5-2013

The Use of Genomic "Knock-In" Strategy to Examine the Role of the Protein Acyl Transferase (DHHC) Family of Enzymes Using Dictyostelium Discoideum

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Recommended Citation

THE USE OF GENOMIC "KNOCK-IN" STRATEGY TO EXAMINE THE ROLE OF

THE PROTEIN ACYL TRANSFERASE (DHHC) FAMILY OF ENZYMES USING

DICTYOSTELIUM DISCOIDEUM

by

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A thesis Submitted in Partial Fulfillment of the
Requirements for a Degree with Honors
(Biology)

The Honors College

University of Maine

May 2013

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Abstract

Palmitoylation is a reversible, post translational, lipid modification performed by proteinacyltransferases (PAT). PATs are membrane-bound enzymes which contain a conserved region that is rich in cysteine residues and contains a DHHC (Asp-His-His-Cys) conserved domain. This region is involved in the transfer of a 16-carbon palmitate from palmitoyl-CoA to a target protein. Palmitoylation plays many important functions such as targeting the protein to a lipid raft, shuttling the target protein or anchoring it to the cell membranes as well as aiding in the three dimensional folding of the protein. This process plays an important role in signal transduction, anchoring and cellular timing. Palmitoylation has been shown to play a role in schizophrenia, hormone dysfunction, intellectual disability, Huntington's disease and cancer.

In order to study PATs, genetic knock-ins were created using YFP (a yellow fluorescent protein) in order to study expression patterns in growing *Dictyostelium discoideum*, a soil amoeba whose multi-stage life cycle is highly dependent on the action of palmitoylated heterotrimeric G-proteins. This method for studying the expression pattern of PATs provides accurate information about both the timing of expression and the location of expression in living dictyostelium.

This research will help to provide a basic understanding of how *Dictyostelium* makes use of its PAT's through providing a 'when' and 'where' for their expression. This data will allow for a more educated inspection of the 'why' and 'how' questions associated with PATs and their substrates.

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Palmitoylation Background

Human cells make wide use of protein palmitoylation. Palmitoylation has been shown to play an important role in T-cell activation in human immune responses (Zhang, 1998). Palmitoylation has also been shown to be an important regulator of cancer cell proliferation (Galluzzo, 2007). Palmitoylation plays an important role in rapid hormone response, such as estrogen, and is a current source of research for metabolic dysfunction (Marino, 2006). There has also been a link to schizophrenia and improper formation of ZDHHC8/DHHC8 (ZDHHC is used when referring to DHHC-CRDs in mammals due to the presence of a zinc finger) (Chen, 2004 and Karayiorgou, 2004). The ZDHHC-Cysteine Rich Domain PATs in humans that relate to intellectual disability, Huntington's disease and cancer proliferation have begun receiving more attention as a potential for therapeutic research (Hemsley, 2012)
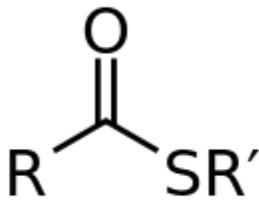
Figure 1 - The thioester linkage
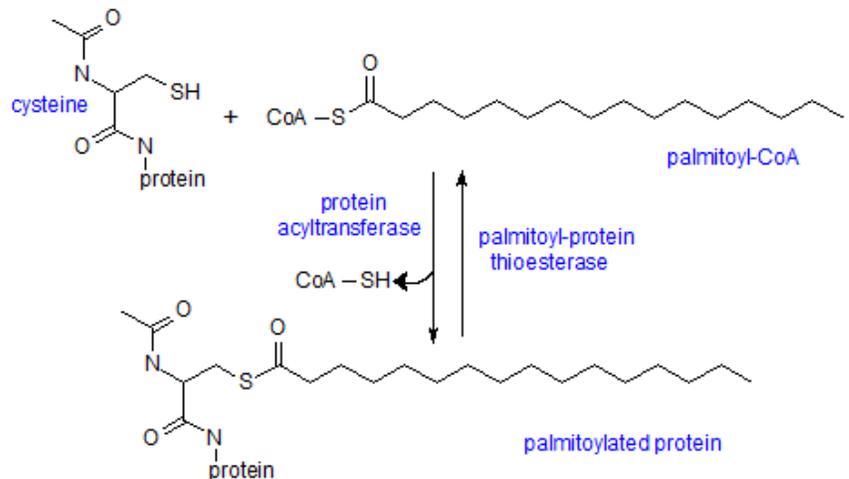
Figure 2 - Reaction of Palmitoylation.

Plants also make widespread use of PAT's for GTP-ase signaling, calcium

signaling, disease resistance, heterotrimeric G-protein signaling and microtubule cytoskeleton formation and microtubule formation (Hemsely, 2013).

Palmitoylation is the biochemical process by which a palmitic acid (16C) is enzymatically attached to a protein. This addition is carried out by forming a thioester linkage (or bond) to the free sulfur of a cysteine residue on the protein to be modified. Palmitoylation is a post translational modification that is reversible due to the low energy level of thioester bonds. This reversibility differentiates palmitoylation from other lipid modifications such as prenylation and myristolation (Iwanaga, 2009). The reversibility of palmitoylation allows for the formation and dissociation of protein complexes such as in the α-subunit of heterotrimeric G-proteins. Palmitoylation is the most common form of post-translational lipid modification and more importantly, it can take place at any point along a protein, whereas all other lipid modifications occur at either the N or C terminus (Hemsley, 2012).

Palmitoylation is performed by a family of enzymes, known as Protein-acyl transferase(PATs) that have a conserved DHHC (Asp-His-His-Cys) cysteine rich domain that appears to act as an intermediary in the creation of the thioester bond. (Resh 2002, Fukata 2004, Roth 2002) Examples of these enzymes have been found in yeast (Deschenes 2002, Roth 2003), plants such as Arabidopsis thaliana and Zea mays (Hemsley 2008) and in mammals (Fukata 2004, Ohno 2006).

Martin et al (2011) were able to identify 34 S-acylation proteins in yeast, 200 in mice neurons, 331 in human prostate cancer cell and 415 in normal human cells through CLICK chemistry analysis.

The precise mechanism of palmitoylation is still a mystery. Purified yeast

Erf2/Erf4, PATs, has been shown to become palmitoylated in the presence of purified palmitoyl-CoA. This spontaneous palmitoylation of a protein is called autoacylation. Palmitoyl-CoA acts as the palmitate donor. Autoacylation does not require enzymatic function. The existence of autopalmitoylation in the presence of palmitoyl-CoA could suggest a two-step process in which palmitate is first transferred to the PATase and then to the protein substrate (Roth, 2002).

Further research into Erf2/Erf4 in yeast by Mitchell et al (2010) was able to elucidate more about its palmitoyl-CoA hydrolase activity. First, when purified Ras protein (a palmitoylated protein) was added to purified Erf2/Erf4 and there was a dramatic increase in the uptake of free palmitoyl-CoA but a decrease in the level of free palmitate. This implied that the palmitate from palmitoyl-CoA was being transferred to the Ras protein. In a second study radio labeled palmitate was attached to Erf2/Erf4 and then the enzyme was exposed to nonpalmitoylated Ras in an excess of unlabeled palmitoyl-CoA. As a result, there was a dramatic loss in radioactivity from the radiolabeled enzyme. As a baseline, the rate of natural radioactivity loss due to hydrolysis of the thioester linkage was measured. These results helped to show that palmitoylation of Eas by Erf2/Erf4 follows a ping-pong kinetic mechanism. (Mitchell, 2010)

Loss of function studies have shown that the mutation of the DHHC-CRD interferes both with autoacylation of the PAT and in the palmitoylation of the target protein substrate. Loss of function studies have also shown that this reduced palmitoylation leads to mislocalization in many instances (Linder, 2013). It should be noted that Linder also questions if the cysteine in the DHHC region is directly involved in an autoacylation event and highlights the need for more active enzyme mapping.

