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Innate Immune Recognition of Candida Albicans in Zebrafish

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INNATE IMMUNE RECOGNITION OF *CANDIDA ALBICANS* IN ZEBRAFISH

by

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ABSTRACT

Candida albicans is an opportunistic fungal pathogen that has the capability to switch from commensal to pathogen in immunocompromised individuals. Recognition of pathogens, like *C. albicans*, during infection is poorly characterized primarily due to the difficulties in visualizing the host/pathogen interaction without killing the host. Transparent animal hosts, such as *Danio rerio* (zebrafish), enable imaging of pathogen recognition while maintaining host viability. For pathogen recognition, zebrafish likely use immune receptors similar to mammalian receptors including C-type lectin receptors. Human C-type lectin receptors have already been shown to be crucial in recognition of fungal pathogens like *C. albicans*, and our goal is to identify and characterize cognate receptors crucial for fungal recognition in zebrafish. Here, I show how I purified fusion proteins of recently identified receptors and began characterizing the binding of these receptors to *Candida albicans*. Determining the specificity of these receptors may enhance our understanding of fungal recognition in the zebrafish host and the evolution of vertebrate immune receptor specificity. In addition, receptors that bind to *C. albicans* could be used as a diagnostic for *C. albicans* infection in patients.

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INTRODUCTION

i. Overview

Opportunistic pathogens have become a major concern in the realm of infectious disease. While asymptomatic in healthy individuals, these pathogens have the ability to cause significant infections in patients undergoing immune-suppressing procedures. *Candida albicans* is an opportunistic fungal pathogen that causes diseases ranging from oral thrush to candidemia in immunocompromised individuals. Recognition of pathogens, like *C. albicans*, during infection is poorly characterized primarily due to the difficulties in visualizing the host/pathogen interaction without killing the host. Transparent animal hosts, such as *Danio rerio* (zebrafish), are necessary to accomplish the task of imaging pathogen recognition while maintaining host viability. In addition to being transparent, zebrafish share genetic relatedness to humans and are easy to manipulate. For pathogen recognition, zebrafish likely use immune receptors similar to mammalian receptors: Toll-like receptors, Nod-like receptors, and C-type lectin receptors. Human C-type lectin receptors have already been shown to be crucial in recognition of fungal pathogens like *C. albicans*. The long-term goal of this research is to identify and characterize potential zebrafish receptors that mediate *C. albicans* recognition. In addition, we hope these studies will provide insight into the evolutionary relationship between zebrafish and mammalian immune systems.

ii. *Candida albicans* - an opportunistic pathogen

Nosocomial candidemia (*Candida* in the bloodstream) is an ongoing public health concern. In 2000, *C. albicans* accounted for 5 – 10% of all nosocomial bloodstream infections in acute care hospitals, with a 25% mortality rate (17). The medical costs associated with hospital acquired candidemia were reported upwards of $34,000 per patient (10,17). Other complications

from infection with *Candida* species include oral, gastrointestinal, and vaginal thrush. Ninety percent of acquired immunodeficiency syndrome patients (AIDS) patients suffer from oral candidiasis, which is the most common oral manifestation of HIV infection (18). *Candida albicans* is also the most common species associated with candidiasis and candidemia (20). *C. albicans* has the ability to switch from commensalistic to pathogenic in immunocompromised hosts (patients with AIDS, undergoing chemotherapy, or receiving immunosuppressant drugs for organ transplants). Determining the mechanisms of this molecular switch has been an ongoing, complex process.

C. albicans possesses morphological plasticity, meaning it can exist in yeast, pseudohyphael, and hyphal forms. Many studies have shown that the hyphal form is responsible for invasive candidiasis. In murine models, prevention of the filamentous form inhibits *C. albicans* growth and hinders dissemination (11). In addition, yeast cells that are engulfed by macrophages switch to hyphal form to escape the macrophage (13). Alternatively, the yeast form of *C. albicans* is responsible for the pathogen's ability to disseminate and cause secondary infections (9). *Candida*'s ability to both invade the mucosal and epithelial barriers and to disseminate and cause systemic infections makes it very difficult to treat.

iii. Innate immunity and pattern recognition receptors (PRRs)

Introduction of foreign invaders into the human body leads to stimulation of the innate immune response via pattern recognition receptors (PRRs). These PRRs are displayed on the surface of specific innate immune cells and bind to pathogen associated molecular patterns (PAMPs). This PAMP-PRR interaction initiates signaling cascades that elicit a nonspecific immune response to the invader. PAMPs can be many different things, including carbohydrates, nucleic acid methylation patterns, lipopolysaccharides, and proteins. There is promiscuity with

PRRs, meaning many PRRs can recognize different PAMPs and many PAMPs stimulate more than one PRR. While the response generated is PAMP/PRR specific, it is not specific to the pathogen and thus does not confer long term immunity.

Pattern recognition receptors are classified based on the class of molecules they recognize and the type of receptor. These include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs). C-type lectin receptors, which are highly conserved across the animal kingdom, are typically expressed on myeloid and mucosal epithelial cells (22). All C-type lectin receptors contain at least one carbohydrate recognition domain (CRD) and can exist as either a soluble or transmembrane receptor. While there are currently seventeen identified C-type lectin groups, only those of Groups II, III, IV, V, and VI have shown important roles in innate immunity. *Dectin-I* (Group V) and *Dectin-II* (Group II) are primarily responsible for recognition of *Candida albicans* in humans.

The cell wall of *C. albicans* serves a greater role than just protection against osmotic offense, and has been shown to be a "dynamic organelle" (7). Approximately 80–90% of the cell wall are carbohydrates, including β(1-3)-glucan and β(1-6)-glucans, *N*-acetyl-D-glucosamine, and mannan. Dectin-I recognizes and binds to β(1-3)-glucans and elicits a proinflammatory response at the site of infection. By masking β-glucan with mannan, *C. albicans* is able to evade immune recognition by *Dectin-1*. However, *Dectin-II*, *Mincle*, and *DCSF* recognize and bind mannan and also elicit a proinflammatory response. Due to its dynamic nature and contribution to virulence, it is important to determine which receptors bind which components of *C. albicans* cell wall in the zebrafish model.

iv. *Danio rerio* as a research tool to study fungal infection

One of the major difficulties with studying host-pathogen interaction in vivo is the necessity for a transparent host. For this reason, the zebrafish model is of extreme benefit as opposed to using the murine model. Using the zebrafish larval model allows for real-time imaging of *C. albicans* infection, both invasive and disseminated. The other commonly known benefits of using zebrafish are their genetic relatedness to humans, large brood size, short generation time, and ease of care and genetic manipulation. Zebrafish are also one of the smallest animal models with both an innate and adaptive immune system (15). The greatest advantage in studying the innate immune-fungal interaction is that zebrafish only have an innate immune system until they reach adulthood (4–6 weeks post fertilization). Since *C. albicans* recognition in humans is a primarily innate response, this makes the zebrafish larval model as an ideal tool for studying *Candida* infection.

v. Current and previous research

It has yet to be determined which innate receptors of zebrafish recognize *C. albicans*. While the downstream signaling of innate receptors is highly conserved across the animal kingdom, the extracellular domains of the receptors have evolved rapidly over time. The evolution of the carbohydrate recognition domain (extracellular portion) of C-type lectin receptors has been driven by pathogenic evolutionary pressures (3). Only the membrane-embedded portion of C-type lectin receptors have been conserved across animal models including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (8). The Group V C-type lectin receptors have not been found in teleost fish, suggesting there is not a

direct *Dectin-1* homolog in these fish. While there are Group II C-type lectin receptors in zebrafish, there has yet to be a *Dectin-2* homolog discovered.

It has been documented numerous times that *C. albicans* recognition in zebrafish is primarily an innate response. NADPH-oxidase dependent phagocyte recruitment during early infection is a critical requirement for pathogen clearance (4). Neutrophil recruitment during early infection is needed to prevent filamentous growth which causes invasive candidiasis (16). For this reason, the purpose of this study has been to identify innate receptor(s) that are responsible for recognition in zebrafish to further the understanding of *C. abicans* infection in this animal model.

The beginnings of this project were conducted by Erin Carter of the Wheeler lab. Using sequence homology, several potential fungal recognition receptors were identified (Fig. 2). Of these, seven receptors were cloned for further studies. The following is the name of each of the receptors and the recognition done by the human ortholog. Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin like F (*DCSIGN-F* or *DCSF*) recognizes high-mannose-containing glycoproteins (14). Cluster of Differentiation 209 (*CD209*) binds carbohydrate ligands on various microbial surfaces including *C. albicans* (5). There is no human homolog, of dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin like G (*DC-SIGN-like-G* or *DSLG*) but it is related to human *DCSIGN,* also known as *CD209* (2). Zebrafish killer cell lectin 1B (*KCL*) shares homology with human *CD209* (6). Immune-related lectin-like receptor 1 (*ILLR1*) possesses an activation motif typically seen in Group V natural killer T-cell receptors (19). Asialoglycoprotein receptor 2 (*ASGPR2*) binds to galactaose residues located on microbial surfaces (12). Salmon C type Lectin Receptor A (*SCLRA*) shares the most homology to human *Dectin-1* (6).

All seven identified receptors were cloned into plasmids with a secretion tag (Fig. 3). This was done by fusing the carbohydrate recognition domain (CRD) to the constant portion of human Fc. The construct contained a pSecTag2 secretion tag which ensured that the protein would be secreted by cells expressing the plasmid. In addition, the receptors were labeled with GFP for fluorescence detection. Following creation of these soluble constructs, the plasmids were transfected into the HEK293T cell culture line. Secreted protein was collected, purified to contain only the receptor, and concentrated. Prior to the research discussed in this thesis, five of the receptors had been purified in this manner.

