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REVIEW

The *Hydractinia* allorecognition system

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Abstract
Hydractinia symbiolongicarpus is a colonial hydroid and a long-standing model system for the study of invertebrate allorecognition. The *Hydractinia* allorecognition system allows colonies to discriminate between their own tissues and those of unrelated conspecifics that co-occur with them on the same substrate. This recognition mediates spatial competition and mitigates the risk of stem cell parasitism. Here, I review how we have come to our current understanding of the molecular basis of allorecognition in *Hydractinia*. To date, two allodeterminants have been identified, called *Allorecognition 1* (*Alr1*) and *Allorecognition 2* (*Alr2*), which occupy a genomic region called the allorecognition complex (ARC). Both genes encode highly polymorphic cell surface proteins that are capable of homophilic binding, which is thought to be the mechanism of self/non-self discrimination. Here, I review how we have come to our current understanding of *Alr1* and *Alr2*. Although both are members of the immunoglobulin superfamily, their evolutionary origins remain unknown. Moreover, existing data suggest that the ARC may be home to a family of *Alr*-like genes, and I speculate on their potential functions.

Keywords Allorecognition · Cnidaria · Homophilic binding · Immunoglobulin domain

Introduction

Allorecognition is an organism's ability to distinguish between its own tissues and those of conspecifics. Within metazoans, allorecognition occurs naturally in sessile, colonial invertebrates when they grow into contact with each other. Natural allorecognition can also occur at the maternal–fetal interface in placental animals (Moffett et al. 2017) and in species with transmissible cancers (Dujon et al. 2020). Experimentally, allorecognition can be induced via tissue grafts in vertebrates (Zhao et al. 2020; Hennessy et al. 2021).

The occurrence of allorecognition in such disparate biological and phylogenetic contexts raises a question: are allorecognition systems related? We know vertebrate allorecognition involves the immune system, and vertebrate immune systems are clearly homologous to each other. But what about invertebrates? Are invertebrate systems homologous to each other? Are they homologous to the parts of the vertebrate immune system that recognize allogeneic non-self? This remains a difficult question to answer because we still do not know the evolutionary history of most invertebrate allorecognition systems, and most of what we do know comes from just a handful of species (Nicotra 2019).

In this review, I will focus on one model system for invertebrate allorecognition, the cnidarian *Hydractinia symbiolongicarpus*. After describing the organism and its allorecognition responses, I will explain what we currently know about the molecular basis of its allorecognition system. I will then speculate about possible functions for some newly discovered *Alr* genes, and end with a brief comment about how to approach the question of homologies between allorecognition systems.

The selective advantage of allorecognition in *Hydractinia*

To understand why allorecognition occurs in *Hydractinia*, it is necessary to explain where they live, how they grow, and a peculiarity of their cell biology.

Hydractinia colonies live on gastropod shells inhabited by hermit crabs (Hauenschild 1954; Buss and Yund 1989).

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Adult colonies are dioecious and release eggs or sperm directly into the water column. After an egg is fertilized, it develops into a larva that crawls on the sea floor until it latches on to the shell of a passing hermit crab (McFadden et al. 1984; Yund et al. 1987). There, it metamorphoses into a polyp. The polyp has one outer, epidermal cell layer and one inner, gastrodermal cell layer, separated by a thin acellular matrix. The gastrodermal cells line a gastric cavity that opens at one end to form a mouth ringed with tentacles. The aboral end of the polyp is attached to the shell. Shortly after metamorphosis, tube-like structures called stolons grow from the polyp's aboral end and extend along the surface of the shell. The stolons are continuous with the gastric cavity of the polyp and are also formed from an epidermal and a gastrodermal cell layer. As the animal matures, additional polyps develop on the stolons, and the spaces between the stolons become filled with a plate of epidermal tissue called the stolonal mat (Fig. 1a). The result is a colony with continuous epidermal and gastrodermal cell layers and a gastrovascular system connecting all polyps and stolons.

