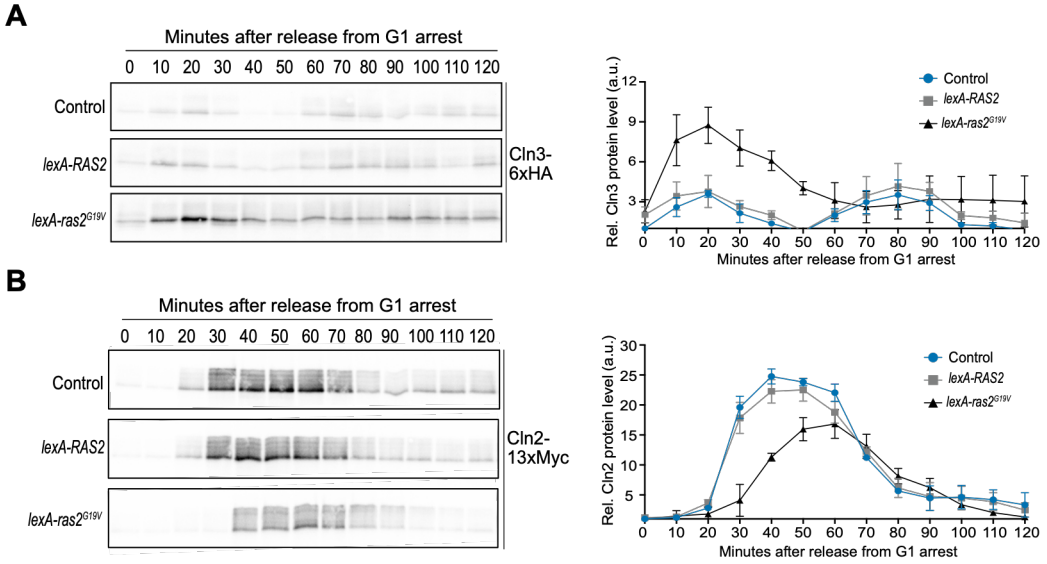

Figure 2.4. *ras2*^{G19V} influences expression of G1 phase cyclins. (A-B) Cells were grown to log-phase overnight in YPD and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC. Expression of *RAS2* or *ras2*^{G19V} was induced with 200 nM estradiol beginning 1.5 hours before release from the arrest. (A) The levels of Cln3-6xHA protein were analyzed by western blot. (B) The levels of Cln2-13xMyc protein were analyzed by western blot. The representative western blots in (A) and (B) are from the same experiment and protein abundance was quantified relative to levels in wildtype cells at time point 0 after first normalizing to a loading control, see Figure 4–figure supplement 1. Error bars represent SEM of three biological replicates. Loading controls are shown in Figure 4 –figure supplement 1. For both plots, protein abundance was plotted as a ratio over the signal in wild type control cells at t=0 after first normalizing to the loading control.

transcription of *CLN2*. *ras2*^{G19V} caused a delay in *CLN2* transcription and a reduction of *CLN2* mRNA levels (**Figure 2.5A**). To test whether *ras2*^{G19V} also

influences expression of *CLN2* via post-transcriptional mechanisms, we replaced the endogenous *CLN2* promoter with the heterologous *MET25* promoter. In this context, *ras2*^{G19V} no longer delayed Cln2 protein expression and caused a small decrease in protein levels, although it was unclear whether the decrease was significant (**Figure 2.5B**). This observation suggests that *ras2*^{G19V} influences *CLN2* expression largely via transcriptional mechanisms.

Induction of late G1 cyclin transcription by Cln3 is thought to be the critical molecular step that initiates cell cycle entry. It is also thought to be the step where cell growth influences cell cycle entry. Thus, the discovery that expression of *ras2*^{G19V} promotes high level expression of Cln3, yet fails to induce normal expression of Cln2, suggests that *ras2*^{G19V} inhibits a key step in cell cycle entry.

ras2^{G19V} causes decreased recruitment of Cln3 to the *CLN2* promoter

Previous work has shown that Cln3 is recruited to promoters controlled by SBF, including the *CLN2* promoter, where it has been proposed to activate Cdk1 to directly phosphorylate RNA polymerase [82,83]. To test whether *ras2*^{G19V} disrupts recruitment of Cln3 to the *CLN2* promoter, we used ChIP to analyze recruitment of Cln3 to *CLN2* promoters in *lexA-ras2*^{G19V} cells. We found that *ras2*^{G19V} caused a 3-fold reduction in the amount of Cln3 that is recruited to the *CLN2* promoter

(**Figure 2.5C**). The mechanisms by which Cln3 is recruited to the *CLN2*

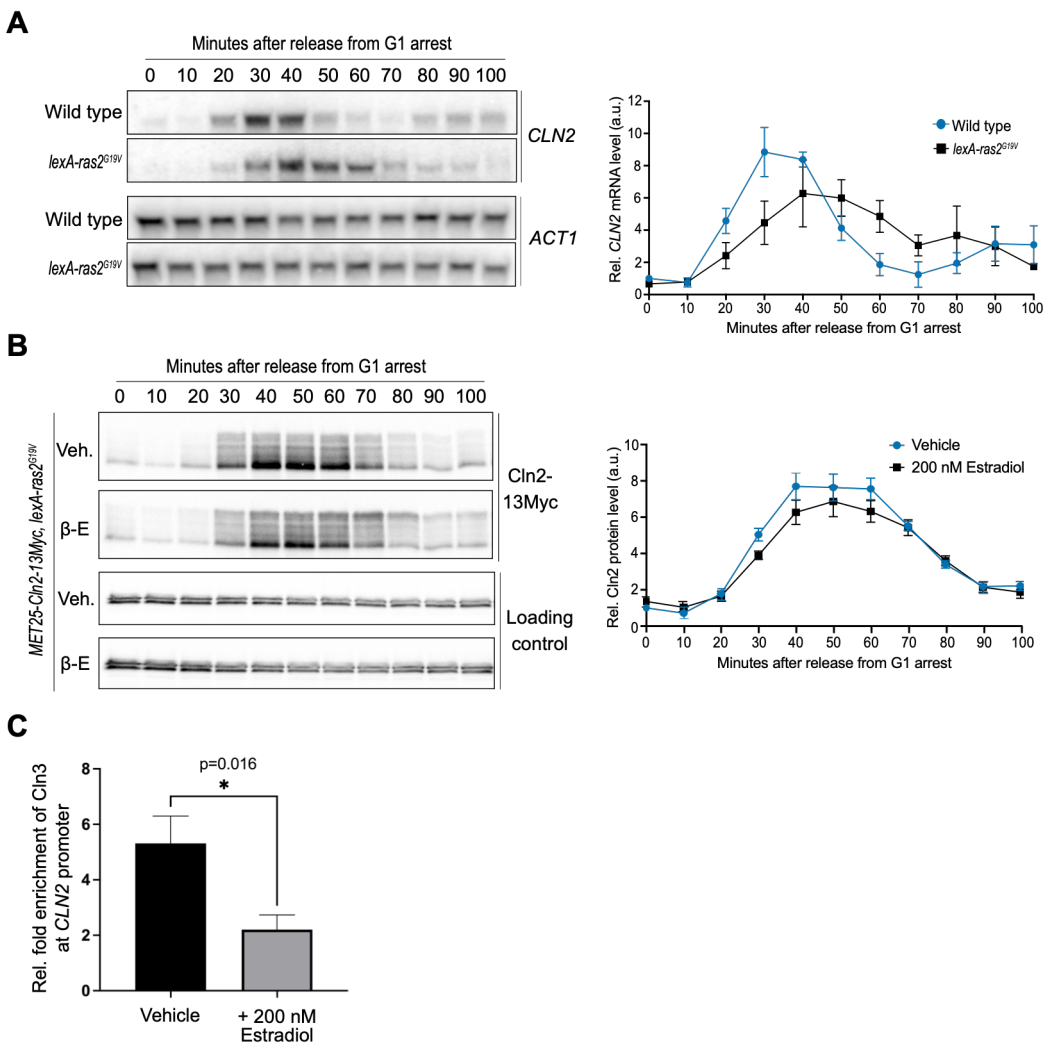

Figure 2.5. Expression of *ras2*^{G19V} modulates CLN2 at the level of transcription. (A-B) Cells were grown to log-phase overnight in YPD and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC. Expression of *RAS2* or *ras2*^{G19V} was induced with 200 nM estradiol beginning 1.5 hours before release from the arrest. (A) Levels of *CLN2* mRNA were analyzed by northern blot. Levels of the *ACT1* mRNA were analyzed as a loading control. After normalizing to the loading control, the *CLN2* mRNA signal was quantified and plotted as a ratio over the signal at t=0 in the wild control cells. Error bars represent SEM of three biological replicates. (B) Levels of Cln2-13xMyc protein were analyzed by western blot. Protein abundance was plotted as a ratio over the signal in wildtype cells at t=0 after first normalizing to the loading control. Error bars represent SEM of three biological replicates. Anti-Nap1 was used for a loading control. (C) For ChIP experiments, cells were grown to log-phase overnight in YPD and expression of *RAS2* or *ras2*^{G19V} was induced with 200 nM estradiol for 3 hours at 30ºC. Cln3-6xHA was immunoprecipitated and qPCR was conducted to determine relative fold enrichment of Cln3-6xHA at the *CLN2* promoter. *p = 0.016 by student's t test.

promoter are unknown so we are unable to define the molecular defect that obstructs Cln3 recruitment.

PKA influences cell size and expression of G1 phase cyclins

The fact that Ras activates PKA suggests that the effects of *ras2*^{G19V} on cell size could be mediated by PKA. Furthermore, it has been proposed that PKA can influence expression of G1 phase cyclins via inhibition of Whi3, an RNA binding protein that is thought to bind and inhibit the expression of dozens of mRNAs, including those for *CLN2* and *CLN3* [71,84,85]. *WHI3* was originally discovered in a screen for loss of function mutants that cause reduced cell size [86]. Although one study found no effect of *whi3*Δ on Cln3 protein expression [87], a more recent study found that *whi3*Δ causes an increase in the abundance and translation efficiency of the *CLN3* mRNA, which could account for the reduced cell size of *whi3*Δ cells [84,85]. Mutation of a PKA consensus site within Whi3 causes reduced binding of Whi3 to *CLN3* mRNA; however, it remains unknown whether this site is phosphorylated by PKA in vivo [71]. Together, these observations suggest a model in which *ras2*^{G19V} influences G1 phase cyclin levels and cell size via PKA-dependent inhibition of Whi3.

To begin to test this model, we first analyzed the effects of modulating PKA activity on cell size and cell cycle progression. Prior work tested the effects of increased PKA activity by deleting the *BCY1* gene, which encodes the inhibitory subunit for PKA [49,50,54,88]. Loss of *BCY1* leads to increased cell size [55,56]. However, we found that *bcy1*Δ is lethal in the strain background used here

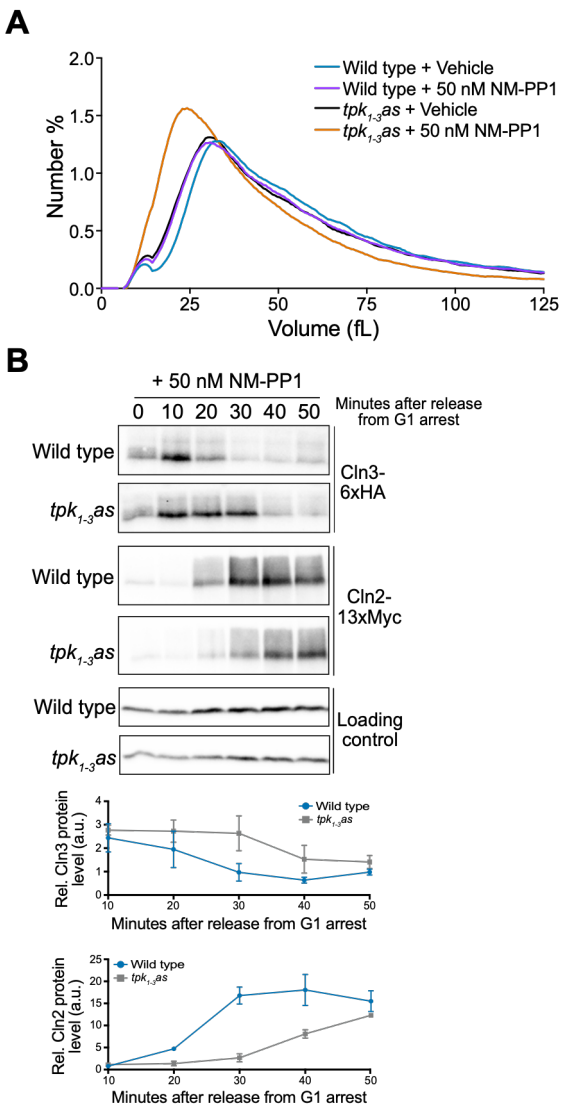

Figure 2.6 PKA activity influences cell size and expression of G1 phase cyclins. (A) Cells were grown to log phase in YPD + vehicle or 50 nM 1NM-PP1 and cell size was measured using a Coulter Counter. (B) Cells were grown to log-phase in YPD and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC and released into YPD with 100 nM 1NM-PP1. Cln3-6xHA and Cln2-13xMyc protein were analyzed by western blot. Protein abundance was plotted as a ratio over the signal in wildtype cells at t=0 after first normalizing to the loading control. Error bars represent SEM of 3 biological replicates. Loading control was anti-Nap1.

(W303). We therefore used an analog-sensitive version of PKA to analyze the effects of decreased PKA activity. PKA in budding yeast is encoded by three redundant genes referred to as *TPK1*, *TPK2* and *TPK3*. A previous study generated cells in which all three *TPK* genes carry mutations that make them sensitive to the adenine analog inhibitor 1NM-PP1 (*pka-as*) [64]. If the effects of $ras2^{G19V}$ are due to hyperactive PKA, then reduced activity of PKA would be expected to cause effects that are opposite to the effects of hyperactive Ras alone.

We found that *pka-as* cells showed substantially reduced cell size in response to a non-lethal dose of inhibitor (50 nM) (**Figure 2.6A**). Inhibition of PKA had little effect on peak levels of Cln3,

although it did prolong the interval of expression of Cln3 in G1 phase **(Figure 2.6B)**. Expression of Cln2 protein was reduced and delayed by inhibition of PKA, similar to the effects of *ras2*^{G19V}.