The research discussed in this thesis begins with purification of the remaining two receptors, *ILLR1* and *ASGPR2*. Following this, characterization using luminescence binding assays were conducted on four previously purified receptors. These studies were done to build upon the fluorescence microscopy experiments conducted by Riju Shresta, who did her capstone in the Wheeler Lab. The goal of this thesis research has been to determine the binding affinities of the zebrafish receptors to *C. albicans* using luminescence high-throughput binding assays. This information will contribute to a broader understanding of innate immune recognition of *C. albicans* in the zebrafish model. In addition, this will allow for *in vivo* studies to be conducted to corroborate the results found.

MATERIALS AND METHODS

i. Cell Culture Maintenance

A Steril Gard II laminar flow hood was used to prevent contamination while conducting the cell culture procedures. HEK293T (Human Embryonic Kidney) cell line was used and grown in T75 flasks that were placed in a 37ºC incubator for overnight storage. When the HEK293T

cells reached approximately 90% confluence, as determined by viewing under an inverted dissecting microscope, the cells were split to double the original number of flasks. Minimum essential medium supplemented with 10% filter-sterilized FBS, 1μM sodium pyruvate, and 1% gentamyacin (to prevent contamination) was used for the cells. HEK293T cells are adherent cells, so the media (~20mLs) was pulled off without disturbing the cells. 4 mL of 0.5% trypsin-EDTA (Gibco by Life Technologies) was added to the flask to un-adhere the cells. The flask containing trypsin was incubated for 5 minutes at 37ºC. Following this incubation period, 16 mL of fresh media was added to the flask. The fresh media, now containing the HEK293T cells, was pulled off and transferred to a 50 mL falcon tube. This tube was centrifuged at 1,000rpm for 3 minutes using a Sorvall ST16 centrifuge (Thermo Fisher Scientific). The pelleted cells were then resuspended in fresh culture media. Using 2 new T75 flasks, 2mL of the resuspended cells were placed in 18 mL of fresh media for each of the flasks. These flasks were then incubated at 37ºC until reaching confluence, at the process was repeated.

ii. Polyplus Transfection of HEK293T cells using jetPRIME

HEK293T cells were transfected using a Polyplus jetPRIME transfection kit. HEK293T cells were taken out of liquid nitrogen storage and grown in a T75 flask. The cells were split into two T75 flasks following the procedure for Cell Culture Maintenance. This was done to ensure the freshly thawed cells would grow properly before conducting a transfection. Media was removed from the flasks, and the HEK293T cells were trypsinized. Following the trypsinization incubation, 16 mL of fresh media was added and the media, now containing the cells, was transferred to a 50 mL falcon tube. Following centrifugation at 1,000 rpm for 3 minutes, the

pelleted cells were resuspended in 4 mL of fresh media and transferred to a new T75 flask. An additional 6 mL of fresh media was added, to bring the total volume to 10 mL.

In a microcentrifuge tube, 500 µL of jetPRIME buffer, 10 µg of receptor DNA (Table 1), and 30 µL of jetPRIME reagent were combined. The microcentrifuge tube was then vortexed for 10 seconds and short spun in a centrifuge. This reaction was allowed to incubate at room temperature for 10 minutes. 200 µL of this transfection mixture was added to the T75 flask containing HEK293T cells. The flask was incubated at 37ºC. Since the soluble receptor constructs induce Zeocin resistance, Zeocin was added the day following transfection to select only transfected cells. Transfections were carried out for approxiametly 4-5 weeks to ensure a stable line had been created.

Name of Receptor	**Concentration (ng/µL)**	**Amount Added (µL)**
ILLR1	775.8	12.9
ASGPR2	187.7	53.6

Table 1. Concentration of Receptors

iii. Bioreactor Collection of Solubilized Protein

Since the receptors contain a SecTag that allows them to be secreted from the HEK293T cells, a CellLINE bioreactor was used to collect large amounts of protein. Using a hemocytometer, cell count was determined of the transfected cells. The desired concentration was 2.5 x 10^6 cells/mL to be placed into the bioreactor. This volume of cells was added to 10 mL of "special media" which contained 10% low IgG FBS instead of 10% HI-FBS. This media

containing the transfected cells was placed into the inner compartment of the biorecactor. 100 mL of regular media was placed into the outer compartment of the bioreactor along with 1 mL of Zeocin (to maintain selection). Every 7 days, the media was collected from the small compartment and spun down. The supernatant containing the soluble protein was kept and stored at -20ºC until the time came for protein purification. The bioreactor was maintained for 5-6 weeks to ensure adequate protein collection.

iv. Receptor Protein Purification

Transfected HEK293T supernatants were thawed to room temperature prior to purification. Receptor purification was done using a Hitrap-Protein A column (GE Healthcare). Hitrap-Protein A column was also brought to room temperature prior to use (stored at 4ºC when not in use). The column was prepared using a 10 mL syringe loaded with 10 mL of binding buffer (1x PBS). The syringe was attached as recommended by the manufacturer with a luer lock adaptor that was also connected to the Protein A column. The flow through was collected in a 50 mL waste Falcon tube. To prevent the introduction of an air bubble, the syringe was disconnected from the adaptor when ~0.5mL of binding buffer remained. Between each loading step, a drop of 1x PBS was added to the top of the column to prevent drying the column. After the column was prepped, a syringe loaded with 10.5 mL of receptor supernatant was applied to the column, stopping at 0.5 mL as before. The flow rate was kept at a constant 1-2 mL per minute. The column was then washed with 10 mL of binding buffer (1x PBS). Following this washing step, 5.5 mL of elution buffer (100mM glycine, 150mM NaCl, 0.05% NaN3, pH 2.8) was loaded into a syringe and 5 mL was pushed through the column. The flow through was collected in a clean 15 mL falcon tube containing 0.75 mL neutralizing buffer (1M Tris, pH 9). A

final washing step with 1x PBS was conducted before the column was placed in a 15 mL Falcon tube containing 1x PBS.

v. Dialysis of Purified Receptor Protein

The purified eluate was dialyzed against 1x PBS using a Slide-a-lyzer 20,000 MWCO cartridge (Thermo Scientific). A plastic beaker was filled with 1 liter of 1x PBS. The Slide-a-lyzer cartridge was soaked in the beaker of 1x PBS for 3 minutes before being loaded with the purified eluate using a syringe and 18 gauge needle. After the sample was loaded, the syringe and needle was used to remove any air from the inside of the cartridge. The cartridge was attached to a floatation device and placed back in the beaker containing 1x PBS along with a stir bar. This beaker was placed on a stir plate at 4ºC. After one hour of dialysis, the 1 liter of PBS was replaced with a fresh liter of PBS. Dialysis was then allowed to continue overnight. The following day, the sample was removed using a syringe and 18 gauge needle and transferred into a 15 mL Falcon tube to be left on ice.

vi. Centrifugal concentration of dialyzed protein receptors

The dialyzed receptor protein was concentrated using 4 Amicon Ultra 0.5 mL 3k membrane microconcentrators (Millipore). To each of the microconcentrators, 500 µL of receptor protein was added. The microconcentrators were centrifuged at 14,000xg for 30 minutes at 4ºC. The microconcentrators were hen inverted and transferred to new microcentrifuge tubes. These were spun at 1,000xg for 2 minutes. This process was repeated until the dialyzed protein

was at a volume of approximately 500 µL. After concentration, 1 volume of 90% glycerol was added to the samples (final concentration of 45% glycerol). To prevent protein degradation, a Pierce protease inhibitor mini tablet, EDTA-free (Thermo Scientific) was added at a final concentration of 1x. The samples was then stored at -20ºC.

vii. Bradford Assay to Determine Protein Concentration

A Bradford Assay was conducted to determine the concentration of the purified receptor protein. 10 2-fold serial dilutions were made of bovine serum albumin (BSA) by adding 25 µL of BSA to 25 µL of 45% glycerol + PBS (what the receptor samples are stored in). This dilution was made 10 times down to a concentration of 9.7×10^{-3} µg/µL. Using a 96-well plate, 10 µL of the BSA dilutions were added to respective wells in duplicates. 10 µL of 45% glycerol + 1x PBS was placed in two other wells for duplicate blanks. Two, two-fold dilutions (1:1 and 1:3 dilutions) were made of the concentrated receptor proteins in 1x PBS. 10 µL aliquots of the undiluted receptor protein, the 1:1 dilution, and the 1:3 dilution were added in duplicate to the 96-well plate. To each of the wells containing protein (BSA or receptor), and the blanks, 90 µL of Bradford solution was added using a multi-channel pipette. The 96-well plate was allowed to sit for 10 minutes at room temperature on a plate revolving apparatus. Absorbance readings were then taken at 595 nm using a plate reader.

A Bradford standard curve was created using the 10 BSA dilution readings, blank subtracted (BSA A_{595} – 1x PBS A_{595}). A linear regression line was created using Excel, and the protein concentrations of the receptor samples were calculated from the linear equation.

viii. Validation of Protein Purification via SDS-PAGE and Western Blot

A SDS PAGE was first conducted to separate the protein based on size. Samples were prepared by adding approximately 1µg of protein to SDS-PAGE preparation buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol). The samples, including an aliquot of Kaleidoscope standard, were boiled for 5 minutes before being centrifuged for 10 seconds. A TGX (tris-glycine) precast gel was added to the gel holder. 10µL of sample was loaded to each well: ASGPR2, ILLR1, Dectin-1 (positive control), and 2 wells of Kaleidoscope standard (Figure 1A). SDS-PAGE buffer was added to the gel compartment. The gel was run for ~50 minutes at 200V to allow for size separation.

After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and assayed using a Western Blot. The Ready Gel Blotting Sandwich was arranged as according to Bio-Rad's instructions (Figure 1B). This sandwich was soaked in Towbin buffer (tris base and glycine), before being run at 25V for 20 hours. The following day, the nitrocellulose membrane now containing the protein, was washed in TBS for 5 minutes (200mM tris and 500 mM NaCl). To prevent any nonspecific binding, the membrane was blocked for 1 hour in blocking solution (40 mL TTBS with 2g of nonfat milk) at room temperature. Following blocking, antibody staining was conducted using 0.04ng/mL HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch) for 30 minutes at room temperature. The membrane was then washed 4 times in TTBS, 5 minutes per wash, to remove any excess blocking solution or secondary antibody. Western Blotting Chemiluminescence Substrate was added before the membrane was imaged.