It has been speculated that there are more active cysteines in the enzyme and that the DHHC cysteine is only one in a chain of cysteines that bring the palmitate from palmitoyl-CoA to the protein-substrate (Jennings and Linder, 2012)

Lack of reliable antibodies for PATs forces researchers to rely on fluorescent tagging to understand PAT expression patterns. Fluorescent tagging has shown that PATs are bound to various subcellular compartments like the Golgi, ER or the plasma membrane (Roth, 2002).
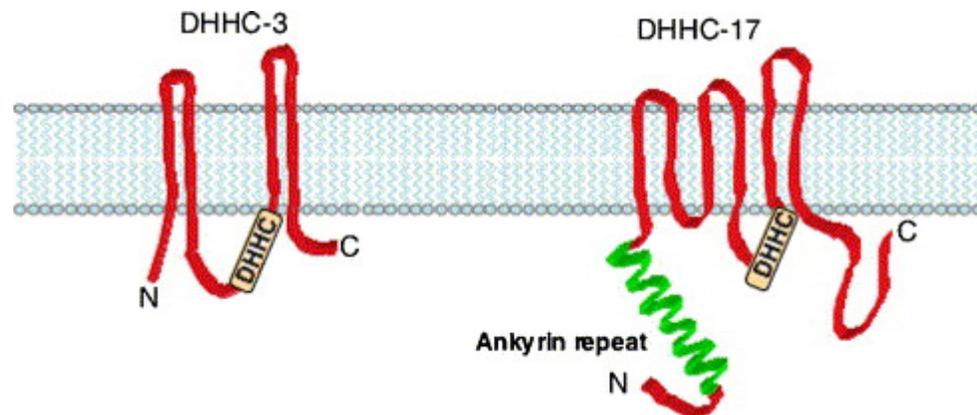
Figure 3 - Topology of DHHC-CRD membrane bound proteins (Fukata, 2006)

In 2005, Politis et al were able to experimentally determine the topology of DHHC proteins. Their research verified that all DHHC proteins are polytopic integral membrane proteins. In addition, they found that all DHHC proteins have either four or six transmembrane domains (TMD). In proteins with 4 TMDs the N and C termini extend into the cytoplasm with the DHHC-CRD projecting into the cytoplasm between the second and third TMD. A somewhat rarer variety of DHHC protein has 6 transmembrane domains and an extended N-terminus that encodes for ankyrin repeats (the founding member of this group was Akr1) (Politis, 2005). Both ends of the polypeptide chain

extend towards the cytoplasm and mediate protein-protein interaction between the DHHC-enzyme and its protein substrate. (Linder, 2013)

Roth et al. (2002) were able to establish that DHHC-CRD enzyme Akr1 is responsible for the palmitoylation and localization of Yck2 to the plasma membrane of yeast *in vivo* and for that purified Akr1 is able to palmitoylate purified Yck2 *in vitro* (Roth, 2002). This finding allows us to assume that the DHHC-containing enzyme must be the enzyme which performs palmitoylation, it is not a subunit of some protein complex. It would appear after much research that Palmitoylation is a conserved function of DHHC proteins in eukaryotic organisms (Fukata, 2004).

The regulation of palmitoylation/depalmitoylation is mostly unknown. This regulation can be envisioned most readily in the activity of G-protein α subunits, which must be tightly regulated in order for G-proteins to function normally, as well as in neuronal scaffolding proteins (El-husseini, 2002)

Factors that influence the activity of palmitoylation include post translational modification of the protein-acyl transferases (PAT's) such as phosphorylation and S-nitrosylation. PAT's can also be regulated by the presence of some secondary messengers such as phospholipids, cAMP and small metal ions. PAT's also appear to be, at least in part, regulated by the actions of other proteins. While PAT's are single protein enzymes they do seem to be influenced by the action of other proteins. ATP itself also seems to play a role in the regulation of palmitoylation as it vastly increases the rate of protein palmitoylation (the cause of this base rate seems to be up for debate) (Roth 2002). Acting as a palmitate analogue, 2-Bromopalmitate acts as an inhibitor of palmitoylation by inhibiting the formation of palmitoyl-CoA. Cerulenin has also been shown to act as a

palmitoylation inhibitor by alkylating protein PAT's (Resh, 2006).

Palmitoylation plays an important role in the functioning of many different types of proteins within the cell. These proteins can include G protein α-subunits, receptors, protein channels, Ras proteins, SNARE complexes, tyrosine kinase and scaffolding proteins (Resh, 2006). The prevalence of this process within the cell implies that it plays a vital role in cell function. This widespread use in the cell, however, does raise the question of PAT substrate specificity. (Linder, 2013)

First, PAT's show a mixed level of acyl-CoA specificity in regards to acyl chain length. This difference is best observed looking at DHHC2 and DHHC3. DHH2 is able to transfer acyl chains that are fourteen or more carbons in length. DHHC3, however, shows far more specificity and will only interact with acyl chains fewer than sixteen carbons in length (Jennings and Linder, 2012). It would appear that this preferential selection of acyl chains leads to the differential S-acylation of proteins observed in cells.. (Lidner, 2013)

The basis for this specificity is unknown, but a similar enzyme, N-myristoyltransferase (NMT), has very strict acyl-CoA specificity. NMT's specificity is caused by its tertiary structure, specifically the distance between an oxyanion hole and the floor of its hydrophobic pocket. Further research into the crystalline structure of PATs would help to determine the level of specificity (Linder, 2013).

The protein substrate specificity of DHHC-PAT's is highlighted in early studies by Fukata on yeast. In yeast, some DHHC enzymes were shown to act on multiple substrates, for example DHHC3 was shown to act on PSD-95, eNOS, DABA-A receptor γ2 subunit, SNAP-25, CSP, G α-subunits, NCAM and GAP-42. In contrast other PAT's seem to show higher levels of specificity, acting on just one or two substrates, for

example, DHHC2 appears to palmitoylate only PSD-95 (Fukata, 2004).

PAT selectivity is still as much a mystery as is the exact enzymatic mechanism and kinetics of PATs. While prenylation and N-myristoylation both have well defined consensus sequences on their target substrate-proteins, palmitoylation, does not. This lack of consensus sequence makes palmitoylation prediction very difficult to impossible (Hemsley, 2012).

Further confounding the issue of PAT protein specificity is the idea that multiple PAT's may act on a single protein. This was seen in yeast by Bartles et al (1999); even after Erf2 was removed through genetic knockout palmitoylation of Ras proteins still occurred. This was expanded upon by Roth and coworkers when they conducted a proteonomic study on yeast to eludicidate the palmitoylproteome through biotin exchange chromatography and mass spectrometry. Strains of yeast were then created with a deletion in the DHHC region of a specified PAT. In the palmitoylation profile they created there was evidence that some proteins are acted upon by only a single PAT while others are acted upon by multiple (Roth, 2006).

The action of multiple PATs on a single protein was examined in mice. Twenty-three DHHC enzymes in mice were individually co-expressed and what emerged were two categories of PATs; one group that palmitoylates a protein substrate and another that enhances palmitoylation through interaction with the PAT (Fukata, 2004).