The observed effects of inhibiting PKA are difficult to reconcile with simple models for *ras2*^{G19V} functions based on previous studies. The discovery that *ras2*^{G19V} and inhibition of PKA have opposite effects on cell size would appear to be consistent with a model in which *ras2*^{G19V} drives an increase in cell size via hyperactivation of PKA. However, the discovery that *ras2*^{G19V} and inhibition of PKA have similar effects on Cln2 protein levels and that inhibition of PKA has little effect on peak levels of Cln3 suggests that the effects caused by hyperactive Ras signaling cannot be explained solely by changes in the activity of PKA. Rather, the data suggest that hyperactive Ras signaling could be influencing Cln2 levels via PKA-independent mechanisms.

The effects of *ras2*^{G19V} are not due solely to inhibition of Whi3

To investigate the relationship between *ras2*^{G19V} signaling and Whi3, we compared cell size, the timing of bud emergence, and expression of Cln3 and Cln2 protein in wildtype, *whi3*Δ, *lexA-ras2*^{G19V}, and *lexA-ras2*^{G19V} *whi3*Δ cells. If *ras2*^{G19V} influences expression of Cln3 or Cln2 via PKA-dependent inhibition of Whi3, one would expect *whi3*Δ and *ras2*^{G19V} to cause similar effects on expression of Cln3 and Cln2 protein levels.

We found that *whi3*Δ accelerated the timing of bud emergence and caused reduced cell size, as expected (**Figure 2.7A,B**). Moreover, in contrast to a previous study [87] we found that *whi3*Δ caused a substantial **1**). increase in

Cln3 protein levels (**Figure 2.7C and Figure 2.7–figure supplement** factor was added back to the cells to prevent a second cell cycle, so the second peak in Cln3 levels corresponds to the second mitotic peak of Cln3 that has been reported previously [73,74]. *whi3*Δ also accelerated expression of Cln2 protein, as expected for increased Cln3 protein levels (**Figure 2.7D and Figure 2.7–figure supplement 1**).

The effects of *lexA-ras2*^{G19V} expression and *whi3*Δ on Cln3 protein levels were similar, as both caused a substantial increase in Cln3 protein levels. Moreover, the effects of *whi3*Δ and *lexA-ras2*^{G19V} on peak Cln3 protein levels were not additive in G1 phase in *lexA-ras2*^{G19V} *whi3*Δ cells. Thus, it is possible that *ras2*^{G19V} drives an increase in Cln3 protein levels via inhibition of Whi3. However, *whi3*Δ and expression of *lexA-ras2*^{G19V} caused opposite effects on the timing of bud emergence, cell size, and accumulation of Cln2 protein. Thus, the effects of *ras2*^{G19V} cannot be explained solely by inhibition of Whi3.

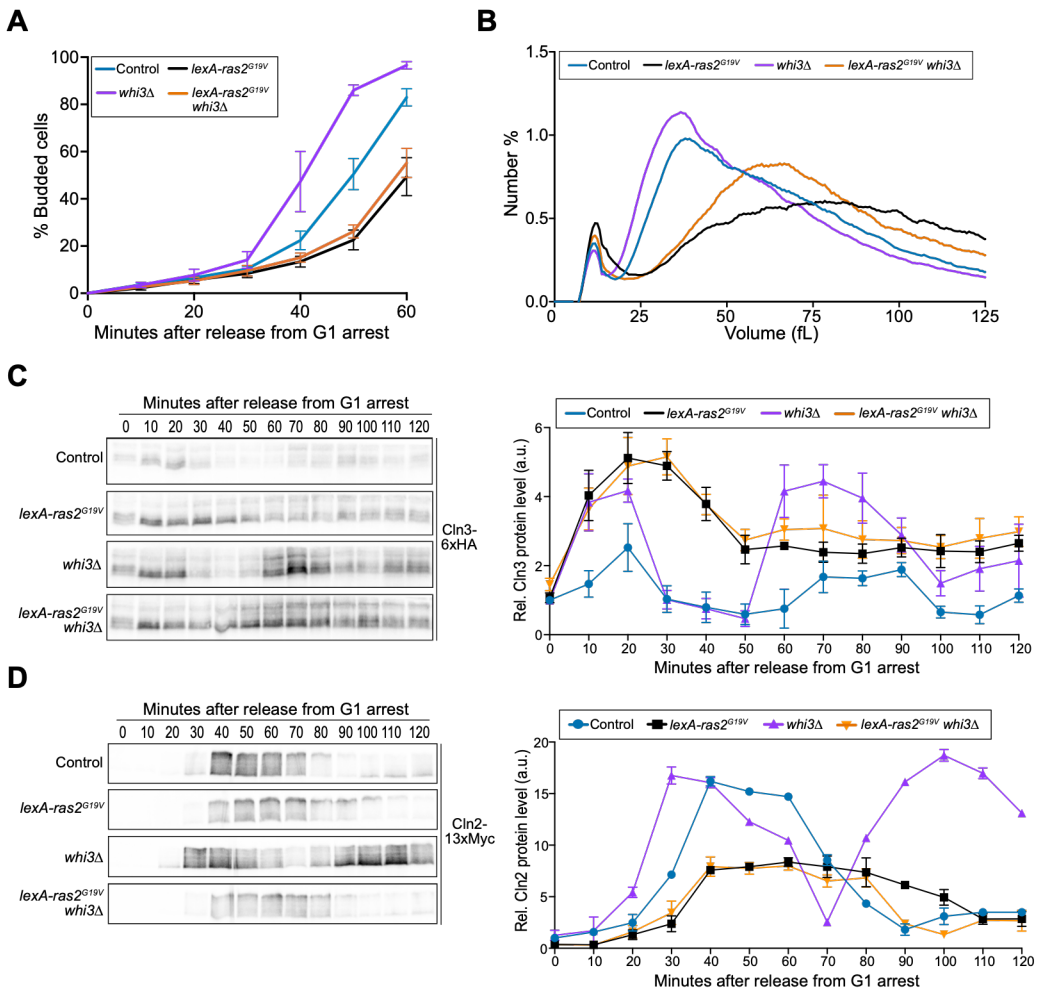

Figure 2.7. The effects of ras2^{G19V} are not due solely to inhibition of Whi3. (A-C) Cells were grown to log-phase overnight in YPD and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC. Cells were treated with 200 nM estradiol beginning 1.5 hours prior to release. (A) The percentage of budded cells as a function of time. (B) The levels of Cln3-6xHA protein were analyzed by western blot and protein abundance was quantified relative to levels of Cln3-6xHA in wildtype cells at t=0 after first normalizing to a loading control. The loading control is shown in Figure 7-figure supplement 1. Error bars represent SEM of three biological replicates. (C) The levels of Cln2-13xMyc protein were analyzed by western blot and protein abundance was quantified relative to levels of Cln2-13xMyc in wildtype cells at t=0 after first normalizing to a loading control. The loading control is shown in Figure 7-figure supplement 1. Error bars represent SEM of three biological replicates. Error bars represent SEM of three biological replicates. (D) Cells were grown overnight to log phase in YPD + 100 nM estradiol and cell size was measured using a Coulter counter.

*whi3*Δ also caused a strong increase in the second peak of Cln3 later in the cell cycle (**Figure 2.7C and Figure 2.7–figure supplement 1**). Note that alpha

The effects of *ras2*^{G19V} on late G1 cyclin expression are dependent upon Whi5

Genetic data suggest that Cln3 promotes transcription of *CLN2* at least partly via inhibition of Whi5, which binds and inhibits the SBF transcription factor that promotes *CLN2* transcription[26,27]. We found that *whi5*Δ largely rescued the delays in Cln2 expression and cell cycle progression caused by *ras2*^{G19V} **(Figure 2.8A)**. This observation provides further support for the idea that expression of *lexA-ras2*^{G19V} blocks a key step in the mechanisms by which Cln3 initiates transcription of *CLN2*, and it suggests that *ras2*^{G19V} may prevent inhibition of Whi5. However, the mechanisms by which Cln3 promotes inhibition of Whi5 are poorly understood[82,89]. Deletion of *WHI5* only partially rescued the cell size defects caused by *ras2*^{G19V}, which suggests that *ras2*^{G19V} influences cell size via mechanisms that operate outside of G1 phase **(Figure 2.8B)**.

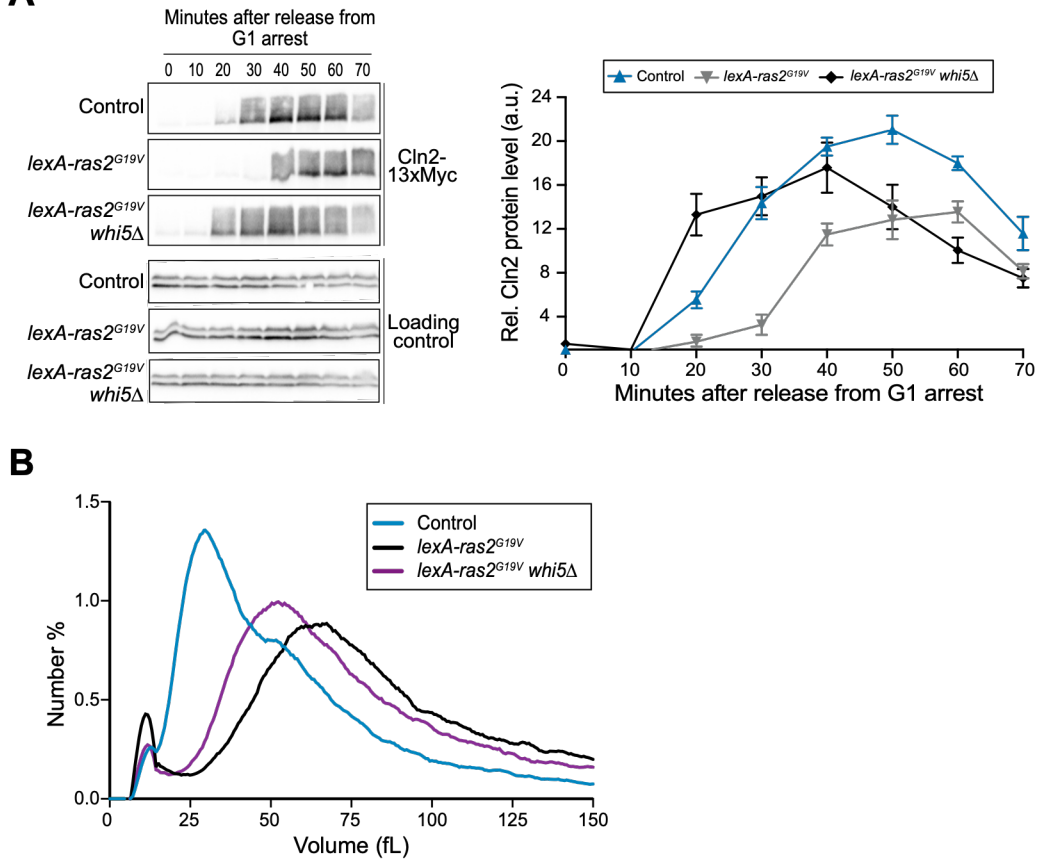

Figure 2.8. The effects of *ras2*^{G19V} on late G1 cyclin expression are dependent upon Whi5. (A) Cells were grown to log-phase overnight in YPD and then arrested in G1 phase with alpha factor for 3 hours at 30ºC. Cells were treated with 200 nM estradiol beginning 1.5 hours prior to release. The levels of Cln2-13xMyc protein were analyzed by western blot and protein abundance was quantified relative to levels of Cln2-13xMyc in wildtype cells at t=0 after first normalizing to the loading control. Error bars represent SEM of three biological replicates. Anti-Nap1 was used for a loading control. (B) Cells were grown overnight to log phase in YPD + vehicle or 100 nM estradiol and cell size was measured using a Coulter counter.

Cln2 may influence expression of Cln3 via negative feedback

ras2^{G19V} and inhibition of PKA both caused Cln3 protein to persist for a longer interval in G1 phase (**Figures 2.4A, 2.6B, and 2.7C**). In each context, prolonged expression of Cln3 protein was accompanied by reduced and delayed accumulation of Cln2. A potential explanation for these observations is that Cln2

is required for downregulation of Cln3. This kind of negative feedback regulation has been observed at other stages in the cell cycle. For example, mitotic cyclins repress expression of cyclins that appear earlier in the cell cycle[90]. To test this idea, we determined whether gain-of or loss-of-function of *CLN1/2* influences expression of Cln3 protein in synchronized cells. We found that over-expression of *CLN2* from the *GAL1* promoter led to substantially lower levels of Cln3 protein during G1 phase, as well as a premature reduction in Cln3 protein levels (**Figure 2.9A**). Conversely, loss of function of Cln1/2 in *cln1*Δ *cln2*Δ cells appeared to cause increased Cln3 protein expression during G1 phase (**Figure 2.9B**). A caveat is that the *cln1*Δ *cln2*Δ cells did not fully synchronize. Therefore, we also analyzed Cln3 levels in unsynchronized cells, which again appeared to show that loss of CLN1/2 causes an increase in Cln3 protein levels (**Figure 2.9C**).