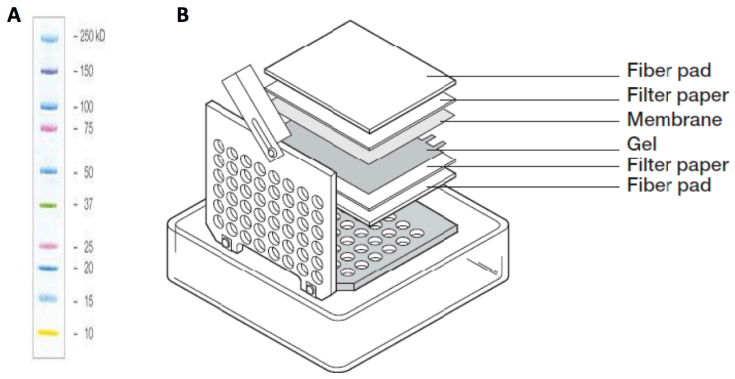

Figure 1. Western Blot was conducted to confirm protein purity. (A) Kaleidoscope standard used for protein size in SDS-PAGE. **(B)** Bio-Rad Western Blotting Sandwich arrangement. Image sources: Bio-Rad (A); ThermoFisher Scientific (B).

ix. High-Throughput Luminescence Staining

Wildtype-EGFP (WT-EGFP) and KAH3-EGFP *C. albicans* strains were streaked for isolation onto YPD plates (2% Bacto Peptone, 1% Bacto Yeast Extract, 2% Glucose, 2% Bacto Agar) and grown for 2 days at 37ºC. A single colony of WT-EGFP and a single colony of KAH3-EGFP were each inoculated into 3 mL of YPD. 1:100 inoculums were done of each by taking 200 µL of the original inoculum and adding it to 1.98 mL of YPD. The original inoculums and the 1:100 inoculums were incubated overnight at 30ºC in a roller drum.

The following day, 1 mL of each overnight inoculum was spun down for 30 seconds at 21,000 xg. The cells were then washed three times with 1 mL of 1x PBS, before being resuspended in 1 mL of 1x PBS. To determine cell count, an OD50 reading was taken. The spectrophotometer was blanked used 1 mL of 1x PBS. Each sample was diluted 1:100 by adding 10 µL of sample to 990 µL of 1x PBS. The volume of the sample to be added to 8 mL of RPMI was calculated using the following equation:

$$(\text{x mL of 1:100})(OD_{50})(10^7)(100) = (\text{8 mL RPMI})(\text{desired cell concentration})$$

Using a multichannel pipette, 160µL of *C. albicans* in RPMI was added to each of the wells in a 96-well cell binding plate. The plate was sealed and incubated at 37ºC for 3-4 hours to allow cell adherence. Following this incubation period, the cells were washed three times using 100 µL of 1x PBS. Cells were blocked for 30 minutes at room temperature using 100 µL of 1x PBS + 2% BSA. Cells were washed three times with 1x PBS. Addition of 50 µL of receptor at a desired concentration was added to all wells except for those chosen to be no-receptor negative controls. Receptor binding occurred at room temperature for 1 hour. After receptor binding, cells were washed three times with 1x PBS. Addition of 50 µL of Biotin-SP-conjugated AffiniPure Donkey Anti-Human IgG FC (Jackson ImmunoResearch) took place for 20 minutes on ice. Following 2º antibody addition, cells were washed three times with 1x PBS. Streptavidin-HRP was added for 20 minutes on ice. Cells were washed three more times with 1x PBS. Using a plate reader, a GFP read was taken of the 96-well plate. A 1:1 dilution of Western Blot Substrate dark solution to bright solution was created. PBS was removed from the wells and 50 µL of this luminescence substrate was added. A luminescence read was then taken using the plate reader.

RESULTS

i. Overview

Zebrafish have proven to be an extremely useful model for studying infection with the opportunistic fungal pathogen *Candida albicans*. While it has been demonstrated that candidiasis in zebrafish leads to a primarily innate immune response, the receptors involved in recognition have yet to be determined. This project has been focused on answering this question, with the goal of furthering understanding of candidiasis in this animal model. The work discussed in this thesis picks up with expression and purification of two potential zebrafish receptors to allow for

receptor purification. Following purification, screening for receptor binding to *C. albicans* was conducted using high-throughput luminescence binding assays.

ii. Expression of zebrafish receptors was achieved using Polyplus Transfection

To identify potential targets for zebrafish pattern recognition receptors, we sought to test the ligand specificity of purified soluble versions of the receptors. Previous work led to the cloning of seven zebrafish Type II C-type lectin carbohydrate recognition domains (CRDs) in a mammalian expression vector. The carbohydrate recognition domains of two zebrafish receptors, *ILLLR1* and *ASGPR2*, had been previously cloned into a mammalian expression vector and these could then be stably transfected into HEK293T cells. HEK293T (*Homo sapiens* Embryonic Kidney) cells are widely used for various cell culture applications. They have the benefits of being adherent, making passaging easy, and transfect very rapidly. The expression constructs used contained the CRD region of the desired receptor, a secretion tag, the constant portion of human IgG Fc, and a gene encoding for zeocin resistance (Fig. 3). The secretion tag allowed for receptor secretion from transfected HEK293T cells. By fusing the CRD to the constant portion of human IgG Fc, the same purification and secondary antibody for receptor screening could be used for all the receptors including the positive control. Lastly, zeocin resistance allowed for positive selection of transfected cells by eliminating nontransfected cells.

Upon transfection, a stable cell line was created by positive selection with zeocin antibiotic. Positive selection initially led to decreased observed growth rate because the majority of cells were killed and HEK293T cells require growth factors from neighbors to grow normally. Thus, cells were allowed to grow for several weeks until they returned to normal growth rate, ensuring the cell line was stable. To make certain the cell line wasn't just evolving from a subset

of inherently zeocin resistant cells, a negative control was conducted at the same time with non-transfected HEK293T cells being treated with the same amount of zeocin as transfected cells. Negative controls typically died within a few days after zeocin treatment had begun (Table 3-6).

Two different transfection protocols were conducted to express the receptor constructs. Originally, two AMAXA nucleofections (Table 2) were conducted with the *ILLR1* receptor construct. However, the cell populations never recovered from the nucleofections. Next, a jetPRIME Polyplus transfection protocol (Fig. 4) was tried with the *ILLR1* receptor construct. The first attempt didn't survive, but the second attempt was successful at creating a stable transfected cell line (Table 3). Some of the transfected cells were frozen down and these were maintained in a CELLine bioreactor (Fig. 5) for 6 weeks. The transfected cells were placed in a small compartment and media was placed in a large compartment of the bioreactor. A low molecular weight filter allowed for passage of nutrients and waste but excluded secreted proteins from leaving the small compartment. This allowed for protein concentration and samples were taken out every week for purification. Due to the success of the jetPRIME Polyplus transfection, this protocol was used for expression of the *ASGPR2* receptor construct (Table 4-6). A stable line was created and maintained for several weeks before being placed in a bioreactor for 7 weeks (Table 6).

iii. Purification of zebrafish receptors was achieved using HiTrap Protein A Columns

Since HEK293T cells secrete proteins other than the receptors, and the growth media contains some bovine proteins, protein purifications were conducted on samples taken from the bioreactor. The secreted protein was applied to a HiTrap Protein A column. Protein A is a surface protein found on *Staphylococcus aureus* that binds to the constant portion of human Fc

(fused to the receptor CRD of the expression constructs). The HiTrap columns were prepared by washing with phosphate buffer solution (PBS). The supernatants of the bioreactor samples were applied to the column to allow the lysate to bind to the Protein A. Any unbound protein was washed off using PBS before an elution buffer was applied. This buffer is highly basic which leads to a conformational change and release of the lysate. The purified protein was dialyzed against PBS to remove the elution buffer and was then concentrated. Bradford assays suggest there was enough protein produced and purified for subsequent analysis (Table 7).

iv. Western Blot was performed to determine purification quality

An SDS-PAGE was performed to separate purified protein based on molecular weight. This was followed by a Western Blot to transfer purified protein onto a Western Blot membrane. Immunodetection was done on the Western Blot membrane to determine if the purified protein was the desired receptor. The expected molecular weights of *Dectin-1* is 49.4 kDa, *ILLR1 is* 45.1, *ASGPR2* is 47.6 kDa, and IgG Fc alone is 27.3 kDa. However, the immunodetection results (Fig. 6) show lower molecular weights for *ILLR1* and *ASGPR2*. The bands are around 27 kDa, which is the molecular weight of Fc alone. This result suggests that only the Fc portion purified of the *ILLR1* and *ASGPR2* receptor constructs. A proteolytic cleavage event likely occurred, separating the Fc and CRD portions.

v. Development of a high-throughput screening assay for measuring binding of CRD-Fc fusions

Previous screenings done with the purified zebrafish receptors utilized fluorescence microscopy. While this technique is useful for visualization, the goal of this part of the project was to obtain large amounts of quantifiable data. Fluorescence microscopy has very low throughput, thus in order to determine binding affinities for each of these receptors a higher throughput protocol needed to be used. Previous research in the Wheeler Lab using 96-well CellBIND plates for screening antifungal compounds against *C. albicans* proved to be an efficient screening method with a large output. CellBIND plates are adherent, which allow the *C. albicans* to bind to the plate with minimal disruption from continued pipetting. Based on these previous studies, a protocol using CellBIND plates was developed to screen the binding affinities of several receptors at once.