It is interesting to note that there appears to be evidence that other lipid modifications may play a role in target identification by PAT's. Nadolski and Linder (2009) were able to remove the targeting determinants from Vac8 (vacuole-related protein 8) that allowed it to be recognized by Pfa3, a PAT, *in vitro*. This allowed them to

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examine what aspects of Vac8 allowed it to be recognized. They found that while myristoylation was not required for Vac8 palmitoylation, it does greatly increase its palmitoylation rate *in vitro*. (Nadolski and Linder, 2009)

With so many different proteins palmitoylated within a cell palmitoylation must play several important roles throughout the cell. Palmitoylation affects the ability of proteins to migrate towards protein rafts, for the anchoring of specific proteins to lipid bilayers and for the translocation of proteins throughout the cell (Linder, 2013).

Palmitoylation play an important role in the targeting of specific proteins to lipid rafts throughout the cell. The addition of the 16 carbon palmitate gives the most efficient rise in hydrophobicity (Iwanga, 2009) of all post translational modifications and this increased hydrophobicity targets these proteins to bilayers. Once attached into the membrane, the palmitoylated proteins tend to accumulate in lipid rafts. In fact, even integral membrane proteins such as NCAM, which by themselves are already hydrophobic enough to be incorporated into the bilayer and palmitoylated. Its palmitoylation increases its affinity to lipid rafts. (Brown and London, 2000)

Furthermore, this was shown by preventing the palmitoylation of NCAM140 in neural cells, which in turn prevented them from associating with lipid raft domains and inhibited cell growth. Iwanga et al (2009) hypothesized that regulation of palmitoylation by PAT's could provide an avenue for cellular regulation of membrane composition and protein activity through protein sequestering.

Another important action of palmitoylation incorporates the transfer of proteins from one membrane to another. This was shown to be the source of specific membrane targeting in H-Ras and N-Ras proteins. Through selective palmitoylation and

depalmitoylation proteins were selected for either the plasma membrane or the golgi

apparatus (Resh, 2006). Later research into the action of palmitoylation on RAS proteins

showed that when palmitoylation was blocked by the palmitoylation inhibitor 2-

bromopalmitate that these RAS proteins distributed themselves randomly throughout

intracellular membranes (Rocks, 2005). This was later extrapolated to mean that this

constant cycling of RAS proteins allowed them to be rapidly mobilized when needed but

kept the supply sequestered when not needed. This same type of cycling regulation has

also been seen with multiple forms of heterotrimeric G-protein α subunits (Chisari, 2007)

and various other proteins (Drenan, 2005)

Heterotrimeric G proteins are important regulators of many cellular processes that allow for the transmission of extracellular signals inside the cell by means of interaction with the three active subunits of the G-protein: α, β and γ. β and γ, themselves, are able to associate with lipid membranes independent of modification. The alpha subunit, however, does not have such a domain and must rely on palmitoylation (and other post translation lipid modifications such as myristoylation and through association with the beta-gamma complex) to associate with lipid membranes (Resh, 2006). The alpha subunit is also involved in selective targeting through means of modifications to its lipid attachment (prenylation or

Figure 4- The Epinephrine receptor requires G protein alpha subunit palmitoylation to interact with Adenylyl Cyclase

palmitoylation), this allows alpha subunits to rapidly move around the cell and interact

with multiple beta-gamma complexes on both the plasma membrane and endomembranes

(Chisari, 2007). G-Proteins are best known for their role in cyclic AMP (cAMP) regulation, in which the alpha subunit interacts with adenyl cyclase (another membrane bound enzyme) to create cAMP. In *Dictyostelium*, G-proteins play an important role in aggregation signaling.

Dictyostelium Background

Dictyostelium discoideum is a haploid soil living amoeba whose complex life cycle makes use of Heterotrimeric G-proteins to transition between its life cycle stages. *Dictyostelium* goes through four stages in its life cycle (Fig 5); vegetative, aggregation, migration, and culmination.
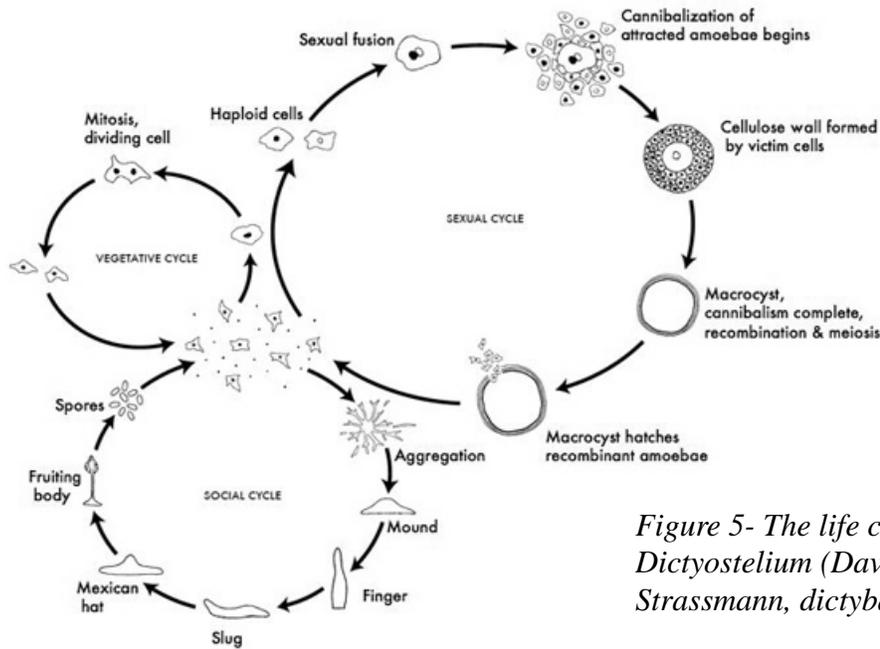
Figure 5- The life cycle of Dictyostelium (David Brown & Joan E. Strassmann, dictybase)

In the vegetative stage, *Dictyostelium* is in its nonsexual reproductive stage. This stage is occupied mostly by the search for food in the form of bacteria and mitotic cell division. When the cells begin to starve they enter the aggregation phase. The transition to aggregation is marked by the creation of biochemical machinery required chemotaxis and adhesion.

During aggregation, G-proteins regulate the production of cyclic-AMP which is secreted by the cells to act as a chemoatractant and signal other cells to aggregate through chemotaxis. Once enough cells have come together, a 'slug' is formed and as a single unit the cells begin to move towards heat and light in search of proper conditions to enter the

culmination phase. The culmination phase begins with the anterior end of the slug adheres to the surface and forms the stalk while the posterior end will become the fruiting body which will create the spores that will begin the new generation of *Dictyostelium* in this new environment

The entire life cycle of *Dictyostelium* takes place in 24 hours and each stage marks major changes in genetic expression.

GA2 is a heterotrimeric G-protein that is involved in the adenylyl cyclase pathway initiated in *Dictyostelium* aggregation. In *Dictyostelium* as in yeast it is actually the $G_{\beta 2}$ is the subunit that is directly responsible for adenylyl cyclase activation.