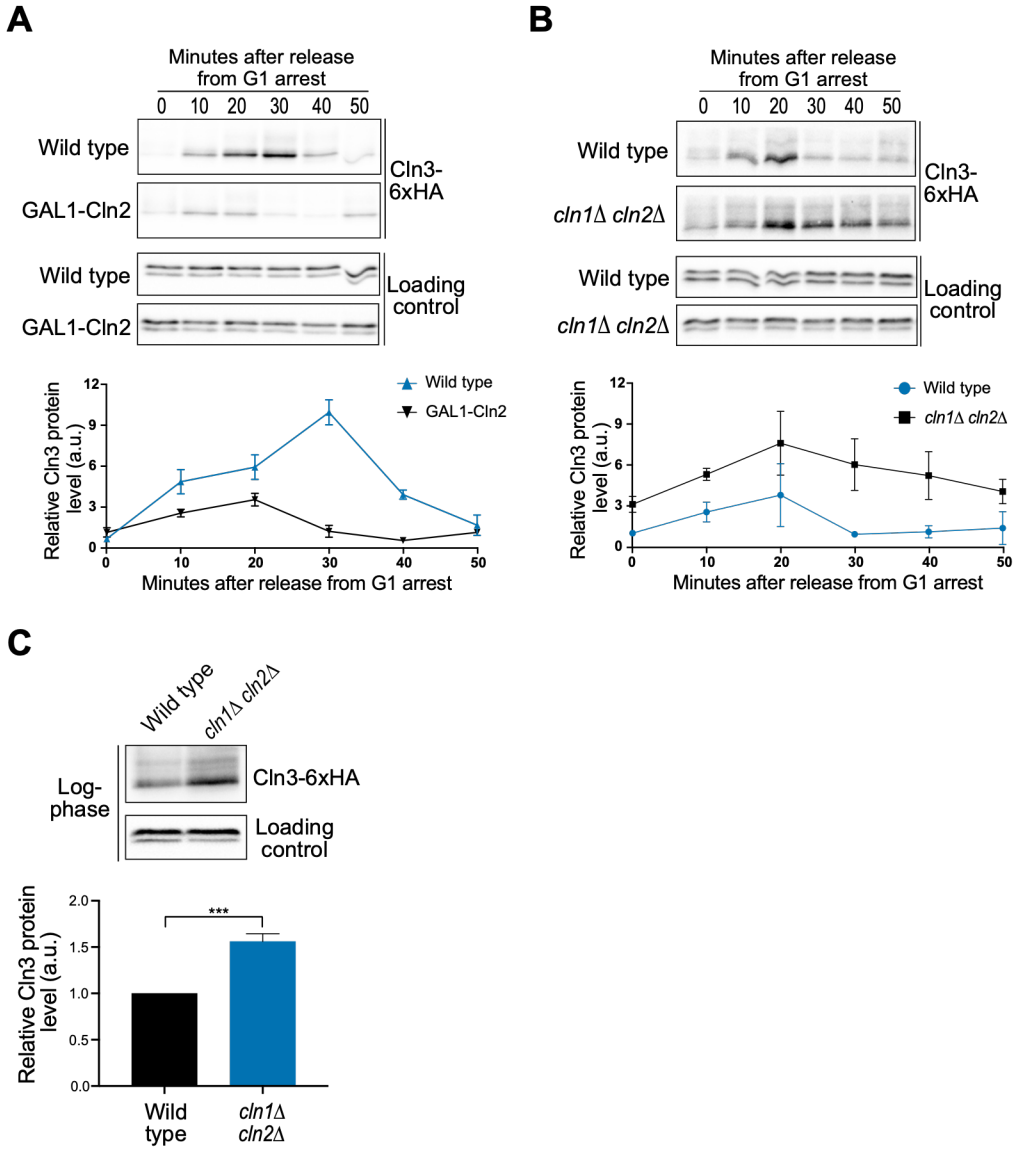

Figure 2.9. Cln2 imposes negative feedback upon Cln3. (A) Cells were grown to log-phase overnight in YPG/E and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC. Expression of GAL1-CLN2 was induced 40 minutes before release by addition of 2% galactose. Cells were washed and released into YEP containing 2% galactose. The levels of Cln3-6xHA protein were analyzed by western blot and protein abundance was quantified relative to Cln3-6xHA levels in wildtype cells at t=0 after first normalizing to the loading control. (B) Cells were grown to log-phase overnight in YPD and were then arrested in G1 phase with alpha factor for 3 hours at 30ºC. The levels of Cln3-6xHA protein were analyzed by western blot and protein abundance was quantified relative to Cln3-6xHA levels in wildtype cells at t=0 after first normalizing to the loading control. (C) Cells were grown to log-phase overnight in YPD. Levels of Cln3-6xHA protein were analyzed by western blot and protein abundance was quantified relative to Cln3-6xHA levels in wildtype. (A-C) Error bars represent SEM of three biological replicates. *** $p < 0.001$ by student's t test.

Discussion

Hyperactive Ras influences cell size in budding yeast and mammalian 3T3 cells

Pioneering work carried out over 20 years ago suggested that Ras influences cell size in yeast. However, technical limitations made it difficult to determine whether hyperactive Ras directly influences cell size. Here, we used modern methods to express Ras from an inducible promoter at endogenous levels, which establishes a powerful new system in which to analyze the effects of hyperactive Ras. This allowed us to clearly establish that cell size defects are an immediate and direct consequence of hyperactive Ras.

We also tested whether oncogenic Ras influences cell size in mammalian cells. Previous work found that cancer cells show severe size defects[14]; however, cancer cells accumulate numerous additional mutations as they undergo extensive evolution, so the primary cause of the defects has remained unknown. We found that expression of oncogenic $HRas^{G12V}$ in NIH 3T3 cells is sufficient to drive a large increase in cell size. Thus, it appears that signals from key oncogenic drivers directly influence cell size. The fact that hyperactive Ras causes increased cell size in both yeast and mammals suggests that Ras could influence cell size via conserved mechanisms. Consistent with this, a previous study found that mammalian $HRas^{G12V}$ causes increased expression of the early G1 phase cyclin D1[91], similar to our discovery that yeast Ras drives an increase in expression of the early G1 phase cyclin Cln3. However, much more work in both yeast and mammalian cells is needed to determine whether Ras influences

cell size via conserved mechanisms. Use of the powerful approaches available in yeast to learn more about Ras signaling could provide a roadmap for future work in mammalian cells.

Expression of *ras2^{G19V}* disrupts a critical step in cell cycle entry

Several previous studies suggested that hyperactive Ras influences cell cycle progression and expression of G1 phase cyclins, which could help explain the size defects of *ras2^{G19V}* cells. However, these studies examined the effects of hyperactive Ras indirectly by manipulating PKA signaling, and in some cases obtained conflicting results[49,50,54–56,60,71]. Here, we directly tested the immediate effects of *ras2^{G19V}* expressed from an inducible promoter, which showed that *ras2^{G19V}* causes a prolonged delay before bud emergence. Growth continues during the delay so that *ras2^{G19V}* cells initiate bud emergence at a substantially larger size than control cells.

To better understand the cause of the G1 phase delay, we assayed expression of early and late G1 phase cyclins and found that *ras2^{G19V}* causes a 3-fold increase in Cln3 protein levels and also prolongs Cln3 expression in G1 phase. Increased Cln3 expression would be expected to accelerate and increase expression of Cln2. However, we found that *ras2^{G19V}* causes delayed and decreased expression of *CLN2* mRNA and protein. The effects of *ras2^{G19V}* on Cln2 protein expression appeared to occur primarily at the level of transcription, since replacing the normal promoter of *CLN2* with a heterologous promoter eliminated much of the effect of *ras2^{G19V}* on Cln2 protein expression. Thus, the data suggest that ras2^{G19V} disrupts the mechanisms by which Cln3

induces transcription of late G1 phase cyclins, which is a critical step in the molecular mechanisms that initiate entry into the cell cycle.

ras2^{G19V} is unlikely to control G1 cyclin expression via a simple PKA-Whi3 signaling axis

Previous studies suggested that *ras2*^{G19V} could influence G1 cyclin expression and cell cycle entry via a signaling axis in which Ras activates PKA to inhibit Whi3, which is thought to bind and inhibit expression of G1 cyclin mRNAs [71,85,87]. We tested this model by comparing the effects of *ras2*^{G19V} to the effects of *whi3*Δ or inhibition of PKA. Expression of *ras2*^{G19V} and *whi3*Δ both caused an increase in Cln3 protein levels and a *lexA-ras2*^{G19V} *whi3*Δ double mutant did not show additive effects on Cln3 protein levels. These observations are consistent with a model in which *ras2*^{G19V} drives an increase in Cln3 protein levels via inhibition of Whi3 but do not rule out alternative models. However, the data do not support the idea that *ras2*^{G19V} influences Cln2 protein levels via a PKA-Whi3 signaling axis. For example, *ras2*^{G19V} and inhibition of PKA caused similar effects on Cln2 protein levels, which would not be expected if *ras2*^{G19V} drives a decrease in Cln2 protein levels via hyperactivation of PKA. Overall, the data are most consistent with a model in which hyperactive signaling from *ras2*^{G19V} influences Cln2 protein levels via a PKA-independent pathway. Testing this model will require additional work to define the signaling steps by which *ras2*^{G19V} blocks normal expression of Cln2.

The effects of *ras2*^{G19V} on late G1 cyclin expression are dependent upon Whi5

Previous studies suggested that Cln3 initiates transcription of late G1 phase cyclins at least partly via inhibition of Whi5 [26,27]. Here, we found that expression of *ras2*^{G19V} does not delay expression of Cln2 in *whi5*Δ cells, which suggests that *ras2*^{G19V} may disrupt the mechanism by which Cln3 inactivates Whi5. Previous studies suggested that a Cln3/Cdk1 complex directly phosphorylates and inactivates Whi5; however, more recent work has definitively shown that Cln3 is not required for phosphorylation of Whi5 during cell cycle entry [82,89]. Thus, the mechanisms that inactivate Whi5 are poorly understood.

Whi5 appears to be functionally similar to Rb in mammalian cells, as both are repressors of late G1 phase cyclin transcription. However, Whi5 and Rb show no sequence homology and recent evidence suggests that they may be regulated via different mechanisms [82,89]. It has therefore remained unclear whether the mechanisms that control Whi5 and Rb are conserved. Further investigation of the mechanisms by which *ras2*^{G19V} influences Whi5 activity could therefore lead to a better understanding of the relationship between signals that control Whi5 and Rb.

Evidence for Cln2-dependent negative regulation of Cln3

Expression of Cln2 protein was reduced and delayed by *ras2*^{G19V} and also by inhibition of PKA. In both contexts, the window of Cln3 protein expression was prolonged, which could be explained by a model in which Cln2 represses expression of Cln3. Consistent with this, we found that overexpression of Cln2 causes reduced expression of Cln3 protein, while loss of Cln1/2 appeared to increase and prolong expression of Cln3. Previous studies have shown that this kind of feedback regulation works at other times during the cell cycle in yeast [90].

The discovery that Cln3 appears to be regulated by Cln2-dependent negative feedback suggests a new entry point for further exploration of the mechanisms that control cell cycle entry.

Analysis of aberrant Ras signaling in yeast may provide new insight into cancer cell biology

Figure 2.10 shows a simplified model of the mechanisms that are thought to control cell cycle entry, in which the steps that appear to be influenced by *ras2*^{G19V} are highlighted. Our discovery that hyperactive *ras2*^{G19V} influences key steps in cell cycle entry, potentially via PKA-independent mechanisms, provides new insight into how *ras2*^{G19V} influences cell size and cell cycle progression. In mammalian cells, there is evidence that constitutively active Ras influences expression of cyclin D via a mechanism that is independent of the canonical MAP

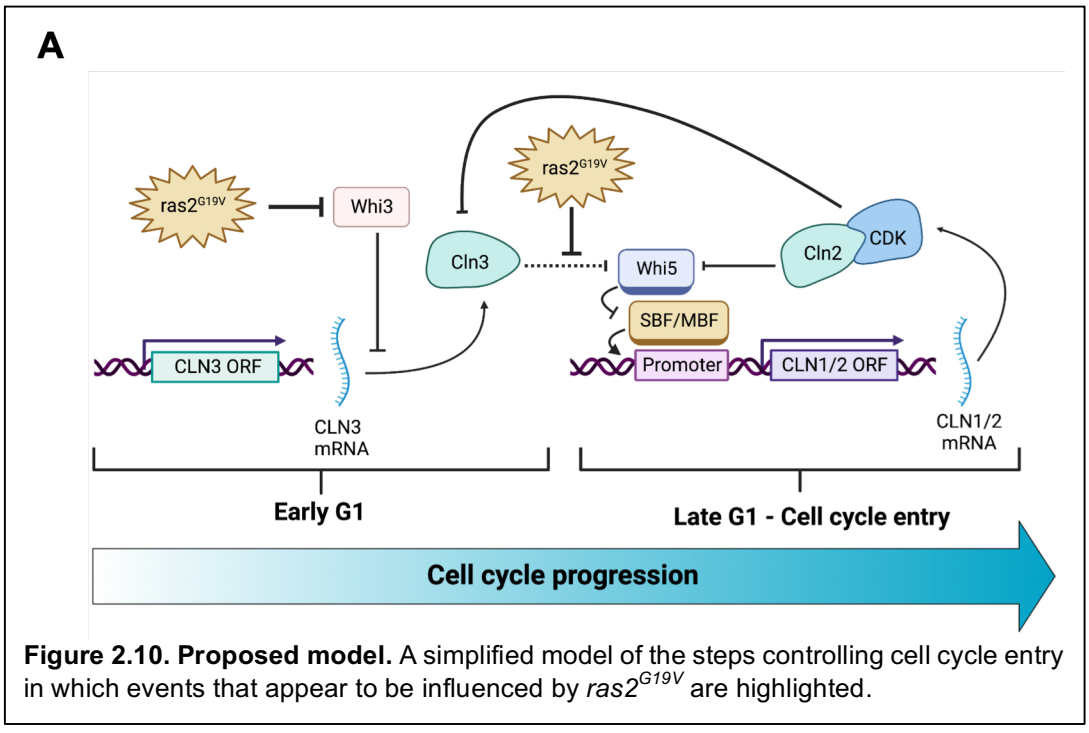

Figure 2.10. Proposed model. A simplified model of the steps controlling cell cycle entry in which events that appear to be influenced by *ras2*^{G19V} are highlighted.

kinase signaling pathway that has been thought to mediate many functions of Ras. Together, these observations indicate that much remains to be learned about how aberrant Ras signaling influences basic cell biology. Further investigation of the mechanisms by which hyperactive Ras influences cell size and the cell cycle in yeast could yield broadly relevant insights that that are relevant to cancer cell biology.

Supplemental Figures

Figure 2.1–figure supplement 1.

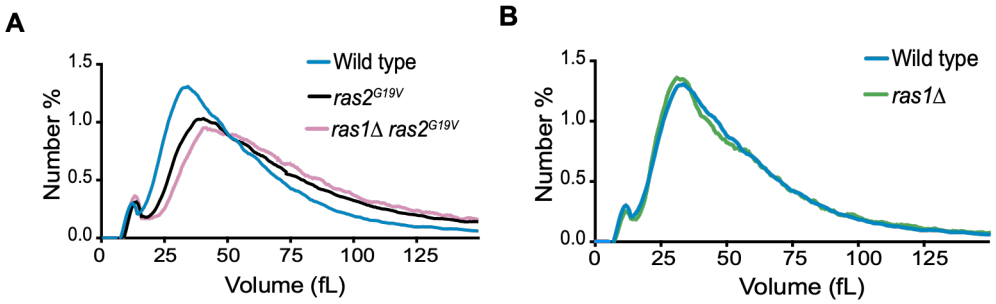

Figure 2.1–figure supplement 1. (A-B) Yeast cells were grown to log phase in YPD and cell size was measured using a Coulter counter. (A) Coulter counter size plots comparing Wild type, *ras2*^{G19V}, and *ras1*Δ *ras2*^{G19V} cells. (B) Coulter counter size plots comparing Wild type, and *ras1*Δ cells. Error bars represent SEM of 3 biological replicates. Loading control was anti-Nap1.

Figure 2.4–figure supplement 1.

