

## ABSTRACT

Title: METABOLIC ENGINEERING OF  
EUKARYOTIC SIGNAL TRANSDUCTION IN  
*DROSOPHILA* SCHNEIDER 2 (S2) CELL  
CULTURE.

John C. March, Doctor of Philosophy in  
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Engineering

Eukaryotic cell culture presents metabolic engineers with opportunities not found in bacterial systems. The cell cycle enables higher order organisms to continually check their growth conditions, taking internal inventory of their general metabolic state before committing to DNA replication or cell division. Cell cycle check points and growth stimuli comprise an intricate feedback loop. Similarly, insulin signaling works to insure cells do not overproliferate when taking advantage of nutrient availability within a developing multicellular organism. However, the insulin signaling pathway is present in *Drosophila* S2 cells as well as whole flies, and may control growth in cell culture. The work described here exploited the insulin signaling pathway and the cell cycle for enhanced cell growth and heterologous protein expression. By using the relatively new approach of RNA interference (RNAi) gene silencing, key intermediates involved in growth and cell cycling were interrupted. This work demonstrates both the potential of RNAi and the utility of

controlling cellular signaling pathways as new and powerful tools in metabolic engineering.

METABOLIC ENGINEERING OF EUKARYOTIC SIGNAL TRANSDUCTION IN  
*DROSOPHILA* SCHNEIDER 2 (S2) CELL CULTURE.

By

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## Dedication

To my loving wife, Anne.

## Acknowledgements

I would like to thank the many people who have made this work possible. These include my labmates in the Bentley lab, my committee members, teachers in Chemical Engineering and the Center for Biosystems Research (CBR), and the staff members of Chemical Engineering, Bioengineering (Julie Holbrook), and CBR.

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Finally, I want to give my loving thanks to my wife, Anne, to whom this work is dedicated. Working a fulltime job to keep her husband in graduate school for most of our marriage is a task that will go largely unsung (at least in print) outside of these pages. Her support of me and our family has been not only the reason for this work's completion, but the inspiration that made completing it a pleasure. She would lift my spirits through months of bad data and raise a glass with me when results finally came in. I cannot express gratitude and love enough to match my heart, but will suffice it to say that I cannot imagine a better life without her in it.

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# Chapter 1: Introduction

## *Summary*

Recombinant protein production has emerged as a major component of the pharmaceutical industry (Thayer 2002). The recombinant product fraction of the pharmaceutical market is expected to increase as the world population ages and new products, such as cosmetic proteins, come online (Class, 2002; Mullin, 2003). Enzymes are used to manufacture specialty chemicals, such as stereo-specific isomers, in addition to pharmaceuticals (Panke and Wubbolts 2002; Zhao et al. 2002). There are now more products than ever produced through recombinant means. The increase in product lines has attracted the interest of greater numbers of manufacturers into the recombinant protein market. With patents running out on as much as \$13 billion worth of recombinant products in the next five years, generic manufacturers are entering the market (Rouhi 2002). With more products and competition, there will be increased pressure for chemical producers to reduce production costs.

The most commonly used organism for synthesizing recombinant proteins is *Escherichia coli* (Lee 1996). *E. coli* is inexpensive to grow, simply transformed, and genetically well-characterized. Its use is well documented in the literature and will not be discussed here. A limitation with *E. coli* is that it lacks the eukaryotic protein processing machinery to properly fold, glycosylate, and amidate many mammalian proteins. This shortcoming has resulted in the necessity to use mammalian cell culture to synthesize proteins that are incorrectly processed, or, in some instances, too

big for *E. coli* to produce effectively. Mammalian cell culture can be far more expensive than *E. coli* fermentations, largely due to slower growth, increased genetic complexity, difficulties with transfection, and lower product yield.

One potential solution to producing high-quality, properly-processed mammalian (and other eukaryotic) proteins is to use *Drosophila* Schneider 2 (S2) cells. S2 cells are a stable cell line derived from embryos of *Drosophila melanogaster* (Schneider 1972). They grow more quickly in culture than mammalian cells and grow optimally at room temperature (25 to 28 °C)(Echalier 1997). The use of S2 cells for the production of recombinant gene products has been well-established (Kirkpatrick and Shatzman 1999). They are almost as genetically well-characterized as *E. coli* (Adams et al. 2000), and it has been demonstrated that *Drosophila* cell cultures are capable of properly glycosylating and amidating mammalian proteins (Aldecoa et al. 2000; Benting et al. 2000; Li et al. 1996; Percival et al. 1997; Tota et al. 1995). Some of the disadvantages of using S2 cells are that they grow more slowly, require greater care, and are more difficult to transfect than *E. coli*. S2 cells also have a lower product yield (g product/L culture volume) than *E. coli*, which can have in excess of 10-fold higher yield than S2 cells (Lee 1996; Olsen et al. 1992; Pfeifer et al. 2001).

The work proposed here will address S2 cell growth and protein production. Specifically, it relates *Drosophila* cell growth, cycling, and proliferation regulation to recombinant protein production. The focus is on the insulin signaling pathway and the cell cycle. Project scope, hypotheses, and the proposed approach follow the literature review.

## *Literature review*

### **Drosophila S2 cells**

*Drosophila melanogaster* Schneider 2 cells were first isolated in 1972 at the Walter Reed Army Institute of Research in Washington, DC (Schneider, 1972). The strain was developed from trypsinized fragments of 20 to 24-hour old Oregon-R *Drosophila melanogaster* embryos. The cells can grow as a loosely attached monolayer in petri plates or in suspension. The cell size can range from 5 to 10  $\mu\text{m}$  in diameter for round cells and up to 35  $\mu\text{m}$  in length for elongated cells, depending on the growth conditions. Tetraploid cells compose approximately 60% to 80% of the culture, with all cells exhibiting XX (female) chromosomes (Echalier, 1997).

S2 cells are among the most frequently used *Drosophila* cell lines (Echalier, 1997). They can be modified to express various biopharmaceuticals and recombinant products, including many mammalian proteins (Lee et al. 2000; McCarroll and King 1997). However, S2 cells do not grow as quickly as *E. coli* and are more difficult to transfect. This places S2 cells somewhere between mammalian cells and *E. coli* in their usefulness for large-scale production of recombinant proteins. Recently, Lee and coworkers (2000) demonstrated that baculovirus-mediated transfection of S2 cells for the production of enhanced green fluorescent protein increased transfection efficiency from 10% to nearly 100%. The transfection left the cells intact and increased the specific protein expression levels (as a fraction of total protein) to well within the range expressed by high five cells (a lepidopteran insect cell line) (Lee et al., 2000). No follow-up work has been published on this approach, and no specific information on cell growth rates post-baculovirus-infection was provided. There has

yet to be a study reviewing S2 cell growth with respect to recombinant protein production.

### **Growth in *Drosophila* cells**

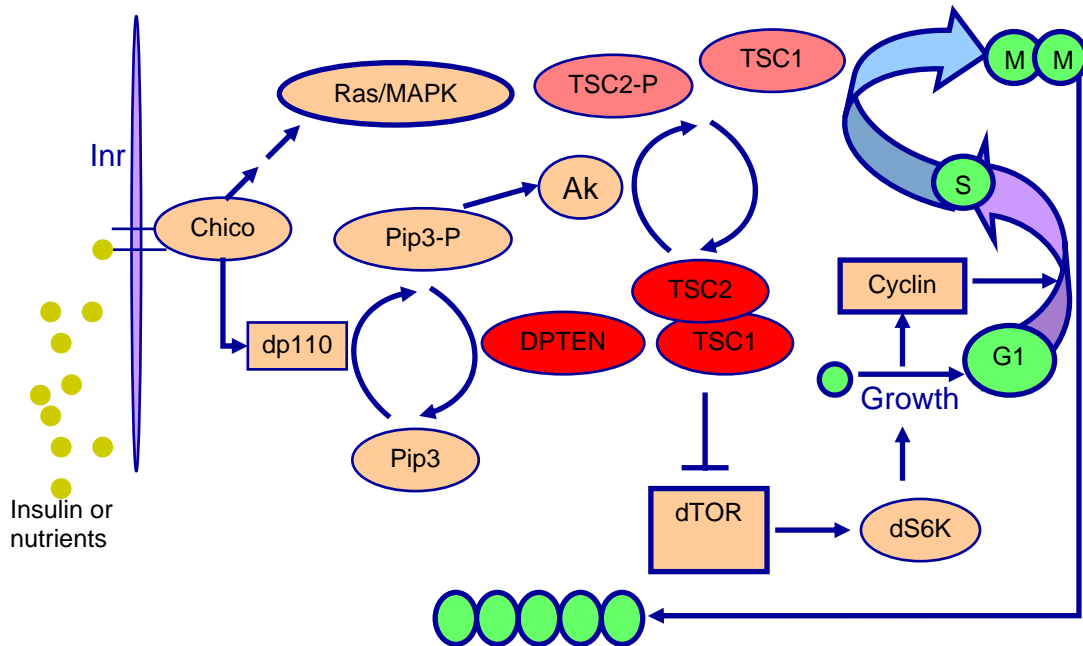
Two primary pathways have been the focus of studies investigating growth control in S2 cells: the insulin signaling pathway and the Ras-MAP kinase pathway. The positive effect of insulin on cell growth and proliferation have long been established (Mosna 1981; Mosna and Barigozzi 1976; Seecof and Dewhurst 1974; Wool et al. 1966; Wyss and Bachmann 1976). The insulin signaling pathway (Fig. 1) is controlled both by the presence of insulin-like growth factors and nutrients (Brogiolo et al. 2001; Gao et al. 2002; Thomas and Hall 1997). Its function in eukaryotes is to maintain balance between cell growth and available substrate. Insulin signaling is found in almost all eukaryotic organisms, and provides cells with a mechanism for halting growth in the absence of nutrients and for stimulating growth in times of excess nutrient availability. Alterations in any component of the insulin pathway in *Drosophila* can affect either cell size or proliferation (Potter and Xu 2001), but do not necessarily affect both (Verdu et al. 1999).

The insulin signaling cascade starts with insulin or an insulin-like-peptide (Brogiolo et al., 2001). There are at least seven known insulin like peptides in *Drosophila* (Claeys et al. 2002). These peptides all stimulate the insulin receptor (Inr, a receptor tyrosine kinase) leading to the downstream activation of Akt, a protein serine/threonine kinase. The Inr, while nominally a tyrosine kinase, cannot initiate the signaling cascade independently. To phosphorylate the first intracellular cascade

receptors, Inr requires the help of a scaffolding adapter molecule known as an insulin receptor substrate (IRS, Chico in *Drosophila*). Chico acts as a docking site for *Drosophila* dp110 (P13K in mammals), which in turn activates downstream cascades leading to both cell proliferation and cell growth (Brogiolo et al. 2001; Claeys et al. 2002; Stocker and Hafen 2000; Tapon et al. 2001b).

**Figure 1.**

**Insulin and cell cycling pathway.** Insulin or nutrients stimulate the insulin receptor (Inr) on the cell membrane. Phosphorylation of Inr (a tyrosine kinase-like receptor) leads to phosphorylation of the insulin receptor substrate, Chico. Chico has numerous downstream phosphorylation targets, including dp110. Nutrient or insulin stimulation leads to phosphorylation of dS6K, which has numerous downstream transcriptional and translational targets that stimulate growth, which shortens G1 of the cell cycle by (in part) synthesizing Cyclin E. Rapidly growing cells become overproliferative (green circles).



The Akt branch of the insulin pathway has been the subject of numerous studies. The Akt pathway is linked to cell growth and proliferation control in whole fruit flies (Brogiolo et al. 2001; Claeys et al. 2002; Ito and Rubin 1999; Kozma and Thomas 2002; Miloloza et al. 2000; Oldham et al. 2000; Potter and Xu 2001; Radimerski et al. 2002; Rintelen et al. 2001; Soucek et al. 1997; Stocker and Hafen 2000; Tapon et al. 2001a; Tapon et al. 2001b), but the phenotypic effects of alterations to this pathway have not been studied in S2 cells. For example, Clemens and coworkers demonstrated that dsRNA (double stranded RNA interference) inhibition of DPTEN (*Drosophila* phosphatase and tensin homologue deleted from chromosome 10) results in a 19-fold increase in Akt activity in S2 cells in the presence of insulin, but never studied cell growth or morphology in the DPTEN knock-outs (Clemens et al. 2000; Leff 2001). By preventing Akt phosphorylation, DPTEN stimulates forkhead transcription factor translocation to the nucleus. When in the nucleus, the forkhead transcription factors activate transcription of cyclin-dependent kinase inhibitors (such as p27Kip1), which inhibit Cyclin E/Cdk2 activity in *Drosophila*. The result is G1 cell cycle arrest (Nakamura et al. 2000) and possibly reduced cell proliferation.

Verdu and coworkers found that overexpression of dp110 (P13K) and Akt in whole flies results in increased cell size, but no increase in cell number (Verdu et al. 1999). However, inhibition of DPTEN has been shown to increase cell growth *and* proliferation in *Drosophila* whole flies (Gao et al. 2000). DPTEN mutant fly eye cells outgrew their non-mutated counterparts three to one, and were 140% larger. Moreover, organism-wide overexpression of DPTEN in whole flies using a *GAL-4*



driver proved fatal for the most part, with those that survived exhibiting severely limited growth (Gao et al., 2000). This suggests that there may be an independent pathway or branch point downstream of DPTEN, independent of Akt that is responsible for proliferation.

An important control point in the insulin pathway occurs with the protein TOR (target of rapamycin) and its interaction with the gene product of *gigas*. *Gigas* is the *Drosophila* homologue of tubular sclerosis gene 2 (*TSC2*) in mammals. In mammals and *Drosophila*, the *TSC2* gene product, tuberin, forms a complex with hamartin, the product of tubular sclerosis gene product 1 (*TSC1*) (Ito and Rubin 1999). When active, the complex antagonizes the insulin signaling pathway by blocking TOR phosphorylation (Ito and Rubin 1999). Human mutants of *TSC1* or *TSC2* develop tubular sclerosis, a disease marked by benign tumor cells called hamartomas, giant-sized cells in the tumorous tissue (Ito and Rubin 1999). The effect of *TSC1* or *TSC2* inhibition in whole flies and mammalian cells is the same, specifically that cells grow two to four times normal size and overproliferate (Aicher et al. 2001; Ito and Rubin 1999; Miloloza et al. 2000; Potter et al. 2002; Potter and Xu 2001; Soucek et al. 1997; Tapon et al. 2001b). Akt phosphorylation regulates the TSC complex (Aicher et al., 2001), and aids it in its role as a tumor suppresser. When phosphorylated by Akt, tuberin disassociates from hamartin (Potter et al., 2002) and loses its ability to limit TOR activity (Gao et al., 2002).

Gao and coworkers found that reducing expression of TOR by 50% recovered the effect of inhibiting TSC gene expression (*i.e.* increased cell size) in *Drosophila* eyes by 15.8% (Gao et al., 2002). In order to ascertain whether the TSC complex

regulates TOR activity, downstream products of TOR were measured (rather than TOR itself) due to the unavailability of antibodies specific to *Drosophila* TOR. dsRNA against *TSC1* or *TSC2* resulted in a 4.5-fold increase in S6K phosphorylation, without a change in the S6K protein levels. dsRNA against TOR had the same effect, regardless of whether dsRNA against TSC genes were used. Further, immunoprecipitation experiments revealed that tuberin (the *TSC2* gene product) interacted with TOR *in vivo*. Taken together these results indicated that *TSC* gene products regulate TOR expression and control of the Akt signaling pathway (Gao et al., 2002).

