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CNIDARIA - AN EMERGING MODEL PHYLUM TO INVESTIGATE THE EVOLUTION OF METAZOAN IMMUNITY

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SUMMARY

The Cnidarian phylum (sea anemones, jellyfish, *Hydra*, corals) is considered to be phylogenetically basal to Bilateria (files, worms and humans). Due to their relative position within the tree of life, Cnidarians provide insight into the genetic toolkit of the last common metazoan ancestor. Their simple body plan consists of only two epithelial cell layers connected by a jelly-like mesoglea. Despite their morphological simplicity, the cnidarian immune system is surprisingly complex, with multiple immune components conserved from Cnidarians to humans. In addition, the cnidarian immune system appears to be more similar to the human immune system compared to canonical model organisms such as *Drosophila melanogastor* and *Caenorhabditis elegans*. Here, I begin with a brief overview of cnidarian immunology and demonstrate that while the field has advanced rapidly in the past decade, large gaps exist that warrant additional investigations. Next, I outline a novel approach to identify predicted immune proteins in Cnidarians that are missed using traditional protein annotation methods. This approach is based on the identification of similarities between viral gene products and host proteins and can be applied to virtually any animal system.

INTRODUCTION

Cnidarians: Aggressors of oceans and protectors of continents

The phylum Cnidaria (sea anemones, jellyfish, *Hydra*, and corals) contains over 10,000 species ranging in size from a few millimetres to over 75 meters and inhabits both fresh and saltwater environments across the globe (*Daly* et al., 2007). The lifestyle of a Cnidarian is either completely sessile or slow moving. Therefore, it would be reasonable to assume that they are relatively docile organisms. However, at the cellular level they are amongst the most aggressive hunters of the aquatic environment. Using phylum-specific molecular harpoons, Cnidarians inject host tissue with toxic venom capable of paralyzing prey and even killing humans (*Beckmann* and *Özbek*, 2012). At the macro-level a specific group of Cnidarians, reef-building corals, are responsible for the only biologically generated structure that can be viewed from space: the coral reef. Despite covering less than 0.5% of the ocean surface, coral reefs support almost one third of all marine fish species, providing both food and coastal-protection against storms (*Moberg* and *Folke*, 1999. Whether attending to a jellyfish sting or consuming one of the many

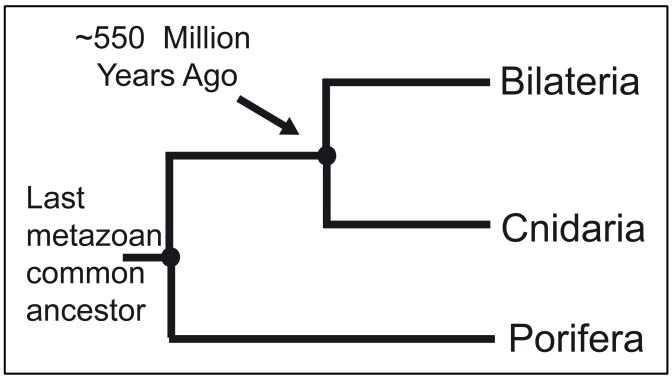

Figure 1: Cnidarians are phylogenetically basal to Bilaterians. The Bilaterian phylum diverged from Cnidarians approximately ~550 million years ago predating the Cambrian Explosion.

species of fish supported by the coral reef ecosystem, millions of people are directly impacted by Cnidarians.

Cnidarians: A basal metazoan phylum

In addition to their modern-day impact, Cnidarians also occupy a basal position within the animal tree of life. The first cnidarian fossils were discovered in Cambrian-era rock and can be traced back ~550 million years ago during a period referred to as the Cambrian Explosion (*Van Iten* et al., 2016). Often referred to as the "Big Bang" of life on Earth, the Cambrian Explosion describes a dramatic period in the fossil record during which the ancestors of all extant animal phyla can be found (*Shu*, 2008). Molecular phylogenetic data combined with fossil evidence suggest the first Cnidarians existed approximately 542-720 Myr ago (*Davidson* and *Erwin*, 2009) and it was during this period that their sister group Bilateria, which includes flies, worms, and humans, diverged from the last common metazoan ancestor (Figure 1) (*Kortschak* et al., 2003; *Hemmrich* et al., 2012). Therefore, Cnidarians are considered to be amongst the most phylogenetically basal to all metazoan life and provide important insight into the ancestral state of the first metazoans.