In order to determine the optimal conditions for these screens, numerous optimization experiments were conducted with the positive control CRD-Fc fusion, *Dectin-1*-CRD-Fc. Two GFP labelled *C. albicans* strains were used: wildtype and KAH3. KAH3 is a strain that has a mutated cell wall with decreased mannan allowing β-glucan to be exposed. By using KAH3 and wildtype, the type of carbohydrate the zebrafish receptor binds to can be inferred (mannan versus β-glucan). KAH3 also allows for detection of other layers of the cell wall besides the top most mannan layer in wild type *C. albicans*. *Dectin-1* is a β-glucan receptor, thus higher levels of binding to the KAH3 strain should be seen, creating an additional parameter to determine the accuracy of the screen. For all the high-throughput experiments discussed in this thesis, *C. albicans* was incubated in the CellBIND plates for 3-4 hours. This length of time was previously determined to be sufficient for cell adherence to the wells. The optical density at 600 nm absorbance was used to achieve the desired *C. albicans* concentration.

a. HRP-conjugated α-human IgG leads to excess background signal

Based on previous studies and for simplification, an Horseradish Peroxidase (HRP) conjugated α-human IgG secondary antibody (Fig. 7) was first used to detect *C. albicans*-bound receptors. Following an incubation with bovine serum albumin (BSA) to prevent non-specific binding, Dectin-1 and HRP-conjugated α-human IgG were added to the wells. This secondary antibody was found to have limitations, however. A high enough concentration of secondary antibody to bind to the Dectin-1 provided too high of a background luminescence. The data was analyzed by taking the luminescence read (corresponds to amount of secondary antibody-receptor bound to the *C. albicans*) and dividing this by the GFP read (amount of *C. albicans* in the respective well). A difference in LUM/GFP could be seen as the secondary antibody concentration increased, but no significant difference could be seen with increasing *C. albicans* concentration (Fig. 8). For this reason, an alternate secondary antibody was needed to eliminate background luminescence that significantly inhibited screening purposes.

b. Biotinylated α-human IgG allows for more specific luminescence detection of Dectin-1

In order to decrease background luminescence from a high HRP concentration, a biotinylated α-human IgG secondary antibody was used. In addition, this antibody allows for bivalent interactions with receptors to increase avidity of the antibody-receptor complex. Streptavidin has an extremely high affinity for biotin, and was thus used to bind to the biotinylated α-human IgG (Fig. 9). A higher secondary antibody concentration could then be used when followed by binding with a lower concentration of Streptavidin conjugated with HRP. The optimal concentration of biotinylated α-human IgG and streptavidin was determined based on which concentration showed a higher luminescence of Dectin-1 with KAH3 as opposed to

wild type. Initially, very high dilutions were made (1:100 – 1:500 biotinylated α-human IgG and 1:500 – 1:1,000 streptavidin-HRP), however these were not concentrated enough for detection. Upon further experimentation, the optimal concentration of biotinylated α-human IgG was determined to be a 1:20 dilution of the secondary antibody (Fig. 10A). The optimal concentration of streptavidin-HRP was determined to be a 1:100 dilution (Fig. 10B). These concentrations were used for the remainder of the high-throughput luminescence binding assays discussed in this thesis.

vi. High-throughput luminescence screening suggests candidate receptors for zebrafish *C. albicans* recognition

After determining the optimal protocol for the high throughput luminescence screen, the four candidate zebrafish receptors were tested (*KCL*, *DCSF*, *CD209* and *DSLG*). *DCSF* did not appear to recognize either wild type or KAH3 *C. albicans* (Fig. 11A). *KCL* showed minimal recognition of the KAH3 strain. *CD209* showed similar recognition to both wild type and KAH3 strains. *DSLG* showed significant recognition of the wild type strain and minimal recognition of the KAH3 strain (Fig 11A). None of the zebrafish receptors reached the level of *Dectin-1* recognition in either the wild type (Fig. 11B) or KAH3 (Fig. 11C) strains of *C. albicans*.

DISCUSSION

i. Overview

Recognition of *Candida albicans* leads to a primarily innate immune response in a human host. In order to study this recognition, the zebrafish larval model is used because zebrafish possess only an innate immune system until adulthood. The receptors that recognize *C. albicans*

in zebrafish have yet to be determined, although this information is necessary to fully understand the infection within this model. In addition, this information could provide insight into the evolution of vertebrate innate immunology and the characteristics of the zebrafish innate immune system as a model for studying mammalian innate immunity. In continuation of a previous project aimed at identifying these cognate receptors, I attempted to purify two zebrafish C-type lectin receptors. I also worked to develop a high throughput method for determining binding affinities of these receptors to *C. albicans* that can be easily adapted to detecting binding affinities for other microbes. These early studies suggest that *DSLG* and *CD209* have the capacity to recognize *C. albicans*.

ii. Transfection of HEK293T cells can be used to express zebrafish receptors

HEK293T cells are widely used for protein production, have a high efficiency for various types of transfection, and reproduce easily and rapidly (21). This cell line was ideal for our purposes of expressing receptor constructs in high volumes. While two different transfection protocols were attempted, Polyplus jetPRIME transfection reagent was the most efficient transfection protocol. Another benefit of the HEK293T cell line for our purposes is that the cells are adherent, and thus were very easy to passage. This especially was useful when the transfected cells were placed into a bioreactor, and allowed us to routinely remove the secreted protein without disturbing the living cells. Based on these results, the Polyplus jetPRIME transfection protocol and CELLine bioreactors can likely be used for future expression and concentration of other zebrafish proteins.

iii. Immunodetection reveals the receptor constructs were not fully purified

Based on the expected molecular weights of *ILLR1* and *ASGPR2*, it does not appear that the receptor constructs were purified fully. The transfection protocol was used previously by another student and led to the purification of two of the other receptor constructs (*CD209* and *DSLG*), and thus it is unlikely that the transfection was unsuccessful. In addition, looking at the time it took the negative control to die (only a few days), it is highly unlikely that the cells evolved from a subset of inherently zeocin resistant cells. The HiTrap purification, dialysis, and concentration protocols were identical to those used for purification of all of the other receptor constructs. It is possible that a protease was introduced, and led to the degradation of part of the protein. However, the fact that all three proteins are at approximately the same weight even though the purifications were done at very different times suggests this is unlikely as well.

It appears that protein was purified that contains the human IgG Fc region, but it isn't possible to determine whether there was something else present before the purification. The sequence of the expression constructs will have to be double-checked to ensure that there wasn't an issue with the constructs. After that, the only other possibility would be that proteolytic cleavage occurred at some point leading to the separation of the CRD and Fc regions of the protein. The secondary antibody used for the western blot, goat α-human IgG-HRP, binds to the Fc portion. Any other protein present will not be detected by immunodetection, and thus another type of protein quantification test would have to be used (i.e. coomassie blue).

iv. High-throughput luminescence assays can be used to measure binding of CRD-Fc fusions

Developing a high-throughput luminescence assay protocol allowed for rapid detection of receptor binding interactions with *C. albicans*. This type of screen is necessary to obtain large amounts of quantifiable data, which can't be done using fluorescence microscopy and also

eliminates user bias. Using *Dectin-1*, a known receptor for *C. albicans* in humans, the optimal conditions for the assay could be determined. Originally, goat α-human IgG-HRP secondary antibody was used because it was the same one used in the Western Blot. However, to reach a high enough concentration of secondary antibody for detectable interaction with the bound receptors, the HRP background signal was too high and interfered with interpretation of results.

Although using a biotinylated secondary antibody and streptavidin conjugated with HRP made a more complex interaction, it allowed for an increased secondary antibody concentration without increasing the HRP concentration. This decreased the background signal and helped with interpretation of results. Using both the wild type and KAH3 strains of *C. albicans* allowed us to use *Dectin-1*-Fc as a positive control. KAH3 has a mutated cell wall with the outer mannan layer removed and the inner layers of the cell wall (including β-glucan) exposed. Knowing that *Dectin-1* is a receptor for β-glucan, the optimal conditions could be determined by which concentrations led to the biggest increase in luminescence of *Dectin-1* bound to KAH3 as opposed to wild type.

Two of the purified zebrafish receptors showed apparent recognition of *C. albicans* using the optimized high-throughput luminescence assay. Luminescence was seen in both the wild type and KAH3 strain with *CD209*. *DSLG* only appears to recognize wild type *C. albicans*. While neither luminescence signal was as strong as *Dectin-1*, these results do suggest that these receptors are worth further characterization.

v. *CD209* in humans recognizes various microbes including *C. albicans*

Both of the zebrafish receptors that showed binding, *CD209* and *DSLG* (*DC-SIGN-like*-G) are related to *CD209* in humans, which is also known as *DCSIGN.* The human homolog of

CD209 has been shown to recognize various microbial ligands of bacteria, viruses, and parasites. Zebrafish Human *CD209* recognizes several *Candida* species, although the ligand that *CD209* binds has yet to be determined (5). In a study to determine the extent to which pathogens have exerted selective pressure on *CD209* in humans, it was found that *CD209* has undergone a strong selective constraint (Barreiro, 2005). Thus, it is likely that recognition of pathogens, including *C. albicans*, by zebrafish *CD209* and *DSLG* will be similar to that of *CD209* in humans.

vi. Current and future research

In order to confirm the results seen with the first luminescence assays, these experiments are being repeated to create a larger sample size. Based on sequence homology, it is highly likely that *CD209* and *DSLG* will recognize *C. albicans,* but there isn't enough data to confirm this at this point. A set of experiments that will be conducted to help form a hypothesis would be to introduce excess ligand into the assay. For example, by adding excess β-glucan to the wild type *CD209* assay, if there is decreased signaling this would suggest β-glucan could be the ligand *CD209* binds to.

Following quantification using the conditions discussed in this thesis and excess ligand assays, visualization of this interaction will need to be recorded. Fluorescence microscopy on its own isn't sufficient for determining binding affinities, but it can certainly be used to collaborate quantified data. In addition, microscopy will be the most likely method to observe the binding interaction *in vivo*. Taking this to the zebrafish model will be the final step in characterization of the role of these receptors in *C. albicans* recognition. Thus having record of what this interaction looks like *in vitro* will make *in vivo* observations easier. Morpholinos are a very common and easy way to knockdown genes in zebrafish and could be used to knockdown the genes encoding

for *CD209* or *DSLG.* Comparing cytokine expression of control and knockdown zebrafish would demonstrate the downstream signaling effects of recognition by the receptors.