TOR has been linked to amino acid sensing (Hara et al. 2002; Miron and Sonenberg 2001) as well as to the insulin cascade (Gingras et al. 2001; Miron and Sonenberg 2001). Insulin stimulates muscle growth (Wool et al. 1966; Wyss and Bachmann 1976), but there is evidence that a significant portion of muscular growth following amino acid starvation stems from the amino acids themselves, independent of insulin (Svanberg et al. 1996). Hara and coworkers investigated S6K and eIF-4E BP1 phosphorylation in mammalian cell lines in response to amino acid abundance (Hara et al. 1998). They determined that there existed an unknown factor between TOR and extracellular amino acids that downregulated TOR activity in the event of amino acid deprivation (Hara et al., 1998). The effect was seen regardless of the presence of insulin. The unknown factor that senses amino acids may be the TSC complex. Through its interactions with TSC gene products, TOR senses extracellular amino acid concentrations and up regulates translational initiation regulators such as S6K and the initiation factor 4E binding protein (4E-BP) in the presence of amino

acids (Gao et al., 2002; Miron and Sonenberg, 2001). The mechanism by which the TSC complex senses amino acid concentrations is unknown. It may be the case that the TSC complex prioritizes stimuli such that amino acid starvation closes the insulin pathway, and restoration of amino acids allows insulin-mediated growth. The work of Hara and others supports this hypothesis. In the presence of insulin and the absence of amino acids, S6K was not phosphorylated. The amino acid concentration required to restore S6K activity was four times lower in insulin-stimulated cells than in non-stimulated cells (Hara et al., 1998).

### **Drosophila cell cycle**

In order to understand eukaryotic cell growth and proliferation, researchers have long focused on the cell cycle. There is a link between the cell cycle and cell growth and proliferation (Tapon et al. 2001b). In higher order organisms such as *Drosophila*, the developmental stage of the organism as well as the organ or system in question play important roles in how the cell cycle relates to cell growth. In *Drosophila* embryos, cells divide rapidly without growing as a result of a shortened cell cycle. In eye imaginal disks cells grow to five or six times their post-mitotic size before dividing (Tapon et al. 2001b). There are some factors, such as cyclins, which are synthesized in close correlation with cellular growth that are responsible for at least some aspects of cell cycle progression (Bock et al. 2001). Weigmann and coworkers, working with *Drosophila* pupal imaginal disc cells, demonstrated that cell size is not controlled by factors effecting cell division, but rather by "physical distance or tissue volume", which could be interpreted as volumetric concentrations of growth-regulating molecules (Weigmann et al. 1997). When the wing imaginal

discs mutant for Cdc2 where arrested in G2 of the cell cycle, the resultant wing size was the same as a non-mutated control, but the size of the cells in the wing were much larger, indicating that growth can proceed in the absence of cell division (Weigmann et al., 1997).

Factors that control the Akt or Ras pathways also control, either directly or indirectly, the synthesis and activation of cell cycle regulators (Tapon et al. 2001b). Reducing function in any of the insulin pathway components Chico, dp110, DPTEN, Akt, TOR, PDK-1, or SK6 slows the cell cycle (Oldham et al. 2000; Stocker and Hafen 2000). The exact pathways are unclear, but Myc, a protein long associated with cell proliferation and death (Coller et al. 2000) also has a strong effect on the cell cycle (Nasi et al. 2001). Induction of Myc expression shortened G1, but caused a concomitant lengthening of G2 in *Drosophila* developing wings (Johnston et al. 1999). Cells overexpressing Myc were smaller than wild-type controls and grew poorly (Johnston et al., 1999). For its role in shortening G1, Myc is thought to increase Cyclin E activity. Increasing dp110 or Ras expression increased growth rates, but did not accelerate the cell cycle in *Drosophila* imaginal discs (Bock et al., 2001).

Some cyclin-containing factors such as the cyclin-independent kinase 4 (CDK4)/Cyclin D complex, which drive cell growth, do not have a direct effect on cell cycling (Datar et al. 2000; Meyer et al. 2000). Datar and coworkers demonstrated that overexpression of Cyclin D/CDK4 resulted in *Drosophila* eye overgrowth and increased proliferation without changing the cell cycle (Datar et al., 2000). In the developing wing, Cyclin D/CDK4 overexpression resulted in higher

proliferation with no significant change in cell size. Sixty-seven hours after induction of Cyclin D/CDK4 overexpression the wing cells filled an area that was 180% of the area occupied by the controls. In the same study it was shown that Cyclin E overexpression truncates G1 and decreases cell size.

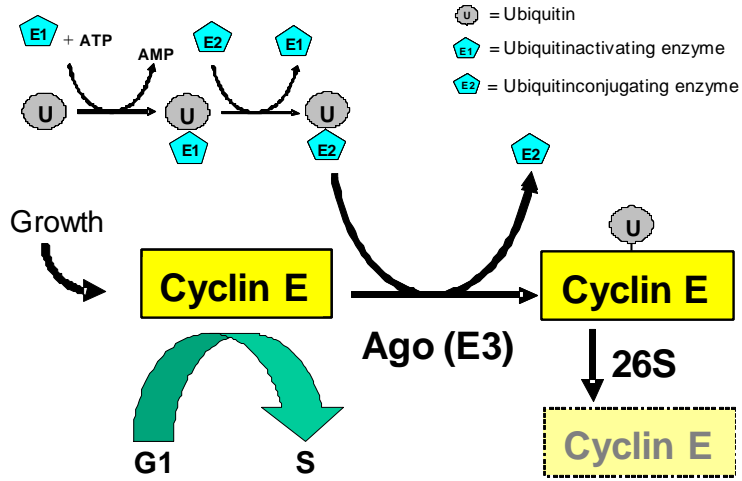
Cyclin E plays an important role in G1 to S phase transition, but that role is not completely understood (Bock et al. 2001; Richardson et al. 1993). Lees and others determined that Cyclin E aids in regulating the tumor suppresser gene, retinoblastoma, which forms a complex with transcription factor E2F and cyclin E during G1 (Lees et al. 1992). Cyclin E-mediated phosphorylation inactivates retinoblastoma and allows the cells to pass to S phase (Richardson et al., 1993). Cyclin E levels are controlled, in part, by ubiquitin-mediated proteolysis. Accumulation of Cyclin E can result in premature S phase entry, genetic instability, and tumorigenesis (Koepp et al. 2001). Destruction of Cyclin E is a function of the 26S proteasome, and is facilitated by the formation of polyubiquitin-protein conjugates. A ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and Archipelago, a ubiquitin ligase (E3) are involved in a cascade of reactions that ultimately form a ubiquitin complex with Cyclin E, marking it for proteolysis (Fig. 2A). Archipelago-mutant *Drosophila* eye cells have elevated levels of Cyclin E and overproliferate (Moberg et al. 2001). Moberg and coworkers successfully inhibited Archipelago synthesis in S2 cells by transfecting cells with dsRNA against *ago*, the gene encoding Archipelago (Moberg et al., 2001). They did not examine the phenotypic effects of the transfection on S2 cells. Hence, it has already been established that ago dsRNA affects Archipelago, that Archipelago affects Cyclin E

accumulation, and that Cyclin E accumulation can lead to overproliferating cells in flies. The three elements have not previously been coordinated or examined as a target for metabolic engineering.

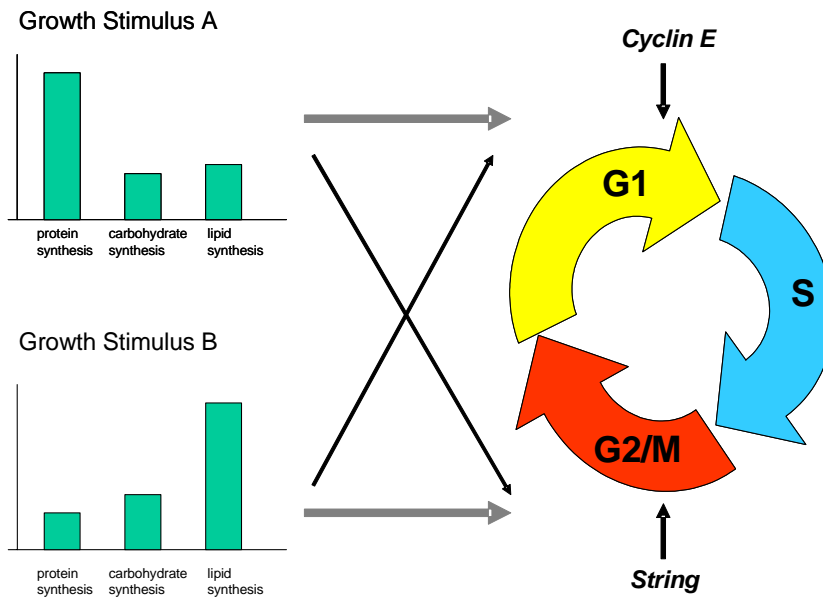
**Figure 2.**

Growth stimulus and the cell cycle. **A.** Many factors stimulate transcription of key signaling molecules, such as Cyclin E. Cyclin E accumulation triggers G1 to S phase transition. Archipelago (Ago) tightly regulates Cyclin E via ubiquitination and proteolysis (26S proteasome). Three enzymes serve to ubiquitinate numerous proteins for proteolysis, a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3), which is archipelago (Ago) in the case of cyclin E. **B.** Proposed model of Tapon, et. al (2001), in which disparate stimuli control the anabolic landscape in favor of protein, carbohydrate, or lipid synthesis. Growth factor A favors protein synthesis, while growth factor B stimulates lipid synthesis. Control could be linked to the cell cycle at checkpoints, such as G1/S (Cyclin E) or G2/M (String).

**A.**



**B.**



Adapted from Tapon et al. (2001) Curr. Opin. Cell Bio. 13:731-737



## **Protein expression and the cell cycle**

Cell growth and protein expression can vary significantly throughout the life of a cell culture. Creanor and coworkers estimated that yeast cells exhibited an increase in protein expression rates for the first 60% of the cell cycle when compared to the remaining 40% (Creanor and Mitchison 1982). The cell cycle phases have numerous overlapping qualities, but in general, G1 is for cell growth and, presumably, protein synthesis. The G1 phase can be divided into two subphases: the post-mitotic phase (G1pm) and the pre-synthesis phase (G1ps) (Abu-Absi and Srien 2000). In G1pm the cell is dependent upon growth factors, and requires growth before it can proceed with the rest of the cell cycle. When the cell is prepared to begin replicating the DNA and continue with the cell cycle it is in G1ps, which is delineated from G1pm by the restriction point (Abu-Absi and Srien, 2000).

Tapon and colleagues (2001) proposed a simple model for the disparate growth stimuli on the cell cycle and anabolic pathways (Fig. 2B). In short, there may be a stimulus that prolongs G1, resulting in heightened protein synthesis, or prolongs G2/M, resulting in the production of more lipid. These scenarios are meant to illustrate that there may exist distinct metabolic states which are more amenable to specific target-compound synthesis.

Cells growing in culture are subject to various stresses, including oxidative stress, heterologous protein expression, shear stress, toxin buildup and nutrient depletion. One cellular response to stress is apoptosis (programmed cell death),

which has overlapping control pathways with the cell cycle (Fussenegger and Bailey 1998). In addition to changing environmental parameters such as nutrient concentrations and impeller speed, approaches to minimizing apoptosis include overexpression of anti-apoptotic genes and injection of chemical antagonists to apoptotic pathways. The gene product of *bcl-2*, a mammalian survival gene, inhibits apoptosis and has been used to increase yield of monoclonal antibody by up to 40% in chemostat cultures of hybridoma cells (Simpson et al. 1997). By adding thymidine to cells expressing *bcl-2*, apoptosis was suppressed longer than without the thymidine addition (Singh et al. 1996). Cells respond to amino acid starvation by inhibiting the insulin signaling cascade, limiting protein translation, and initiating apoptosis.

Mazur and coworkers demonstrated the use of controlled proliferation in the production of secreted alkaline phosphatase (SEAP) in Chinese hamster ovary (CHO) cells (Mazur et al. 1998). Cells grew until they reached early stationary phase and then were induced to synthesize SEAP concurrently with the p27 gene product. P27 is a mammalian cell-cycle-arresting gene, which allowed the cells to remain in G1 of the cell cycle. This approach is similar to that used frequently in bacterial fermentations for anaerobic fermentation products. There, cells are grown aerobically to an optimum density and then allowed to synthesize products anaerobically, with very little cell growth. The biphasic approach for eukaryotic systems takes advantage of cell cycle controls to achieve the same stationary state exhibited in bacterial fermentations. By using a tetracycline-dependent promoter, Mazur and coworkers coordinated G1 rest with optimal cell density. Tetracycline degraded (with or without cells present) at a rate that correlated with first order kinetics. Given an initial

concentration of 20 ng/mL, the concentration of tetracycline in the media was low enough after 48 hours to prevent promoter inhibition. With tetracycline below the threshold value, the promoter allowed for synthesis of p27 and SEAP concurrently. The result was a 15-fold increase in SEAP expression in biphasic cells over proliferation competent controls (Mazur et al., 1998). Biphasic protein expression control has not yet been demonstrated in S2 cells. Although S2 cells have a cell cycle distinct from mammalian cells, they still have a presumed increase in protein synthesis in G1pm, and are subject to similar stresses in expressing heterologous proteins (Echalier, 1997).

### **Double-stranded RNA interference**

In 1998, Fire and coworkers reported on the use of short sequences of double-stranded RNA for interfering with gene expression in *Caenorhabditis elegans* (Fire et al. 1998b). The method has since been used in fungi, plants, fish, and *Drosophila* among other organisms (Fire 1999; Ullu et al. 2002). The mechanism by which dsRNA inhibits gene expression is not entirely known, but the most generally accepted model can be described as follows. Short sequences of double-stranded RNA enter the cytoplasm of a cell, either by injection or diffusion, where they are cleaved by an enzyme (Dicer in *Drosophila*) or enzyme complex to 22 nucleotide RNA fragments with short 3' overhangs. The fragments then find and bind to the mRNA to which they are homologous. This step likely takes place on the cleaving enzyme, which is thought to possess a helicase domain. Once the mRNA and short dsRNA fragment are bound, the cleavage enzyme degrades the mRNA, rendering it useless for translation (Bass 2000; Fire 1999; Ullu et al. 2002).

dsRNA is an effective tool for targeted inhibition of specific gene expression in S2 cells (Caplen et al. 2000; Clemens et al. 2000). Clemens and coworkers used dsRNA to investigate the insulin-signaling pathway. By inhibiting expression of Chico, DPTEN, and dp110, researchers confirmed the order of these components in the insulin pathway and demonstrated the use of dsRNA in S2 cells. Similarly, Caplen and coworkers demonstrated dsRNA efficacy against heterologous protein expression in S2 cells (Caplen et al., 2000). In transient and stable transfections, green-fluorescent-protein-specific dsRNA inhibited green fluorescent protein (GFP) expression without interfering with expression of the recombinant control product, chloramphenicol acetyltransferase (CAT). While the inhibition was not as pronounced as it was in the Clemens study, it demonstrated the very specific nature of the knockout. These two examples are likely only a small fraction of the studies using dsRNA to investigate gene expression in S2 cells currently being undertaken. The technology is simple to use and can theoretically be targeted against any gene expressed in S2 cells.