The relatively simple Cnidarian body plan consists of two cell layers, an endoderm and ectoderm, held together by the jelly-like mesoglea. Due to the morphological simplicity and basal status, the Cnidarian phylum has traditionally been viewed as "primitive" and less complex than "higher" organisms such as flies and worms. However with the genomic sequencing of *Nematostella vectensis* (*Putnam* et al., 2007), *Hydra magnipapillata* (*Chapman* et al., 2010), and *Acropora digitifera* (*Shinzato* et al., 2011), as well as numerous functional studies, it has become clear the "primitive" hypothesis is consistently unsupported with regards to development (*Kusserow* et al., 2005; *Desvignes* et al., 2010) and immunity (*Miller* et al., 2007; *Zmasek* et al., 2007; *Franzenburg* et al., 2012; *Quistad* et al., 2014). In addition, many gene families that have been lost in canonical model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* are present in Cnidarians (*Miller* et al., 2005; *Technau* et al., 2005).

Model organisms have provided invaluable insight into the origin of immunity and how the human immune system functions. For example, studies in chickens led to the identification of

T and B cells, while self-versus non-self was first investigated in starfish (*Cooper* et al., 1965; *Litman* and *Cooper*, 2007). However, our current perspective of the evolution of immunity is largely based on only three of the thirty extant phyla (Bilateria, Nematoda, Arthropoda), therefore, a broader representation of phyla is required (*Litman* and *Cooper*, 2007)**.** Here, I argue that the conservation and complexity of the Cnidarian immune system, combined with well-developed molecular tools, strongly supports Cnidarians as a valuable and emerging model phylum to investigate the ancestral state of the animal immune system. First, I briefly review the status of cnidarian immune research focusing on studies that involve direct experimentation. Then, I present a novel approach to investigate the immune systems of Cnidarians based on predicted viral-host interactions that can be applied to virtually any animal system.

THE CNIDARIAN IMMUNE SYSTEM - ANCIENT AND CONSERVED

Mucosal surfaces: Dynamic regions of host-microbe interactions

Mucosal surfaces likely appeared with the first Cnidarians and can now be found throughout the metazoan tree of life (*Lang* et al., 2007). The Surface-Mucus Layer (SML) is composed of a multi-layered matrix of glycoproteins (mucins), located at the apical region of specific epithelial cell types (*Bäckhed* et al., 2005). Glycosylation of mucins is tightly controlled by the host repertoire of glycosyltransferases resulting in a diverse array of mucin macromolecules that either remain tethered to the cell surface or is secreted into the surrounding environment (*Ferez-Vilar* and *Hill*, 1999; *Hang* and *Bertozzi*, 2005). These energy-rich mucins not only provide food to commensal bacteria (*Derrien* et al., 2010) but also serve as a particle trap for their major predators: the bacteriophage (phage). Despite high mucin turnover, metazoans maintain specific phage and bacteria species within the SML (*Lozupone* et al., 2012; *Grasis* et al., 2014) and alterations of the SML microbiome have been associated with various disease states from Cnidarians (*Closek* et al., 2014) to humans (*Johansson* et al., 2013). Resident bacteria protect their hosts via niche exclusion of pathogenic species or strains while phage can directly lyse invading pathogens that traverse the SML (*Barr* et al., 2013). In the Bacteriophage Adherence to Mucus (BAM) model, mucus-adherent phages bind to host mucins and provide the host with immune protection (*Barr* et al., 2013, 2015). For an in-depth discussion of the origin and evolution of microbiome selection within the first metazoan SML the readers are pointed to the article of *Quistad* et al. (2016a). Taken together the physical properties of the SML combined with the host-specific microbiome provide the first line of immune protection against invading pathogens.