It is highly unlikely that all of the purified receptors recognize fungal pathogens. Fortunately, adapting the high-throughput luminescence assay to detection of other microbes would be relatively simple. As long as the microbe being tested is adherent, the assay will remain the same. Determining the specificity of these receptors for various microbes will provide important information for understanding zebrafish innate immunity. Comparing sequence homology of these receptors to human innate immune receptors and the results observed from the binding assays will give insight into how vertebrate innate immunity has evolved from teolest fish to mammals.

vii. Conclusion

Candida albicans is the third leading cause of nosocomial infections in hospitalized patients in the United States. This opportunistic pathogen can cause various infections, including lethal invasive candidiasis in immunocompromised patients. In order to develop treatments for *C. albicans* infection, the role of specific immune cells in recognition needs to be characterized. The larval zebrafish model is ideal for studying *C. albicans* infection, because they only possess an innate immune system. However, in order to understand how *C. albicans* is recognized in this model, the receptors involved need to be elucidated. The goal of this research project has been to determine which zebrafish C-type lectin receptors bind to *C. albicans*. This project also has two

broader implications: understanding how vertebrate innate immunology has evolved from teolest fish to humans and assessing the benefit of using zebrafish for fungal immunology research.

WORKS CITED

1. **Barreiro L, Patin E, Neyrolles O, Cann H, Gicquel B, Quintana-Murci L**. 2005. The Heritage of Pathogen Pressures and Ancient Demography in the Human Innate-Immunity CD209/CD209L Region. *The American Journal of Human Genetics*. 77:869-886.
2. **Bennett K**. 2014. Binding specificity of zebrafish immune receptors. Unpublished bachelor's independent research project – University of Maine, Orono, Maine.
3. **Bowie AG, Unterholzner L**. 2008. Viral Evasion And Subversion Of Pattern-Recognition Receptor Signalling. *Nat Rev Immunol.* 8.12:911-922.
4. **Brothers KM, Gratacap RG, Barker SE, Newman ZR, Norum A, Wheeler RT.** 2013. NADPH Oxidase-Driven Phagocyte Recruitment Controls Candida Albicans Filamentous Growth And Prevents Mortality. *PLoS Pathog* 9.10:e1003634.
5. **Cambi A, Gijzen K, de Vries I, Torensma R, Joosten B, Adema G, Netea M, Kullberg B, Romani L, Figdor C**. 2003. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. *European Journal of Immunology.* 33:532-538.

6. **Carter EV**. 2013. Evolutionary and molecular analysis of conserved vertebrate immunity to fungi. Unpublished bachelor's thesis – University of Maine, Orono, Maine.
7. **Chaffin W, Lopez-Ribot J, Casanova M, Gozalbo D, Martinez J.** (1998) Cell wall and secreted proteins of Candida albicans: identification, function, and expression. *Microbiol Mol Biol Rev.* 62:130-180.
8. **Dodd RB, Drickamer K**. 2001. Lectin-Like Proteins In Model Organisms: Implications For Evolution Of Carbohydrate-Binding Activity. *Glycobiology* 11.5:71R-79R. Web.
9. **Gow NA, van der Veerdonk FL, Brown AJ, Netea MG.** 2011. Candida Albicans Morphogenesis And Host Defence: Discriminating Invasion From Colonization. *Nature Reviews Microbiology*.
10. **Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, Phelan M, Morgan J, Lee-Yang W, Ciblak MA, Benjamin LE, Sanza LT, Huie S, Yeo SF, Brandt ME, Warnock DW**. 2004. Incidence Of Bloodstream Infections Due To Candida Species And In Vitro Susceptibilities Of Isolates Collected From 1998 To 2000 In A Population-Based Active Surveillance Program. *Journal of Clinical Microbiology* 42.4:1519-1527.
11. **Lionakis M, Lim JK, Lee CC, Murphy PM**. 2011. Organ-Specific Innate Immune Responses In A Mouse Model Of Invasive Candidiasis. *J Innate Immun* 3.2: 180-199.
12. **Li Y, Huang G, Diakur J, Wiebe LI**. 2008. Targeted Delivery Of Macromolecular Drugs: Asialoglycoprotein Receptor (ASGPR) Expression By Selected Hepatoma Cell Lines Used In Antiviral Drug Development. *Current Drug Delivery* 5.4:299-302.
13. **Lorenz MC, Bender JA, Fink GR**. 2004. Transcriptional Response Of Candida Albicans Upon Internalization By Macrophages. *Eukaryotic Cell* 3.5:1076-1087.
14. **Lozach PY, Amara A, Bartosch B, Virelizier JL, Arenzana-Seisdedos F, Cosset FL, Altmeyer R**. 2004. C-Type Lectins L-SIGN And DC-SIGN Capture And Transmit Infectious Hepatitis C Virus Pseudotype Particles. *Journal of Biological Chemistry* 279.31:32035-32045.
15. **Meeker ND, Trede NS**. 2008. Immunology And Zebrafish: Spawning New Models Of Human Disease. *Developmental & Comparative Immunology* 32.7: 745-757.
16. **Moyes DL, Wilson D, Richardson JP, Mogavero S, Tang SX, Wernecke J, Höfs S, Gratacap RL, Robbins J, Runglall M, Murciano C, Blagojevic M, Thavaraj S, Förster TM, Hebecker B, Kasper L, Vizcay G, Iancu SI, Kichik N, Häder A, Kurzai O, Luo T, Krüger T, Kniemeyer O, Cota E, Bader O, Wheeler RT, Gutsmann T, Hube B, Naglik JR.** (in press). Candidalysin: A fungal peptide toxin critical for mucosal infection. *Nature*.
17. **Homei A.** 2006. Medical mycology development and epidemiology in the USA, UK and Japan. *Med Mycol*. 44:39-54.
18. **Palmer G, Robinson P, Challacombe S, Birnbaum W, Croser D, Erridge P, Hodgson T, Lewis D, McLaren A, Zakrzewska J.** 2008. Aetiological factors for oral manifestations of HIV. *Oral Diseases*. 2:193-197.
19. **Panagos P, Dobrinski K, Chen X, Grant A, Traver D, Djeu J, Wei S, Yoder J**. 2006. Immune-related, lectin-like receptors are differentially expressed in the myeloid and lymphoid lineages of zebrafish. *Immunogenetics* 58:31-40.
20. **Sudbery P, Gow N, Berman J**. 2004. The distinct morphogenic states of Candida albicans. *Trends in Microbiology.* 12:317-324.

21. **Thomas P, Smart T.** 2005. HEK293 cell line: A vehicle for the expression of recombinant proteins. *Journ of Pharm and Toxi Methods.* 51:187-200.
22. **Vautier S, MacCallum DM, Brown GD**. 2012. C-Type Lectin Receptors And Cytokines In Fungal Immunity. *Cytokine* 58.1: 89-99. Web.

TABLES

	Experiment Started
	Experiment terminated
	Zeocin added
	Negative control died
N.D.	Not determined
Surface Area	**Container**
2.0 cm^2	1 24-well plate well
3.7 cm^2	1 12 well plate well
9.61 cm^2	1 6 well plate well
75 cm^2	T75 flask

Key for Table 2 (below)

Day	**AMAXA Nucleofection ILLR1 Attempt 1**	**AMAXA Nucleofection ILLR1 Attempt 2**

	Cell Container/ Surface Area	Confluence	Cell Container/ Surface Area	Confluence
-2	1 75cm^2 flask	90%	1 75cm^2 flask	90%
-1	2 75cm^{2}flasks	Split	2 75cm^2 flasks	Split
0	3 9.61 cm^2 wells	N.A.	3 9.61 cm^2 wells	N.A.
1	3 9.61 cm^2 wells	40%	3 9.61 cm^2 wells	N.D.
2	3 9.61 cm^2 wells	60%	3 9.61 cm^2 wells	40%
3	3 9.61 cm^2 wells	90%	3 9.61 cm^2 wells	N.D.
4	6 9.61 cm^2 wells	Split	6 9.61 cm^2 wells	Split
5	6 9.61 cm^2 wells	30%	6 9.61 cm^2 wells	50%
6	6 9.61 cm^2 wells	40%	10 9.61cm^2 wells	Split
7	6 2.0 cm^2 wells	Condensed	10 9.61cm^2 wells	70%
8	6 2.0 cm^2 wells	N.D.	1 75cm^2 flask	Combined
9	6 2.0 cm^2 wells	40%	6 9.61 cm^2 wells	Condensed
10	6 2.0 cm^2 wells	40%	12 3.7 cm^2 wells	Condensed
11	6 2.0 cm^2 wells	20%	12 3.7 cm^2 wells	30%
12	6 2.0 cm^2 wells	Cells died	12 3.7 cm^2 wells	N.D.
13			12 3.7 cm^2 wells	Cells died

Table 2. AMAXA Nucleofection was unsuccessful at expressing the *ILLR1* receptor construct in the HEK293T cell line. In an attempt to create a stable cell line expressing the *ILLR1* receptor construct, two AMAXA Nucleofections were attempted.