Recent work has focused on *in vivo* synthesis of double-stranded (dsRNA) or short interfering RNA (RNAi) in *Drosophila* (Giordano et al. 2002), plants (Wesley et al. 2001), and mammalian cells (Brummelkamp et al. 2002). RNAi usually refers to the *in vivo* synthesis of dsRNA with homology to a target mRNA. The length of the dsRNA is sometimes only 22 nucleotides, the same size that larger dsRNA is believed to be fragmented into. While short, interfering RNA is effective in mammalian systems, it has been demonstrated that in S2 cells larger (>500 nt) dsRNA is significantly more effective (Hammond et al. 2000). In *Drosophila* whole

flies Giordano and coworkers expressed dsRNA against *white*, a color marker in *Drosophila* eyes, using the pUAST vector to express sense and antisense strands of *white* under GAL-4 UAS promoter (Giordano et al., 2002). Sense and antisense strands synthesized under the same promoter were expected to anneal to form the dsRNA. It was estimated by the researchers that the sense and antisense strands did not anneal as readily as had been expected, and therefore the construct was less efficient of a tool for gene inactivation than a similar construct carrying inverted repeats of *white* with 200 bp spacing between them. In both constructs *white* mRNA was diminished, however, the effect was more pronounced in the case of the inverted repeats. The use of *in vivo* dsRNA synthesis has not yet been demonstrated in S2 cell culture.

### ***Hypothesis***

The work described above regarding *Drosophila* growth, proliferation, and cell cycle regulation has demonstrated that inhibition or overexpression of certain key factors in *Drosophila* can change cell size, proliferation, and cell cycling. More specifically, inhibition of the insulin pathway regulators, *DPTEN* and *TSCI* result in overgrowth and overproliferation of *Drosophila* imaginal discs. Inhibition of Cyclin E results in cell cycle arrest in *Drosophila*. Inhibition of Archipelago synthesis results in accumulation of Cyclin E. There is a potential link between amino acid availability and insulin signaling that may implicate the TSC complex as the central component to both pathways.

The amino acid signaling pathway and the insulin signaling pathway, which are important for enhancing cellular growth, have been linked to cell cycle control

(Conlon et al. 2001; Oldham et al. 2000). As cells grow in G1pm (under the influence of the insulin pathway), they synthesize Cyclin E. When Cyclin E reaches a threshold concentration (up to eight times its mitotic level), the cells can enter S phase (Richardson et al., 1993). These observations give potential targets for metabolic engineering through both up- and down-regulation.

The hypothesis for this work was as follows:

1. Inhibition of *DPTEN*, *TSC1*, and *ago* will result in increased S2 cell proliferation and protein synthesis.
2. S2 cells that are enhanced for growth and proliferation will reach early stationary phase sooner than cells that have not been enhanced as described in 1. above.
3. S2 cells can be maintained in G1 of the cell cycle by inhibiting Cyclin E expression.
4. S2 cells maintained in G1 will synthesize recombinant protein at a faster rate and realize a higher yield than cells not maintained as in 1. and 3. above.

## Chapter 2: Insulin Stimulates Uptake of dsRNA by *Drosophila* Cells

### ***Introduction***

The advent of RNA interference has facilitated a boom in biochemical pathway analysis, and functional genomics. RNAi refers to any RNA molecule that interferes with the expression of its homologous gene product (Fire et al. 1998a; Rocheleau et al. 1997; Timmons and Fire 1998). Also referred to as post-transcriptional gene silencing, RNAi is exquisitely specific for the targeted gene and encompasses sense, antisense, or double-stranded RNA (dsRNA) molecules, although it is commonly attributed with dsRNA as single-stranded RNA (ssRNA) effects have been traced to low levels of contaminating dsRNA (Fire et al. 1998a; Rocheleau et al. 1997).

Double-stranded RNA of 220 to 700 basepairs has been shown to significantly reduce levels of mRNA transcript in *Drosophila* cell culture for a wide range of genes including insulin signaling pathway components (Clemens et al. 2000), recombinant GFP (Caplen et al. 2000), and for 91% of the genes associated with proliferation and survival (Boutros et al. 2004). Double-stranded RNA of 500 to 700 basepairs can be transfected into *Drosophila* Schneider 2 (S2) cells by incubating them with fetal bovine serum (FBS) following serum starvation (Clemens et al. 2000). Unfortunately, FBS has numerous stimulatory effects and can greatly complicate metabolic studies, due its poorly characterized and variant composition.

## ***Materials, Methods and Results***

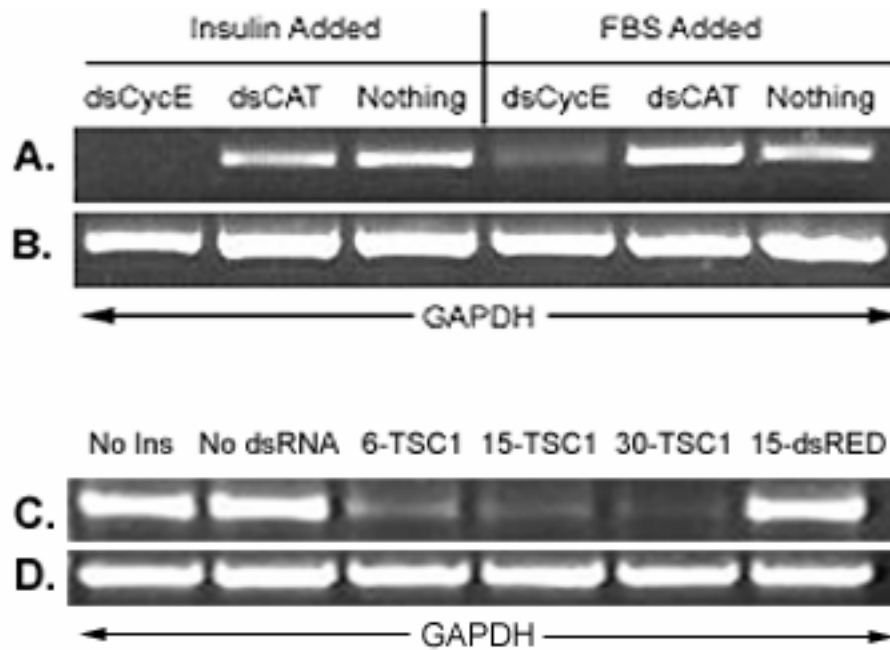
We were able to remove FBS completely from dsRNA studies by replacing it with porcine insulin. To test the effect of trying to replace FBS with insulin, we selected Cyclin E as a target gene for silencing. S2 cells were grown in *Drosophila* serum-free media (SFM) (Invitrogen, Carlsbad, CA). Double-stranded RNA was synthesized following a modified version of the method developed by Clemens and coworkers (Clemens et al. 2000). Briefly, S2 cells were grown to  $5 \times 10^6$  cells/mL, and RNA was extracted using an RNAqueous® kit (Ambion, Houston, TX) according to the manufacturer's protocol. First strand templates of Cyclin E DNA were synthesized from the total mRNA using oligo-dT primers and a Retroscript® kit (Ambion) as per the manufacturer's instructions. A 630 basepair region of first strand DNA template was PCR amplified using Cyclin E specific primers (5'-ATG GGT TTA AAT GCC AAG AGT GTT TGT TC; 3'-CAC CAC CAC TGG CGT CTG CTT GCT TCC ACG. T7 sequences (TAA TAC GAC TCA CTA TAG GGA) were added to each Cyclin E template using PCR, making T7 templates. To transcribe single-stranded RNA (ssRNA), T7 templates were used with the Megascript™ kit (Ambion) as per the manufacturer's instructions. The ssRNA synthesized was extracted using phenol/chloroform and resuspended in nuclease-free water to a concentration of approximately 3.3 µg/µL. Single-stranded RNA was incubated at 65 °C for 30 minutes before being allowed to cool to room temperature on the benchtop. Subsequent to this annealing step, dsRNA was checked for size and integrity using agarose gel electrophoresis.



Cells were seeded in triplicate to  $1 \times 10^6$  cells/well in 0.7 mL SFM in 12-well plates and incubated with 15  $\mu\text{g}/\text{mL}$  dsRNA against cyclin E, chloramphenicol acetyl transferase (CAT), or an equal volume of nuclease-free water. CAT was used as a control because it has no significant similarity to any genes in the *Drosophila* genome (BLAST search). The CAT dsRNA was 630 basepairs long. After 1 hour incubation, SFM containing either 10% FBS (Sigma, St. Louis, MO) or 1.75  $\mu\text{M}$  porcine insulin (Sigma) was added to a final volume of 2.1 mL per well. The plates were incubated for 60 hours at 27 °C. RNA was extracted from the cells and RT-PCR was performed as described above for a 630-basepair segment of Cyclin E and a 866-basepair segment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Each treatment's triplicate RNA samples were pooled before reverse transcription. Results (Fig. 3) indicate that insulin worked as well or better than FBS at facilitating post-transcriptional gene silencing in S2 cells.

**Figure 3.**

Insulin stimulates uptake of dsRNA in S2 cells. **A.** RT-PCR of total RNA from S2 cells using oligo-dT primers for reverse transcription, and *Cyclin E*-specific primers for PCR. Conditions are listed across the top with cells either receiving dsRNA treatment against *Cyclin E* (dsCycE), *CAT* (dsCAT), or no dsRNA treatment (Nothing). Either insulin or FBS was used to stimulate dsRNA uptake. **B.** Reverse transcripts were also subject to PCR using GAPDH primers as a loading control. **C.** RT-PCR of total RNA from S2 cells using oligo-dT primers for reverse transcription, and *TSC1*-specific primers for PCR following treatment with nuclease-free water and no insulin (No Ins), nuclease free water and 1.75  $\mu$ M insulin stimulation (No dsRNA), 6  $\mu$ g/mL dsRNA against *TSC1* (6-TSC1), 15  $\mu$ g/mL dsRNA against *TSC1* (15-TSC1), 30  $\mu$ g/mL dsRNA against *TSC1*, and 15  $\mu$ g/mL dsRNA against dsRED (15-dsRED). **D.** Reverse transcripts were also subject to PCR using GAPDH primers as a loading control. PCR products are aligned with the appropriate treatment.



**Acknowledgements**

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## Chapter 3: Engineering Eukaryotic Signal Transduction with RNAi: Enhancing *Drosophila* S2 Cell Growth via TSC1

### ***Abstract***

RNAi has been useful in the study of biochemical pathways, but has not been widely used as a tool in metabolic engineering. The work described here makes use of double-stranded RNA (dsRNA) for the post-transcriptional gene silencing of *TSC1* in *Drosophila* S2 cells. *TSC1* downregulates the insulin pathway, and serves as a metabolic control to guard against cellular overproliferation and tumorigenesis in both flies and mammals. By silencing *TSC1* with *in vitro*-synthesized dsRNA, we have created a tunable and specific metabolic “throttle” that apparently circumvents deleterious and pleiotropic effects of excess insulin addition (eg. which lead to lysis), while significantly increasing the specific growth rate of S2 cells in a dose dependent manner. During the period wherein dsRNA was active, cell growth rate was increased by 11 % by the addition of 15  $\mu\text{g}/\text{mL}$  dsTSC1 and by over 20% by the addition of 30 $\mu\text{g}/\text{mL}$  dsTSC1. Potential applications for improving eukaryotic cell culture are anticipated.

**Keywords:** *Drosophila*, S2, SI-2, cell culture, insulin, harmartin, *TSC1*, tubular sclerosis, metabolic engineering, RNAi.

## ***Introduction***

The use of RNA molecules to inhibit gene expression is referred to as RNA interference (RNAi) or antisense RNA. Antisense RNA refers to RNA that is single-stranded, complementary to a target sequence, and mostly used in prokaryotic organisms. RNAi refers to either long (500-800 bp) or short (21-23 bp) fragments of double-stranded RNA that also inhibit complementary genes, although through a different mechanism, and exclusively in eukaryotes. The specific and tightly regulated nature of RNAi has made it indispensable as a tool in the biological sciences, but RNAi has thus far found limited use in metabolic engineering, perhaps due to its transient nature (when synthesized *in vitro*), relatively inefficient delivery methods in mammalian cell culture, and the more typical metabolic engineering objective functions based on permanent changes in genotype. Noteworthy examples of gene silencing in the metabolic engineering literature employing *antisense* RNA have appeared, however, resulting in the formation of novel glycosyltransferase regulators in CHO cells (Prati et al. 2002; Prati et al. 1998), increased yield of metabolites acetone and butanol in *C. acetobutylicum* (Desai and Papoutsakis 1999) and increased yield and activity of an organophosphorus hydrolase expressed in *E. coli* (Srivastava et al. 2000). In the latter case, antisense RNA was used to downregulate the heat shock response during protein production and the desired transient effects were a result of the relative instability of ssRNA in *E. coli*.

In eukaryotes with significantly longer process time constants (e.g. tissue or organism generation rate), and with different silencing mechanisms, single-stranded antisense RNA is less effective. Correspondingly, researchers have used dsRNA to

target genes in several organisms, including *C. elegans* (Fire et al. 1998a; Fire et al. 1998b; Montgomery and Fire 1998; Sharp 1999), *Drosophila* (Caplen et al. 2000; Clemens et al. 2000; Matsushima et al. 2004; Misquitta and Paterson 1999), trypanosomes (Ngo et al. 1998), cultured mammalian cells (Krichevsky and Kosik 2002; Sui et al. 2002; Xia et al. 2002), plants (Angell and Baulcombe 1997; Klahre et al. 2002; Voinnet and Baulcombe 1997; Voinnet et al. 1998) and mice (Xia et al. 2002). Importantly, it has been reported that dsRNA and antisense RNA directly or virally delivered to *Lepidopteran* insect and insect cells can suppress the expression of housekeeping genes such as Bmwh3 (*B. mori* white eye, (Quan et al. 2002), hemolin (*H. cecropia* hemolin, (Bettencourt et al. 2002) and JH esterase (*H. virescens* JH esterase, (Hajos et al. 1999). The recent emergence of *in vivo* RNAi synthesis techniques (Matsushima et al. 2004; Sui et al. 2002; Xia et al. 2002) will surely lead to an array of host functions that are stably suppressed to alter the protein synthesis landscape.

*Drosophila melanogaster* Schneider 2 cells were first isolated in 1972 at the Walter Reed Army Institute of Research in Washington, DC (Schneider 1972). The strain was developed from trypsinized fragments of 20 to 24-hour old Oregon-R *Drosophila melanogaster* embryos. The cells can grow as a loosely attached monolayer in petri plates or in suspension. The cell size ranges from 5 to 10  $\mu\text{m}$  in diameter for round cells and up to 35  $\mu\text{m}$  in length for elongated cells, depending on the growth conditions. Tetraploid cells compose approximately 60% to 80% of the culture, with all cells exhibiting XX (female) chromosomes (Echalier 1997). S2 cells are among the most frequently used *Drosophila* cell lines (Echalier 1997). They can

be modified to express various biopharmaceuticals and recombinant products, including many mammalian proteins (McCarroll and King 1997). S2 cells compare favorably with other expression systems in that they are simple to manipulate, genetically well characterized, and can grow without CO<sub>2</sub> supplementation.