Detection and response to microbes: The Toll-Like Receptors (TLRs)

While the SML provides a dynamic layer of immune defence, pathogens have evolved various mechanisms to traverse the SML and invade host cells. To detect and respond to extracellular and intracellular microbes, metazoans utilize a class of Pattern Recognition Receptors (PRRs) that rely on Microbe-Associated Molecular Patterns (MAMPs). One of the primary classes of PRRs are the Toll-Like Receptors

(TLRs) (*Muzio* et al., 2000). Upon activation by MAMPs, TLRs recruit the adaptor protein MyD88 resulting in a range of host responses promoting either cell-survival (*Iwasaki* and *Medzhitov*, 2004; *Redfern* et al., 2011) or elimination of the pathogen via programmed cell death (apoptosis) (*Aliprantis* et al., 2000).

The Toll-pathway was first described in *Drosophila* (*Anderson* et al., 1985) and has been shown to be involved with both development (*Wang* et al., 2005) and pathogen defence (*Rosetto* et al., 1995). Additional work in *C. elegans* demonstrated components of the Toll-signalling pathway are present, however, the central proteins involved with TLR-signalling are lacking. Based on these data, the TLR-pathway and its role in immunity was proposed to have evolved within Bilaterians (*Kim* and *Ausubel*, 2005). However, the *Nematostella*, *Hydra*, and *Acropora* genomes revealed that the major components of TLR-signalling are present. To investigate whether the TLR-signalling pathway is functional in *Hydra*, MyD88-deficient and germ-free *Hydra* were generated and the transcriptional profiles were determined. Multiple components central to TLR-signalling were down-regulated in MyD88-deficient and germ-free *Hydra* including members of the TRAF family, MAP-kinase p38, and the kinase TAK1. To determine the role of TLR-signalling in the establishment of the resident microbiome, germ-free MyD88 and wild-type *Hydra* were generated and reinfected with complex microbial communities. MyD88-deficient *Hydra* were found to exhibit a delayed response in bacterial recolonization suggesting TLR-signalling plays a role in the establishment of *Hydra*-associated microbiome (*Franzenburg* et al., 2012). While functional studies have yet to be performed in other cnidarians, the *Acropora digitifera* genome suggests the TLR repertoire is significantly more complex than *Nematostella* or *Hydra* in terms of total TLR proteins present and associated protein domains (*Shinzato* et al., 2011). Future work should focus on determining the binding partners of other cnidarian TLRs, the associated signalling cascades, and the resulting cellular response. Taken together these data support the hypothesis that TLR-signalling and its role in immunity predates the evolutionary split between Bilaterians and Cnidarians. For a more detailed discussion of host-microbe interactions in *Hydra* the reader is pointed to the review by *Schröder* and *Bosch* (2016).

Detection of intracellular microbes: The Nod-Like Receptors (NLRs)

If a microorganism is able to successfully traverse the SML and enter a host cell, Nod-Like Receptors (NLRs) are involved with the detection of MAMPs and activation of the associated cellular response. In humans, NLRs consist of a central NACHT domain, a N-terminal effector domain, and a C-terminal Leucine-Rich Repeat (LRR) that directly binds to bacterial MAMPs (*Hansen* et al., 2011). Activation of NLRs results in the formation of a specialized structure called the inflammasome which is involved with caspase activation and subsequent processing of proinflammatory cytokines (*Franchi* et al., 2009).

While cnidarian intracellular NLRs are relatively understudied compared to the mucosal immune system, *in vitro* experimentation in *Hydra* suggests NLRs may play a conserved role in inflammasome formation. Specifically, the HyNLR Type I protein was found to co-immunoprecipitate with the *Hydra* Caspase 1, a central component of the mature inflammasome. In addition, RT-PCR analysis detected an up-regulation of the HyNLR Type I gene

in response to bacterial stimulation (*Lange* et al., 2011). Bioinformatic analysis of the *Acropora* NLR repertoire identified more NACHT-domain encoding genes (~500 predicted proteins) than any metazoan investigated thus far, including humans, and includes many novel domain combinations (*Hamada* et al., 2013).