	Experiment started
	Experiment terminated
	Zeocin added
	Negative control died
	Bioreactor started
	Cells frozen down
	Thawed frozen cells
N.D.	Not determined

Key for Tables 3-6 (below)

Day	Polyplus Transfection ILLR1 Attempt 1		Polyplus Transfection ILLR1 Attempt 2	
	Number of T-75 Flasks	Confluence	Number of T-75 Flasks	Confluence
-1	1	40%	1	40%
0	1	80%	1	80%
1	1 → 2	Split	1 → 2	Split
2	2	40%	2	N.D.
3	2	Cells died	2	70%
4			2 → 4	Split
5			4	40%
6			4	N.D.
7			4 → 8	80%
8			8 → 10	Two were split
9			10	40%
10			10 → 5	Condensed 6 to 1
11			5 → 10	Split
12			10	70%
13			10	90%
14			2 → 4	Two not frozen down were split
15			4	N.D.
16			4 → 8	Split
17			8	40%
18			8	70%
19			8 → 16	Split
20			16	50%
21			16	90%
22			4	Froze down all but 4
23			4 →8	Split
24			8	Bacterial Contamination
25			1	Thawed cells from freezer
26			1	50%
27			1 → 2	Split
28			2	60%
29			2 → 4	Split
30			4	Placed in Bioreactor

Table 3. Polyplus Transfection was successful at expressing the *ILLR1* receptor construct in the HEK293T cell line. The second attempt at using the Polyplus transfection

protocol was successful at creating a stable cell line expressing the *ILLR1* receptor construct. The transfected cells were placed into a CellLINE bioreactor to concentrate the secreted protein.

Day	**Polyplus Transfection ASGPR2 Attempt 1**		**Polyplus Transfection ASGPR2 Attempt 2**	
	Number of T-75 Flasks	Confluence	Number of T-75 Flasks	Confluence
-1	1	40%	1	40%
0	1	80%	1	80%
1	1 → 2	Split	1 → 2	Split
2	2	40%	2	N.D.
3	2	70%	2	60%
4	2 → 4	Split	2 → 4	Split
5	4	30%	4	40%
6	4	N.D.	4	30%
7	4	40%	4 → 2	Condensed
8	4	40%	2	30%

9	4 → 1	Condensed	2	Cells Died
10	1	30%		
11	1	20%		
12	1	Cells Died		

Table 4. Polyplus Transfection was initially unsuccessful at expressing the *ASGPR2* receptor construct in the HEK293T cell line. In the initial attempts to transfect HEK293T with the *ASGPR2* receptor construct, the cells didn't make it through the positive selection phase.

Day	**Polyplus Transfection ASGPR2 Attempt 3**		**Polyplus Transfection ASGPR2 Attempt 4**	
	Number of T-75 Flasks	Confluence	Number of T-75 Flasks	Confluence
-1	1	40%	1	40%
0	1	80%	1	80%
1	1 → 2	Split	1 → 2	Split
2	2	40%	2	40%
3	2	N.D.	2	80%
4	2	20%	2 → 4	Split
5	2	Cells Died	4	40%
6			4	N.D.
7	Experiment Note: trypsin volume wasn't high enough to remove cells		4 → 8	80%
8			8	N.D.

	from flask			
9			3 → 2	Froze down 5 flasks
10			2	30%
11			2	Bacterial contamination
12			1	Thawed Vial from freezer
13			1	Bacterial contamination
14			1	Thawed another vial from freezer
15			1	Bacterial Contamination
16			Experiment Note: Killed experiment and threw out remaining vials in the freezer; likely contamination came before freezing	
17				
18				

Table 5. Bacterial contamination killed cells transfected with the *ASGPR2* receptor construct. Introduction of a bacterial contaminant inhibited the generation of a stable *ASGPR2* transfected cell line.

Day	**Polyplus Transfection ASGPR2 Attempt 5**	
	Number of T-75 Flasks	Confluence
-1	1	40%
0	1	80%
1	1 → 2	Split
2	2	40%
3	2 → 4	Split
4	4	50%
5	4 → 8	Split
6	8	N.D.
7	8	50%
8	8	70%

9	8 → 16	Split
10	16	60%
11	1	Froze down 15 flasks
12	1	N.D.
13	1 → 2	Split
14	2	50%
15	2 → 4	Split
16	4	60%
17	4	Placed in Bioreactor

Table 6. An *ASGPR2* transfected HEK293T cell line was created using the Polyplus Transfection protocol. The final attempt at transfecting cells with the *ASGPR2* receptor construct was successful. The transfected cells were placed into a CellLINE bioreactor to concentrate the secreted protein.

Receptor	**Concentration**
***ILLR1* Purification #1**	0.459 mg/mL
***ILLR1* Purification #2**	0.236 mg/mL
***ASGPR2* Purification #1**	0.34 mg/mL

Table 7. Bradford assays suggest there was enough purified protein for subsequent analysis. Bradford assays were conducted to determine the concentration of purified protein.

FIGURES

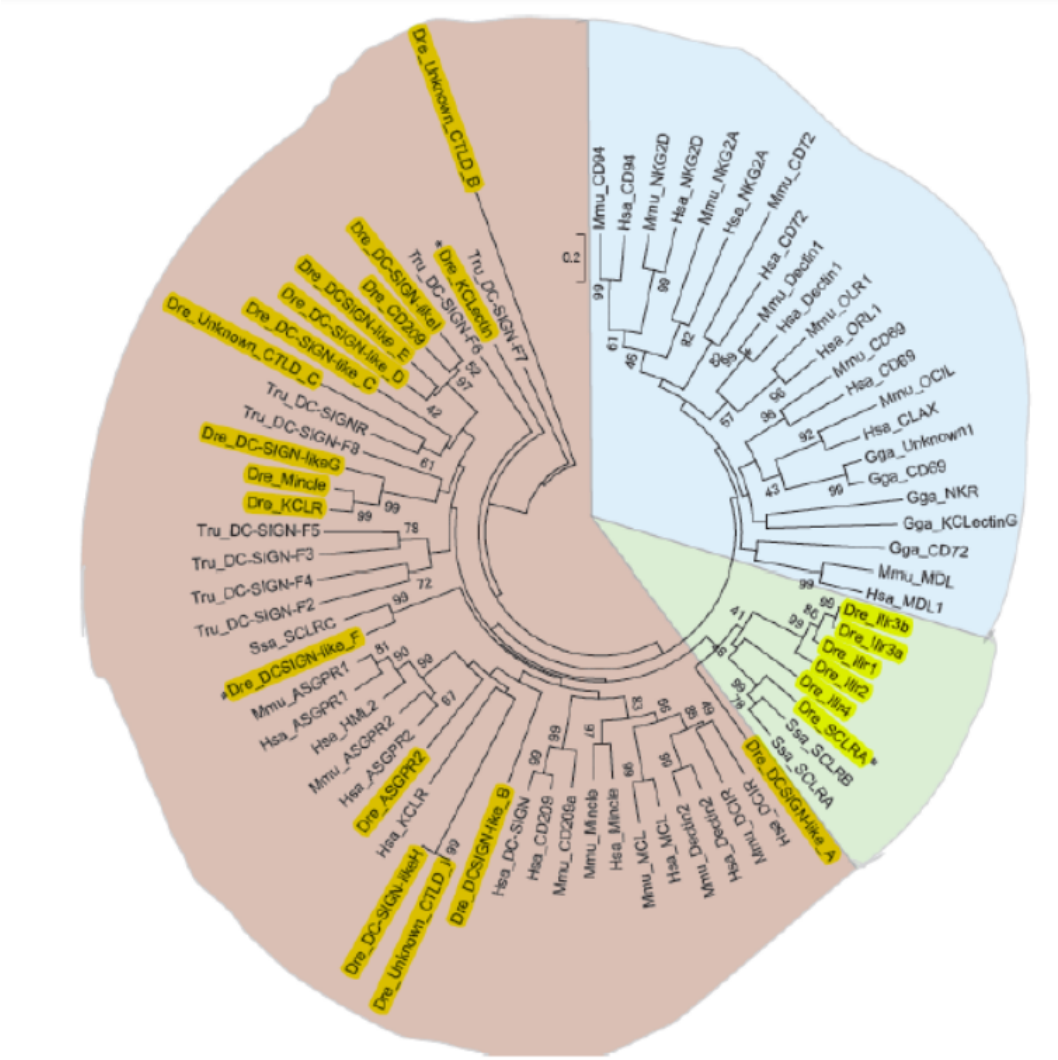

Figure 2. Association between various C-type lectin receptors. Image shows C-type lectin receptors belonging to group II (red), group V blue), NK-like receptors (green) in *Danio rerio* (Dre), *Takifugu rubripes* (Tru), *Salmo salar* (Ssa), *Gallus gallus* (Gga), *Mus musculus* (Mmu), and *Homo sapiens* (Hsa) aligned using Clustal W. The zebrafish receptors are highlighted in yellow. (Source: Erin Carter unpublished thesis, 2014)

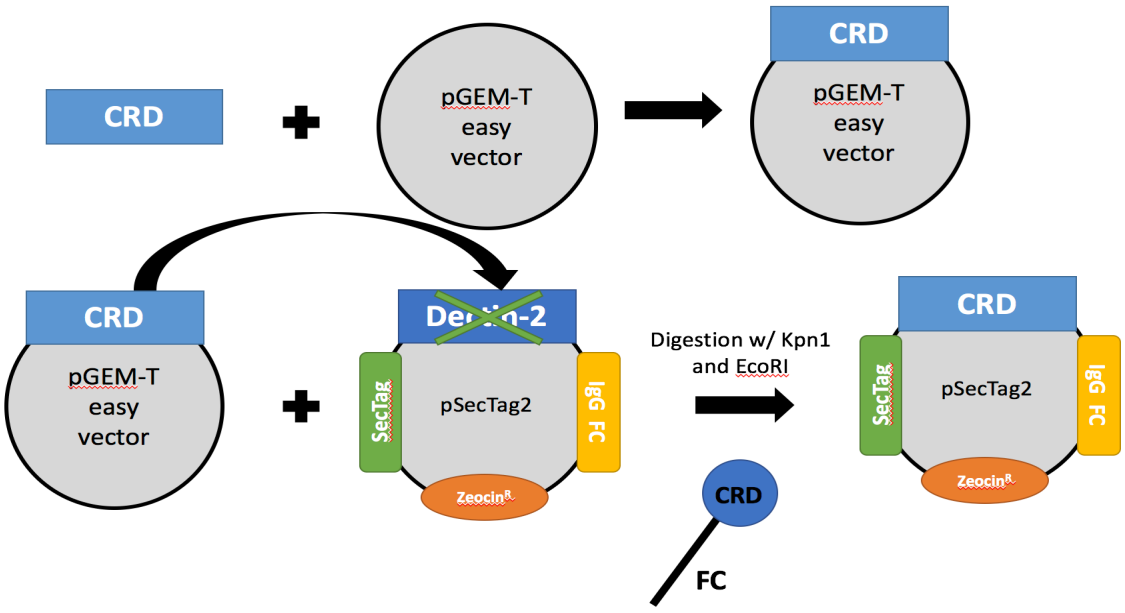

Figure 3. Generation of plasmids containing zebrafish receptor carbohydrate recognition domain. Previous work done by Erin Carter of the Wheeler Lab led to the generation of seven zebrafish receptor constructs. The carbohydrate recognition domain (CRD) of each zebrafish receptor was fused with the constant portion of human IgG Fc, a gene coding for zeocin resistance, and a secretion tag.