One of the objectives for metabolic engineering of eukaryotic cell culture is to increase the specific growth rate of cells grown in suspension. To this end, several media formulations used in industrial settings include insulin as a growth stimulant. The positive effect of insulin on cell growth and proliferation has long been established (Mosna 1981; Mosna and Barigozzi 1976; Seecof and Dewhurst 1974; Wool et al. 1966; Wyss and Bachmann 1976). The insulin signaling pathway is controlled both by the presence of insulin-like growth factors and nutrients (Brogiolo et al. 2001; Gao et al. 2002; Thomas and Hall 1997). Its function in developing eukaryotes is to maintain balance between cell growth and available substrate. Insulin signaling is found in almost all eukaryotic organisms, and can provides cells with a mechanism for halting growth in the absence of nutrients and for stimulating growth in times of excess nutrient availability. Alterations in any component of the insulin pathway in adult *Drosophila* can affect either cell size or proliferation (Potter and Xu 2001), but do not necessarily affect both (Verdu et al. 1999).

An important control point in the insulin pathway occurs with the protein TOR (target of rapamycin) and its interaction with the gene product of *gigas*. *Gigas* is the *Drosophila* homologue of tubular sclerosis complex gene product 2 (*TSC2*) in mammals. In mammals and *Drosophila*, the *TSC2* gene product, tuberin, forms a complex with hamartin, the tubular sclerosis complex gene product 1 (*TSC1*) (Ito and

Rubin 1999) (Fig 1). When active, the complex antagonizes the insulin signaling pathway by blocking TOR phosphorylation (Ito and Rubin 1999). Human mutants of *TSC1* or *TSC2* develop tubular sclerosis, a disease marked by benign tumor cells called hamartomas, giant-sized cells in the tumorous tissue (Ito and Rubin 1999). The effect of *TSC1* or *TSC2* inhibition in whole flies and mammalian cells is the same, specifically that cells grow two to four times normal size and overproliferate (Aicher et al. 2001; Ito and Rubin 1999; Miloloza et al. 2000; Potter and Xu 2001; Soucek et al. 1997; Tapon et al. 2001b). Akt phosphorylation regulates the TSC complex (Aicher et al. 2001). When phosphorylated by Akt, tuberin disassociates from hamartin (Potter et al. 2002) and loses its ability to limit TOR activity (Gao et al. 2002).

Gao and coworkers found that reducing expression of TOR in *Drosophila* eyes partially recovered mutations in *TSC2* (Gao et al. 2002). In order to ascertain whether the TSC complex regulates TOR activity, downstream products of TOR were measured (rather than TOR itself) due to the unavailability of antibodies specific to *Drosophila* TOR. dsRNA against *TSC1* or *TSC2* resulted in a 4.5-fold increase in S6K phosphorylation, without a change in the S6K protein levels. dsRNA against TOR had the same effect, regardless of whether dsRNA against TSC genes were used. Further, immunoprecipitation experiments revealed that tuberin (the *TSC2* gene product) interacted with TOR *in vivo*. Taken together these results indicated that *TSC* gene products regulate TOR expression and control of the Akt signaling pathway (Gao et al. 2002).

Moreover, Kapahi and coworkers recently found that the TSC complex was linked to extension of lifespan in *Drosophila*. They compared mutated flies to flies expressing *TSC2* and *TSC1* under the constitutive GAL4 promoter and found that overexpressing *TSC2* and *TSC1* extended lifespan beyond that of the mutated control by 12% and 14%, respectively (Kapahi et al. 2004). To determine if nutrient stimulation had an effect on lifespan they fed the flies nutrient-rich media. The lifespan of control flies was reduced in rich media, but overexpression of *TSC2* mediated the effects of nutrient stimulation. According to the authors, *TSC2* protects the cells from the deleterious effects of nutrient overstimulation by mimicking dietary restriction (Kapahi et al. 2004). In effect, the TSC complex appears to keep cells from overreacting to positive growth stimuli.

This study investigated the use of dsRNA as a tool for metabolic engineering of S2 cells. Specifically, the large and overproliferating cells noted by previous work inhibiting *TSC1* function were attractive from our application-specific perspective: increased growth and potentially, protein synthesis. Thus, we examined the effect of various growth conditions on *TSC1* mRNA expression and tested whether *in vitro*-synthesized dsRNA against *Drosophila TSC1* could be used to increase the specific growth rate of S2 cells grown in shake-flask cultures.

## ***Materials and Methods***

### **Cell culture**

*Drosophila melanogaster* Schneider 2 (S2) cells were grown in *Drosophila* serum-free media (SFM) (Invitrogen, Carlsbad, CA) in 250 mL shake flasks at 27 °C and 50 rpm in an orbital shaker. Cells were maintained at approximately  $5 \times 10^5$



cells/mL to  $3 \times 10^7$  cells/mL for all experiments. Cell density was determined using a standard hemocytometer and trypan blue exclusion staining. For dsTSC1 experiments, a pulse of insulin was added to the shake flasks to a final concentration of 2.5  $\mu$ M at 60 hours post-transfection.

For insulin concentration experiments, insulin was added to the SFM media to final concentrations of 0.05, 0.25, 2.5, 5, 7.5 and 15  $\mu$ M. Each test was evaluated in at least duplicate flasks, each with duplicate measurements.

For *TSC1* expression experiments, cells were grown in 35 mm petri dishes and incubated (27 °C) either with or without orbital shaking (50 rpm) overnight after supplementation with 2.5  $\mu$ M insulin or an equal volume of nuclease-free water.

#### **dsRNA synthesis and transfection**

dsRNA was prepared following the method of Clemens *et al.* (2000). Briefly, S2 cells were stimulated with bovine insulin (2.5  $\mu$ M) to transcribe components of the insulin signaling pathway. After 0.5 to 2 hours, RNA was extracted using an RNAqueous kit (Ambion, Houston, TX) according to the manufacturer's protocol. First strand copy DNA (cDNA) templates of *TSC1* were transcribed from the mRNA using OligodT primers and the Retroscript® kit (Ambion) as per the manufacturer's instructions. A 700 basepair region of *TSC1* from the ATG start codon to 700 nt downstream was PCR amplified from first strand cDNA templates using the PCR MasterMix™ kit from Promega (Madison, WI) and *TSC1* specific primers (5'-ATG ACG CTG GAG AAC GAG GAG GCC AAG CGC-3'; 3'-CCA TCT CCT TCC ATC GCG TAT TGT TTA CCT-5'). T7 templates were prepared from the *TSC1* 700 nt PCR fragment using PCR amplification and T7 primers (5'-TAA TAC GAC TCA

CTA TAG GAT GAC GCT GGA GAA CGA GG-3'; 3'-TAA TAC GAC TCA CTA TAG  
GCC ATC TCC TTC CAT CGC G-5'). To make the dsRNA, T7 templates were used  
with the Megascript™ kit (Ambion) as per the manufacturer's instructions. The  
single-stranded RNA (ssRNA) synthesized was extracted using phenol/chloroform  
and resuspended in nuclease-free water to a concentration of approximately 3.3  
µg/µL. ssRNA was incubated at 65 °C for 30 minutes before being allowed to cool to  
room temperature and anneal on the benchtop. dsRNA was digested with RNase and  
DNase for 30 minutes to remove any contaminating single-stranded nucleic acids.  
dsRNA was checked for size and integrity using agarose gel electrophoresis.

For dsRNA experiments, S2 cells were grown in 250 mL shake flasks to a  
density of approximately  $1 \times 10^6$  cells/mL in SFM. dsRNA was added directly to 6  
mL of cells at final concentrations of 15 and 30 µg/mL. After incubating in an orbital  
shaker (27 °C, 50 rpm) for 1 hour with dsRNA, 12 mL of fresh media containing  
insulin to a final concentration of 2.5 µM was added to the cells. Aliquots were then  
taken for determining initial cell counts. Treatments were tested in at least duplicate  
flasks with triplicate measurements.

### **Differential display (semi-quantitative RT-PCR)**

For determining relative transcript levels, S2 cells were lysed and RNA  
extracted using an RNAqueous kit (Ambion) as per the manufacturer's instructions.  
RNA concentration was determined by measuring the absorbance of a diluted sample  
at the 260 nanometer wavelength in a UV spectrophotometer (Beckman). Total RNA  
was subject to reverse transcription using oligo-dT primers to obtain the first-strand

DNA template. The DNA template was subject to PCR with sequence-specific primers. PCR products were run on a 1% agarose gel to compare band intensities.

### **Western blot analysis**

In order to insure that cells transfected with dsTSC1 exhibited the anticipated phenotype, total protein was extracted as described previously (Clemens et al. 2000) from cultures exposed to 30  $\mu\text{g/mL}$  dsTSC1 or an equal volume of nuclease-free water (as a control) for 96 hours. Protein extracts were measured for total protein concentration using a Pierce BSA protein assay kit as per the manufacturer's instructions. Fifteen micrograms of total protein was loaded onto an SDS-Page gel. Following electrophoresis, the gels were blotted onto nitrocellulose and probed with monoclonal antibodies against S6K and S6K phosphorylated at Thr389 (Cell Signaling Technology, MA). Blots were subsequently washed and probed with alkaline phosphatase-conjugated secondary antibody (Sigma, MO) before being probed with a solution of 18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt in 67% (DMSO) (v/v) (NBT/BCIP) (Roche, IN). Following color development blots were scanned and analyzed with Scion Image software to determine the relative difference in S6K phosphorylation between treatments.

## ***Results and Discussion***

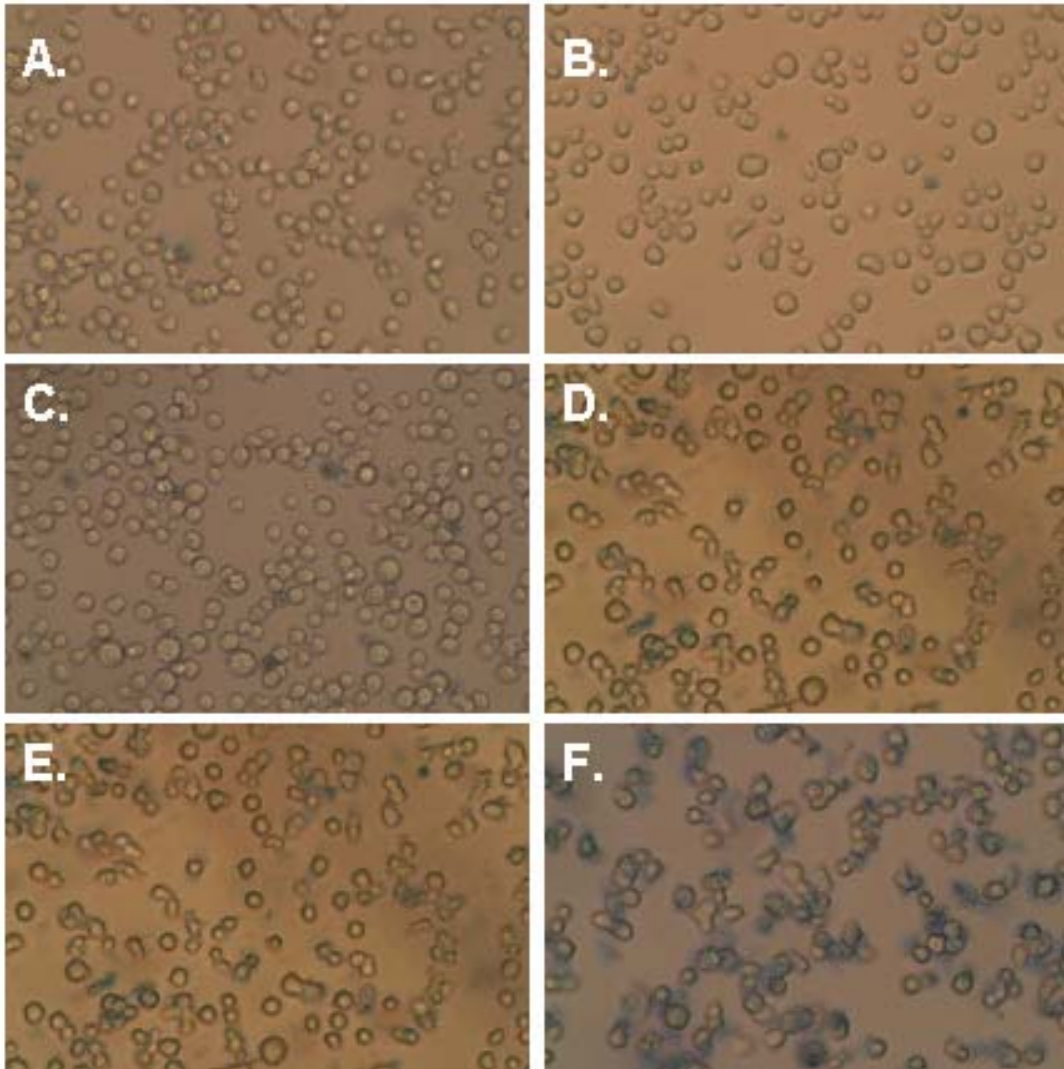
### **Insulin effects on growth**

In order to determine the effect various concentrations of insulin have on S2 cell growth in suspension culture, we incubated cells in 0.05, 0.25, 2.5, 5.0, 7.5, and

15  $\mu\text{M}$  insulin-containing medium without serum. Insulin addition to 5, 7.5, and 15  $\mu\text{M}$  resulted in cessation of growth after 1 to 2 days. Trypan blue exclusion staining of these cells resulted in cell disruption (Fig. 4).

**Figure 4.**

Insulin concentration can affect S2 cell membrane integrity. S2 cells were incubated for up to 48 hours with various concentrations of insulin and stained with trypan blue. Blue stain smudges indicate cell membrane disruption. Concentrations of insulin were as follows: **A.** 0.05  $\mu\text{M}$ ; **B.** 0.25  $\mu\text{M}$ ; **C.** 2.5  $\mu\text{M}$ ; **D.** 5  $\mu\text{M}$ ; **E.** 7.5  $\mu\text{M}$ ; and **F.** 15  $\mu\text{M}$ .