The Cnidarian complement system: Symbiosis or cell lysis?

In addition to being directly targeted by antimicrobial peptides, pathogenic microorganisms can also be targeted for destruction by the complement system. Upon activation, the complement cascade labels foreign cells for direct lysis by host proteins. While multiple pathways can lead to complement activation, all converge on the central effector molecule C3, which is cleaved into C3a and C3b by the C3 convertase complex (*Gros* et al., 2008). C3a subsequently activates an inflammatory response while C3b remains attached to the microbe leading to phagocytosis by the host cell and ultimately cell lysis by the membrane attack complex (MAC) (*Noris* and *Remuzzi*, 2013). Three major pathways lead to complement activation: the classical pathway involving antigen-antibody complexes, the lectin pathway involving Mannose-Binding Lectins (MBL) and the alternative pathway. In Cnidarians C3 and components of both the MBL and alternative pathways are present however, many of the proteins involved with the formation of the MAC are absent, suggesting that the ancestral role of complement may have been the promotion of phagocytosis rather than lysing of invading cells (*Cerenius* et al., 2010). This hypothesis is supported by investigations into the role of the complement pathway in the establishment and maintenance of cnidarian-algal symbiosis. In the reef-building coral *Acropora millepora*, the MBL Millectin was found to bind directly to symbiotic *Symbodinium* species *in vivo* (*Kvennefors* et al., 2010). In addition, members of the complement pathway were activated in response to bacterial challenge in corals though direct opsonisation of cells was not observed (*Kvennefors* et al., 2008, 2010). To determine whether the ancestral role of the metazoan complement system was lysis, symbiosis, or both, future work should directly test the impact of complement binding on cellular viability across a range of symbionts, pathogens, and cnidarian species.

Apoptosis: The final defence against invading microorganisms

In response to an invading pathogen, the host cell can also elect to undergo apoptosis or programmed cell death in an effort to prevent further dissemination of the pathogenic entity (*Barber*, 2001). While many types of apoptosis exist, metazoans appear to be unique through their use of Tumour Necrosis Factor Receptors (TNFRs) (*Quistad* and *Traylor-Knowles*, 2016). The TNF Receptor-Ligand superfamily (TNFRSF) is a central mediator of apoptosis and misregulation of the TNFRSF is involved in a variety of inflammatory disorders including multiple sclerosis, type 2 diabetes, and rheumatoid arthritis (*Bahia* and *Silakari*, 2010). Upon ligand binding, TNFR activation can lead to activation of the NF-κB transcription factor (among others), promoting cell survival, or associate with the Fas-Associated Death domain Protein (FADD) resulting in caspase activation and apoptosis (*Lin* et al., 1999; *Micheau* and *Tschopp*, 2003).

Prior to the sequencing of the *Acropora* genome, expansion of the TNF ligand-receptor superfamily was predicted to have occurred with the emergence of adaptive immunity in