The term "colony" can often confuse those encountering *Hydractinia* for the first time. Is the colony a group of distinct individuals, like a colony of ants? If so, what is the "individual"? For *Hydractinia*, we use the term colony to refer to the continuous system of polyps, stolons, and mat tissues. Because a colony derives from a single zygote, all polyps and other tissues have the same genotype. Thus, it also makes sense to also think of the colony as an individual, at least from a genetic perspective. Colonies can reproduce asexually, but they can also create new colonies by fragmentation. In nature, this probably occurs when parts of a colony are separated due to injury or disease. Since the colonies

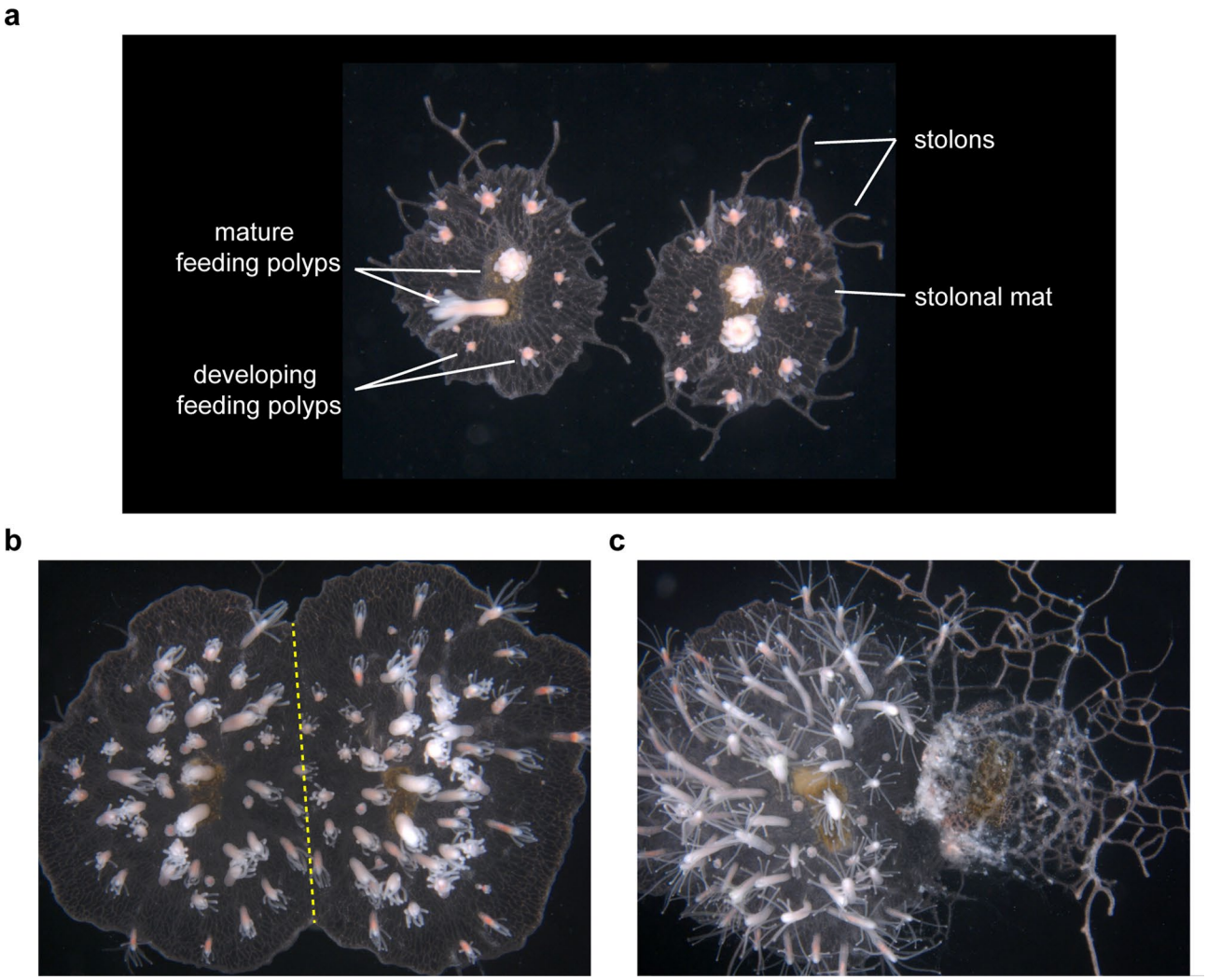

Fig. 1 **a** Two *Hydractinia* colonies growing next to each other on a glass microscope slide. **b** Two colonies that have fused permanently to form a chimeric colony. The yellow dashed line indicates the approximate border between the tissues of each original colony. **c** End stage of aggressive rejection. The colony on the left has overgrown and killed the colony on the right

grow as a single layer that is firmly attached to the shell, it seems unlikely—but not impossible!—that colonies spread to new shells via fragmentation. In the laboratory, however, one can easily excise a small piece of a colony and attach it to a new substrate (typically a glass microscope slide). Thus, it is possible to obtain multiple "copies" or "clones" of the same genotype.