Due to the aggregation phase's use of G-proteins, this stage is the prime source of PAZ gene (the palmitoylating enzymes of *Dictyostelium*) activity observation. If $G_{\alpha 2}$ is not properly palmitoylated then there is no aggregation signaling upon starvation especially at high temperatures (above $29^{o}C$) (Kageyama, MS UMaine 2010). This reliance on palmitoylation is why *Dictyostelium* was chosen as a model organism.
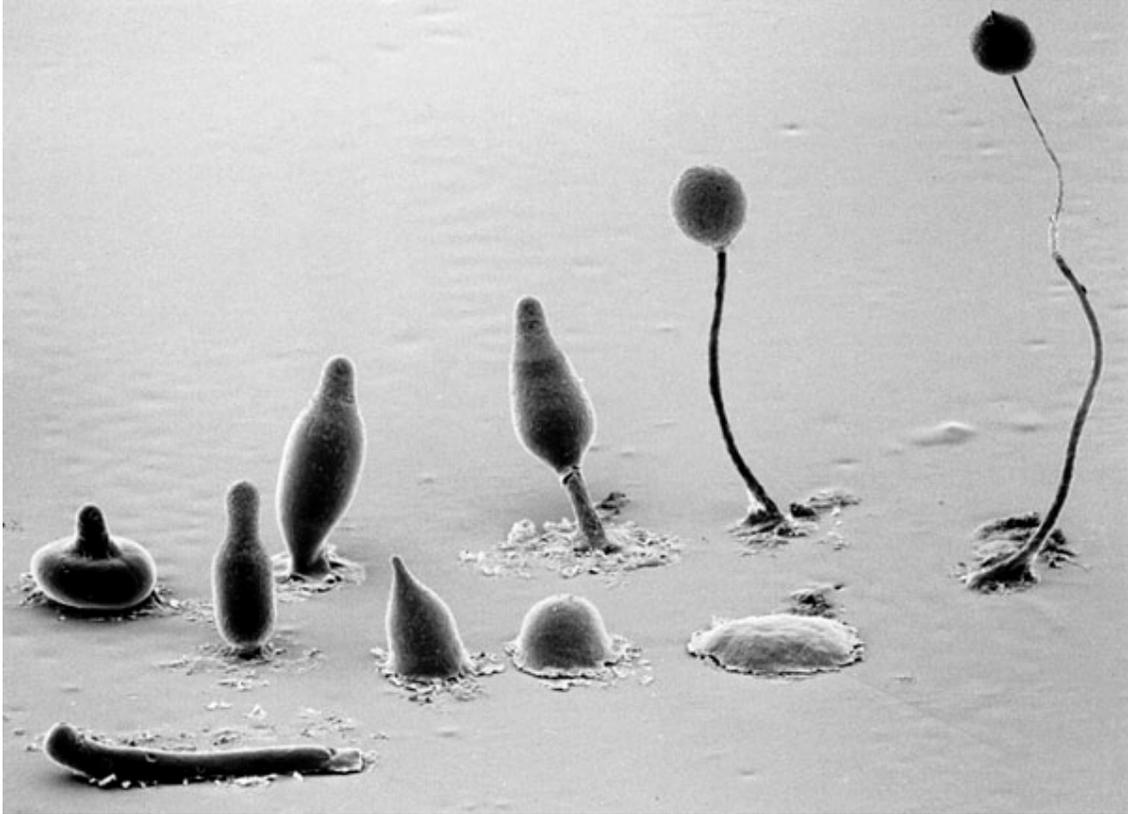
Figure 6 - The various stages of the *Dictyostelium* life cycle starting with the slug and ending with the fruiting body (M.J. Grimson & R.L. Blanton, dictybase)

Materials and Methods

Primer Creation

All primers were made by Integrated DNA Technologies, Inc. All primers made for this project were designed by Joshua Little using the Primer3 (http://frodo.wi.mit.edu/) and the ClustalW2 alignment tool provided by Expasy.org.

PCR Reaction

Polymerase Chain Reactions were carried out using a tabletop thermal cycler (Mj mini/PTC-1148) available from BIORAD. All PCR reactions were done in 50 µl. PCR was used to verify the presence of the knock-in cassette in isolated genomic DNA. Site directed mutagenesis PCR was also used to correct an error in PAZ9-YRP, a plasmid.

All PCR reactions were done in a total volume of 50 µl using New England Biolabs Hotstart Phusion polymerase and its associated 10% buffer, 1µmoles of each primer, 1µM of free nucleotides and over 200ng of template DNA.

E. coli Cell Culture

All *E. coli* cells were grown in a Luria Broth medium supplemented (http://www.microbelibrary.org) with either Kanamycin or ampicillan as determined by the plasmid design. All cultures were grown at 37º C on a rotating wheel.

E. coli cell Preservation

E. coli samples were preserved by first concentrating them using centrifugation then frozen in a 16% sterile glycerol solution at -80º C.

E. coli transformation and cloning

All *E. coli* transformations were done using a TOPO TA cloning kit with competent cells available from http://products.invitrogen.com/ivgn/product/K456001.

DNA was added to a vial of 'One shot *E. coli*'; the mixture was incubated on ice for 30 minutes. After incubation the cells were heat shocked to 42ºC for 30 seconds. After heat shock, the cells were give 250 µl of provided S.O.C. medium and incubated while shaking for one hour at 37º C. After incubation the cells were spread on an LB plate that was previously spread with a 50µg solution of Kanamycin.

Plates were left to incubate over night at 23 º C. After 24 hours, colonies were selected and grown in normal cultures.

DNA Precipitation

DNA Precipitation was accomplished by using one volume of 3M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol. Samples were then placed in a -20º C for 1 hour. After the sample was centrifuged; the supernatant was discarded and the pelleted DNA was rinsed with 70% ethanol and then suspended in a variable volume of provided EB buffer.

DNA Purification by Phenol-Chloroform treatment

DNA purification from protein and RNA was done using phenol-Chloroform extraction. The DNA sample was diluted to 200µl, then an equal volume of phenol-chloroform was added and vortexed. Once centrifuged at 10 RPM for 5 minutes the sample separated into an aqueous phase, which contained the nucleotide fraction (DNA/RNA), and a lower organic layer, which contained protein. The top layer was pulled off and placed in a new sterile centrifuge tube and combined with 200µl of 1:1 isoamylalcohol and chloroform. This solution was vortexed and centrifuged again. The top layer is pulled off and concentrated through DNA precipitation to give concentrated, purified DNA.

DNA Quantification

All DNA quantification was done using a NanoDrop Full Spectrum Flourospectrometer available from nanodrop.com

Dictyostelium **Cultures**

Dictyostelium cultures were grown in a HL5 solution (http://dictybase.org). Knock-in transformed cells were supplemented with Blastocidin-S at 10 µg/ml for selection. All cultures were housed in a clear bottom, plastic petri dish and incubated at 22ºC. Medium was changed every five to seven days.

Preserving *Dictyostelium*

Dictystelium cultures were preserved using 20% DMSO to prevent water crystal formation and fetal bovine serum albumin. All samples were stored at -80 degrees.

DNA Isolation from *E. coli* Cells

All DNA isolation was completed using the Qiagen Spin Miniprep Kit using the

manufacturers recommended protocol found at

(http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-

[Technologies/Plasmid-DNA/QIAprep-Spin-Miniprep-Kit#resources](http://www.qiagen.com/Products/Catalog/Sample-Technologies/Plasmid-DNA/QIAprep-Spin-Miniprep-Kit#resources))

DNA Linearization

Plasmid linearization was accomplished using digest with restriction enzyme KpnI. Cells

were incubated under manufacturers provided buffer at 37 C for 1 hour.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was done using a 1% mixture of agarose in TPE buffer (Tris

base, phosphoric acid and EDTA). All gels were run between 90 and 100 millivolts for 60

minutes in TPE buffer with a 1KB+ ladder (Invitrogen) using 6x loading dye. All gels

were then rocked in Ethidium Bromide (1µg/ml) for 15 minutes. Gels were photographed

using transluminescent ultraviolet light using an alpha innotech corporation imager

system.