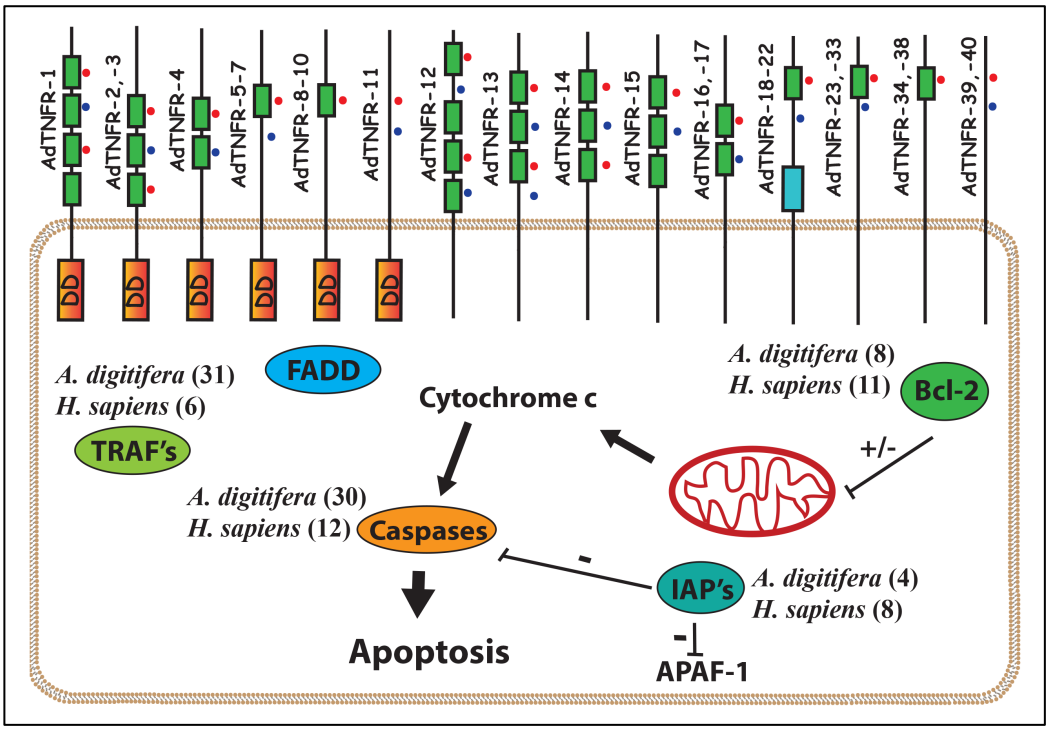

Figure 2: The coral apoptotic repertoire. The putative TNFR repertoire of *Acropora digitifera* (top) with Death Domain (DD), Cysteine Rich Domain (green boxes), Immunoglobin Domain (blue box), 50s loop-TNF binding site (red dot), and 90s loop TNF binding site (blue dot) indicated. Members of the Death Receptor Signalling Pathway (bottom) found in the *Acropora digitifera* genome with number of proteins within a specific protein family indicated for both *Acropora digitifera* and *Homo sapiens* including TNF-Receptor Associated Factors (TRAFs), B-Cell Lymphoma family members (Bcl-2), Inhibitor of Apoptosis proteins (IAP's), FADD, APAF-1 and Caspases. (From: *Quistad* et al., 2014).

vertebrates (*Wiens* and *Glenney*, 2011). Corals and other Cnidarians were predicted to have one, if any, members of the TNFRSF. However, genomic analysis of the *Acropora digitifera* genome led to the discovery of 40 predicted TNFRs compared to 29 found in humans (*Quistad* et al., 2014). In addition to TNFRs, the *Acropora* genome was found to have all of the central components of the canonical apoptotic cascade including three TRAF proteins, thirty caspases, and eight members of the Bcl-2 family (Figure 2) (*Shinzato* et al., 2011). This conservation led us to wonder what might happen if coral cells were exposed to a human TNF. Exposure of coral cells to Human TNFα was found to cause apoptotic blebbing, caspase activation, cell death, and finally coral bleaching. Next, human T-cell lymphocytes were exposed to a member of the coral TNFSF and it was found that coral TNF also caused apoptosis in humans, demonstrating remarkable conservation of TNF-induced apoptosis across 550 million years of evolution (*Quistad* et al., 2014). Further investigations into the origin of TNFRs suggests their role in apoptosis evolved before the Cnidarian-Bilateria split, though the individual domains of the TNFR protein are even more ancient (*Quistad*

and *Traylor-Knowles*, 2016).