Given the chance, colonies will grow until they cover their shell entirely. In addition, stolons branch as they grow. This means tissues from the same colony—primarily stolons and mat—frequently encounter each other as they grow. When this happens, the epidermal and gastrodermal cell layers fuse to create newly connected stolons or mat with continuous gastrovascular canals (Fig. 1b) (Buss et al. 1984; Lange et al. 1989). Fusion also occurs whenever tissues grow around a three-dimensional surface or regrow after being fragmented by injury or disease.

But a shell can have more than one colony on it. In fact, most colonies encounter conspecifics as they grow (Yund et al. 1987). When this happens, they compete for space with an aggressive rejection response. They differentiate specialized fighting stolons, called "hyperplastic" stolons, which rapidly branch and elongate, lifting off the shell and growing over their opponent (Buss et al. 1984; Lange et al. 1989). These stolons have a high density of nematocytes, cells that carry harpoon-like organelles called nematocysts. The nematocysts fire into the opposing tissue and damage it. Eventually one colony completely overgrows and eliminates its opponent (Fig. 1c) or the colonies establish a "no-man's-land" in which they have cleared each other's tissues.

Less aggressive responses can also occur. Colonies with morphologies that are biased toward the formation of mat will sometimes fail to develop hyperplastic stolons and reject passively (Buss and Grosberg 1990). Over time, one or both colonies may develop hyperplastic stolons. More rarely, two colonies may initially fuse but separate their tissues after a period ranging from days to months (Hauenschild 1954; Shenk and Buss 1991). Permanent fusion is exceedingly rare. (When it does, we refer to the result as a "chimeric colony.") Studies have shown that only 0–2% of colonies selected from the field will fuse or display transitory fusion to each other (Mokady and Buss 1996; Grosberg et al. 1996; Nicotra and Buss 2005). Competition appears to be the norm.

Colonial invertebrates like *Hydractinia* compete for space because larger colonies have greater access to nutrients, lower mortality, and higher fecundity, all of which contribute to higher fitness (Jackson 1977, 1985; Sebens 1987; Hall and Hughes 1996). So why do not colonies fuse and share the benefits of increased size? That turns out to be risky because *Hydractinia* colonies have mobile stem cells. These stem cells, called interstitial cells, move through a colony along the space between epidermal and gastrodermal cells. When colonies fuse, the interstitial cells can migrate from one colony into the other (Kunzel et al. 2010). There, they might invade the somatic tissues of reproductive structures, differentiate into germ cells, and contribute to that colony's gametic output. This stem cell parasitism has the potential to dramatically reduce the potential fitness of the invaded colony.

The requirement that colonies fuse with self tissues as they grow, recognize conspecific competitors, and limit the risk of stem cell parasitism creates selective pressure to accurately distinguish self from non-self (Buss 1987; Feldgarden and Yund 1992; Aanen et al. 2008). This pressure drives the evolution of the *Hydractinia* allorecognition system. At the moment, *Hydractinia* is the only cnidarian for which we have a (partial) understanding of how the allorecognition system works at a molecular level. And this is largely because it is easy to culture and breed *Hydractinia* in the laboratory.

Our current understanding of the allorecognition system

Hydractinia allorecognition was first described nearly 70 years ago (Hauenschild 1954). From the outset, allotypes—the functional identities that determine how colonies respond to each other—were clearly under genetic control. Incompatible colonies could be collected from nature and bred to produce offspring that fused to both of their parents and most of their siblings (Hauenschild 1954, 1956; Grosberg et al. 1996). The most likely explanation seemed to be a system with one or more polymorphic loci. Yet models for how genotype led to allotype could never fully accommodate the phenotypes of all offspring from such crosses.

To deal with this complexity, the Buss laboratory established inbred lines with defined allotypes (Mokady and Buss 1996). These allotypes were shown to be determined by a single locus with alternative alleles. The locus was later revealed to be at least two linked loci, called *Allorecognition 1* (*Alr1*) and *Allorecognition 2* (*Alr2*) (Cadavid et al. 2004). Colonies would fuse if they shared at least one allele at both loci, reject if they shared no alleles at either locus, and exhibit transitory fusion if they shared one or more alleles at only one locus.