DNA transformation into *Dictyostelium*

Knock-in constructs were introduced to *Dictyostelium* using electroporation at 1.2 KV,

3µF and 200 Ohmns. The τ (time interval) used was .4 to .6. Cells were then allowed to

grow in HL5 medium. Within 24 hours of electroporation, heat killed cells (*E. coli*) were

added to the medium to improve growth. After 24 hours, Blasticidin S (10ug/ml) was

added to select for transformed cells.

Genomic DNA Purification

Genomic DNA from *Dictyostelium* was obtained using the Dneasy Blood and Tissue Kit

available from QIAGEN (http://www.qiagen.com)

Dictyostelium **Starvation**

      Dictyostelium cells were prepared for examination by starvation on 1.5% agar

plates with development buffer (10nM phosphate buffer, 2mM $MgSO_4$ and .2mM $CaCl_2$)

for between one and twenty-four hours. Plates were stored at 23º C. Cells were

transferred to a glass bottom microculture plate with 1 ml of development buffer before

examination

Fluorescent Microscopy

      Knock-in *Dictyostelium* cells were examined using an Olympus IX81 flourescent

microscope and examined using Fluoview ASW V.3.1. The cells were exposed to 514-

127 nm light.

Results

In order to create a knock-in (KI) construct the target gene must first be available in a plasmid; this makes the gene exposed and easily modifiable. Our first step was to introduce a Not1 restriction site and to remove the stop codon from the end of the target gene. The Not1 site is used to ligate the YFP/BSR cassette and removal of the stop codon allows for our cassette to be read in frame with the target gene to create a PAZ-YFP fusion protein.

The next step was to check that the Not1 site was functioning properly. In the case that the knock-in was not in frame an additional mutation may be required.

Figure 7- knock-in creation scheme
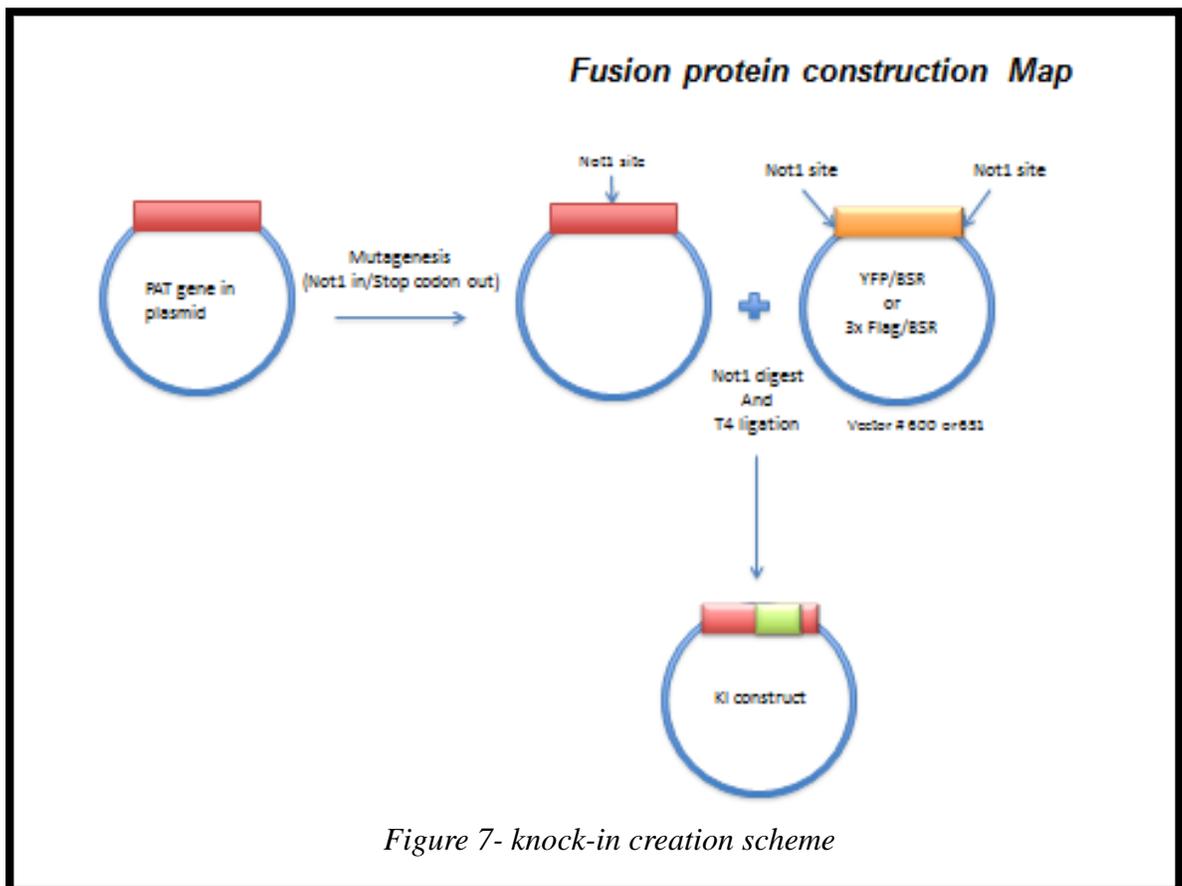
The third step was to cut the DNA at the Not1 restriction site. The YFP/BSR cassette, already in a plasmid, is bookended by Not1 sites and was also cut using the not1 restriction enzyme.

The now linearized gene and the YFP/BSR cassette are ligated together using T4 DNA ligase.

Penultimately, the new construct must be transformed into *Dictyostelium* using electroporation.

Lastly, transformed *Dictyostelium* must be examined using PCR and confocal microscopy to verify knock-in validity.

1) **Not1 restriction site addition/Stop Codon removal**

First, primers were constructed that began about 20 bases before and after the stop codon of PAZ9; in place of the stop codon in the primers, however, was a Not1 restriction site. PCR was done using a table top PCR machine to produce a plasmid of PAZ9 with no stop codon and NotI restriction

Original Paz9 sequence with stop codon highlighted in red

GTTTACTCTCATATTATTAATTGGTTGGTTTACCGACCGATCAAAAATAAAAAATAAAAAAAAAA
CAAATGAGAGTATAATAATTAACCAACCAAATGGCTGGCTAGTTTTTATTTTTTATTTTTTTTTT

GTTTACTCTCATATTAT   TTGGTTGGTTTACCGACCGATCAAAAATAAAAAATAAAAAAAAAA
        TCTCATATTATGGCGGCCGCTTGGTTGGTTTACCGACCGAT

Alignment with paz9-stop+not1 primer with not1 site in red and extra guanine in blue

Figure 8-paz9 stop codon removal and NotI site addition

site. Removal of the stop codon allowed for the knock-in to be read continuous with PAZ9 by the ribosome.