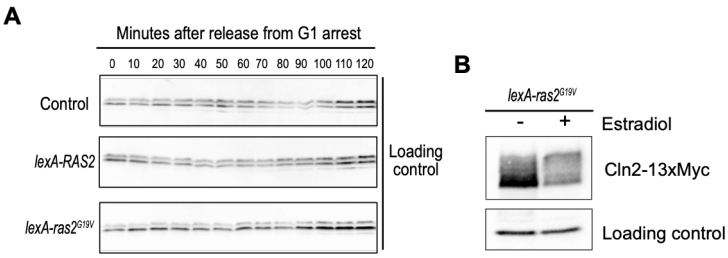

Figure 2.4–figure supplement 1. (A) Loading controls for the data in Figures 4A,B. Levels of Nap1 protein were analyzed for a loading control. (B) Cells were grown to log-phase overnight in YPD and then treated with vehicle or 200 nM estradiol for 1.5 hours. The levels of Cln2-13xMyc protein were analyzed by western blot.

Figure 2.7–figure supplement 1.

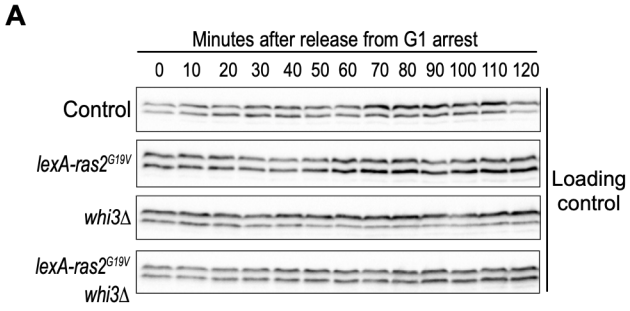

Figure 2.7–figure supplement 1.
Loading controls for the data in Figures 7C,D. Levels of Nap1 protein were analyzed for a loading control.

Materials and Methods

Yeast strains and media

The genotypes of the strains used in this study are listed in Table 1. All strains are in the W303 background (*leu2-3,112 ura3-1 can1-100 ade2-1 his3-11,15 trp1-1 GAL+, ssd1- d2*). Genetic alterations, such as addition of epitope tags, promoter swaps, and gene deletions were carried out using homologous recombination at the endogenous locus (Longtine et al., 1998; Janke et al., 2004). Strains that express *RAS2* or *ras2*^{G19V} from the estradiol-inducible lexA promoter at the *RAS2* locus were generated by homologous recombination as previously described [70]. Briefly, *lexA* promoter elements were amplified and integrated upstream of wild type *RAS2* locus (oligos: *TAACCGTT TTCGAATTGAAAGGAGATATACAGAAAAAAAACGAGAGCTTGCCTTGTCCCC* and *GTACTCTCTTATGTTCGACTTGTTCAAAGGCATAAGCTTGATATCGAATT CCTG*). The *ras2*^{G19V} mutation was incorporated into the 3' oligo (oligos: *TAACCGTTTTCGAATTGAAAGGAGATATACAGAAAAAAAACGAGAGCTTGCC TTGTCCCC, ATTGGGTCAATTGTATGGTCAAAGCAGATTTACCAACACCAAC ACCACCAACGACGACTAGCTTGTACTCTCTTATGTTCGACTTGTTCAAAGGC ATAAGCTTGATATCGAATTCCTG*). The *ras2*^{G19V} mutant was verified by Sequencing.

Cells were grown in YP medium (1% yeast extract, 2% peptone) that contained 40 mg/L adenine and a carbon source. Rich carbon medium (YPD) contained 2% dextrose, while poor carbon medium (YPG/E) contained 2% glycerol and 2% ethanol. In experiments using the ATP analog inhibitors 1-NM-PP1 or 3-MOB-PP1 no additional adenine was added to the media. All ATP

analog inhibitors were solubilized in 100% DMSO. 3-MOB-PP1 was a gift from Kevin Shokat (UCSF). β-estradiol (#50-28-2 from Acros) was added to cultures from a 10 mM stock in 100% ethanol.

Mammalian cell lines and media

Wild type and $HRAS^{G12V}$ NIH3T3 cells were generated by Dr. Alice Berger's lab [69]. NIH 3T3 Cells were cultured at 37ºC/5% CO_2 in DMEM high glucose medium with L-Glutamine, no Sodium PyruvateEM (Cytiva #SH30022.01) supplemented with 10% bovine calf serum (Sigma #12133C) and 1% penicillin + 1% streptomycin (Thermo #15-140-122). We routinely test for mycoplasma contamination via standard PCR.

Cell cycle time-courses and serial dilution assays

Cell cycle time courses were carried out as previously described [92]. Briefly, cells were grown to log phase at room temperature overnight in YPD or YPG/E medium to an optical density (OD^{600}) of 0.5 - 0.7. Cultures were adjusted to the same optical density and were then arrested in G1 phase with alpha factor at room temperature for 3 hours. *bar1* strains were arrested with 0.5 µg/mL alpha factor, while *BAR1*+ strains were arrested with 15 µg/mL alpha factor. Cells were released from the arrest by washing 3 times with YPD or YPG/E. All time courses were carried out at 30˚C unless otherwise noted, and alpha factor was added back at 40 minutes to prevent initiation of a second cell cycle. For experiments involving induced expression of *RAS2* or $ras2^{G19V}$, cells were arrested for 3 hours and β-estradiol (200 nM) was added 1.5 hours before release. Cells were released from the arrest by washing 3 times with fresh YPD containing 200 nM β-estradiol.

Serial dilution assays were carried out by growing cells overnight in YPD to an OD^{600} of 0.4. A series of 10-fold dilutions were prepared, spotted on YPD plates, and grown for 2 days at 30˚C.

Northern blotting

Gel-purified PCR products were used to generate radio-labeled probes to detect *CLN2* and *ACT1* mRNAs by Nothern blotting (oligonucleotides: TCAAGTTGGATGCAATTTGCAG, *TGAACCAATGATCAATGATTACGT*; *ACT1* oligonucleotides: TCATACCTTCTACAACGAATTGAGA and ACACTTCATGATGGAGTTGTAAGT. Probes were labeled using the Megaprime DNA labelling kits (GE Healthcare). Northern blotting was carried out as previously described (Cross and Tinkelenberg, 1991; Kellogg and Murray, 1995). *CLN3* blots were stripped and reprobed for *ACT1* to control for loading.

ChIP and qPCR

ChIPs were performed as previously described (Voth et al., 2007). Yeast cells were collected at an OD of 0.6–0.8 and cross-linked in 1% formaldehyde for 20 min at room temperature. Cross-linking was quenched with 0.125 M glycine for 5 min, and cells were washed twice with 1X TBS (0.05 M Tris, 0.15 M NaCl, pH 7.6). Cell pellets were resuspended in lysis buffer (0.1% deoxycholic acid, 1 mM EDTA, 50 mM Hepes, pH 7.5, 140 mM NaCl, 1% Triton X-100 supplemented with protease inhibitors) and were lysed with 0.5-mm glass beads (BioSpec #11079105) in a cell disrupter (Mini-Beadbeater; BioSpec). After centrifugation, the pellet was washed with lysis buffer and sonicated to a shearing size of <500 nucleotides using a bath sonicator (Biorupter XL; Diagenode). The sonicated

material was centrifuged, and the supernatant was collected for immunoprecipitation.

Chromatin immunoprecipitations were performed overnight at 4°C using 500–700 µg of chromatin and a mouse monoclonal anti-HA antibody (12CA5, Gift of David Toczyski, University of California, San Francisco) bound to Protein A Dynabeads (Thermo Fisher #10001D). The beads were washed twice with lysis buffer (50 mM HEPES-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate), twice with high salt buffer (lysis buffer with 500 mM NaCl), twice with LiCl buffer (0.5% deoxycholic acid, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 10 mM Tris-HCl, pH 8.0), and once with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Cross-links were reversed overnight in elution buffer (5 mM EDTA, 0.5% SDS, 0.3 mM NaCl, 10 mM Tris-HCl, pH 8.0) at 65°C. DNA was purified using the ChIP DNA Clean & Concentrator purification kit (Zymo #D5201). Quantitative PCR reactions were performed using a detection system (LightCycler480 II; Roche). A standard curve representing a range of concentrations of input samples was used for quantifying the amount of product for each sample with each primer set. All ChIP samples were normalized to corresponding input control samples, to a genomic reference region on chromosome I, and to a genetically identical untagged strain as a control. (ChIP primers for the *CLN2* promoter region: *CAATTCATGCGCGCTTTACC, TCTTCGCTAGGTATCCGCAT.* ChIP primers for the chromosome I control region: *GTTTATAGCGGGCATTATGCGTAGATCAG* and *GTTCCTCTAGAATTTTTCCACTCGCACATT*.)

Western blotting and quantification

For western blotting, 1.6 ml samples were taken from cultures and pelleted in a microfuge at 13,200 rpm for 30 sec before aspirating the supernatant and adding 250 µL of glass beads and freezing on liquid nitrogen. Cells were lysed in 140 µL of 1X SDS-PAGE sample buffer (65 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 100 mM βglycerophosphate, 50 mM NaF, 5% β-mercaptoethanol, 2 mM PMSF, and bromophenol blue) by bead beating in a Biospec Mini-Beadbeater-16 at 4ºC for two minutes. The lysate was centrifuged for 15 seconds to bring the sample to the bottom of the tube and was then incubated in a 100ºC water bath for 5 minutes followed by a centrifugation for five minutes at 13,200 rpm. Lysates were loaded onto 10% acrylamide SDS-PAGE gels that were run at a constant current setting of 20 mA per gel at 165 V. Gels were transferred to nitrocellulose membrane in a BioRad Trans-Blot Turbo Transfer system. Blots were probed overnight at 4ºC in 4% milk in western wash buffer (1x PBS + 250 mM NaCl + 0.1% Tween-20) with mouse monoclonal anti-HA antibody (12CA5, Gift of David Toczyski, University of California, San Francisco), mouse monoclonal anti-Myc (2276S from Cell Signaling), polyclonal anti-Clb2 antibody, polyclonal anti-Nap1 antibody, polyclonal anti-Ypk1 antibody, or polyclonal rabbit anti-T662P antibody (Gift from Ted Powers, University of California, Davis). Western blots using anti-T662P antibody were first blocked using TBST (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) + 4% milk, followed by one wash with TBST, then overnight incubation with anti-T662P antibody in TBST + 4% BSA. Western blots were incubated in secondary donkey anti-mouse (GE Healthcare NA934V) or donkey anti-rabbit (GE Healthcare NXA931 or Jackson Immunoresearch 711-035-152) antibody conjugated to HRP

at room temperature for 60-90 min before imaging with Advansta ECL chemiluminescence reagents in a BioRad ChemiDoc imaging system. Western blots were quantified using BioRad Imagelab software v6.0.1. Relative signal was calculated by normalizing to a loading control and then setting all other samples to a reference of either the zero-minute time point for time-course experiments or to wild type for log-phase comparisons (see figure legends for details).

Cell size analysis by Coulter Channelyzer and bud emergence

Yeast cells were grown overnight at 22ºC to mid-log phase (OD600 less than 0.7). Cells were fixed with 3.7% formaldehyde for 30 min and were then washed with PBS + 0.02% Tween-20 + 0.1% sodium azide before measuring cell size using a Z2 Coulter Channelyzer as previously described (Lucena et al. 2018) using Z2 AccuComp v3.01a software. For log phase cultures, each cell size plot is an average of three independent biological replicates in which each biological replicate is the average of two technical replicates. The percentage of budded cells was calculated by counting >200 cells at each time point using a Zeiss Axioskop 2 (Carl Zeiss).

Cells were seeded at ~20-30% confluence and grown for 2 days at 37ºC with 5% CO2 before harvesting cells for size analysis by Coulter Channelyzer and were never allowed to grow beyond ~90% confluence. In brief, cells were trypsinized and then quenched with serum prior to centrifugation for 3 minutes at 2300 RPM. Cells were fixed with 3.7% formaldehyde for 30 min and were then washed with PBS + 0.02% Tween-20 + 0.1% sodium azide. Cells were then filtered through a 40 µM nylon membrane (Corning ref.# 352340) to eliminate

clumped cells before measuring cell size using a Z2 Coulter Channelyzer using Z2 AccuComp v3.01a software.

Experimental replicates and Statistical analysis

All experiments were repeated for a minimum of 3 biological replicates. Biological replicates are defined as experiments that are carried out on different days with different cultures. Figures present data from representative biological replicates and Coulter Counter data represent the average of biological replicates. For the statistical analyses, one-tailed unpaired t test was performed using Prism 9 (Grahpad). p values are described in each figure legend.

Additional contributions to published work

During my time as a graduate student in the Kellogg lab, I had the opportunity to work on a fellow graduate student's project. My contributions earned second authorship for the following publication: Robert A Sommer, **Jerry T DeWitt**, Raymond Tan, Douglas R Kellogg (2021) Growth-dependent signals drive an increase in early G1 cyclin concentration to link cell cycle entry with cell growth eLife 10:e64364. https://doi.org/10.7554/eLife.64364. My contributions to this work can be observed in the following figure panels: Figure 1. Panels F and G, Figure 3. Panel D, and Figure 7. Panel E.

Chapter 3: Unpublished experiments

Aberrant Ras signaling may influence TORC2 signaling networks in yeast and mammalian 3T3 cells.

The results detailed in Chapter 2 focus on how hyperactive Ras signaling affects growth and size control with respect to cell cycle progression, particularly during G1. However, throughout these investigations, I began to think about known growth controlling networks, and *how* aberrant Ras signaling could be potentially influencing growth control. Previous works from our lab, and others, have found that the evolutionarily conserved Target of Rapamycin kinase Complexes (TORC1/2) to be critical for cell growth and size control[38,41,42,93–96].

TOR can exist in two complexes, referred to as TORC1 and TORC2, that play distinct roles in control of cell growth and proliferation[93–96]. TORC1 is potently inhibited by rapamycin, which has facilitated discovery of many of its roles in normal and transformed cells. Much less is known about TORC2 and the mechanisms by which it controls growth. Previous work from our lab discovered that a TORC2 signaling axis governs cell growth and size in budding yeast[41]. In mammals, TORC2 directly phosphorylates and partially activates the Serum and Glucocorticoid-regulated Kinases (SGKs); in yeast, SGK homologs are called Ypk1/2. However, full-activation of SGK, requires further phosphorylation by a conserved phosphoinositide-dependent kinase (PDK1). Activation of SGK by TORC2 and PDK1 is conserved from yeast to mammals[39]. Additionally, previous reports have found that oncogenic forms of Ras have the capacity to bind to and stimulate TORC2[97]. Therefore, I set out to test if/how aberrant Ras signaling can modulate the TORC2-SGK signaling axis in yeast and mammalian

3T3 cells.