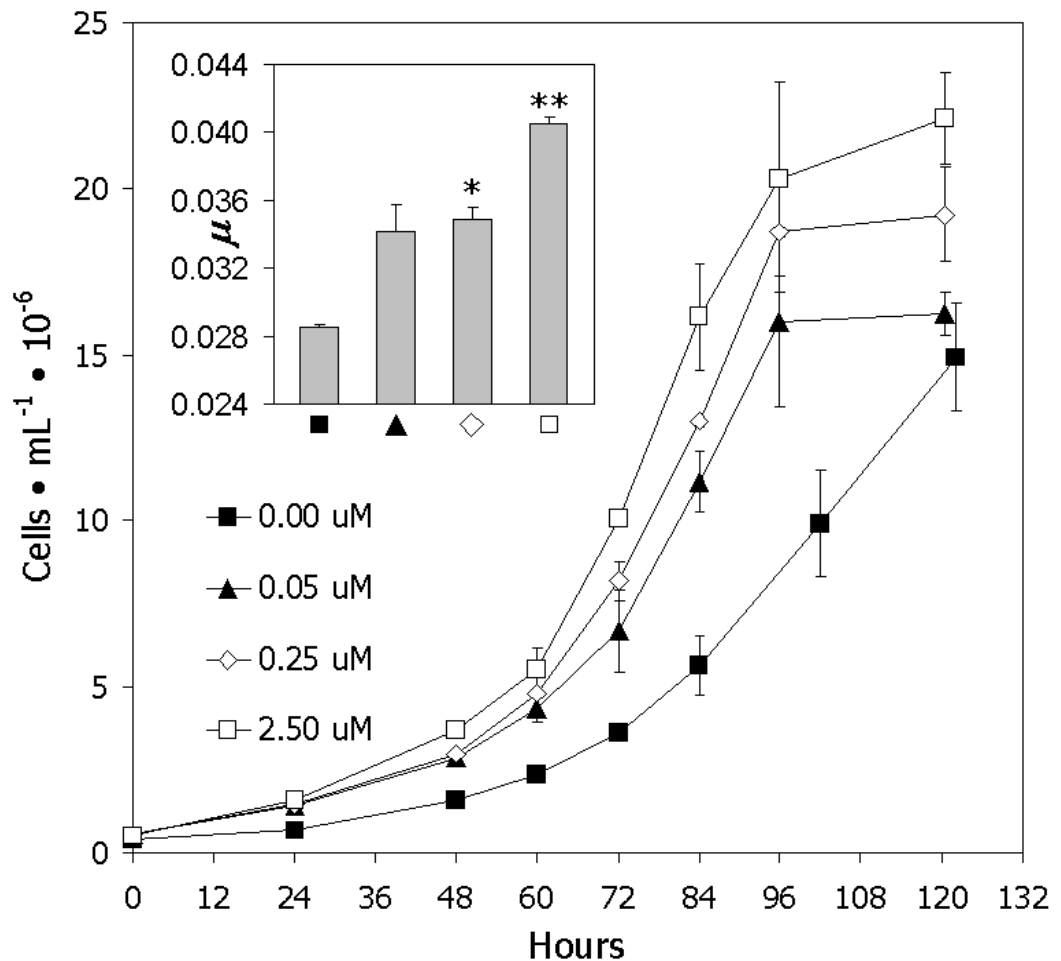


In panels A, B and C, very little cell disruption can be seen. In panel D, some disruption is present, and this effect increases in panels E and F. Panel F cells (15  $\mu\text{M}$  insulin) were almost all completely lysed following trypan blue staining. Of the remaining lower concentrations, 2.5  $\mu\text{M}$  appears to have the most positive effect on growth (Fig. 5), while having no readily visible negative effect on physiology (Fig. 4, panel C). One possible explanation for the apparent weakening of the cell membrane at higher concentrations of S2 cells could be that insulin stimulation encourages growth at a rate that supercedes that of lipid synthesis. It has been shown that interrupting insulin signaling in adult *Drosophila* results in mutants that have almost twice as much lipid as controls (Bohni et al. 1999). However in KC cells, the data regarding insulin effects on lipid synthesis are inconclusive (Ceddia et al. 2003). It remains to be seen whether or not lipid levels are directly controlled by insulin signaling, but the images presented are consistent with this possibility.

Cells grown in serum-free medium supplemented with 2.5  $\mu\text{M}$  insulin exhibited a significantly increased growth rate and an apparent increase in the maximum cell number (Fig. 5). The specific growth rate of cells supplemented with 2.5  $\mu\text{M}$  insulin was 46 % higher than that of cells without insulin. Correspondingly, cells with 2.5  $\mu\text{M}$  insulin grew 21 % and 17 % higher than cells with 0.25  $\mu\text{M}$  insulin and 0.05  $\mu\text{M}$  insulin, respectively (Fig 5, inset). Overall, the growth rate of the cells increased in accordance with the supplemented level of insulin.

**Figure 5.**

Insulin increases the cell growth rate in S2 cell culture. Insulin supplementation level is indicated in the legend. Cells were grown in shake flasks (28 °C, 50 rpm) for the time indicated. Cell counts were performed in duplicate for duplicate flasks using trypan blue exclusion staining. ***Inset:*** Insulin supplementation increases the specific growth rate,  $\mu$  during the first 48 hours of growth. Double stars (\*\*) indicate significant difference ( $p \leq 0.001$ ) for the 2.5  $\mu\text{M}$  insulin-supplemented cells ( $\times$ ) versus the non-insulin supplemented control ( $\diamond$ ). A star (\*) indicates significant difference ( $p \leq 0.01$ ) for the 0.05  $\mu\text{M}$  ( $\blacksquare$ ) versus the non-insulin-supplemented control. For 0.25  $\mu\text{M}$  insulin-supplemented cells ( $\blacktriangle$ ),  $\mu$  was also significantly greater than it was for non-supplemented cells ( $p \leq 0.1$ ).

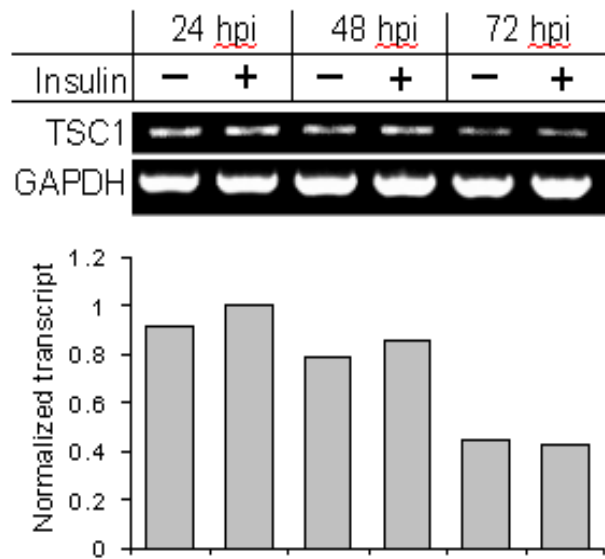


## **Tsc1 expression and inhibition**

*TSC1* transcription was investigated under various conditions. Cells were grown in serum-free media with and without insulin supplementation. Differential display after 24 hours indicated that *TSC1* transcription is only slightly influenced by insulin supplementation (2.5  $\mu$ M) (Fig. 6). After 48 hours, when the culture is still growing exponentially, the transcription level of *TSC1* is relatively unchanged from 24 hours. By 72 hours the 35 mm plate cultures are, by in large, growing more slowly and the transcription of *TSC1* is reduced relative to the 24-hour transcript. These data may indicate the important role *TSC1* plays in regulating cell growth in the presence of nutrient stimulation, which has a greater effect on *TSC1* transcription than insulin addition (Fig. 6). *TSC1*'s role as a controller of the insulin signaling pathway and its implication in cancerous growth when mutated, point to a potential role for *TSC1* in regulating metabolism in response to environmental stimulus: a role that was elucidated for *TSC1* and its co-suppressor, *TSC2* by Gao and coworkers (Gao et al., 2002). In that study, loss of *TSC1/TSC2* mediated the effects of amino acid starvation, which normally would inactivate downstream components of the insulin signaling pathway, slowing growth. Our results, where the depletion of nutrients and concurrent growth retardation over time tracks with lower *TSC1* transcription, further implicates *TSC1* as a metabolic "throttle" that is itself regulated by cell growth or the *potential* for cell growth detected by environmental cues.

**Figure 6.**

*TSC1* mRNA expression in S2 cell culture. Mid-exponential growth S2 cells were grown in 35 mm culture plates either in an incubator (27°C) in SFM with (+) or without (-) insulin supplementation (2.5  $\mu$ M). Total RNA was extracted after 24, 48, and 72 hours and differential display of *TSC1* mRNA was performed as described in the Materials and Methods.

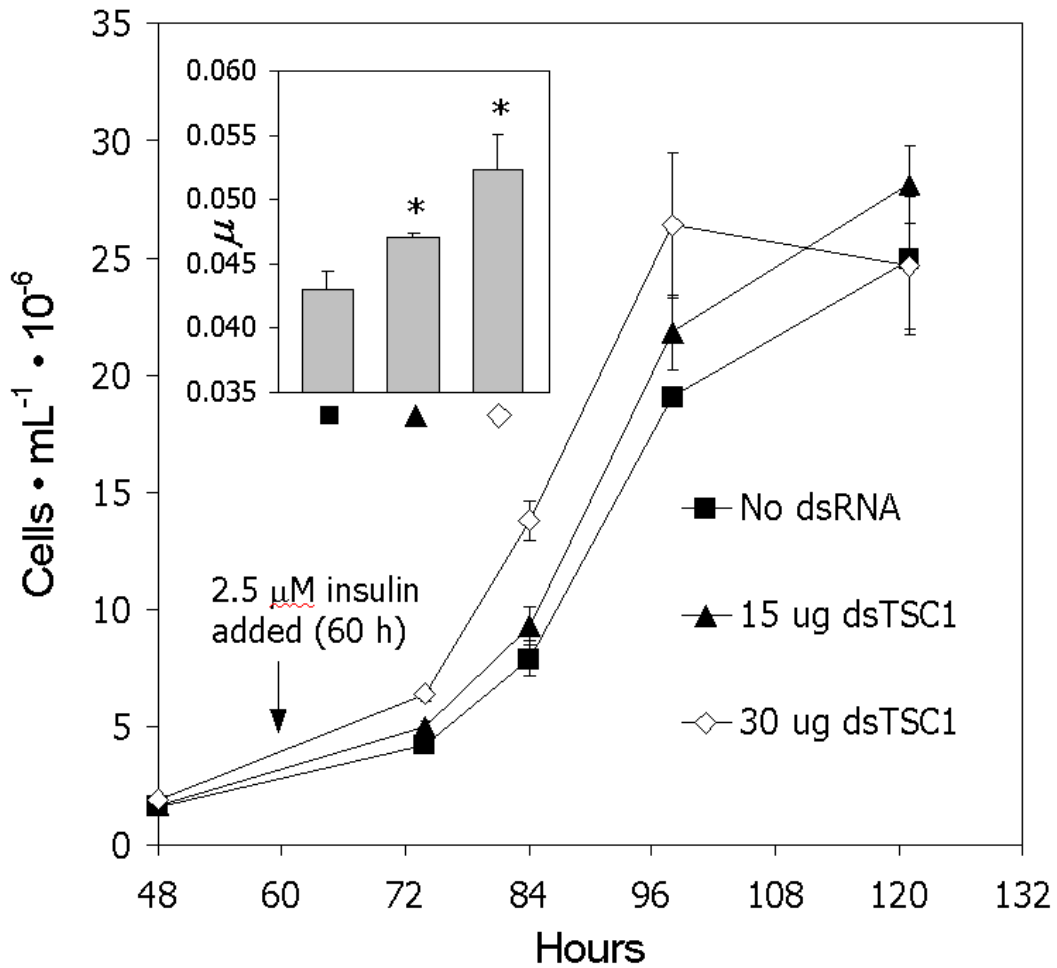




To determine the *direct* effect that inhibiting *TSCI* has on cell growth, we transiently blocked *TSCI* transcription using dsRNA. Our idea was that we could stimulate growth beyond what had been seen in the previous insulin experiments without causing cell lysis. That is, if *TSCI* was acting as a down-regulator of cell growth in the presence of insulin, it is possible that by targeting the specific controller, one would avoid the wider-reaching effects from increased insulin (such as altered lipid synthesis and propensity to lyse), effectively “tuning” the metabolic response. S2 cells were transfected with either 15  $\mu\text{g}/\text{mL}$  dsTSC1, 30  $\mu\text{g}/\text{mL}$  dsTSC1, or no dsRNA and were counted at regular intervals to measure cell growth (Fig. 7). These cells were transfected at time zero, then stimulated with an insulin pulse of 2.5  $\mu\text{M}$  at 60 hours post-transfection. The addition of this amount of insulin was determined from our previous experiments to stimulate growth but *without lysis*, and the timing is comparable with the timing shown to inhibit apoptosis in recombinant cell culture (Yun et al. 2003). In both cases of dsTSC1 addition, a significant acceleration of the growth rate was observed. Notably, this growth rate increase was apparently linear with dsRNA concentration.

**Figure 7.**

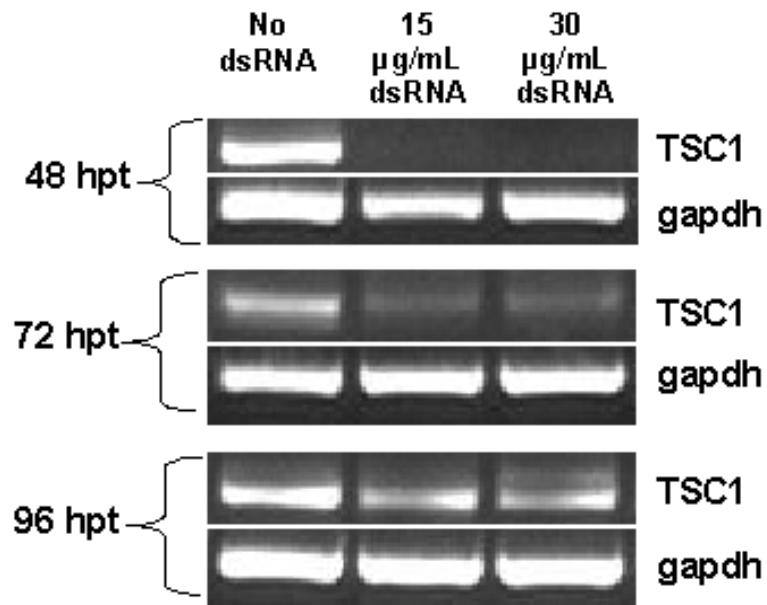
Double-stranded RNA against *TSC1* increases growth in S2 cell culture. Cells were incubated with either 15  $\mu\text{g}/\text{mL}$  or 30  $\mu\text{g}/\text{mL}$  dsRNA against *TSC1* for 1 hour before adding insulin to 2.5  $\mu\text{M}$  in fresh serum-free media. Arrow indicates an addition of 2.8  $\mu\text{g}/\text{mL}$  insulin 60 hours after the initial incubation with dsRNA. ***Inset:*** dsRNA against *TSC1* increases the specific growth rate,  $\mu$  during the time from 48 hours to 84 hours after initial incubation with dsRNA. This was thought to be the time that *TSC1* gene expression was reduced based on differential display data. Star (\*) indicates significant difference at the  $p \leq 0.02$  level for the 30  $\mu\text{g}/\text{mL}$  ( $\diamond$ ) and the 15  $\mu\text{g}/\text{mL}$  dsRNA-supplemented cells ( $\blacktriangle$ ) versus the control without dsRNA ( $\blacksquare$ ).



RNA extracts were analyzed with differential display to determine the efficacy and duration of the transcriptional silencing. The level of *TSC1* mRNA decreased significantly for up to at least 72 hours, and had apparently recovered by 96 hours (Fig. 8). This transient effect has been reported in the literature and was expected due to the proposed mechanism of dsRNA post transcriptional gene silencing in which the antisense strand of dsRNA combines stoichiometrically with complimentary native mRNA to form a complex for degradation (Bernstein et al. 2001). Thus, after considering the potential time required for its degradation and subsequent synthesis, it was estimated that the time over which the cells were least likely to have active hamartin was between 48 and 84 hours post-transfection. Even after 96 hours, however, western blot analysis of downstream TOR-regulated S6K indicated that S6K phosphorylation was still considerably higher in the dsTSC1 cells (30  $\mu\text{g}/\text{mL}$ ) than in the non-dsRNA control (Fig. 9). This represents independent confirmation of dsRNA activity towards *TSC1*, and further suggests that timing the insulin pulse at 60 hours appeared appropriate for stimulating S6K phosphorylation. The specific growth rate ( $\mu$ ) was calculated over this time and was found increased by 11 % for the 15  $\mu\text{g}/\text{mL}$  dsTSC1-treated cells and by 20 % for the 30 $\mu\text{g}/\text{mL}$  dsTSC1-treated cells versus the cells with only insulin supplementation (Fig. 7, inset).