Once PCR finished, the resulting DNA was treated with DPN1; a restriction
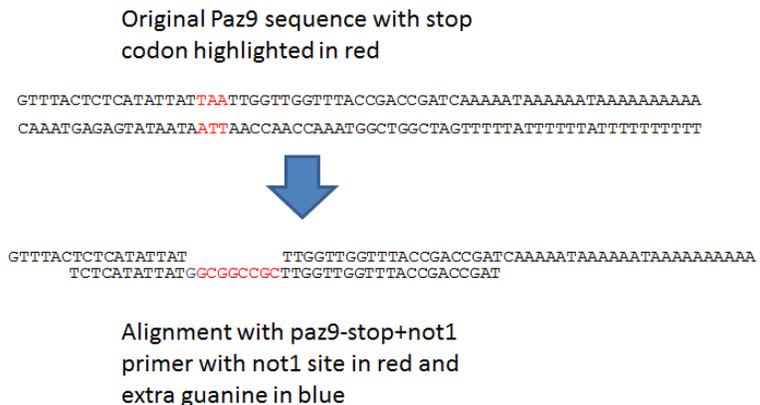
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enzyme that digests methylated DNA, in this case the original, unmodified DNA (PCR product does not have a chance to be methylated). The modified PAZ9 was then purified using phenylchloroform.

Once precipitated the concentrated modified PAZ9 was inserted into *E. coli* using the TOPO cloning kit and heat shock. *E. coli* was then grown in order to amplify the modified PAZ9. Modified PAZ9 was isolated from the *E. coli* using the Qiagen miniprep kit.

Once amplified and isolated, the PAZ9 plasmid was then digested using Not1 to verify the mutagenesis.

Results of this initial Not1 digest were inconclusive and further testing of the original genomic PAZ9 sample have yielded confusing restriction enzyme analysis results.

2) **Point mutation to ensure proper reading frame of Not1**

Paz10 had been brought to this point previously by the Gundersen lab. PAZ10 was first sequenced in order to check the state of its Not1 site. Sequencing revealed that the Not1 site was out of frame and therefore would not create the proper fusion protein of PAZ10 and YFP. In order to make PAZ10 serviceable, two primers were created that mirrored the 30 bases surrounding the Not1 site but the primers inserted an extra guanine base before the Not1 site to correct the reading frame.

Original paz10 sequence
with Not1 site out of frame
(blue)

ATA CTG ATG AAT ATA ATA GGC ATA AAA AAG CGG CCG CAT GGT GAG CAA GGG CGA GGA GCT GTT
TAT GAC TAC TTA TAT TAT CCG TAT TTT TTC GCC GGC GTA CCA CTC GTT CCC GCT CCT CGA CAA

paz10-not1fix-a primer
ATTATCCGTATTTTTTCCGCCGGCGTACCACTCGTTCC
ATACTGATGAATATAATAGGCATAAAAAAGGCGGCCGCATGGTGAGCAAGGGCGAGGAGCTGTT
TATGACTACTTATATTATCCGTATTTTTTCCGCCGGCGTACCACTCGTTCCCGCTCCTCGACAA
TAATAGGCATAAAAAAGGCGGCCGCATGGTGAGCAAGG
Paz10-not1fix-s primer

Figure 9-paz 10 frame shift correction using 2 custom primers

After PCR with the new primers PAZ10 was digested by Dpn1 to remove the template DNA and treated with phenylchloroform to purify the modified DNA.

The purified modified PAZ10 was then transformed into E. coli using heat shock and the TOPO cloning kit. *E. coli* containing the PAZ10 plasmid was grown on agar plates and colonies were selected and allowed to multiply.

Once amplified in *E. coli*, the modified PAZ10 was isolated from the *E. coli* and sequenced to verify the correction.

3) **Not1 digest and Ligation with YFP/BSR Cassette**

In order to introduce the YFP/BSR cassette into the target gene the gene must first be opened using the previously added NotI site. Using the NotI enzyme, the enzyme can be linearized and prepared to be ligated with the YFP/BSR cassette which has

also been digested by NotI. The gene and the cassette share complimentary ends now

due to the action of NotI. These ends can be ligated together to

form an in frame connection between the gene and the YFP/BSR

cassette.

Not1 digest and ligation with the YFP/BSR cassette for

PAZ13 was done previously by the Gundersen lab. Not1

digest and ligation with the YFP/BSR cassette for PAZ5 was

done previously by the Gundersen lab.

The Not1 site creates overlapping ends on both the

YFB/PSR cassette and the newly linearized gene when cut

with a Not1 restriction enzyme. In solution, these ends will

tend to realign and can be reformed using T4 DNA ligase.

This creates a knock-in construct with the YFP running in

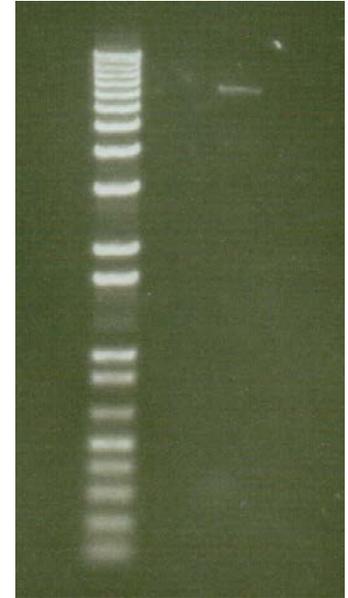frame with the target gene and the BSR behind it (under its

own promoter).

Figure 11 - Paz13 linearization check using gel electrophoresis. The single band indicated a total digest

4) Knock-in transformation into *Dictyostelium*

Now that the knock-in is created it needs to be introduced to *Dictyostelium*. This

is done using electroporation; a mechanical process through which the plasma

membrane of the *Dictyostelium* is opened using electric current. The knock-in can

then be introduced directly into the genome of *Dictyostelium* where a crossover event

will lead to the incorporation of the knock-in into the genome of the cell.

The PAZ13 plasmid was isolated from *E. coli* using the Qiagen miniprep kit.

PAZ13 was then linearized using Kpn1 to allow incorporation into the genome

through a double cross-over event. Linearized PAZ13 was purified using phenol-

chloroform and then precipitated to concentrate the linearized DNA.

Electrophoresis was then used to verify the linearization of PAZ13.

The linearized PAZ13 plasmid was then inserted into *Dictyostelium* using

electroporation. These pores allow for the linearized plasmid to pass through the

plasma membrane into the cytosol where they will be inducted into the genome of

Dictyostelium through double crossover during mitotic division.

PAZ5 introduction to *Dictyostelium* was done previously by the Gundersen lab.

5) Verification by Genomic PCR and Fluorescence

In order to verify that the
knock-in has been successfully
incorporated into the genome of
Dictyostelium by double
crossover there must be proof
that both the YFP/BSR cassette


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CGATTATTTAGACACAAAGCTTAAAGATGATAAATTATTTGGCGGCAATGAGAGACTCTA 2100
-----------ACACAAAGCTTAAAGATGATAAATTATTTG------------------ 30

TCATAGATTTTGGACAGTGATGGGTGTGTTGACAGTATTGGTGCCAACCTGGATTCTATG 2160
------------------------------------------------------------ 