To test this, I created a strain of budding yeast that expresses either Wild Type Ras2, or hyperactive $Ras2^{G19V}$ using an estradiol-inducible system for gene expression; this expression system and these strains are discussed in detail in chapter 2. I found that induction of hyperactive $Ras2^{G19V}$ for cells in log-phase growth leads to a decrease in the expression of Ypk1 protein levels, and compromises TORC2-dependent phosphorylation of Ypk1 at T662 and to a lesser extent compromises Pkh(PDK1)-dependent phosphorylation of Ypk1 at T504. However, it appears that the phosphorylation signaling ratio (T662/Ypk1 and T504/Ypk1) may be increased, due to the decrease in Ypk1 protein levels following induction of hyperactive $Ras2^{G19V}$. Although, future repetitions would be required to determine if these effects are significant **(Figure 3.1)**. However, if so,

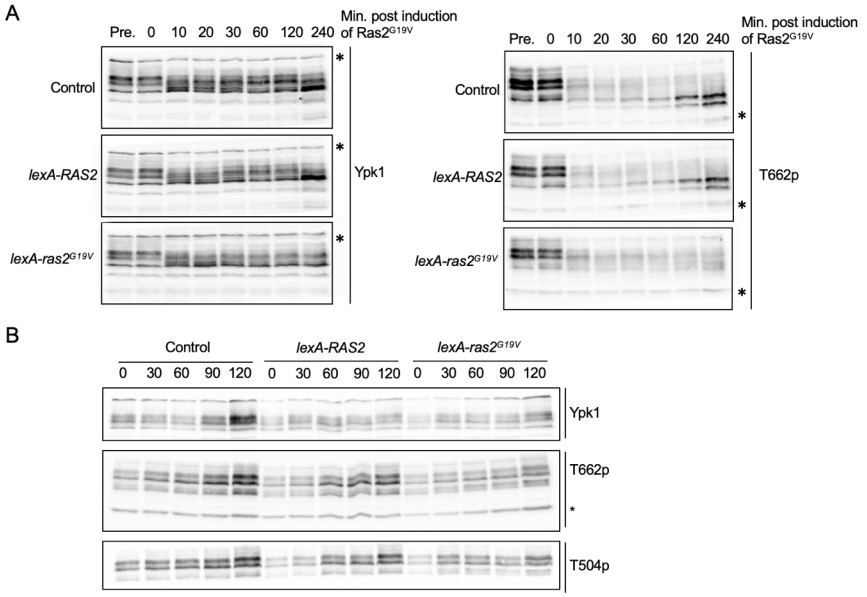

Figure 3.1. Aberrant Ras signaling may influence TORC2 signaling networks in budding yeast. (A-B) Cells were grown to log-phase overnight and treated with 200nM estradiol (to induce expression of Ras2, $Ras2^{G19V}$) for the indicated amount of time. The levels of Ypk1 protein, T662 phosphorylation, and T504 phosphorylation were analyzed by Western blot. (*) background band

then these data would argue that hyperactive Ras influences TORC2 signaling during the cell cycle.

Previous work has found that TORC2 activity peaks in mitosis, and hyperactive Ras can delay cell cycle progression. Therefore, I next tested if expression of hyperactive Ras2 can have differential effects on PDK1 signaling, TORC2 phosphorylation and Ypk1 levels throughout the cell cycle.

To test this, I conducted Western blot analysis of cell cycle time courses with and without induction of Ras2 or Ras2^{G19V} expression. I found that TORC2 signaling (T662p) normally appears to increase throughout the cell cycle, peaking at time points that correlate with mitosis, which is consistent with prior reports that showed TORC2 activity increasing in mitosis **(Figure 3.2B)**. However, cells expressing Ras2^{G19V}, fail to increase TORC2 signaling and levels appear constant throughout the duration of the cell cycle. The results for PDK1-dependent phosphorylation of Ypk1 were markedly different. I found that PDK1

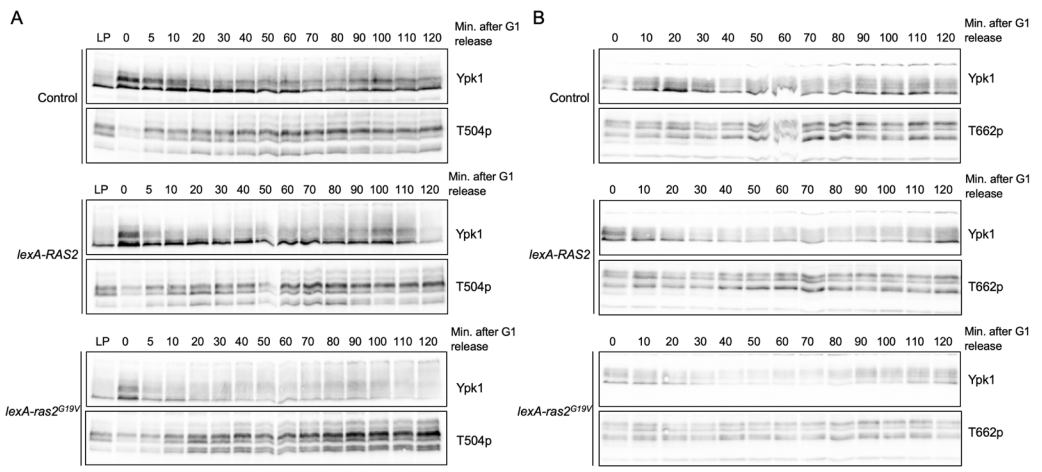

Figure 3.2. Aberrant Ras signaling may influence TORC2 signaling during cell cycle progression. (A-B) Yeast cells were grown to log-phase overnight in YPD, then arrested in G1 phase with alpha factor mating pheromone for 3 hours at 30ºC. Cells were treated with 200 nM estradiol beginning 1.5 hours prior to release. The levels of Ypk1, T504p, and T662p were analyzed by Western blot.

phosphorylation of Ypk1 at T504 appears to increase substantially, while Ypk1 levels decrease **(Figure 3.2A)**. Because T662 phosphorylation is a priming phosphorylation needed for PDK1 to phosphorylate Ypk1 at T504 and lead to its full activation, these data suggest that the ratio of fully-activated and phosphorylated Ypk1 protein may be increased upon aberrant Ras signaling.

I examined how aberrant Ras signaling ($HRas^{G12V}$) affects the TORC2-SGK signaling axis in mammalian NIH 3T3 cells. I found that Sgk1 is rapidly dephosphorylated and degraded within 10 minutes following serum-withdrawal, consistent with Sgk being positively regulated by serum **(Figure 3.3A)**. I further found that expressing hyperactive HRas in NIH 3T3 cells appears to promote TORC2-dependent phosphorylation of Sgk1 and Akt at S422 and S473,

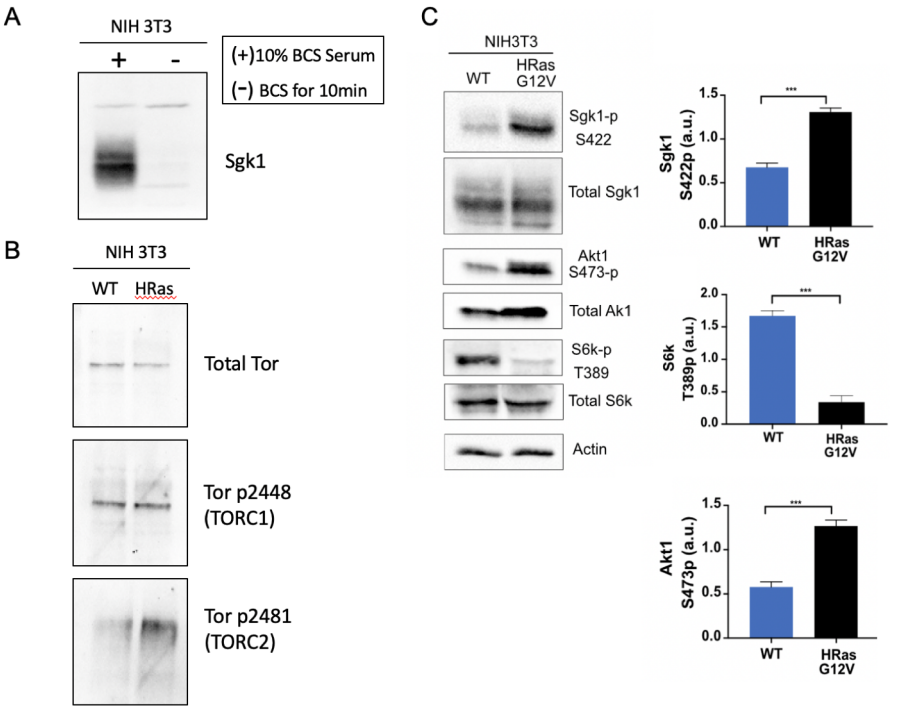

Figure 3.3. Aberrant Ras signaling may influence TORC2 signaling networks in NIH 3T3 cells. (A-C) NIH 3T3 cells harboring a hyperactive allele (G12V) of HRas or Wild Type control cells were cultured at 37ºC with 5% CO_2 and 1% Pen/Strep for 48 hours prior to Western blot analysis.

respectively **(Figure 3.3C)**. What's more, is that TORC1-dependent phosphorylation of its canonical substrate, S6k at T389, was reduced **(Figure 3.3C)**. Together, these data suggest that $HRas^{G12V}$ may promote TORC2 activity, while decreasing TORC1 activity. Additionally, TOR is differentially phosphorylated when in complex 1, and 2. Therefore, I tested if $HRas^{G12V}$ affects TOR complex formation in 3T3 cells. I found that $HRas^{G12V}$ appears to increase the amount of TOR-phosphorylation that is associated with TORC2 complex formation (2481) while total TOR protein remains unchanged **(Figure 3.3B)**. Altogether, these results suggest an interesting possibility that hyperactive Ras signaling may modulate the conserved signaling axes surrounding SGK (including TORC2 and PDK1) in yeast and mammalian cells. Further exploration into these possibilities would provide better insight into oncogenic signaling surrounding Ras in the control of cell growth and size.

Prolonged induction of hyperactive Ras alters TORC2 signaling and proliferation.

The aforementioned results suggest that short-term induction of hyperactive Ras (2-3 hours of expression) reduce Ypk1 protein levels while maintaining relatively constant TORC2 activity, by T662 phosphorylation of Ypk1. However, in testing long-term expression of hyperactive Ras (4-7 hours of induction) we found that TORC2 signaling is prolonged, relative to induction of a Wild type allele of Ras2 **(Figure 3.4A)**. Normally, after several hours of log-phase growth in undiluted media, cells undergo a change in nutrient signaling called a diauxic shift. A diauxic shift allows for yeast cells to alter their metabolism to utilize alternative carbon sources, other than the preferred carbon molecule,

glucose. Prior to a diauxic shift, TORC2 signaling is reduced and cells momentarily enter a period of stationary growth **(Figure 3.4)**. However, it appears that for cells expressing hyperactive Ras, there is a delay in the reduction of TORC2 signaling **(Figure 3.4A)**. In these cells, TORC2 signaling persists for an additional hour of growth; however, hyperactive Ras reduces the rate of proliferation within 3-4 hours post induction **(Figure 3.4)**. These data suggest that TORC2 signaling persists and rate of cell proliferation decreases following long-term induction of hyperactive Ras, as cells approach a diauxic shift.

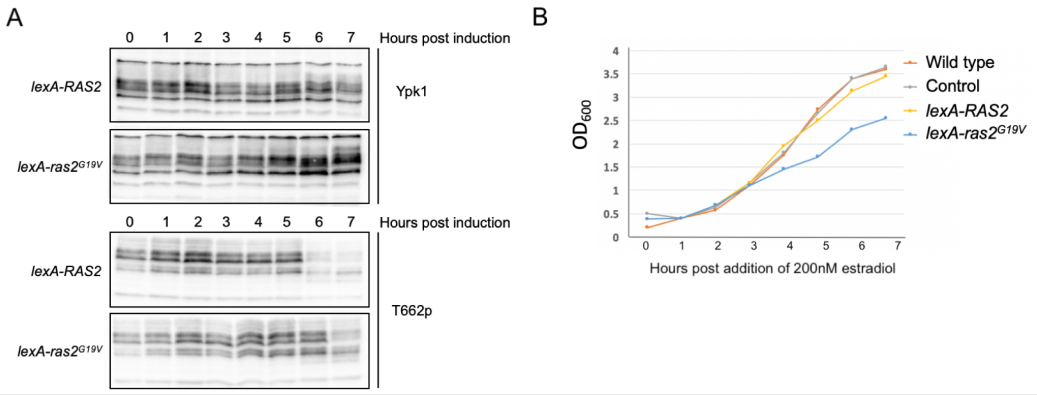

Figure 3.4. Prolonged induction of hyperactive Ras alters TORC2 signaling and proliferation. (A-B) Cells were grown overnight in YPD and treated with 200 nM estradiol. After 1 hour of growth in estradiol-treated YPD media, cultures were all set to an optical density (OD_{600}) of 0.4, and allowed to grow for a total of 7 hours. At each hour, optical density was measured and plotted (B). Additionally, hourly samples were collected for western blot analysis of Ypk1 protein and T662 phosphorylation (A).

Ras signaling is important for nutrient modulation of cell size in budding yeast.

Previously our lab found that budding yeast modulate their cell size in response to changes in carbon source and perturbations of TORC2 signaling

axes prevents nutrient modulation of cell size. Therefore, I set out to investigate if Ras signaling is important for this process.

Ras signaling is essential, and in budding yeast deletion of either paralog RAS1 or RAS2 is viable, but deletion of both is lethal. Previously, I found that the hyperactive allele (G19V) of Ras2 causes substantial increases in cell size and that deletion of either Ras paralog causes little-to-no change in cell size dynamics. Therefore, I tested how gain-of-function and loss-of-function of Ras2 may affect nutrient modulation of cell size. I found that loss-of-function of Ras2 prevents nutrient modulation of cell size **(Figure 3.5A)**. Somewhat surprisingly, I found that cells harboring the gain-of-function allele, $Ras2^{G19V}$, maintain the ability to modulate size in response to a change in carbon source, albeit at an overall increased general cell size **(Figure 3.5B)**. These data suggest that hyperactive Ras increases overall cell size, but the processes underlying nutrient modulation of cell size are seemingly intact. Because expression of Ras1 is controlled by glucose, Ras2 becomes essential when cells are grown in alternative carbon sources. Therefore, the result that *ras2*Δ cells fail to modulate

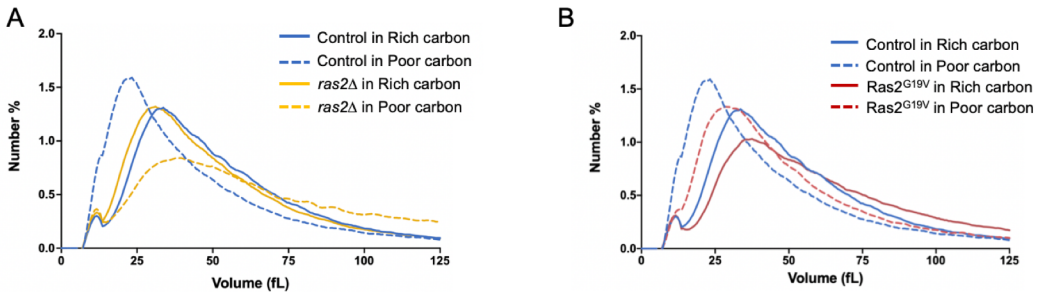

Figure 3.5. Ras signaling is important for nutrient modulation of cell size in budding yeast. (A-B) Cells were grown overnight in YPD (Rich carbon) or YEP+2% glycerol (Poor carbon) media. Samples were collected on the following day and cell size was measure by Coulter channelizer.

size in response to a change in carbon source, may be an artifact of synthetic lethality.