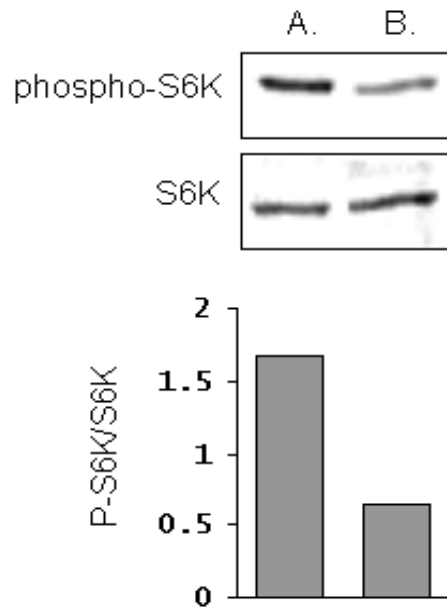
**Figure 8.**

Double-stranded RNA against TSC1 (dsTSC1) specifically inhibits *TSC1* transcription for at least 72 hours in shake flask cultures of S2 cells. Cells were transfected with 15  $\mu\text{g}/\text{mL}$  dsTSC1, 30  $\mu\text{g}/\text{mL}$  dsTSC1, or No dsRNA. Differential display of mRNA from cell extracts measured relative expression of *TSC1* and the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) at 3 timepoints: 48, 72, and 96 hours post-transfection. Extracts are from at least duplicate pooled samples.



**Figure 9.**

Western blot analysis of S6K and phosphorylated S6K levels in cells treated with dsTSC1. Cells were transfected with dsTSC1 (**A.**) or an equal volume of nuclease-free water (**B.**). Equal amounts (15  $\mu$ g) of protein extract were loaded onto SDS-page gels and subsequently blotted onto nitrocellulose and probed with monoclonal antibodies to either phosphorylated S6K (phospho-S6K) or S6K (S6K). Western blots were scanned and analyzed for relative band intensity. Each bar in the chart indicates the ratio of phospho-S6K band intensity divided by S6K band intensity for the specified treatment.



We have shown here that RNAi has tremendous potential for the metabolic engineering of *Drosophila* S2 cells. *TSCI* transcription was higher in exponentially growing cells passaged in fresh media. That *TSCI* expression was higher under these conditions suggests that the cells temper the benefits of nutrient addition by using *TSCI* as a metabolic throttle. We found that targeted inhibition of the *TSCI* increased the specific growth rate of S2 cells beyond that of insulin addition, without apparent side effects. Our results are consistent with what is already known about insulin signaling and importantly, provide a framework from which to explore more completely the area of cellular signaling in the context of metabolic pathway manipulation.

***Acknowledgements***

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## Chapter 4: Increasing protein yield through metabolic engineering of signal transduction and cell cycling in *Drosophila* S2 cells

### **Abstract**

We have demonstrated the interruption of cellular controllers to increase product yield in *Drosophila* Schneider 2 (S2) cell culture. Components of insulin signaling (*TSCI* and *dPTEN*) and cell cycling (*CycE* and *Archipelago*) were silenced using RNA interference (RNAi). Double-stranded RNA (dsRNA) against *TSCI* and *CycE* showed the most significant level of silencing, while dsRNA against *ago*, *TSCI*, and *CycE* resulted in the highest increases in product yield: 84%, 50%, and up to 400% respectively. Complete silencing of *CycE* resulted in no significant change in expression after 24 hours, and a decrease in expression after 72 hours. This is likely due to the fatal effects of *CycE* inhibition. By lowering the level of silencing for *CycE* we were able to increase protein synthesis substantially (400%).

**Keywords:** *Drosophila*, S2, S1-2, cell culture, insulin, *dPTEN*, harmartin, *TSCI*, tubular sclerosis, RNAi, *CycE*, Cyclin E, Archipelago, *Ago*, cell cycle, GFP

## ***Introduction***

Metabolic engineering has, in recent years, turned a corner with respect to the level of sophistication by which the cell's metabolism is viewed and what constitutes an appropriate level of manipulation with cellular processes. Previously, researchers focused on central metabolic pathways for the re-distribution of carbon toward product or away from harmful secondary metabolites. This represented a one dimensional view of biochemical reaction pathways. The advent of more cellularly benign and user-friendly techniques, such as RNAi coupled with the increase in biophysical and biochemical information being generated over the past decade, has shifted focus from central metabolism to cellular signal transduction and other cellular functions including subcellular assemblies. Recent examples include interrupting viral cellular receptor proteins to combat AIDS (Anderson et al. 2003) and interrupting tumor development in cancer (Sumimoto et al. 2004a; Sumimoto et al. 2004b). These studies made use of RNAi to inhibit pathogenicity through the signaling cascades that initiate or regulate an infection or tumorigenesis.

For metabolic engineers, this fits into the greater trend of thinking about cellular function more in terms of discrete events, rather than in terms of steady-state kinetics. After pioneering work that took advantage of varying growth states in the cell cycle to enhance recombinant protein synthesis (Abu-Absi and Srienc 2000; Fussenegger and Bailey 1998; Fussenegger et al. 1998), control of protein synthesis regulation has become an important tool for enhancing recombinant protein yield, and RNAi in particular has emerged as a simple, yet powerful method for controlling gene expression (Hacker et al. 2004; Kramer and Bentley 2003).



The transient nature, seemingly low metabolic burden, relative ease of use, and high efficacy of RNAi make it attractive from the standpoint metabolic engineering. RNAi can be synthesized *in vivo* or synthesized *in vitro* and transfected into cells. Both methods have proven effective for gene silencing. Work in our laboratory used *in vitro*-synthesized double-stranded RNA to silence various genes in *Tricopusa ni* larvae (Kramer and Bentley 2003) and *Drosophila melanogaster* S2 cells (this work) for increased green fluorescent protein (GFP) expression. Other studies have investigated the use of *in vivo* RNAi synthesis for increasing transient protein expressing in HEK293 cells (Hacker et al. 2004).

Here we investigated the use of *in vitro*-transcribed RNAi for metabolic engineering of specific cellular controllers in S2 cells. *Drosophila*, like vertebrates, are capable of developing tubular sclerosis complex (TSC): a disease marked by benign tumors called hamartomas. The disease results from mutation of either *TSC1* or *TSC2*, the genes encoding for hamartin and tubulin, respectfully. These proteins form a complex that inhibits downstream phosphorylation of S6K, which has downstream transcriptional and translational targets. Once activated S6K can increase the transcription of the 4E-BP transcriptional activator, leading to increased protein synthesis and cell growth. The effect of *TSC1* or *TSC2* mutation in whole flies and mammalian cells is the same, specifically that cells grow two to four times normal size and over proliferate (Aicher et al. 2001; Ito and Rubin 1999; Miloloza et al. 2000; Potter and Xu 2001; Soucek et al. 1997; Tapon et al. 2001b). TSC is an important element in the insulin signaling pathway, and acts there to inhibit cellular overgrowth when the cells are stimulated by insulin. In addition, it is believed that

TSC acts as a sensor of nutrient availability and down-regulates growth in the absence of excess amino acids. We recently demonstrated that S2 cells in culture also increase their growth rate when stimulated by insulin, an effect that is amplified by the mutation of *TSCI* (in review).

Another regulator of insulin signaling in *Drosophila* is *dPTEN*, which encodes a phosphatase that responds to insulin stimulation by dephosphorylating PIP3, and thereby inhibiting downstream growth stimulation. Cells mutated for *dPTEN* exhibit increased cell size and proliferation (Gao et al. 2000). Some models of insulin signaling put *dPTEN* directly upstream from *TSCI*, making the regulation redundant. However, a recent review hypothesizes that *dPTEN* and *TSCI* may be part of independent pathways that respond to nutrient levels separately, but these genes respond to insulin through the same pathway. This hypothesis is only supported for whole flies, which likely have greater crosstalk capability than do cells in culture (Pan et al. 2004).

It is widely accepted that the eukaryotic cell cycle can be divided into regions of enhanced protein synthesis, DNA replication, and finally, division. The G0/G1 phase of the cell cycle is thought to be the point at which cells produce the most protein and, as a consequence, the most translational machinery. It is therefore been of interest to metabolic engineers to control cycling cells to remain in G0/G1 to increase synthesis of a recombinant protein (Abu-Absi and Sreenc 2000). Successful attempts have been reported (Fussenegger et al. 1998; Lee et al. 1996; Mazur et al. 1998), and models of cell cycle regulation for enhancement of particular biomolecules have been proposed (Tapon et al. 2001b). In *Drosophila*, progression

from G1 of the cell cycle is mediated by Cyclin E. Regulation of Cyclin E is complicated and involves transcriptional, translational, and degradative elements (Qu et al. 2003a; Qu et al. 2003b). Degradation of Cyclin E is mediated by Archipelago (encoded by *ago*) and carried out by the ribosomal 26 S protease (Moberg et al. 2001). Mutations in archipelago result in elevated growth of human cell lines and *Drosophila* whole flies (Moberg et al. 2001). For this reason, Archipelago was selected as a potential target for silencing by RNAi. The silencing of *ago* and *CycE* have beneficial effects on recombinant protein synthesis, presumably through different mechanisms. By inhibiting Cyclin E synthesis, the cells are forced to remain in G1, and not expend energy on cell division. By inhibiting *ago* expression, the culture is forced into a higher growth state, possibly increasing pools of protein synthesis machinery and thereby producing more recombinant product. *TSC1* and *dPTEN* were equally of interest as silencing targets, since they also worked to slow growth and proliferation. This work investigated the targeted inhibition of these growth regulators in an attempt to increase the cell's capacity to synthesize proteins of interest.

## ***Materials and Methods***

### **Cell culture, stable cell line, and induction**

For all experiments, *Drosophila melanogaster* Schneider 2 (S2) cells were grown in *Drosophila* serum-free media (SFM) (Invitrogen, Carlsbad, CA) in untreated 35 mm petri plates at 27 °C and 50 rpm in an orbital shaker. Cells were maintained at approximately  $5 \times 10^5$  cells/mL to  $6 \times 10^6$  cells/mL. Cell density was determined using a standard hemocytometer and trypan blue exclusion staining.

GFP was PCR amplified from pGFP (Invitrogen) and ligated into the pMtV5hisB *Drosophila* expression vector (Invitrogen, Carlsbad, CA) downstream of the metallothioneine (Mt) promoter to make pMt-GFP. S2 cells were co-transfected with pMt-GFP and pCoHygro (Invitrogen) encoding hygromycin resistance, at a ratio of 44:1 in twelve-well plates. Cellfectin (Invitrogen) was used to transfect the cells as per the manufacturers instructions. Cells were incubated for 12 hours in SFM plus 10% FBS and then washed once in SFM without FBS before being covered with SFM plus 10% FBS and 250  $\mu\text{g}/\text{mL}$  hygromycin. After three to five weeks of hygromycin selective pressure, stable cells expressing GFP were selected and transferred to 75-mL T-flasks. These cells (S2-GFP) were thereafter maintained in SFM plus 250  $\mu\text{g}/\text{mL}$  hygromycin.

For protein expression experiments,  $\text{CuSO}_4$  was added to the media to a final concentration of 10  $\mu\text{M}$  to induce transcription of GFP. In all experiments GFP expression was measured within 24 hours of induction. For cells transfected with dsTSC1, dsDPTEN, dsAgo, dsCycE, or nuclease-free water, incubation was carried out for 48 hours before induction with  $\text{CuSO}_4$ . For other cells transfected with dsCycE or nuclease-free water, incubation was carried out for two hours before induction with  $\text{CuSO}_4$ .

### **dsRNA synthesis and transfection**

dsRNA was synthesized following the method of Clemens *et al.* (2000). Briefly, S2 cells were stimulated with bovine insulin (2.5  $\mu\text{M}$ ) to synthesize components of the insulin signaling pathway. After 0.5 to 2 hours, RNA was extracted using an RNAqueous kit (Ambion, Houston, TX) according to the

manufacturer's protocol. First strand copy DNA (cDNA) templates of *TSCI*, *DPTEN*, *CycE*, and *Ago* were synthesized from the mRNA using OligodT primers and the Retroscript® kit (Ambion) as per the manufacturer's instructions. 700 basepair regions of first strand cDNA templates were PCR amplified using the PCR MasterMix™ kit (Taq DNA polymerase) from Promega (Madison, WI) and *TSCI* (5'-ATG ACG CTG GAG AAC GAG GAG GCC AAG CGC-3'; 3'-CCA TCT CCT TCC ATC GCG TAT TGT TTA CCT-5'), *dPTEN* (5'-TAA TGT CCA ACG TGA TAC GCA ATG-3'; 3'-GCA AGG TTT TCA GTC TAT CTG-5'), *CycE* (5'-ATG GGT TTA AAT GCC AAG AGT-3'; 3'-CAC CAC CAC TGG CGT CTG CTT-5'), and *ago* (5'-ATG AGG AAC CCG AGC CGG AGG-3'; 3'-AGG TTG AGC TGG AGT TGG AAG-5')-specific primers. T7 sequences were added to the templates (making them T7 templates) using T7 primers, which were comprised of the first 15 to 18 nt of the template primers (described above) downstream from a T7 promoter sequence (5'-TAA TAC GAC TCA CTA TAG GG - 3'). To make the dsRNA, T7 templates were used with the Megascript™ kit (Ambion) as per the manufacturer's instructions. The single-stranded RNA (ssRNA) synthesized was extracted using phenol/chloroform and resuspended in nuclease-free water to a concentration of approximately 3.3 µg/µL. dsRNA was checked for size and integrity using agarose gel electrophoresis, after digestion with RNAase and DNAse for 30 minutes to remove any contaminating single-stranded nucleic acids.

For dsRNA experiments, S2 cells were plated in 35-mm untreated petri plates to a density of approximately  $1.5 \times 10^6$  cells/mL in SFM. dsRNA or an equal volume of nuclease-free water was added directly to 1 mL of cells at final concentrations of

30 µg/mL. After incubating in an orbital shaker (27 °C, 50 rpm) for 1 hour with dsRNA, 2 mL of fresh media containing insulin to a final concentration of 2.5 µM was added to the cells. Treatments were tested in at least triplicate plates with triplicate measurements.

### **Differential display (semi-quantitative RT-PCR)**

For determining relative transcript levels, S2 cells were lysed and RNA extracted using an RNAqueous kit (Ambion) as per the manufacturer's instructions. RNA concentration was determined by measuring the absorbance of a diluted sample at the 260 nanometer wavelength in a UV spectrophotometer (Beckman). Total RNA was subject to reverse transcription using oligodT primers to obtain the first-strand cDNA template. The cDNA template was subject to PCR with sequence-specific primers: *TSCI* (5'-ATG ACG CTG GAG AAC GAG GAG GCC AAG CGC-3'; 3'-CCA TCT CCT TCC ATC GCG TAT TGT TTA CCT-5'), *dPTEN* (5'-TAA TGT CCA ACG TGA TAC GCA ATG-3'; 3'-GCA AGG TTT TCA GTC TAT CTG-5'), *CycE* (5'-ATG GGT TTA AAT GCC AAG AGT-3'; 3'-CAC CAC CAC TGG CGT CTG CTT-5'), and *ago* (5'-ATG AGG AAC CCG AGC CGG AGG-3'; 3'-AGG TTG AGC TGG AGT TGG AAG-5'). PCR products were run on a 1% agarose gel stained with ethidium bromide to compare band intensities under fluorescent light. Primers against a 900 bp section of glyceraldehyde dehydrogenase (*GAPDH*) (5'-CCA GAA GAT CAC CGT GTT-3'; 3'-CCC TTG CGG ATT ATG CAA-5') were used to PCR amplify a loading control from the reverse transcript. For attenuated silencing of *CycE*, photographs of agarose gels under UV light were analyzed for band intensity using Image J software from NIH.