Additional work involving other cnidarian apoptotic proteins has further supported a Precambrian origin for the canonical apoptotic cascade (*David* et al., 2005; *Moya* et al., 2016). For example, expression of a coral caspase induced cell death in mammalian cells and coral FADD protein was found to directly associate with a zebrafish caspase (*Sakamaki* et al., 2014). TNFRs have also been implicated in the ability of coral to resist heat stress which is expected to increase in frequency with future climate change (*Hoegh-Guldberg* et al., 2007). Specific TNFR genes were found to be "front-loaded" in corals that were naturally heat-resistant compared to corals of the same species that were heat-sensitive revealing a genomic basis of coral resilience (*Barshis* et al., 2013). While functional investigations into cnidarian apoptosis are still in their infancy they have already revealed novel and exciting insights into the origins and evolution of apoptosis.

The Cnidarian immune system: Complex, conserved, minimally explored

Many publications to date investigating Cnidarians use the term "unexpected complexity" however it is now clear this phrase is no longer appropriate. At the molecular level Cnidarians have proven to be extremely complex and often more similar to humans when compared to established model organisms such as *Drosophila* and *C. elegans*. The basic properties and processes of the SML and mucosal immunity in general began in Cnidarians and continue to operate in the human gut (*Schröder* and *Bosch*, 2016). Immune receptors such at NLRs are more diverse in Cnidarians than any animal investigated thus far (*Lange* et al., 2011). Investigations into the origin and evolution of fundamental immune processes such as TLR-signalling and apoptosis have forced us to rethink multiple decade-long hypotheses (*Kim* and *Ausubel*, 2005; *Wiens* and *Glenney*, 2011). In summation, the Cnidarian phylum has already provided us with unexpected and novel insights into the ancestral state of the first animals, however, all investigations thus far have relied on previously characterized immune proteins described in other systems. How can we begin to understand immune processes that may be phylum-specific or completely novel?

USING VIRUSES TO PREDICT NOVEL IMMUNE PROTEINS

Viruses are the master manipulators of host immunity (*Tortorella* et al., 2000). As obligate intracellular parasites, viruses must be able to complete their life cycle while avoiding immune detection. To successfully invade and replicate within a host cell, viruses express proteins that mimic host immune proteins (*Liang* et al., 2008; *Hagai* et al., 2014). For example, if a virally encoded cytokine was compared to the human proteome *in silico*, then the corresponding human cytokine could be identified without any *a priori* knowledge of the human immune system. An expansion of this concept led to the hypothesis that an *in silico* comparison of all viral gene products against the host proteome would identify both known and potentially novel immune-associated proteins. To test this hypothesis we created a mock viral metagenome using 16 human viruses and compared them against the human proteome using tBLASTn revealing multiple proteins involved with