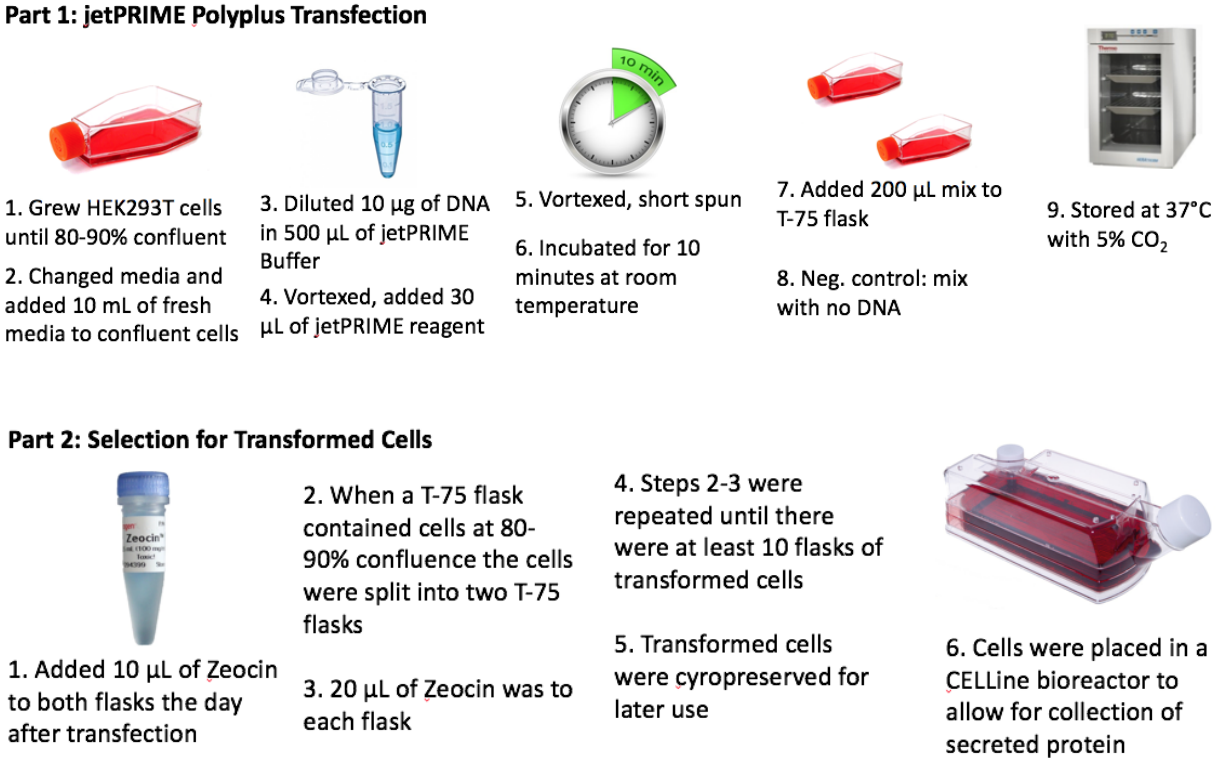

Figure 4. Polyplus Transfection was used to express the *ILLR1* and *ASGPR2* receptor constructs in HEK293T cells. The transfection protocol was divided into two parts: initial transfection with jetPRIME reagents and the plasmid DNA containing the receptor constructs; positive selection for transformed cells using zeocin. Cells were placed in a bioreactor for collection of secreted protein. A no DNA transfection was used as a negative control.

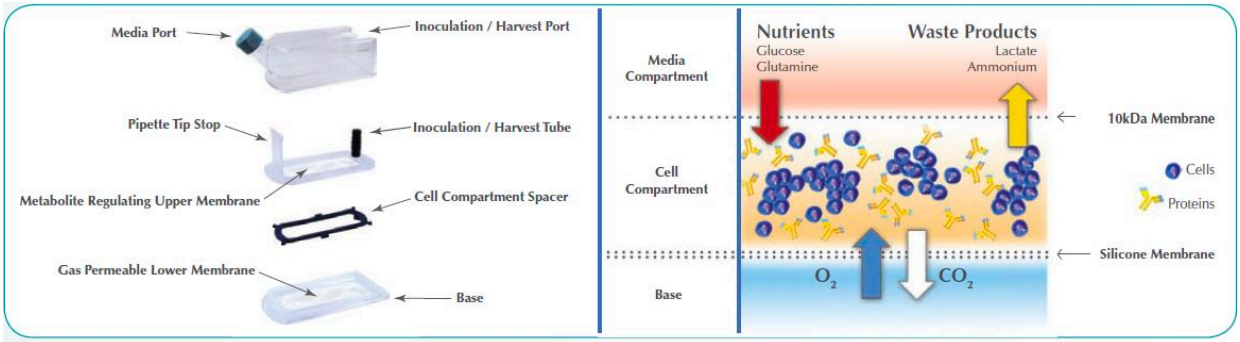

Figure 5. CELLine bioreactor was used to concentrate transfected HEK293T secreted protein. The transfected HEK293T cells are placed in a small compartment in the bioreactor which is separated from the large compartment holding cell culture media by a low molecular weight filter. This filter allows for passage of nutrients and waste but inhibits secreted protein from exiting the small compartment. The secreted protein becomes concentrated and was removed every week for purification. Image source: The Lab Depot.

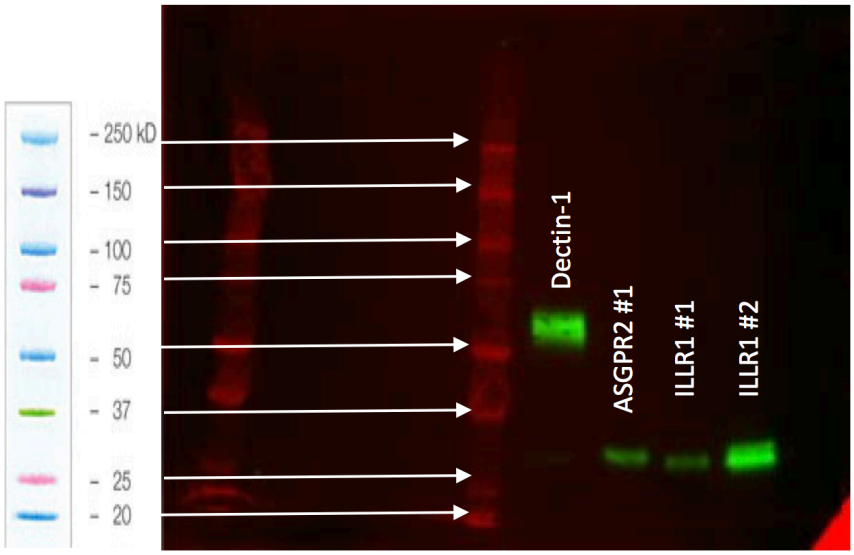

Figure 6. Immunodetection of *ILLR1* and *ASGPR2* receptor constructs suggests that the Fc portion was all that was purified. An SDS-PAGE and Western Blot were conducted to determine if the protein expression and purification was successful. By looking at the corresponding molecular weights of the Kaleidoscope standard, it appears that only the Fc portion was purified of the *ILLR1* and *ASGPR2* receptor constructs.

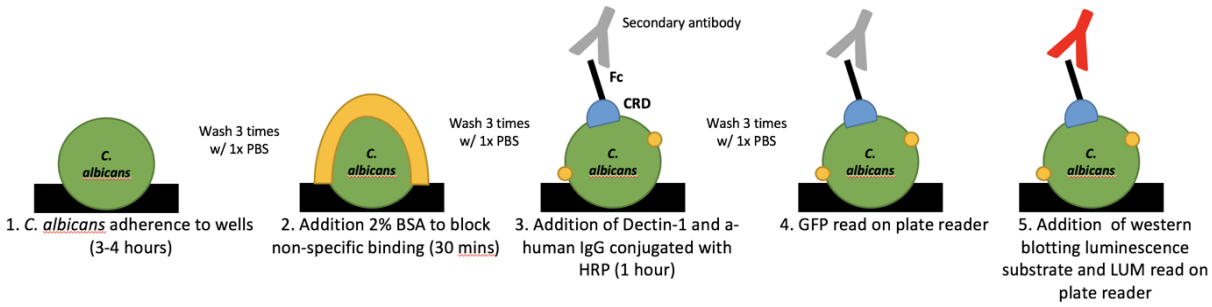

Figure 7. HRP conjugated α-human IgG secondary antibody was initially used for detection of *Dectin-1*-Fc in high-throughput luminescence screen. Initially, an HRP conjugated α-human IgG secondary antibody was used to detect levels of *Dectin-1*-Fc binding to wild type and KAH3 strains of *C. albicans*. BSA was used to prevent any nonspecific receptor binding. Addition of a western blotting substrate leads to a chemical reaction with the HRP that allows for chemiluminescence screening.