## **FACS analysis**

Following 24 hours of induction, the S2 cells were centrifuged ( $400 \times g$ , 4 min.,  $4^{\circ}\text{C}$ ) and washed in ice-cold PBS before fixing in ice-cold PBS with 4% formaldehyde. The fixed samples were immediately subject to flow cytometry (FACSCalibur, BD Bioscience, NJ). To determine GFP expression, 20,000 cells were excited at 488 nm and emission was measured at 530 nm. Samples were gated using forward- and side-scatter dot plots to remove cell fragments and aggregates from the analysis. Histograms were generated by plotting counts against fluorescence intensity.

To determine cell cycling, S2 cells transfected with dsCycE, dsAgo, or nuclease-free water were centrifuged ( $400 \times g$ , 4 min.,  $4^{\circ}\text{C}$ ) and washed in ice-cold PBS before fixing (70% ethanol, 30% PBS,  $-20^{\circ}\text{C}$ ), and stored overnight at  $-20^{\circ}\text{C}$ . Fixed cells were incubated with 20  $\mu\text{g}/\text{mL}$  RNaseA (30 min,  $37^{\circ}\text{C}$ ) and, subsequently, 83  $\mu\text{g}/\text{mL}$  propidium iodide (PI) (5 minutes, room temperature) before being analyzed on the flow cytometer (FACSCalibur, BD Bioscience, NJ). To determine PI incorporation into genomic DNA, 20,000 cells were excited at 488 nm and emission was measured at 618 nm. Because of the rapid turnover rate of Cyclin E, dsCycE cells and a no-dsRNA control were fixed 26 hours after transfection with dsRNA. dsCycE cells and a no-dsRNA control were induced to produce GFP 2 hours after transfection with dsRNA, allowing 24 hours to synthesize GFP prior to analysis. The dsAgo cells were grown for 72 hours post transfection to allow for Ago turnover and GFP synthesis.

## **Western blot analysis**

In order to insure that cells transfected with dsTSC1 and dsDPTEN exhibited the anticipated phenotype, total protein was extracted as described previously (Clemens et al. 2000) from cultures transfected with dsTSC1, dsDPTEN, or an equal volume of nuclease-free water (as a control) for 72 hours. Protein extracts were measured for total protein concentration using a Pierce BSA protein assay kit as per the manufacturer's instructions. Fifteen micrograms of total protein was loaded onto an SDS-Page gel. Following electrophoresis, the gels were blotted onto nitrocellulose and probed with monoclonal antibodies against S6K and S6K phosphorylated at Thr389 (Cell Signaling Technology, MA). Blots were subsequently washed and probed with alkaline phosphatase-conjugated secondary antibody (Sigma, MO) before being probed with a solution of 18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt in 67% (DMSO) (v/v) (NBT/BCIP) (Roche, IN). Following color development blots were scanned and analyzed with Image J software (NIH) to determine the relative difference in S6K phosphorylation between treatments.

## ***Results and Discussion***

### **Gene silencing**

After transfection with 30 µg/mL dsRNA against specific targets: *dPTEN* (dsDPTEN), *TSC1* (dsTSC1), *cycE* (dsCycE), and *ago* (dsAgo), total RNA was



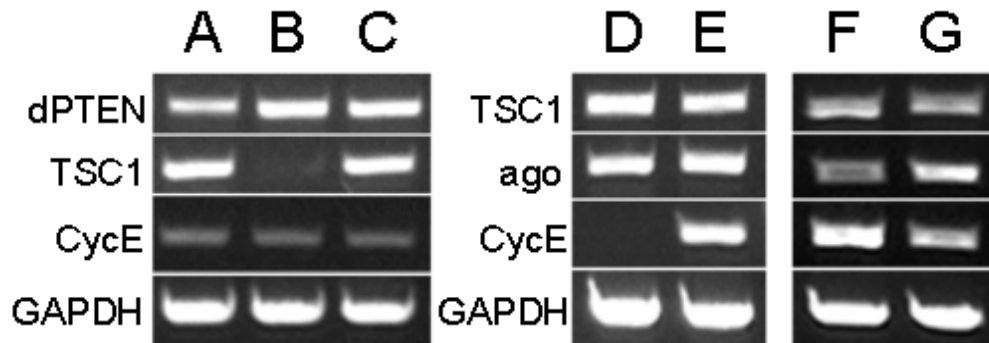
extracted from the cultures and digested with DNAase prior to differential display analysis as described in Materials and Methods. Each column in Fig. 10 represents either a dsRNA treatment or a control. The primers used in the differential display are listed along the left of each agarose gel. Differential display of dsDPTEN-, dsTSC1-, and dsAgo-transfected cells was performed 48 hours post-transfection (Fig. 10A, B and F, respectively), while for dsCyclinE-transfected cells RT-PCR was conducted 26 hours and 72 hours post-transfection. With each experiment, a control transfected with nuclease-free water instead of dsRNA was used to compare relative gene transcription levels. For dsDPTEN- and dsTSC1-transfected cultures, the control can be seen in Fig. 10C. For 26-hour dsCycE-transfected cells, the control is in Fig. 10E. For dsAgo-transfections, the non-transfected control is in Fig. 10G. PCR products of GAPDH are provided as a loading control for each sample (Fig. 10A-G). The 72 hour dsCycE-transfected cells, which received less than 30  $\mu\text{g/mL}$  dsCycE, are discussed in detail in another section (*Tuning gene silencing*) and are not shown in Fig. 10.

Gel band intensities indicated that *TSC1* and *CycE* were almost completely silenced by 30  $\mu\text{g/mL}$  dsRNA (Fig. 10B and Fig. 10D, respectively). However, *dPTEN* and *ago* were not as completely silenced (Fig. 10A and F, respectively) relative to their controls as *TSC1* and *CycE* (Fig. 10B and D, respectively) but still exhibited some reduction in their transcription. Interestingly, a decrease in the transcript levels of *ago* resulted in an increase in *CycE* transcript levels (Fig. 10F and D, respectively). This was reasonable, since Cyclin E transcription can be controlled via a positive feedback loop involving endogenous levels of Cyclin E (Jones et al.

2000). In developing flies (from which S2 cells were derived) Cyclin E and other proteins phosphorylate retinoblastoma (RB), causing RB to disassociate from dE2F1 (a transcriptional activator). dE2F1 is thought to stimulate the transcription of *CycE* (Brumby et al. 2002; Lee and Orr-Weaver 2003). Hence, a reduction in Cyclin E degradation stemming from the silencing of *ago* would lead to an increase in *CycE* transcription.

**Figure 10.**

Silencing of target genes. Reverse transcription and subsequent PCR (differential display) of target genes was carried out from total RNA after DNAase digestion using oligodT primers. The samples were gathered 48 hours post-transfection with dsRNA unless otherwise noted. The gene specific primers used for the PCR are listed at the far left of each figure. (A) Cells transfected with dsRNA against *dPTEN* (dsDPTEN), (B) Cells transfected with dsRNA against *TSC1* (dsTSC1), (C) Cells transfected with water as a control, (D) Cells transfected with dsRNA against *cycE* (dsCycE) 24 hours post-transfection, (E) Cells transfected with water as a control 24 hours post-transfection, (F) Cells transfected with dsRNA against *ago* (dsAgo), (G) Cells transfected with water as a control. Primers against *GAPDH* were used as a loading control for all samples.



### **Physiological effects of silencing**

To determine the effect of silencing cell cycling–related genes on cell cycling, samples were subject to propidium iodide (PI) staining. Cells were harvested from each treatment (dsAgo and dsCycE) and control and fixed in ice-cold ethanol overnight before incubation with ribonuclease A and subsequent staining with PI. PI-stained cells were analyzed with a flow cytometer to determine DNA content. After gating the samples to remove aggregates and lysed cells, histograms were generated from 20,000-cell samples for their emission intensity at 618 nm. The histograms were analyzed using Cellquest software for the approximate percent distribution of cells in three phases of the cell cycle (G1, S, or G2/M). Figure 11 gives typical results for PI staining histograms.

DNA staining indicated that dsCycE-transfected cells at the highest concentration (30  $\mu\text{g}/\text{mL}$ ) (Fig. 11B) were almost 2-fold more in G1 of the cell cycle than the non-transfected control (Fig. 11A) after 26 hours of transfection. After 72 hours of the most concentrated transfection with dsCycE (30  $\mu\text{g}/\text{mL}$ ) cells were mostly dead so that no RNA sample was gathered (data not shown). The histogram in Fig. 2B indicates that the silencing of Cyclin E had its desired effect after 24 hours. The speed with which this occurred may stem from the need to reset Cyclin E levels after S phase, in order that cells can spend sufficient time in G1 following mitosis for biosynthesis of cellular components and replication machinery. Cell death after 72 hours of treatment with 30  $\mu\text{g}/\text{mL}$  dsCycE points to a vital function for Cyclin E, perhaps outside of its role in cell cycling. Holding cells in G1 is not of itself fatal, but

genome-wide microarray analysis has demonstrated that silencing Cyclin E can be (Boutros et al. 2004). The interaction of Cyclin E with the transcriptional activator dE2F1 may result in activation of essential targets that are needed to maintain cell viability, even when the cell is not dividing.

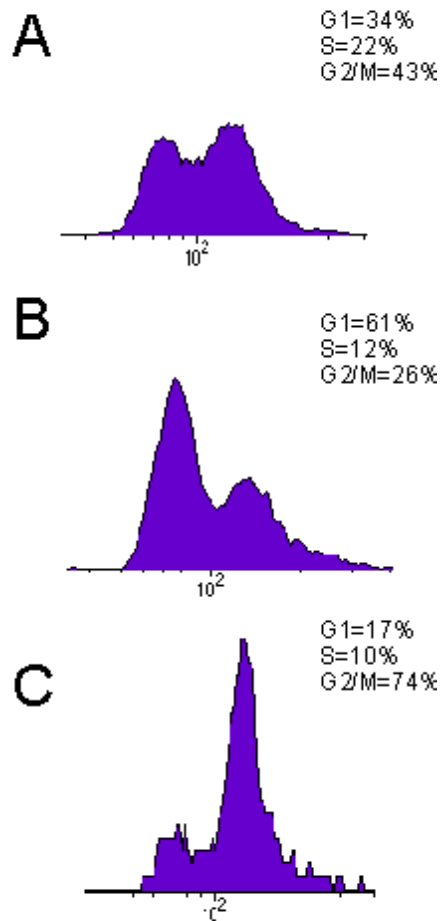
dsAgo-transfected cells were 74% in G2/M after 72 hours (Fig. 11C). These data lend support to the hypothesis that accumulation of Cyclin E results in more rapidly dividing cells. In fact, elevated levels of Cyclin E have been linked to tumorous growth in adult flies (Jia et al. 2003). Transfection of cells with dsAgo may prove a useful tool in future studies to hold cells in G2 of the cell cycle.

The target of insulin signaling gene silencing (*dPTEN* and *TSC1*) was the transcriptional and translational activator dS6K, since phosphorylation of dS6K results in increased cell growth and proliferation. Normal function of dPTEN and TSC1 mediate dephosphorylation of dS6K. Protein extracts were prepared as described in Materials and Methods for cultures treated with dsDPTEN, dsTSC1, or nuclease-free water. Western blot analysis with antibodies specific for phosphorylated S6K or S6K demonstrated an increase in S6K phosphorylation for the dsTSC1-transfected cells (Fig.12B), and almost no change in phosphorylation for dsDPTEN-transfected cells (Fig. 12A) when compared to the nuclease-free water control (Fig. 12C). These data corroborated that silencing of insulin-signaling pathway components leads to increased phosphorylation of dS6K. That dsDPTEN-transfected cultures did not exhibit as much S6K phosphorylation as dsTSC1-transfected cultures was not surprising given the difference in silencing achieved

between the two conditions (Fig. 10A and B, respectively). The difference between these silencing efficiencies was further seen in recombinant protein expression levels.

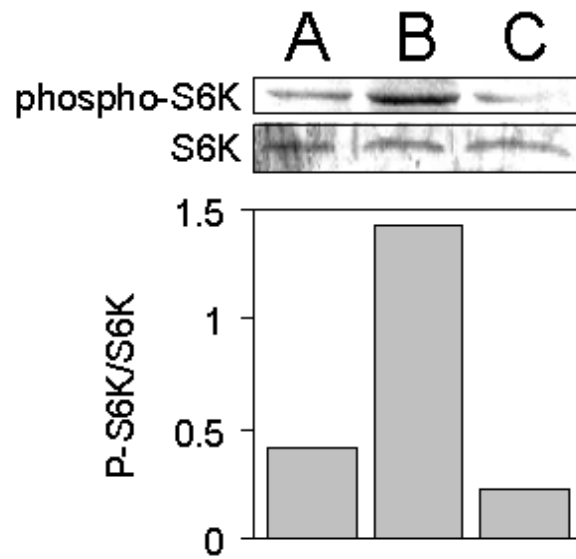
**Figure 11.**

Propidium iodide (PI) staining of cells transfected with dsRNA. Cells transfected with dsRNA against (A) water as a control, (B) *CycE* (dsCycE), and (C) *ago* (dsAgo) were fixed in ethanol, stained with PI and analyzed on a FACSCalibur flow cytometer to determine DNA content. After gating to remove cell debris and aggregates, histograms were generated for counts (at least 20,000 cells) versus emission intensity (618 nm). Cell cycle estimates (G1, S, or G2/M: inset for each histogram) were made using Cellquest software. Cell cycle results are representative of at least triplicate samples (inset for each).



**Figure 12.**

Western blot of S6K phosphorylation. S2 cells were transfected with dsRNA against *TSC1* (dsTSC1), *dPTEN* (dsDPTEN) and water as a control (Control). After 72 hours, the cells were lysed and their protein extracted. 25  $\mu$ g total protein was run on a denaturing SDS-page gel, blotted onto a nitrocellulose membrane, and probed with antibodies to either S6K (S6K) or S6K phosphorylated on Thr389 (phospho-S6K). (A) dsDPTEN, (B) dsTSC1, (C) Control.





## Recombinant protein expression

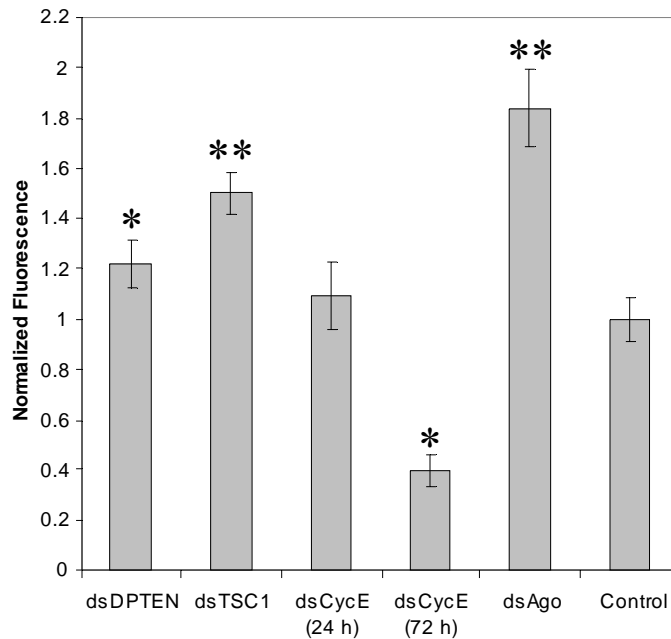
Stable S2 cell lines were constructed expressing recombinant GFP (S2-GFP). S2-GFP cultures were transfected with up to 30  $\mu\text{g}/\text{mL}$  dsRNA against specific genes (*dPTEN*, *TSC1*, *Cyclin E*) and allowed 48 hours for recovery and protein turnover before being induced to express recombinant GFP for 24 hours. After extraction and fixing in 4% formaldehyde, 20,000 cells per treatment were analyzed by flow cytometry for GFP fluorescence (530 nm emission). Cytometry samples were gated to remove fragmented and aggregated cells. Histograms relating counts of cells versus emission intensity were generated, and further gated to account for an M1 population of 20% of the total that exhibited the highest fluorescence intensity. This percentage was calculated by examining samples from the total population using a hemocytometer under a fluorescent microscope.