The *Alr1* and *Alr2* genes were then identified by fine scale mapping and positional cloning via chromosome walks with BAC libraries (Fig. 2a) (Powell et al. 2007; Nicotra et al. 2009; Rosa et al. 2010). Both genes were discovered to encode type I transmembrane proteins (Fig. 2b). Alr1 had two tandem N-terminal Immunoglobulin (Ig) domains, followed by a 125 amino acid (aa) region dubbed the "extracellular spacer," a transmembrane helix, and a 156 aa cytoplasmic tail. Alr2 had three tandem N-terminal Ig domains, a 106 aa extracellular spacer, transmembrane helix, and a

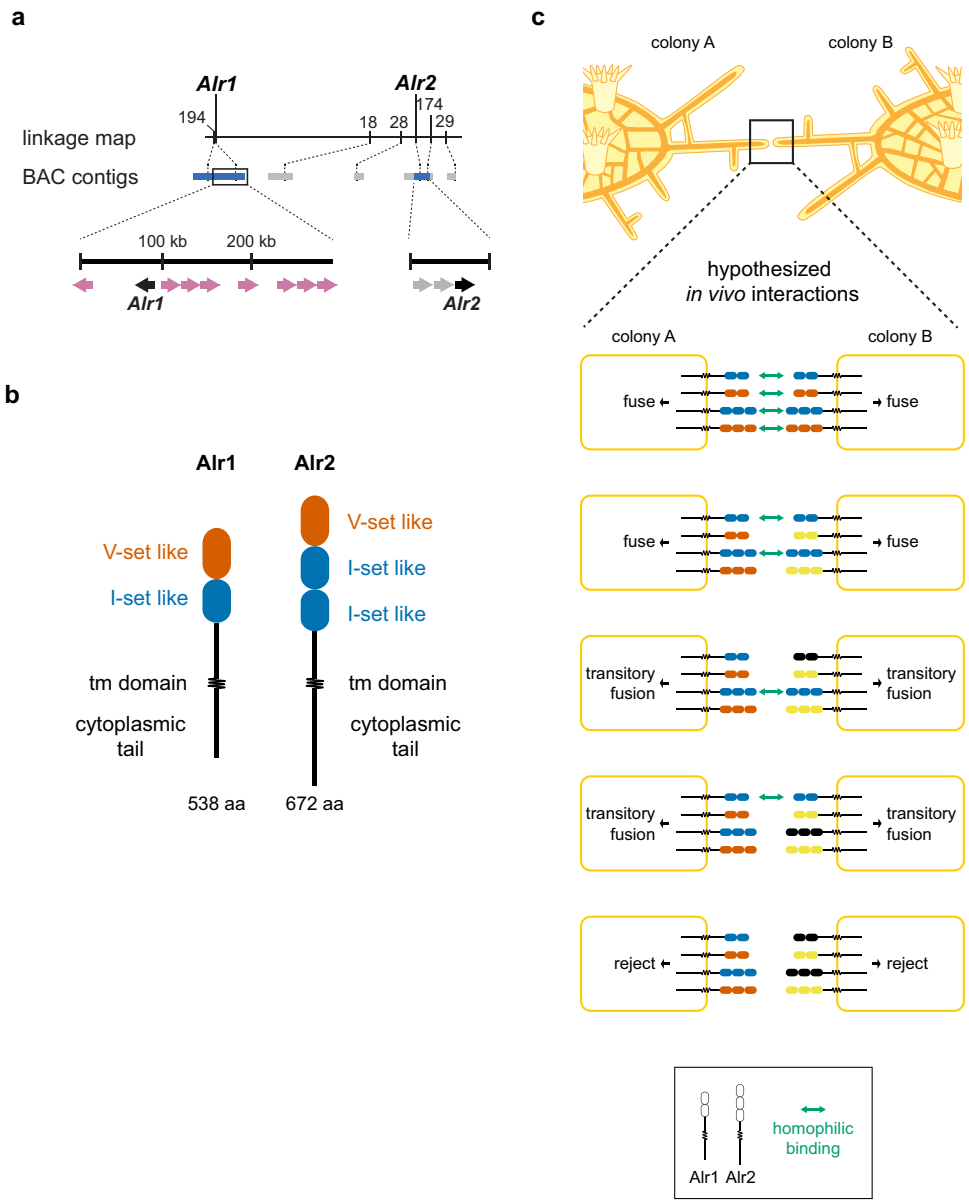

Fig. 2 **a** Linkage map of the ARC, showing the regions that have been sequenced using BAC contigs. *Alr1* is surrounded by several *Alr*-like sequences (purple arrows). *Alr2* is downstream of two *Alr2* pseudogenes (gray arrows). **b** Domain architecture of Alr1 and Alr2 proteins. The proteins are drawn to scale. **c** Hypothesized in vivo interactions that occur when two *Hydractinia* colonies meet. The schematic shows the expression of Alr1 and Alr2 on the surface of a *Hydractinia* cell. Co-dominant expression leads to gene products of both alleles on the cell surface. These are color coded to indicate different alleles. Fusion occurs when colonies have at least one allelic isoform capable of homophilic binding for both Alr1 and Alr2. Transitory fusion occurs when either Alr1 or Alr2 cannot find a binding partner. Rejection occurs when there is no homophilic binding