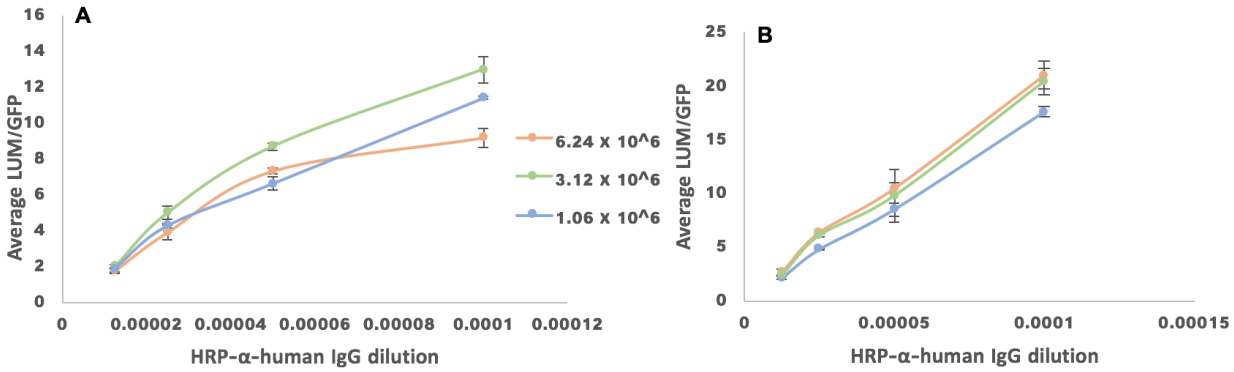

Figure 8. Increased secondary antibody concentration leads to apparent increase in detection of Dectin-1-Fc binding. By increasing the HRP conjugated a-human IgG concentration, there is an increase in level of luminescence per GFP. However, increasing the *C. albicans* concentration shows no significant difference in luminescence in either the wild type (A) or KAH3 (B) strains (the legend corresponds to cells/mL). This suggests that the increased secondary antibody concentration is providing excess background luminescence and false positive reads.

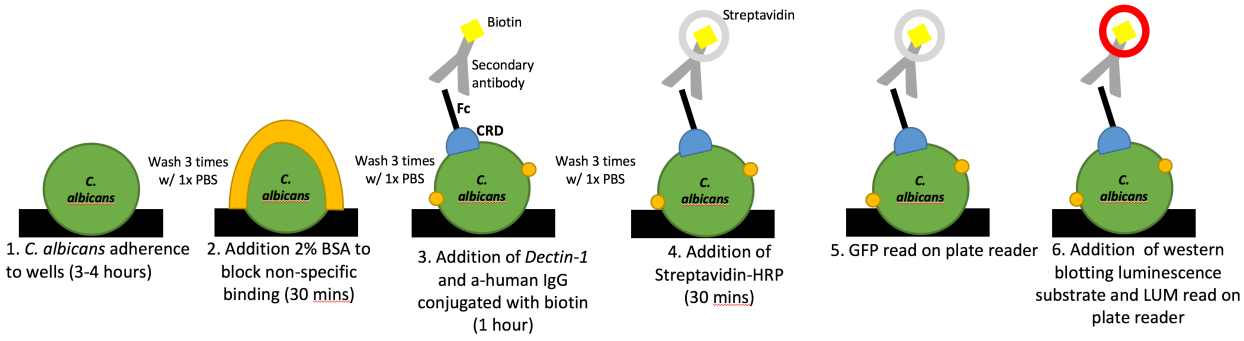

Figure 9. Biotin conjugated α-human IgG secondary antibody was used for detection of *Dectin-1*-Fc in high-throughput luminescence screen. A biotin conjugated α-human IgG secondary antibody was used to detect levels of *Dectin-1*-Fc binding to wild type and KAH3 strains of *C. albicans*. BSA was used to prevent any nonspecific receptor binding. Streptavidin conjugated with HRP (has a strong affinity for biotin) was used for chemiluminscence detection when treated with a western blotting substrate.

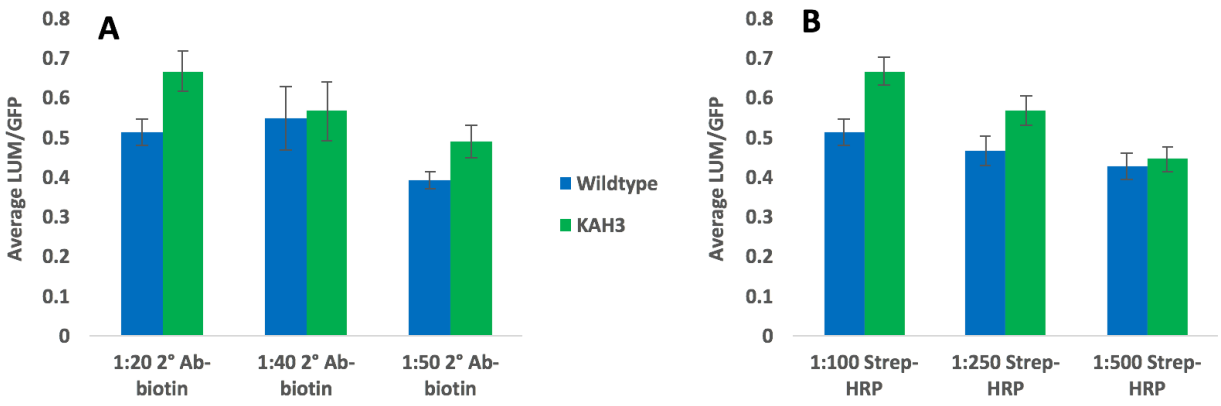

Figure 10. A 1:20 dilution of biotin conjugated with α-human IgG secondary antibody (A) and a 1:100 dilution of streptavidin conjugated with HRP (B) showed the greatest difference in luminescence between wild type and KAH3 strains of *C. albicans*. In order to determine the optimal concentrations of secondary antibody and streptavidin-HRP, the luminescence of *Dectin-1* binding to wild type and KAH3 strains of *C. albicans* were compared. *Dectin-1* is a receptor for β-glucan, which is more exposed in the KAH3 mutant strain than the wild type. Thus, the optimal concentrations were determined to be 1:20 of secondary antibody and 1:100 of strepatividin-HRP because these concentrations showed the greatest increase in luminescence in the KAH3 strain compared to the wild type strain.

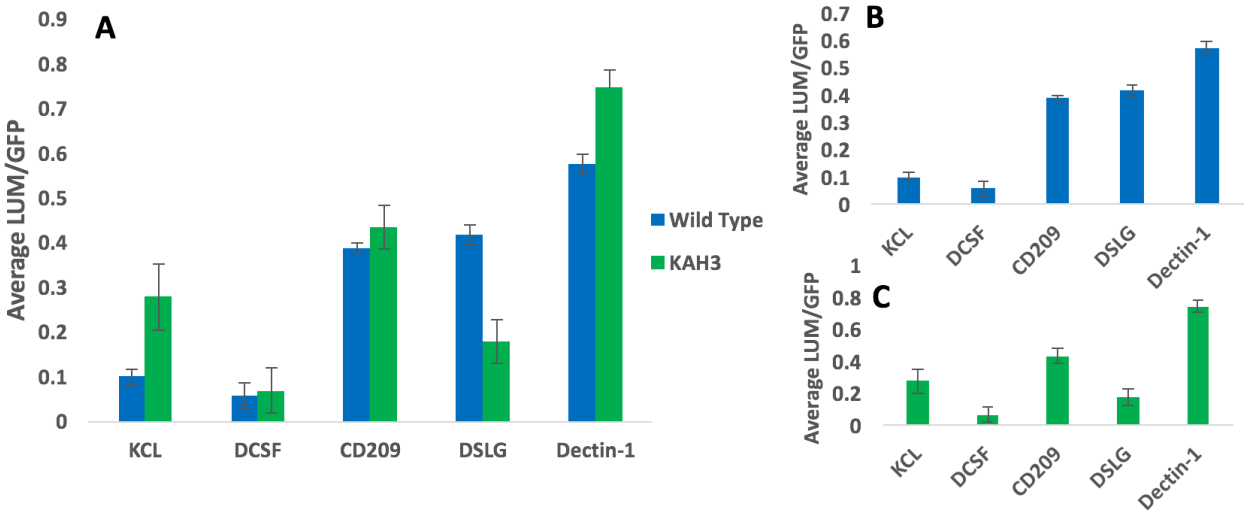

Figure 11. High-throughput luminescence screen shows significant recognition of *C. albicans* by two candidate zebrafish receptors. (A) *CD209* showed similar recognition of wild type and KAH3 strains of *C. albicans*. *DSLG* showed higher recognition of wild type strain than KAH3. *KCL* and *DCSF* didn't appear to recognize *C. albicans*. **(B)** Of the zebrafish receptors, *CD209* and *DSLG* showed the highest recognition of the wild type strain. **(C)** Of the zebrafish receptors, *CD209* showed the highest recognition of the KAH3 strain. Three experiments consisting of quadruplicate wells for each sample condition were conducted (n = 12). Error bars represent standard error.

AUTHOR BIOGRAPHY

Monique is originally from Howland, ME where she graduated from Penobscot Valley High School in 2012. She did her first research internship through the MERITS program the summer before her senior year of high school. This was done at the University of Maine in the chemistry department under the mentorship of Dr. James Killarney. She worked on this project for over two years, before doing an internship at Mount Desert Island Biological Laboratory which was her first experience with biomedical research. Finally, she spent two academic years under the mentorship of Dr. Robert Wheeler, which is when her passion for infectious diseases ignited. While an undergraduate, she presented her work at four different symposia and received several fellowships including the INBRE Functional Genomics and Carolyn Reed Pre-Med senior honors thesis fellowships. Monique is graduating with Highest Honors from the University of Maine.

Monique plans to earn her PhD in Microbiology at Cornell University, focusing on infectious disease. Her academic experiences and time spent at MDIBL have inspired her to pursue academia as well as community outreach through science education as a career. When not in the lab, Monique spends her time skiing, hiking, kayaking, and any other outdoors activity.