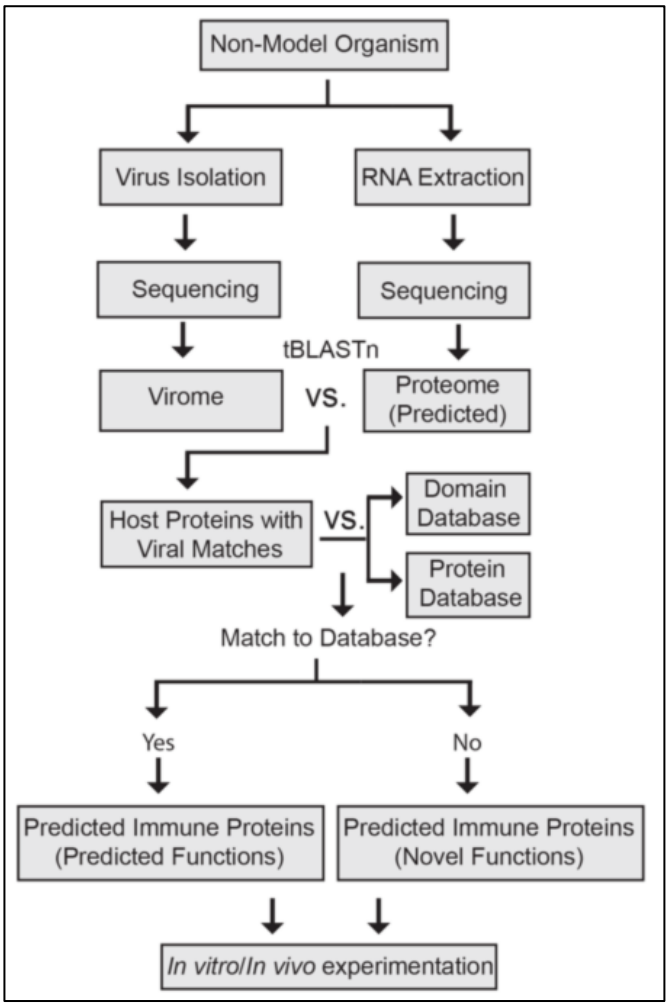

Figure 3: Proposed pipeline to predict immune proteins in uncharacterized systems. Viruses are extracted from the non-model organism of interest using a Virus-Like Particle extraction protocol of choice and nucleic acid is sequenced. If a gene model is not available total RNA is also extracted from host tissue and an assembled transcriptome is prepared. Viral gene segments are compared to the translated transcriptome through tBLASTn to identify matches to host proteins. These proteins are further analysed through comparison to a well-characterized immune system using BLASTp (e.g., human or mouse) and domain prediction database (e.g., Conserved Domain CDD, Pfam). Proteins that lack hits to either database represent predicted immune genes with unknown function and are selected for further biochemical investigations using *in vitro* and *in vivo* experimentation. (From: *Quistad* et al., 2016b).

complement activation, apoptosis, and cytokine signalling (*Quistad* et al., 2016b). Next the same analysis was performed using a coral viral metagenome. As expected, the group of coral proteins identified through the virome analysis were significantly enriched for pathogen-sensing and apoptotic proteins. In total 159 coral proteins were identified that contain no known protein domains, suggesting that they may be involved in novel immune processes. Based on these results, a general pipeline to predict immune proteins was created that can be applied to virtually any animal

system. First, viruses and total RNA are extracted from the organism of interest and nucleic acid is sequenced. Next the virome is compared to an assembled host transcriptome and host proteins matching viral gene segments are identified. The group of proteins with viral matches are then compared to well-characterized host proteomes (i.e. model organisms) and protein domain databases to determine if their function can be predicted. Proteins that fail to match previously characterized proteins or contain previously annotated domains are candidate genes predicted to be involved with phylum-specific immunity or novel immune processes. These candidate proteins can be investigated further using *in vivo* and *in vitro* experimentation (Figure 3). The proposed pipeline combines the power of existing databases with new predictions generated by viral communities providing a more comprehensive prediction of the host immune repertoire (*Quistad* et al., 2016b). While the conserved portion of the Cnidarian immune system has already been identified based on protein annotations from other systems, this domain-independent approach provides novel protein targets to potentially discover cnidarian-specific immune processes.

FUTURE DIRECTIONS

The majority of published work focusing on cnidarian immunity has been performed in the *Hydra* system due to its ease of culture, fully sequenced genome, and well-developed tools for genetic manipulation (*Bosch*, 2013). Cnidarians are divided into two subphyla: Medusozoa (*Hydra* and jellyfish) and Anthozoa (corals and sea anemones) therefore our current understanding of cnidarian immunity is currently biased towards the Medusozoans. While there have been many important discoveries in cnidarian immunology using the coral system, determining the underlying mechanisms is challenging due to lack of molecular tools. To broaden our understanding of cnidarian immunity Anthozoans with developed molecular tools such as *Nematostella vectensis* should also be utilized (*Darling* et al., 2005). Currently, the *Nematostella* system provides a sequenced genome (*Putnam* et al., 2007), gene-knockout techniques (*Ikmi* et al., 2014), and the ability to create transgenic animal lines (*Renfer* et al., 2010) yet, essentially the entire field uses these tools to address development-based questions, leaving the *Nematostella* immune system uncharacterized. By continuing to expand research into *Hydra* and coral immunity as well as developing additional species to investigate mechanistic questions such as *Nematostella*, we will obtain a more comprehensive understanding of the cnidarian immunity and thereby better understand the ancestral state of the animal immune system.

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