CTATGTGCCAGTGATATTTGGGTTACCATTGTTGGCTGTCGGTGGCTACTTTTTAAAGAA 2220
------------GATATTTGGGTTACCATTGTTGGCAG-------------------- 26
```
147 bp product expected

Figure 12-Half forward and half reverse primer alignment shows a 147 bp product.

is present in the genome by PCR and that both are functional by antibiotic selection

and fluorescence.

To verify knock-in presence in Paz5 and Paz13 first the integrity of the genomic DNA was tested using specific PCR primers created from Paz5. (Paz13 genomic DNA contains the unmodified Paz5) Primers were expected to create a product that was 170 base pairs in length

With the integrity verified, two different PCR checks were used to verify the knock-in. YFP/BSR presence was checked using specific primers with the YFP/BSR sequence and primers that ran from the genomic DNA through the YFP/BSR cassette were used to ensure that the cassette knocked into the proper place on the gene.

First, in order to verify the presence of the YFP and BSR cassette a PCR was done using primers created both from the YFP and BSR sequence.
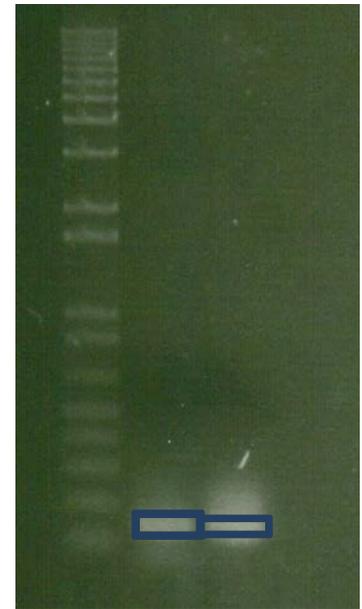

Figure 13- Paz5/Paz13 genomic integrity check using half forward and half reverse primers. Product is between 100 and 200 bp long
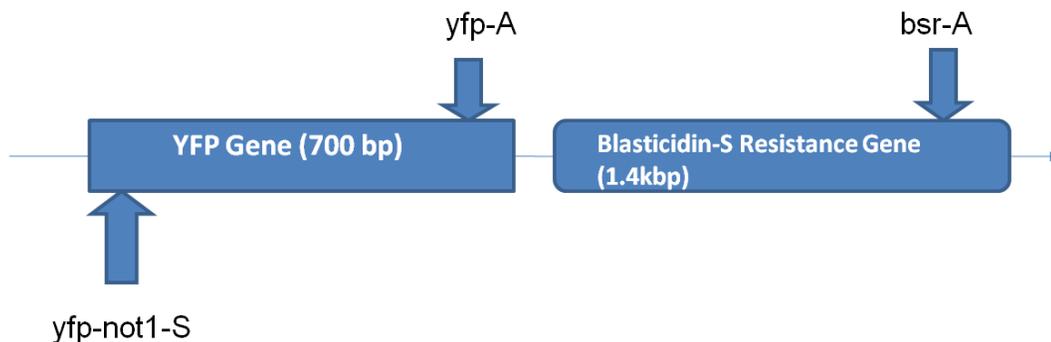

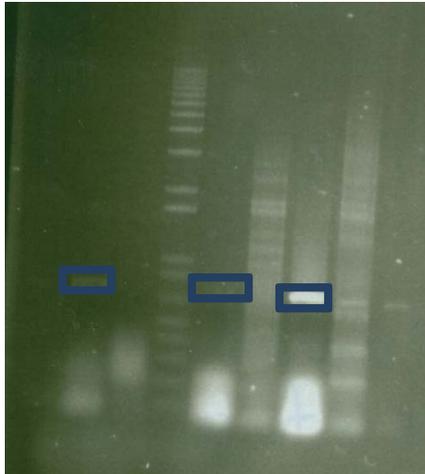*Figure 14-YFP and BSR primer alignment*

Figure 15-Gel electrophoresis shows YFP primer product that is the expected 700bp long

PCR was done using the genomic DNA preparations from PAZ5 and PAZ13 as well as the original PAZ13 plasmid as a control. The YFP-Not1-s primer was used in both cases with the YFP-A being used to verify YFP alone and the BSR-A primer being use to show that BSR cassette was in place directly behind the YFP.

PCR was then used to verify the BSR placement. Because products could not be made using the antisense primer from BSR a complimentary sense primer was create and used. This primer, however, did not create product.

Following verification of the YFP/BSR cassette's incorporation into the gene its proper placement needed to be verified using primers which ran into the YFP gene from the PAZ gene (Fig. 16). From this we expected to see a product that was around 1.4kbp in length but we were unable to produce any product.
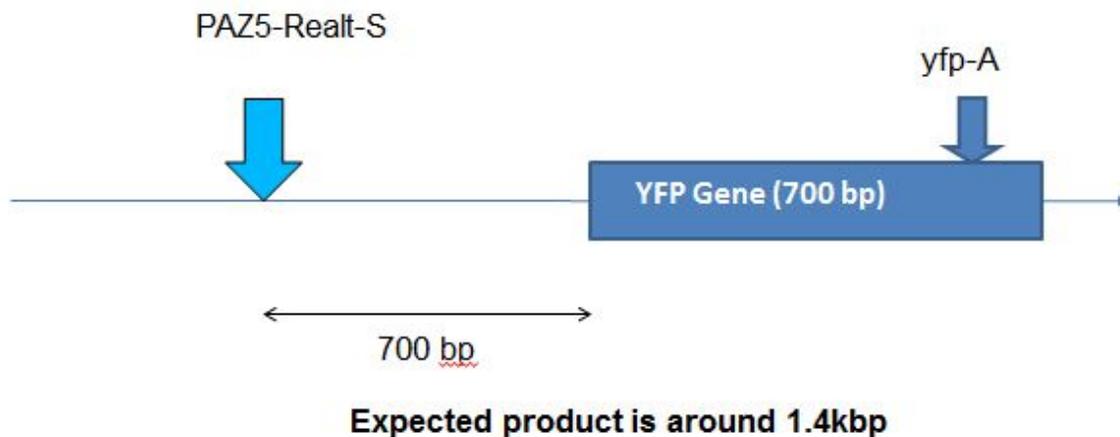
Figure 16- YFP placement check using one primer from genomic and one primer from the YFP.

Lastly, fluorescence was checked using confocal microscopy. When viewed using the Eyfp setting of Fluoview-ASW. Three different PAZ13 clones and one PAZ5 transformation were examined starved for 24 hours, 6 hours and 1 hour. All samples showed no fluorescence. Samples of stalks, spores and slugs were examined.

Figure 17 - example of fluorescence. $G_{\alpha 2}$-YFP fusion protein expressed in wild type Dictyostelium

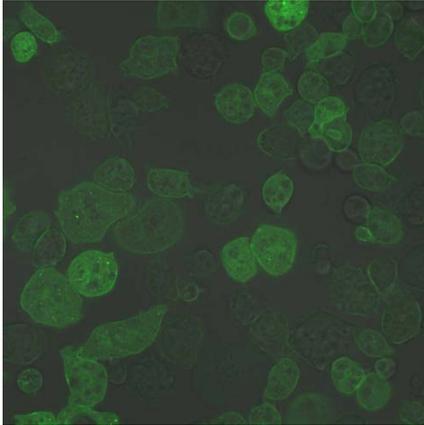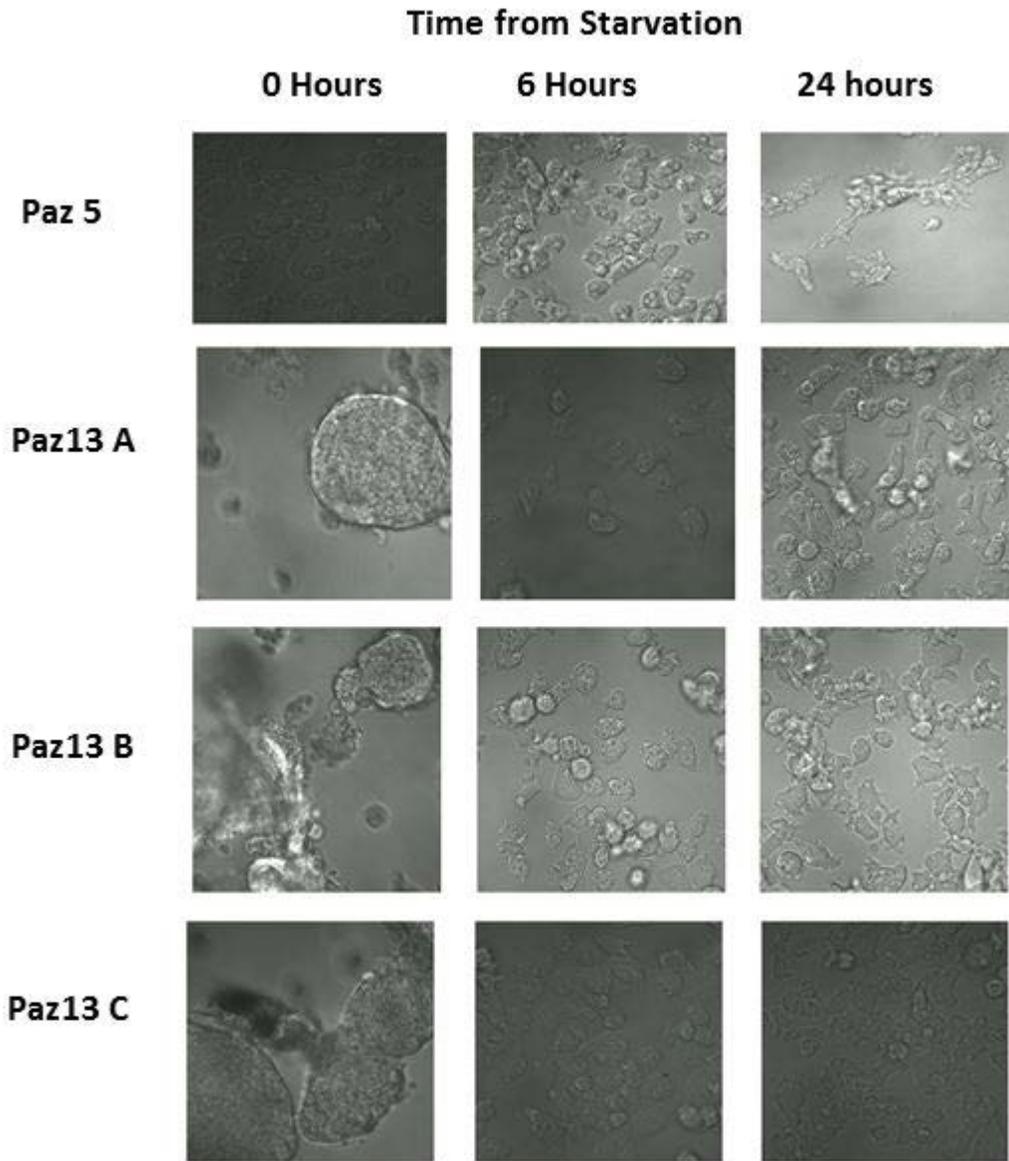*Figure 18-Confocal Microscopy results for Paz5 and three clones of Paz13. Images are composites of both light and confocal images*

Discussion

Analysis of the above outlined process for knock-in creation has shown that each step is feasible and can be used to progress a wild type gene to a complete knock-in construct in *Dictyostelium*. While time did not allow for one gene to be taken through the entire process each step was undertaken in the completion of this thesis so that the entire process could be experienced and analyzed.

Paz5 and Paz 13 were brought to verification using the outlined procedure to mixed results. While PCR showed that both YFP and BSR genes were present in the knock-in construct's genomic DNA, no fluorescence was observed using confocal microscopy. BSR knock-in was also verified using Blasticidin-S antibiotic selection.

PAZ10 was successfully corrected using point mutation PCR and verified using targeted DNA sequencing of the gene. This correction will allow for proper fusion protein with the YFP/BSR cassette.

PAZ9, however, represents a conundrum. At present, PAZ9 is being reevaluated for possible exchange into a new vector with better suited restriction sites.