Inhibition of PKA fails to dramatically alter Ypk1 phosphorylation and mobility.

Previous works suggest that the main output of Ras signaling in yeast is to promote PKA activity. However, the work presented in this thesis argue that Ras may have additional signaling targets beyond PKA.

Induction of hyperactive Ras appears to increase the phosphorylation ratio of Ypk1 protein. Therefore, I sought to test if inhibition of PKA can modulate Ypk1 mobility and phosphorylation. Based on the presence of a background band in the Ypk1 blot, it appears that PKAas samples have less protein loaded, therefore it is difficult to interpret changes in protein levels relative to Wild Type. However, what is apparent is that inhibition of PKA fails to modulate the electrophoretic mobility pattern of Ypk1 or TORC2 signaling **(Figure 3.6)**. These data are

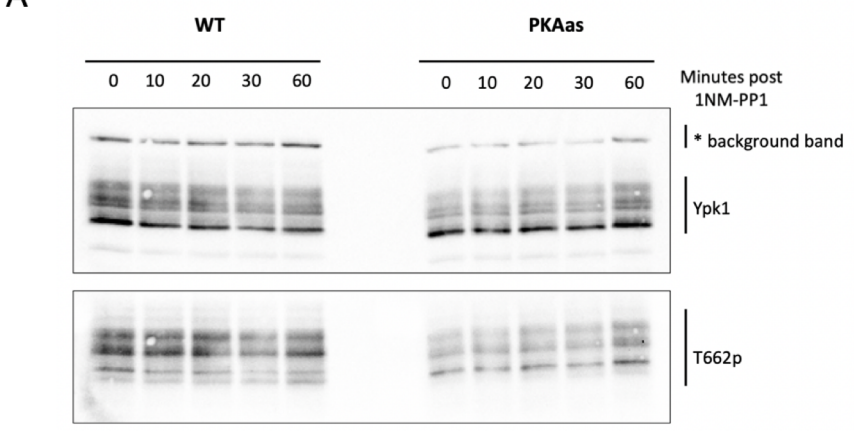

Figure 3.6. Inhibition of PKA fails to dramatically alter Ypk1 phosphorylation and mobility. (A) Cells were grown overnight in YPD and treated with 50 nM of the analog inhibitor, 1NM-PP1, for the indicated amount of time prior to collection for Western blot analysis.

consistent with the notion that Ras signaling may have targets beyond PKA in the regulation of Ypk1 phosphorylation and activity. Although further repetitions of this experiment are required in order to quantify significance between protein levels.

Hyperactive Ras affects the Forkhead box transcription factors FoxO3a and Hcm1 in mammalian 3T3 cells and budding yeast, respectively.

In mammalian cells, Forkhead box (FoxO) transcription factors are tumor-suppressors that are tightly linked with cell cycle progression. Additionally, several groups have previously implicated TORC2-dependent signaling in the control of FoxO transcription factors. In particular, TORC2-dependent regulation of Akt and Sgk1 have been previously shown to promote nuclear export and turnover of FoxO3, a known regulator of cell cycle arrest and apoptosis.

Furthermore, Ypk1 (the budding yeast homolog of mammalian Sgk) was previously determined to have the substrate recognition motifs, “RXRXX(S/T)”. Not surprisingly, the same substrate recognition motifs are shared by the mammalian Sgk/Akt counterparts. In mammals, a key output of Sgk/Akt signaling is inhibition of the FoxO family of tumor-suppressive transcription factors. Interestingly, a previously compiled comprehensive list of predicted ypk1 substrates, in budding yeast, implicated two forkhead box transcription factor homologs: Hcm1 and Fkh1.

Hcm1 has been shown to be important for cell cycle progression at the G1/S transition, metabolism and implicated in cell size control; a deletion of Hcm1 has been previously shown to produce a notable increase in cell size. Altogether, supporting the notion that Hcm1 was a worthy candidate for further

investigation. Preliminary investigations found that Hcm1 is regulated by nutrients (carbon source) and expression of $Ras2^{G19V}$, significantly lowers Hcm1 protein levels **(Figure 3.7A).** Hcm1 protein is known to promote expression of genes required for S-phase. Therefore, the result that Hcm1 levels are decreased when hyperactive Ras2 is expressed further suggests that hyperactive Ras2 hinders the G1/S transition. We further found no effect on the abundance of Fkh1 forkhead box protein (Data not shown). Additionally, we observed cell cycle-dependent protein expression of Hcm1, peaking as cells enter mitosis **(Figure 3.7B)**. It is apparent that hyperactive Ras delays Hcm1 protein expression in G1 and further produces mitotic delays as well **(Figure 3.7B)**. Although, it remained to be seen whether or not Hcm1 expression is modulated in a TORC2-Ypk1 dependent manner. Therefore, we next tested for the epistatic relationship of Ypk1 activity on Hcm1 abundance in budding yeast. To do this, we utilized a

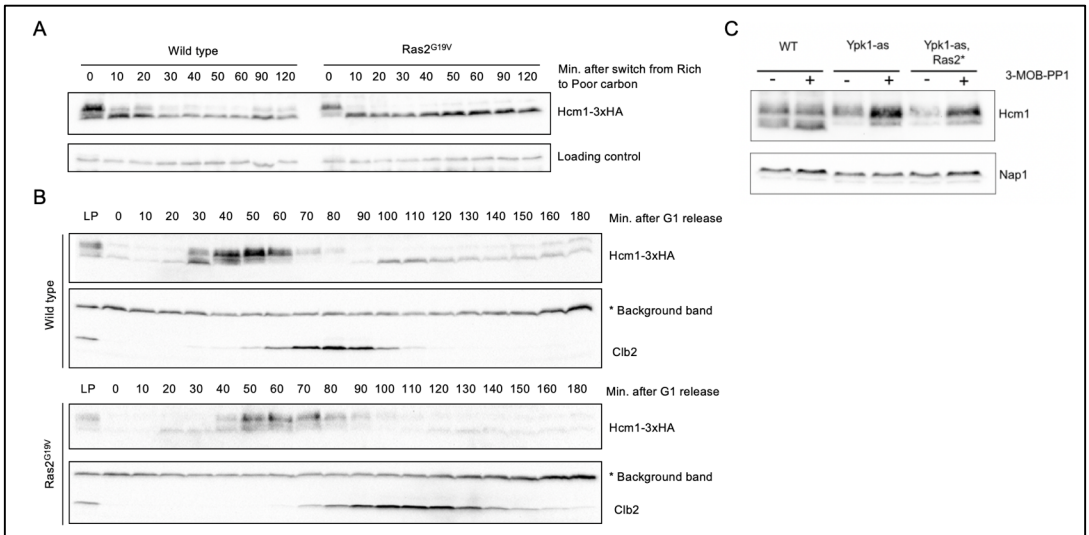

Figure 3.7. Hyperactive Ras and Ypk1 modulate the Forkhead box transcription factor, Hcm1 protein mobility and abundance. (A-C) Western blot analysis of yeast cell lysates. (A) Cells were grown overnight in YPD and switched to YEP+2% glycerol (Poor carbon). Cells were grown overnight in YPD and arrested G1-phase with alpha-factor mating pheromone then released. (C) Cells were grown overnight in YPD media then treated with vehicle or 100 nM of the analog inhibitor, 3-MOB-PP1, for 1 hour prior to collection for Western blot analysis.

previously validated analog sensitive allele of Ypk1. Addition of 3-MOB-PP1 in cells harboring both hyperactive Ras and the Ypk1-as allele resulted in a similar enrichment of Hcm1 protein abundance in less than 30 minutes **(Figure 3.7C)**. Collectively, these data are consistent with a model whereby hyperactive Ras may stimulate a TORC2-Ypk1 signaling axis to decrease levels of the forkhead-box transcription factor, Hcm1, and perturb cell size control.

Because hyperactive Ras also seems to modulate TORC2 signaling in our NIH 3T3 cell model, we next aimed to test if $HRas^{G12V}$ alters expression of the FoxO3 (an Hcm1 homolog) tumor suppressor protein. Consistent with

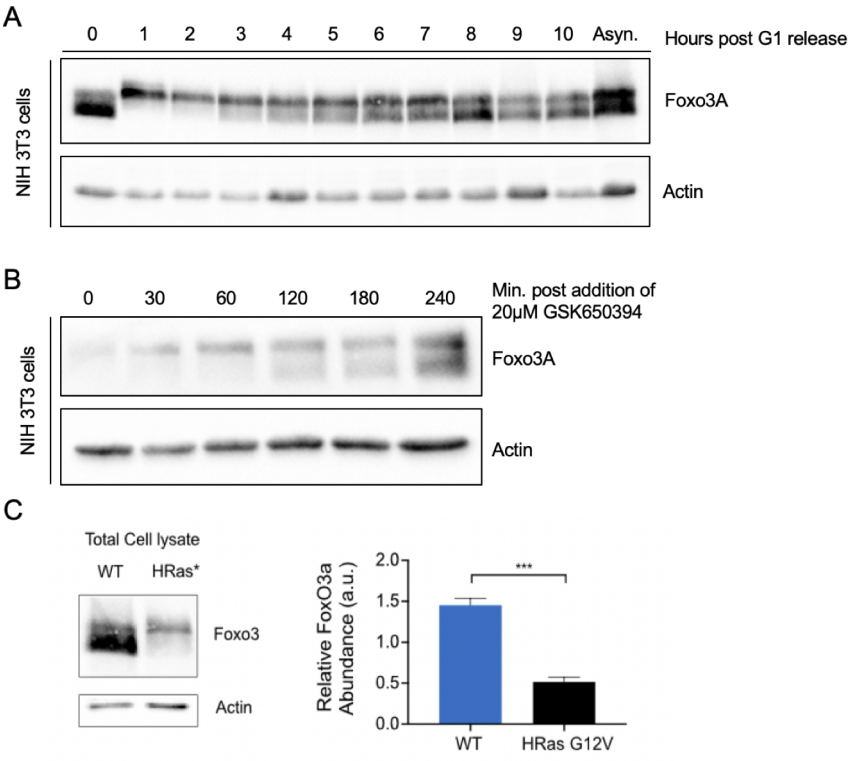

Figure 3.8. The forkhead box homolog, Foxo3A, is regulated by cell cycle progression, SGK signaling, and HRas. (A-C) Western blot analysis of mammalian NIH 3T3 cells. (A) Cells were arrested in G1 using 30-hour serum deprivation, and released into 10% FCS media. (B) Cells were treated for the indicated amount of time with the Sgk1 inhibitor GSK650394. (C) Cells were grown for 48 hours prior to collection for Western blot analysis.

previous findings, and my work conducted in budding yeast, FoxO3a undergoes electrophoretic mobility/phosphorylation changes that are correlative with cell cycle progression **(Figure 3.8A).** Additionally, we found that inhibition of Sgk1 with the small-molecule inhibitor, GSK650394, increases the abundance of FoxO3a protein levels **(Figure 3.8B)**. Furthermore, NIH 3T3 cells expressing oncogenic $HRas^{G12V}$ have significantly reduced levels of FoxO3 protein relative to a genetic control **(Figure 3.8C).** These data are consistent with, and build upon, the previous findings in the field which suggest that Akt and SGK1 stimulate FoxO nuclear export and protein turnover. Altogether, these data suggest that hyperactive Ras limits the expression of forkhead box transcription factors from yeast to mammals.

Whole genome sequencing and preliminary results from synthetic genetic array analysis implicate novel factors underlying hyperactive Ras2 signaling in yeast.

From the results depicted in Chapter 2, we found that expression of hyperactive Ras2 seems to decouple G1 cyclin expression, and increase cell size. These results argued that hyperactive Ras may operate independent to the canonical downstream target, PKA. Therefore, to further investigate the underlying mechanisms by which aberrant Ras signaling may be influencing cell size and cell cycle progression, we conducted whole genome sequencing (WGS) and a genetic analysis by synthetic genetic array (SGA) with the aim to identify novel candidates underlying these Ras-dependent defects.

We noticed that expression of hyperactive Ras2 in different strain backgrounds of budding yeast presented different severities of effects. The strain

background W303, was very sensitive to Ras induction and had a lower threshold of lethality for Ras2 expression. Whereas S288C and the highly related BY4741 strain backgrounds were more tolerant to Ras expression. Although, it is important to mention that all strain backgrounds (S288C, W303, and BY4741) presented the same cell size defects when Ras expression is induced. These observations suggested the possibility that the subtle genetic differences between strain backgrounds may differentially influence and mediate Ras signaling. It has been previously reported that there are over 9000 allelic single nucleotide polymorphisms (SNPs) between various yeast strain backgrounds. Therefore, we set out to determine if we could identify SNPs that segregate with a Ras-sensitive phenotype following a mating cross between W303 (Ras-sensitive) and S288C/BY4741 (Ras-insensitive). To do this, we conducted whole genome sequencing of W303, S288C/BY4741, and pooled DNA from 24 independent segregates that display the Ras-sensitive phenotype. Then we worked with Sol Katzman from the UCSC Genomics Institute to utilize the publically available bioinformatic tool, UnifiedGenotyper (UG), from GATK V3 to analyze and compare the genomes to identify consensus SNPs that are predominately present in in the Ras-sensitive population. This analysis identified 8146 differential SNPs between W303 and S288C/BY4741 strain backgrounds, which is largely consistent with previously published data[98]. To filter through these data, we further restricted our analyses to only include SNPs that are a minimum of 85% homogeneous (refFrac score). This resulted in a list of SNPs present in the Ras-sensitive population. In summary, we found 7 genes containing SNPs that are nearly homogeneous and present in the W303 strain

background, and 12 genes that are nearly homogeneous and present in the S288C/BY4741 background **(Table 3.1)**.