The greatest increase in relative GFP expression for cells transfected with 30  $\mu\text{g}/\text{mL}$  dsRNA was exhibited by the dsAgo-transfected cells, which were 83% more fluorescent than the controls (Fig. 13). G1 of the cell cycle is the state at which most protein synthesis occurs, and we confirmed that cells silenced for *ago* spend less time in G1 than do control cells (Fig. 11A and C). Indeed, much of the literature regarding the cell cycle and heterologous protein expression has demonstrated that holding cells in G1 greatly increases specific product synthesis. However, in order for tissue mutant for *ago* to overproliferate, as has been described (Moberg et al. 2001), there must be increased protein synthesis to meet the biosynthetic demands of the rapidly growing cell. While this demand is not met through the mechanism of holding cells in G1, it could be met in part by downstream targets of dE2F1. As mentioned

previously, Cyclin E and dE2F1 can form a complex that not only facilitates the exit of the cell from G1 into S, but that also facilitates the transcription of downstream targets (Lee and Orr-Weaver 2003). By allowing the accumulation of Cyclin E, silencing *ago* may have stimulated cell growth through a Cyclin E-dependent mechanism.

**Figure 13.**

Protein expression in transfected cells. S2 cells stably expressing GFP were subject to transfection by dsRNA for 72 hours (unless otherwise indicated), fixed in 4% formaldehyde and counted on a flow cytometer to determine GFP fluorescence. Each treatment was compared to a control of stably expressing cells that were not subject to dsRNA treatment. The error bars represent the standard deviation of at least three independent experiments, all normalized by dividing by the fluorescence of the control. Results of the student's T-Test comparing each treatment to the non-transfected control are indicated by stars as follows: \*\* indicates  $p < 0.001$ , \* indicates  $p < 0.05$ .



The method of almost completely inhibiting *CycE* transcription did not significantly change GFP expression after 24 hours, and resulted in a significant decrease in GFP fluorescence after 72 hours (Fig 13), at which point more than 80% of the culture stained dead under trypan blue exclusion staining (data not shown). It was unclear why the nearly complete silencing of *CycE* would prove ineffective at increasing GFP synthesis, and would moreover prove fatal. A recent genome-wide RNAi study confirmed that nearly complete silencing of *CycE* was fatal to *Drosophila* cell culture (Boutros et al. 2004), but the mechanism for cell death was not investigated. Considering that there must be at least one vital function for Cyclin E, attenuated silencing of *CycE* was investigated to hold cells in G1 while allowing for some Cyclin E function.

Silencing insulin signaling down-regulators *dPTEN* and *TSC1* increased GFP fluorescence by 20% and 50%, respectively. The lower yield resulting from transfecting with dsDPTEN (Fig. 13) was in accordance with the level of silencing achieved (Fig. 10A). The 50% increase in yield for dsTSC1-transfected cultures was significantly higher than the control ( $p \leq 0.001$ ); however, the level of increase in GFP expression did not reflect the more than 3-fold increase in dS6K phosphorylation (Fig. 12B and C) for dsTSC1-transfected cultures. There are no indications from the literature that the level of dS6K phosphorylation can serve as a predictor of protein synthesis, and it is unlikely that all of the downstream targets of dS6K are beneficial to recombinant product yield. Yet, this result demonstrates the potential of controlling insulin signaling for improving performance of eukaryotic cell culture.

## ***Tuning CycE transcription***

To examine the effect of tunable *CycE* silencing, various concentrations of dsCycE were incubated with S2-GFP cells for 48 hours prior to induction of GFP expression for 24 hours (72 hours post-transfection). Cells were fixed in 4% formaldehyde and analyzed with flow cytometry. After gating the samples to remove aggregates and lysed cells, histograms were generated from 20,000-cell samples for their emission intensity (530 nm). The silencing of CycE transcript (Fig. 14B) tracked with a significant increase in GFP expression relative to the control transfected with nuclease-free water (Fig. 14A). The greatest increase in fluorescence was seen for cultures transfected with 9  $\mu\text{g/mL}$  of dsCycE, which expressed 4-fold greater GFP than the control (Fig. 14A). For cultures transfected with 12  $\mu\text{g/mL}$  and 30  $\mu\text{g/mL}$  dsCycE, emission intensity was 22% and 90% lower, respectively than for cultures transfected with 9  $\mu\text{g/mL}$  dsCycE. Transfecting with 30  $\mu\text{g/mL}$  dsCycE proved to be fatal (data not shown). Differential display data demonstrated that the silencing tracks in accordance with the amount of dsCycE added (Fig. 14B), although the transcript difference between 3  $\mu\text{g/mL}$  and 6  $\mu\text{g/mL}$  was not pronounced (Fig. 14B). Similarly, the level of GFP expression between these two treatments was not significantly different (Fig. 14A).

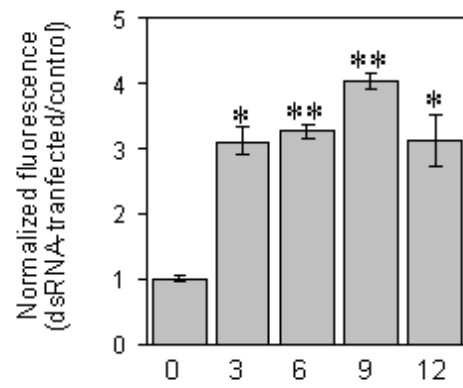
To examine the effect of attenuated silencing of *CycE* on cell cycling, cells were fixed in ethanol and stained with propidium iodide (PI) and analyzed using flow cytometry as described in the Materials and Methods. Histogram plots were generated from 20,000-cell samples after gating to remove fragmented and aggregated cells from the analysis. Plotting of intensity (618 nm) versus cell counts

revealed that even for low levels of dsRNA (3 to 6  $\mu\text{g/mL}$ ) the cell cycle shifts dramatically from predominantly G2- to mostly in G1- or S-phase of the cell cycle (Fig. 14C). At concentrations of dsCycE above 6  $\mu\text{g/mL}$ , the shape of the intensity histogram became more mounded as cells were more clumped and perhaps apoptotic. These profiles may serve as an indicator as to what point the silencing of *CycE* begins to have a detrimental effect on cell growth and survival. While the silencing at the 9  $\mu\text{g/mL}$  level yielded the most GFP fluorescence, it may have been the threshold silencing level at which the overall health of the cell was beginning to be compromised.

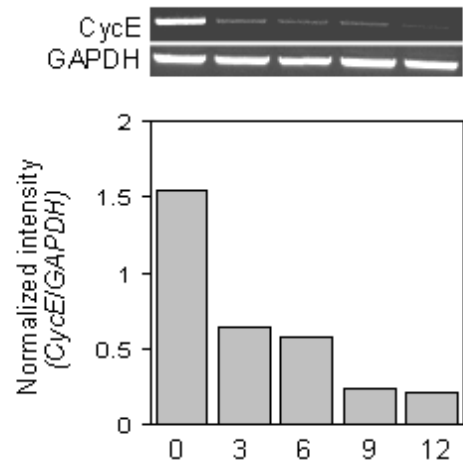
**Figure 14.**

GFP and *CycE* mRNA expression in transfected cells. S2 cells stably expressing GFP were subject to transfection by various concentrations of dsRNA against *CycE* for 72 hours. **A.** For fluorescence measurements each treatment was compared to a control of stably expressing cells that were not subject to dsRNA treatment. The amount of dsRNA ( $\mu\text{g/mL}$ ) transfected into each treatment is listed below its fluorescence (numbers on the X-axis: 0, 3, 6, 9, 12). Error bars represent the standard deviation of at least two independent experiments, all normalized by dividing by the fluorescence of the control. Results of the student's T-Test comparing each treatment to the non-transfected control are indicated by stars as follows: \*\* indicates  $p < 0.001$ , \* indicates  $p < 0.05$ . **B.** Representative differential display of the level of *CycE* transcript is shown for each treatment below the corresponding relative fluorescence. The plot under the gel display indicates the normalized (*CycE* band intensity/*GAPDH* band intensity) intensity for each treatment. The primers used for differential display PCR are indicated at the left of each row. **C.** Profiles of PI-stained cells after 72 hours treatment by dsRNA against *CycE*. Amounts of dsRNA transfected are inset for each profile (0, 3, 6, 9, or 12  $\mu\text{g/mL}$ ). Cells were fixed in 70% ice cold ethanol overnight before staining with propidium iodide and reading on the flow cytometer. Images indicate the shift of cycling from most G2 (control) to mostly G1 (3, 6, and 9  $\mu\text{g/mL}$  dsRNA), to non-discernable (12  $\mu\text{g/mL}$  dsRNA).

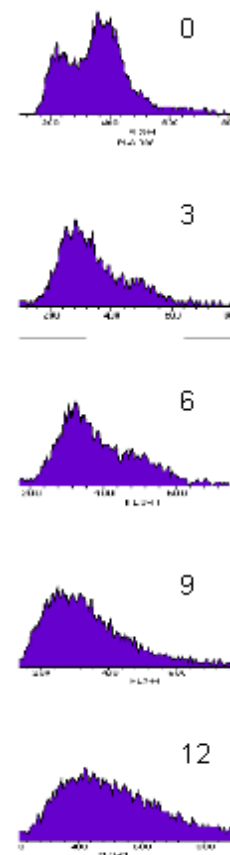
A.



B.



C.





This result clearly marks RNAi a powerful tool in metabolic engineering of eukaryotic systems. With tunable silencing such as exhibited here in the case of Cyclin E, it will be possible to modulate gene expression without having to synthesize additional proteins and imparting the consequent metabolic burden. As the comparison of more fully silenced (30  $\mu\text{g/mL}$ , Fig. 13) to more fully expressed *CycE* (9  $\mu\text{g/mL}$ ) illustrates, there are conditions within a culture that require optimized gene expression for maximized system performance. With this relatively simple system of dsRNA *in vitro* enzymatic synthesis, and subsequent insulin or FBS-mediated transfection, several expression scenarios are possible and can be rapidly screened in a high throughput platform. Furthermore, for complete silencing the cost of developing knockout strains is circumvented, and *Drosophila* S2 lines provide an industrially attractive organism from which to work.

We have shown here the expansion of the technique of using RNAi for metabolic engineering of eukaryotic cell culture. In particular, it has been shown that silencing genes related to tumorigenic growth in whole animals can have a positive effect on recombinant protein expression. The genes *TSCI* and *ago* are potent down-regulators of cellular growth in whole animals. In cell culture they play a similar role, but it may be unnecessary. Cell cultures grow in well maintained environments that are intended to encourage maximum growth and protein synthesis. In such conditions, we have shown here that removing cellular controllers may increase cellular capacity to produce protein, including recombinant product. By controlling the amount of silencing, we have also shown that gene regulation can be tuned

extraneously to have a range of physiological effects from cell death to greatly increased protein expression.

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## Chapter 5: Conclusions

### *Insulin effects on physiology and dsRNA uptake*

The work presented in chapter one investigated the use of insulin to stimulate dsRNA uptake by S2 cells growing in *in vitro*. The results indicated two important aspects of dsRNA gene silencing: 1) that insulin could stimulate S2 cells growing in serum-free media to take up dsRNA of 700 basepairs, and 2) that the amount of dsRNA added to the culture determines the level of transcript expressed after a defined period. These results are important to metabolic engineers studying insulin signaling, in that they enable the removal of fetal bovine serum (FBS) from the transfection process, and they indicate potential for tuning gene expression in recombinant eukaryotic culture.

In Chapter 2 the effects of insulin addition to shake flask cultures of S2 cells were investigated. In that study it was shown that the supplementation of insulin resulted in an increase in the specific growth rate of the culture. Additionally, the effect of insulin supplementation on cell physiology (membrane integrity) following trypan blue exclusion staining was examined. It was found that cells incubated with  $\geq 5$   $\mu\text{M}$  insulin would lyse (indicating a loss of membrane integrity) when stained with trypan blue, and that the amount of lysis increased with insulin concentration. Further it was found that high concentrations of insulin  $\geq 15$   $\mu\text{M}$  resulted in cell death (data not shown). This study marks the first time that the effects of insulin on cell growth rate and physiology in cell culture have been investigated.

### ***TSC1 expression and effect on cell growth***

Chapter 3 describes an investigation into how *TSC1* transcription can be manipulated to change cell growth. As would perhaps be expected, the level of *TSC1* transcription did not significantly change as a function of insulin stimulation. *TSC1*'s role as signal transduction regulator makes its continued presence necessary and its regulation dependent on phosphorylation, rather than transcription. Only when the growth conditions were significantly changed did the transcription of *TSC1* markedly alter. As nutrients were depleted and the growth rate of the culture declined, the transcription of *TSC1* was reduced.

The *TSC1* complex has to be able to respond quickly to changes in nutrient availability. Glucose availability in the adult fly is an example. There may not be sufficient time for cells to react with transcription and translation of a regulating protein. Instead, the transcription level remains constant, and the regulation is accomplished through phosphorylation. In the case of this work, the nearly complete removal of hamartin by *TSC1* silencing had an effect because there was no possibility for growth retardation through this mechanism, as was evidenced by the phosphorylation of dS6K and the change in growth rate. Cells were forced into a higher proliferative state by removal of a complex that normally interrupts growth-promoting signaling cascades.

### ***Insulin and cell cycling targets effect on recombinant protein synthesis***

In Chapter 4 the effects that silencing specific targets in the insulin and cell cycling pathways was investigated. Physiologic effect and recombinant protein

synthesis seemed to correlate with gene silencing efficacy for targets in the insulin signaling pathway (*dPTEN* and *TSCI*), but silencing of cell cycling targets had varying effects, depending on the target. For example, silencing *ago* increased protein synthesis by 83%, yet silencing *CycE* had a gradient effect (from no difference in GFP expression to 4-fold higher expression of GFP) depending on the level of silencing. Propidium iodide staining revealed that transfections of more than 6 µg/mL of dsRNA against *CycE* resulted in non-distinct cell cycling profiles: perhaps indicating the onset of cell death. It was concluded from these results that *CycE* had alternate roles in the cell which were not performed under the condition of more complete silencing, but that attenuating *CycE* transcript could hold cells in G1 of the cell cycle while still allowing for Cyclin E functionality.

Overall these studies substantiate the control of signaling pathways as a viable approach to engineering cells for increased growth and protein production. RNAi has been shown to be an effective tool for mediating gene silencing for these purposes. Future work will endeavor to make stable cell lines expressing dsRNA under control of environmentally sensitive promoters to facilitate as-needed gene silencing. For example, cells could be modified to react to low oxygen concentrations in a large reactor by expressing RNAi against oxidative phosphorylation decoupling targets. There are numerous potential applications that can be developed for controlling time- and environment-sensitive gene repression. This work demonstrates that such gene control is possible and can increase product yields in recombinant culture.

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