The value of this thesis comes in the creation progression of four different PATs of *Dictyostelium* towards becoming YFP/BSR knock-in constructs. These knock-in constructs allow for the expression timing and location to be visualized in living cells. Real time PCR can be used to determine the expression timing of protein synthesis but these results cannot tell us in what specific cells or in what part of an organism. Most importantly real time PCR cannot be used to watch how the expression develops in the living cell.

With the information about the timing and placement of PAT gene expression in growing cells we can begin to learn more about the function and purpose of each PAT in

the cell. The purpose of these PATs can then be used to begin looking at substrates and the functional importance of palmitoylation to their task.

The basic mechanism and function of palmitoylation is still mostly unknown. Basic knowledge such as expression patterns about specific PATs can help us to begin understanding palmitoylation as a conserved process across all taxa.

With luck, by expanding the zeitgeist of palmitoylation we can begin to utilize this research to tackle the wealth of human tribulations associated with palmitoylation such as developmental disorder, cancer proliferation and hormone imbalance.

Connected research being done in the Gundersen lab with genetic knock-outs will help to further elucidate the nature of the PAZ proteins by showing how their absence will impact the development and survival of *Dictyostelium*.

Lack of fluorescence in PAZ13 and PAZ5 constructs presents a puzzle for further research. While PCR was able to verify that the YFP is present in the construct, the lack of fluorescence would imply that the gene is either not being expressed or that once expressed secondary folding of the protein is being inhibited.

Data gained from this thesis is also limited by its use of a haploid bacterial organism like *Dictyostelium* and the direct correlation to palmitoylation in humans is clouded.

Given more time I would like to conduct additional PCR experiments from the end of the YFP to the beginning of the BSR in an attempt to create a successful product under the assumption that a smaller product should be easier to create. I would also like to begin looking at the sequenced genome of *Dictyostelium* in an attempt to identify alternate knock-in sites and test these with PCR.

In the future, all the PAZ genes in *Dictyostelium* must have knock-in constructs made in order to understand their unique expression patterns. PAZ10, now corrected, requires insertion of the YFP/BSR cassette and introduction into *Dictyostelium*. PAZ5 and PAZ13 expression data can now be used to begin examining their importance to *Dictyostelium*.

This data and more like it must be used to continue researching the aspects of palmitoylation that next to nothing it known about such as its kinetic mechanism, target protein consensus sequences, energetic costs and benefits of palmitoylation and, broadly, the costs and benefits of palmitoylation to cells and eventually to the multicellular organism.

Palmitoylation represents a highly important cellular process that research has overlooked in its attempt to combat human illness. Modern biochemistry cannot continue to ignore palmitoylation when it has been linked to such serious human conditions. Research from this thesis and, more importantly, the Gundersen lab as a whole, can begin to unravel the process of palmitoylation. Integrated with the work of other labs around the world, the successes of each small research project can begin to form a comprehensive knowledge base about palmitoylation that can eventually be applied to that most basic of scientific goals; to allay the human condition.

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Author's biography

Joshua Little was born in Norway, Maine on December 14, 1990. Raised in the same house that had held four generations of his family, Josh was encouraged from a young age to love science and knowledge by his grandmother, Evangeline little, and his grandfather, Bradley Record; to whom this work is dedicated. Josh has spent the last four years studying biology at the University of Maine where he has worked towards a minor in music and has worked with the Department of Resident's Life for the last three.

After graduation, Josh will be attending the University of New England to study Physical Therapy.