Table 3.1. List of filtered genes with SNPs present in the W303 Ras-sensitive population. SNP refFrac scores are a corrected ratio that measures that homogeneity of an identified SNP present in the Ras-sensitive population. refFrac values that are close to zero are SNPs that are more present in W303 and refFrac scores that are close to 1 are more present in S288C. Each refFrac score for a respective gene entry represents a single SNP.

Name	Gene ID	SNP refFrac score						
Arp2	YDL029W	0.1	0.11	0.12	0.14	0.15		
Gpr1	YDL035C	0.14	0.15					
Rtk1	YDL025C	0.15						
Sal1	YNL083W	0.08						
Sir2	YDL042C	0.15						
Stp4	YDL048C	0.15						
HIS3	YOR202W	0.1						
Name		refFrac						
AAD3	YCR107W	0.85						
Hap1	YLR256W	0.9	0.9					
Imp4	YNL075W	0.88	0.88	0.9				
Mdr1	YGR100W	0.85						
Mks1	YNL076W	0.85	0.86	0.86	0.87	0.87	0.88	0.88
Mkt1	YNL085W	0.89						
Nis1	YNL078W	0.85	0.85					
Pms1	YNL082W	0.92						
Prm7	YDL039C	0.87						
Sal1	YNL083W	0.87	0.89	0.91				
Spt6	YGR116W	0.85						
sws2	YNL081C	0.85						

From the SNP analysis, we identified several genes of interest. However, the SNPs depicted in **Table 3.1** need further validation to test if they are contributing to the Ras-sensitive phenotypes. For example, 2 genes of interest that are worth further exploration are Sal1 and Gpr1.

Sal1, which contains several different SNPs from both S288C and W303, is an ADP/ATP transporter and critical for viability. Although it is currently unclear how these SNPs affect protein function, it presents novel avenues for further exploration into the mechanisms by which hyperactive Ras may be influencing viability and cell size control. For example, Gpr1 is a G protein coupled receptor that is involved with cAMP synthesis and PKA signaling in yeast and is

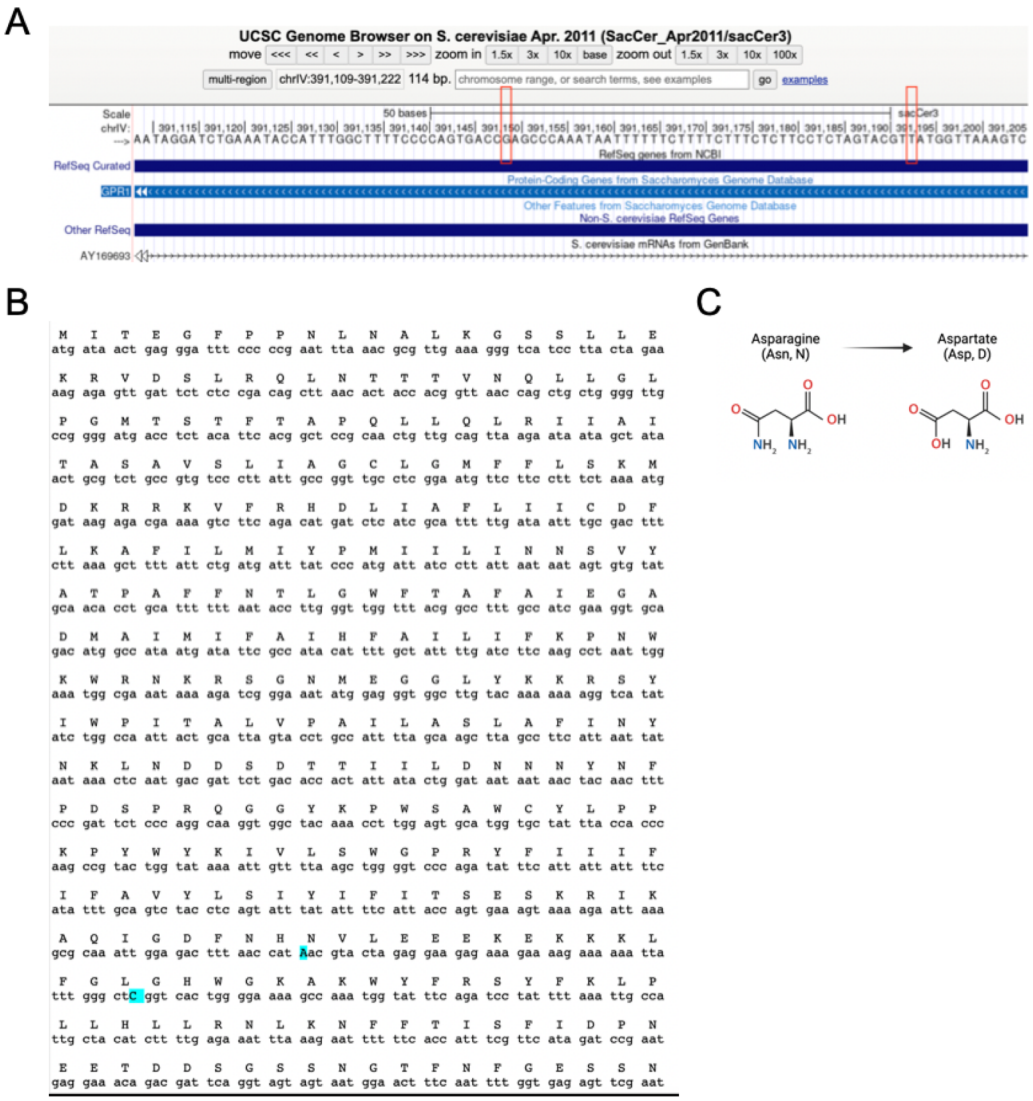

Figure 3.9. Preliminary mapping of Gpr1 SNPs identified by whole genome sequencing. (A) An image from the UCSC genome browser identifying the 2 SNP locations within the Gpr1 coding sequence (Red boxes). (B) Gpr1 protein sequence overlaid above the coding mRNA sequence. Capitalized letters that are highlighted in blue indicate the codons containing a SNP. (C) Amino acid models to illustrate the amino acid substitution present in Gpr1 at position 391193 on chromosome IV.

conserved in mammals. From our bioinformatics analysis, we found 2 different SNPs identified in Gpr1 that segregate with the Ras-sensitive phenotypes. Furthermore, preliminary investigation into the SNPs present in Gpr1 the found that 1 SNP present at position 391193 on chromosome IV contains a nucleotide substitution from A-to-G and ultimately results codon change. In S288C the

codon is AAC (for Asn), and the codon for W303 is GAC (for Asp) **(Figure 3.9)**. This is a strong change in amino acid properties as well because Asp is strongly negatively charged but Asn is uncharged-polar. Therefore, this SNP could likely alter protein function and perhaps influence Ras signaling and sensitivity. Further exploration into Gpr1, and other SNP hits, could result in novel understandings of Ras signaling in yeast, with possible applications to mammalian cell biology and cancer signaling.

In addition to whole genome sequencing, I also established a collaboration with the Boone lab at University of Toronto to utilize their synthetic genetic array (SGA) platform in order to test for genetic interactions with *ras2*^{G19V}. From SGA analysis of TS-alleles for essential and nonessential genes, identified 197 unique genes that result in a negative genetic interaction when combined with *ras2*^{G19V} compared to wild type *RAS2.* Further annotation and analysis of these 197 unique genes identified in the SGA screen found ~95 unique genes that are involved with cellular processes critical for cell growth, metabolism, and the cell cycle **(Table 3.2)**.

Many of the hits that were revealed by SGA analysis are cell cycle regulators with most known to be regulators for DNA replication and DNA repair. This is of particular importance because our previous work, depicted in chapter 2, found that expression of *ras2*^{G19V} delays cell cycle progression at cell cycle entry. Therefore, it suggests that cells expressing *ras2*^{G19V} have severe problems with coordinating cell cycle progression beginning at the G1/S transition and continuing into S-phase. Additionally, these data suggest further cell cycle perturbations extend into M-phase including: G2/M transition, activation of APC,

the mitotic exit network, and cytokinesis. Further investigation into some of these SGA candidates could reveal novel factors underlying Ras signaling in yeast, and may have relevance to cancer signaling in mammalian cells.

Table 3.2. SGA analysis of TS-alleles for essential and nonessential genes revealed ~95 unique genes that have a negative genetic interaction with *ras2^{G19V}*. Genes with TS-alleles that result in negative genetic interactions with ras2^{G19V} are listed in alphabetical order based on the cellular process(es) in which they regulate. * indicates that a gene is present multiple times in the table because it regulates multiple cellular processes. All genes represented in the table meet the SGA threshold for statistical significance i.e. p<0.05 and SGA score> 0.08.

Cellular Process							
Cell cycle							
	G1	*CDC1*	*CDC4**	*CDC19**	*CDC28**	*CKS1**	*MCM7**
	DNA replication	*ABF1**	*AME1**	*ASK1**	*CDC6**	*CDC13*	*CDC15**
		CDC20	*CDC24*	*CDC45*	*CTF4*	*CTF8*	*CTF18*
		DBF4	*ECO1*	*IPL1**	*MCM7**	*MCM10*	*MRE11**
		*NSE1**	*NSE3**	*NSE4**	*NSE5**	*ORC4*	*PAT1**
		POB3	*PRE10*	*QRI1*	*RAD5*	*RFA3*	*RCF2*
		RPN4	*RPN6*	*RPN11*	*RPT6*	*RSC4*	*SMT3*
		SNF4	*TPS1*	*UBC4*	*YCG1*	*YKL069W*	
	G2/M	*CAK1*	*CDC28**	*CDC4**	*CKS1**	*IPL1**	*ULP1**
	APC	*APC2*	*CDC16*	*CDC20*	*CDC27*	*SMT3*	*UBC4*
	MEN and cytokinesis	*CDC3*	*CDC6**	*CDC10*	*CDC11*	*CDC12*	*CDC14**
		*CDC15**	*CDC28**	*CDC48*	*DBF2**	*SPC110*	*ULP1**
DNA repair							
		*ABF1**	*MRE11**	*NSE1**	*NSE3**	*NSE4**	*NSE5**
		PRI2	*RAD5*	*RAD27*	*RFA3*	*RTT101*	*SCC2*
		SCC4	*SMC1*				
Lipid biogenesis							
		*DEP1**	*INO2*	*LCB2*	*SCS7*	*SUR1*	
Growth signaling							
	TORC1/2	*CDC14**	*TAP42*	*TSC11**			
	MAP kinase	*SLT2*					
Metabolism							
		CDC19	*DEP1**	*GLC7*	*TSC11**		
Kinetochore							
		*AME1**	*ASK1**	*CEP3*	*DAD1*	*DAD2*	*DAM1*
		DSN1	*IPL1*	*OKP1*	*SPC24*	*SPC105*	*STU1*
		TID3					
mRNA stability							
		AFG2	*CDC39*	*CDC42*	*CET1*	*CFT2*	*DBF2**
		KIN28	*LSM6*	*PAT1**	*RPB7*		

Chapter 4: Additional contributions to the scientific community

oSTEM at UC Santa Cruz

Introduction: Queer people pursuing STEM need better ally-ship.

Across the globe, queer people are a historically marginalized group. The biases against the queer community have pervasive effects. In fact, in the US, queer people are at significantly higher risk for: substance use, sexually transmitted diseases (STDs), cancers, cardiovascular diseases, obesity, bullying, isolation, rejection, anxiety, depression, and suicide[99]. Furthermore, from a study conducted in Washington DC it was found that 90% of queer youth reported "reservations" about seeking medical support and 68% reported that they choose to not discuss their sexual orientation with their clinicians. Not only is this a medical disparity, but biases against queer-identifying individuals permeates into the academic realm, as well. A 2021 article from Science Advances reported that ~33% of queer respondents (across 25 STEM professional societies) experienced one or more forms of social exclusion in the workplace[100]. UC Santa Cruz is no exception to these statistics. I have personally been affected by academic exclusion on multiple occasions. The most aggressive instance was at a departmental retreat, in which a fellow graduate student (who is a white-passing, cis, straight man) told me in the middle of a group conversation about romantic relationships during graduate school, that my opinions and experiences were not valid **because** I am queer. The statement was shocking and everyone else in the group was obviously uncomfortable. However, the group was utterly silent, until I physically left the conversation. Although one individual later approached me privately to voice concern and sympathy, the lack of ally-ship in

the moment will forever be a lasting memory. Interactions like this happen, and that is a problem, because it enforces a sense of not belonging. In fact, a 2018 study published in AAAS found that queer people are ~8% less likely to be retained in STEM, even after controlling for differences in socioeconomic status. The same study found that the single-largest positive impact on queer scientist retention was a positive STEM-Identity, which increases retention likelihood by ~13%[101].

How can institutions work to better facilitate a positive STEM-Identity for queer people? Being a queer scientist, I felt it necessary to begin to address this question, in addition to my scientific work during my graduate career. This led me to the organization, Out in STEM (oSTEM) which is an international professional association with over 100 chapters.

Description and track-record for oSTEM at UCSC

When I initially set-out to create an oSTEM chapter in the Fall of 2018, I had 2 aims: increase representation/visibility of queer people in STEM, and provide a stronger sense of community/belonging. I first began by recruiting a leadership team of 3 additional graduate students from the Department of MCD Biology: Hannah M. Newby, PhD., Stephanie Nystrom, PhD., and Oarteze Hunter, PhD. Together, we created a chapter of oSTEM that initially functioned as a seminar series open to all in the STEM community. We worked iteratively to recruit openly queer-scientists from all levels and backgrounds to come to the campus and give a 2-part seminar. The first of which being a research-focused talk of their current work, followed by a brief networking lunch, provided by the oSTEM chapter. Then, those interested, could participate in follow-up discussion

of personal experiences, being a queer person navigating various aspects of STEM. From the Fall of 2018 through the Fall of 2022, oSTEM at UCSC have had several community building activities and fundraising events, including a 2022 June Pride event for all. Additionally, we successfully coordinated 4 seminar events for the UCSC STEM community **(Table 4.1)**. Making oSTEM at UCSC sustainable for future cohorts of students is a top priority, and I am excited to know that, oSTEM will continue to be led the UCSC graduate students across the STEM fields. The new leadership is preparing to expand community building efforts to better include undergraduates and I am excited to see the chapter grow in the future.

Graduate STEM Outreach (Biol 220)

Table 4.1. Track-record for UCSC community seminars hosted by oSTEM.

Name	Position & Institution	Date
Lauren Esposito, PhD.	Curator and Schlinger Chair of Arachnology at the California Academy of Sciences.	05/2022
Melissa Moore, PhD.	CSO of Moderna, Eleanor Eutis Farrington Chair in Cancer Research and Professor at UMass Chan Medical School	03/2021
Brian Castellano, PhD.	Post-doctoral Researcher at Genentech	02/2020
Lauren Burrus, PhD.	Professor and chair at San Francisco State University	04/2019

A pilot to increase graduate student involvement in STEM Outreach.

Abstract

Institutions and graduate training programs need to get creative in order to better promote and sustain ODEI in STEM. Outreach is an aspect of ODEI that is often forgotten, but in many ways outreach is a pillar for successfully promoting positive change for DEI. As institutions are becoming more aware of the importance for valuing DEI, training programs need to invest in educating and exposing their graduate students to the concept of STEM outreach. The perspectives presented here aim to highlight the need for promoting outreach in STEM, and provides a meaningful path for training programs to do their part in closing the outreach-gaps.

The larger problem

The US is falling behind other countries in science education[102,103]. The lag in science education in the US converges with growing scientific distrust amongst the general public[104,105]. If the scientific community wants the public to support research initiatives, then it is the responsibility of scientists to be better engaged and communicative with the general public. One way that the scientific community can promote positive change for science education, trust, and support is by increasing and normalizing graduate student involvement in STEM outreach[106,107].

As emerging scientists, graduate students provide important links between senior-level scientists, aspiring scientists, and the broader general public. Yet, the scientific culture remains stagnant, in an elitist *Ivory Tower*, where graduate students are largely dis-incentivized to engage in outreach. The

experiences and values that graduate students receive from their training programs will ultimately shape their future endeavors once they leave their training programs. Therefore, encouraging graduate student involvement in outreach is a critical step that is needed to help repair science education, rebuild trust, and create public support for research.

The ideas and data presented here aim to better understand the lack of graduate student involvement in STEM outreach, and put forward a potential path for other institutions to expand from in order to mindfully and effectively increase graduate student involvement in STEM outreach.

A case study for addressing STEM Outreach

A preliminary interest poll of 33 graduate student participants in the PBSE umbrella program at UC Santa Cruz, found that ~94% of participants are interested in, and want to learn about, engaging in STEM outreach. However, many students convey institutional barriers to exploring outreach **(Figure 4.1).** Furthermore, this interest poll found that 75% of participants fear a delay in their graduation timeline if they were to participate in outreach. Additionally, 58% of participants felt a general lack of support and resources for navigating outreach opportunities **(Figure 4.2).**

Therefore, the MCD department at UC Santa Cruz and I worked to develop a graduate-level STEM outreach course to begin to bridge these gaps at the graduate level. In this course pilot, graduate students can earn elective course credit that can be applied towards graduation requirements. In addition, upon completion of the course, students can also obtain a certificate in *Mindful Practices for STEM Outreach*. These innovations work to incentivize advisors

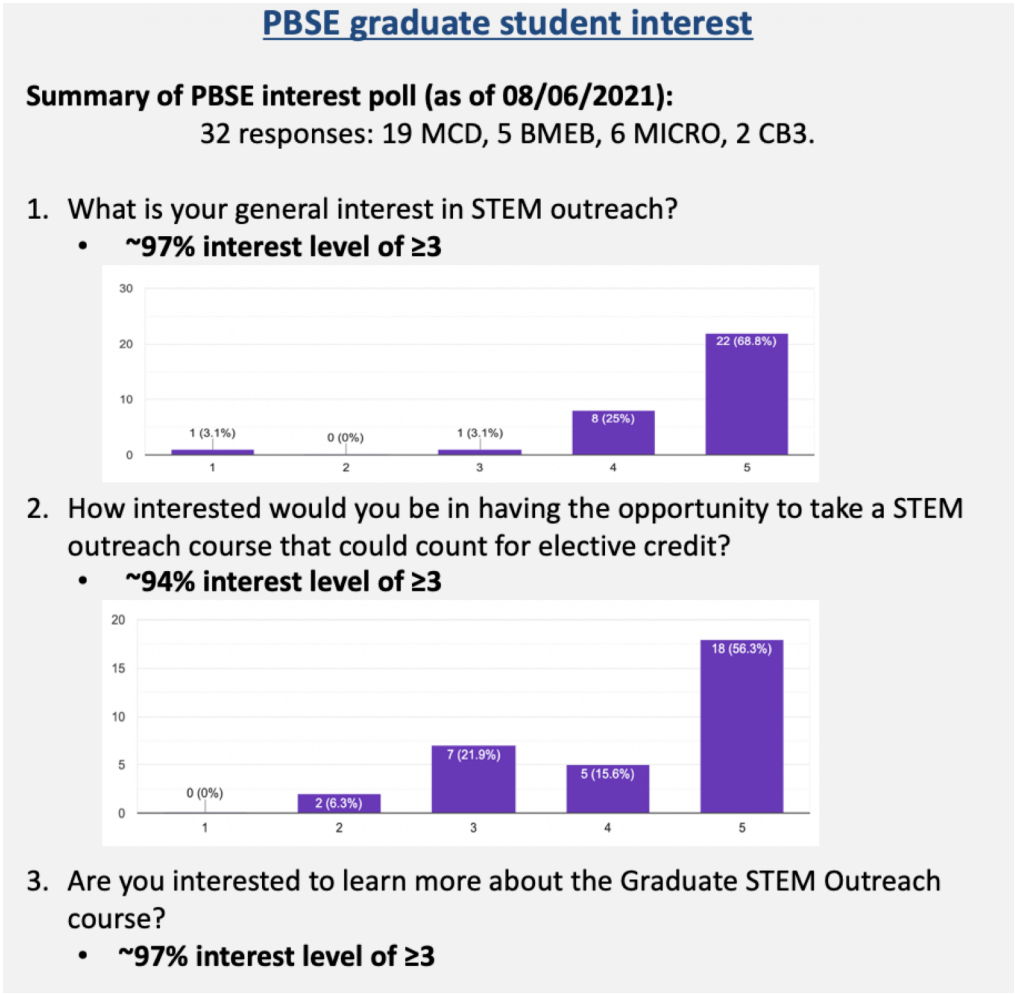

Figure 4.1. Summary of PBSE graduate student interest-poll for Biol 220, STEM Outreach

and institutions at large to allow graduate students the opportunity to explore their interests in outreach. Furthermore, providing students an ability to earn course credit that can be directly applied to their graduation requirements works to offset the fear and stigma that outreach engagement will delay a graduation timeline, a common fear shared amongst graduate students **(Figure 4.2).**

A pilot of the STEM Outreach course (Biol 220) was officially offered for the first time during the Fall of 2022. Throughout the course, students worked collaboratively to discuss outreach possibilities, expand their understanding on

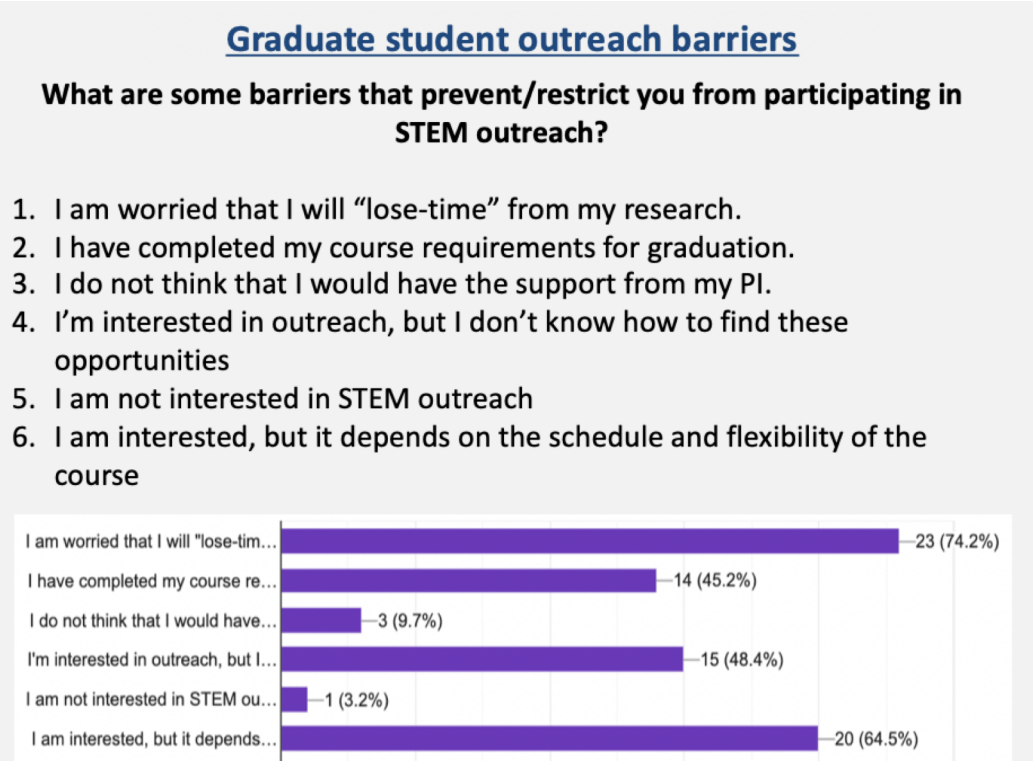

Figure 4.2. Barriers for graduate student participation in STEM outreach

the breadth of opportunities, and focus on ways to engage. There is much more to outreach than any one activity, and graduate students need to be exposed to these opportunities: writing blogs, newspaper articles, lesson plans for educators, podcasts, school visits and demo's, public lectures, discussion panels, participation in workshops, advocacy initiatives, and more. Additionally, during the course, students work to create a proposal that outlines an outreach idea/initiative. Students are not graded on completion of the outreach activity, but are graded on the quality of their proposal, and the ability to integrate aspects from the course (reading materials and guest-lectures) into the proposal. This allows the focus to be on creating a mindful outreach plan that is well thought out to be as effective as possible.

One way that this course helps students learn about the diverse and creative ways to engage in outreach is by having professionals give guest-lectures. Over the 10 weeks we hosted 4 guest-speakers from various academic and professional areas in STEM to share their unique experiences and perspectives on engaging in outreach. Not only does this expand knowledge and tools for mindful outreach practices, it also diversifies the breadth of outreach opportunities available. Additionally, incorporating outside professionals into the course as guest-speakers provides some relief on the instructor who may not be an expert on all-things STEM outreach related. This is important, because common concerns of faculty often surround the time commitment needed for facilitating a course, and not having an expertise in STEM outreach. However, these concerns can be largely mitigated by incorporating outside assistance, as is often the case when conducting scientific research, in general. Therefore, if faculty are accustomed to forming academic collaborations when conducting scientific research, then the same approach can be leveraged when facilitating a course that seems beyond an area of expertise.

Ultimately, it seems likely that graduate students at other institutions are similarly interested in STEM outreach. Therefore, it is important for training programs to explore paths forward that allow students the opportunity to learn about and hopefully engage in outreach.

Abbreviations used:

STEM	Science, Technology, Engineering and Mathematics
UCSC	University of California, Santa Cruz
PBSE	The Graduate Program in Biomedical Sciences and Engineering

MCD	Molecular, Cell, and Developmental Biology
METX	Microbiology and Environmental Toxicology
CB3	Chemical Biology, Biochemistry and Biophysics
BMEB	Biomolecular Engineering and Bioinformatics
ODEI	Outreach, Diversity, Equity, and Inclusion

Course description

Biol 220 (STEM Outreach) provides students an opportunity to plan, discuss, and structure a STEM outreach proposal for elective course credit. The course is designed to provide students with the foundation and support necessary to develop a STEM outreach proposal. The STEM outreach proposal aims to provide organization for the proposed STEM outreach event/activity. Outreach proposals will be focused on engaging communities from under-represented backgrounds as described by NIH guidelines.

Students will work iteratively throughout a quarter to develop a STEM outreach plan. This process will require biweekly updates with the course advisor and/or graduate student co-instructor, who will track progress of the outreach plan. Students are required to obtain permission from the course advisor, prior to registering for the course. Students interested in enrolling in this course should submit a Letter of Intent to course advisor for enrollment approval.

The course will include biweekly reading materials, discussions, guest lectures, and iterative writing. Students will be expected to demonstrate how the STEM outreach can/will impact under-represented communities in addition to effectively incorporate aspects of the reading materials into the final STEM Outreach proposal.

The course will allow students to critically develop, refine, and practice science communication skills with an emphasis on fostering positive STEM-identities for individuals from under-represented backgrounds.

Implementation

Students will attend class weekly and work iteratively throughout the 10-week quarter to create a STEM Outreach Proposal that incorporates aspects from the required course readings into the final document. Biweekly round table/discussions will encompass reading materials and updates regarding the STEM Outreach proposal. Week 5, will be an opportunity for all students to share working drafts of the STEM Outreach proposal with the course advisor, and receive advise & feedback prior to the final submission in Week 9. Additionally, the course will contain 4 supplemental guest lectures where professionals with relevant STEM outreach experience will share their input and participate in open dialogue with the class.

Table 4.2. Course schedule for Biol 220 during Fall, 2022.

Week 1	**In class:** Course overview and Introduction **Homework:** Reading 1, "Demographics of STEM in American Higher Education"
Week 2	**In class:** Reading 1**,** Round table and discussion **Guest speaker:** Yulianna Ortega, UCSC STEM Diversity Programs Director
Week 3	**Homework:** Reading 2, "Diversity and Equity in the Lab: Preparing Scientists and Engineers for Inclusive Teaching in Courses and Research Environments" **Guest speaker:** Amanda Brambila, PhD. SD IRACDA Postdoctoral Fellow
Week 4	**In class:** Reading 2**,** Round table and discussion **Guest speaker:** Zoe Petroff, Operations Coordinator at Scientific Slug Magazine
Week 5	**In class (optional):** Open office hours to review STEM Outreach Proposal drafts **Homework:** Reading 3, "Using Active Facilitation Strategies to Transfer Ownership in Teaching and Mentoring Contexts"
Week 6	**In class:** Reading 3, Round table and discussion

Week 7	**Homework:** Reading 4, “Poor kids, Limited Horizons” (4) & Reading 5, “Teaching First-Generation College Students” **Guest speaker:** Katrina Learned, Data Coordinator at Treehouse Childhood Cancer Initiative
Week 8	**In class:** Reading 4 & 5, Round table and discussion
Week 9	**LAST CLASS:** Submit final written STEM outreach plan to Course advisor

Upon successful completion of the course, students are eligible to earn a Certificate of Completion in Mindful Practices for STEM Outreach **(Figure 4.3)**.

Figure 4.3. A sample Certificate of Completion that is available to all students who successfully complete Biol 220.

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