220 aa cytoplasmic tail. The proteins could only be aligned across their extracellular Ig domains, and only with 25% sequence identity. This suggested they were either distant paralogs, had experienced rapid sequence evolution, or, perhaps, both. Neither protein was obviously homologous to sequences in Genbank. However, their domain architecture, and the presence of many tyrosines in their cytoplasmic tails, suggested that they could be cell surface receptors that engaged in extracellular protein–protein interactions.

Homologs of *Alr1* and *Alr2* have yet to be detected outside of the genus *Hydractinia*. Sequence-based searches against the non-redundant protein database at NCBI typically return alignments to just the extracellular Ig domains, but these hits have marginally significant *e*-values and < 25% sequence identity (Nicotra et al. 2009; Rosa et al. 2010). The Alr cytoplasmic tails do not resemble any known proteins, nor do they contain any recognizable domains. This is true even when searches are restricted to just those of other cnidarians. Adding to the challenge of homology detection is the fact that the Alr Ig domains have very unusual sequences. In particular, they lack the two cysteines that form a disulfide bond at the core of the fold and the highly conserved tryptophan that packs against it (Nicotra et al. 2009; Rosa et al. 2010). In spite of this, sequence-based homology searches, such as HMMER, Interproscan (Jones et al. 2014; Blum et al. 2021), and HHpred (Zimmermann et al. 2018), identify the N-terminal Alr Ig domain as a V-set domain and their neighboring Ig domains as I-set domains (Aidan Huene and Matthew Nicotra, unpublished data). Moreover, the predicted three-dimensional structures of

these domains aligned well to the solved structures of V-set and I-set Ig domains in the Protein Data Bank (Aidan Huene and Matthew Nicotra, unpublished data).

One of the most striking characteristics of *Alr1* and *Alr2* is that they are extremely polymorphic. Most of this polymorphism is in the coding sequence of the extracellular region. At *Alr1*, the two alternative alleles from the inbred strains code for proteins with 25 amino acid differences in their extracellular domains, but only 1 difference in their cytoplasmic tails (Rosa et al. 2010). At *Alr2,* the two alleles encoded proteins with 18 amino acid differences in their extracellular domains, but only 4 differences in the tails (Nicotra et al. 2009). A subsequent study focusing on *Alr2* showed that a single population of *Hydractinia* had at least 194 distinct alleles (Gloria-Soria et al. 2012). When translated, these were predicted to code for 181 distinct amino acid sequences. Most sequence variation was concentrated in the N-terminal Ig domain. The average percent identity between any two allelic isoforms of this domain was only 73%. In addition, most alleles were found to be at relatively equal frequencies. Although not studied as extensively as *Alr2*, *Alr1* is thought to be similarly diverse. Consequently, most colonies are heterozygous and do not share alleles at either *Alr1* or *Alr2*. This provided a genetic explanation for why only 0–2% of colonies selected at random will fuse or display transitory fusion.

These characteristics—a large number of highly diverse alleles at relatively even allele frequencies—are the hallmark of a form of balancing selection called negative frequency-dependent selection acting on the allorecognition genes (Gloria-Soria et al. 2012). Low-frequency alleles have a higher fitness than common ones because they reduce the odds that a colony will mistake non-self for self. Consequently, rare alleles accumulate in the population, and common ones, having lower fitness, decrease in frequency as the population approaches equilibrium.

But how might sequence differences in Alr proteins be recognized? In vitro experiments provided a clue. When either Alr1 or Alr2 was ectopically expressed in mammalian cell lines, the protein could bind homophilically across opposing cell membranes (*in trans*) (Karadge et al. 2015). Moreover, most allelic isoforms of Alr1 or Alr2 would bind only to themselves. This isoform-specific homophilic binding of Alr1 and Alr2 and their extreme allelic polymorphism provided a potential molecular mechanism for self/non-self discrimination in vivo (Fig. 2c). Homophilic binding of at least one allelic isoform of Alr1 and one of Alr2 would be sufficient to signal a match and allow fusion to occur. Partial matches, where only one of the two proteins found a binding partner, might provide enough of a signal to permit fusion initially but not to maintain it over time. Lack of binding led to rejection. This model, while plausible, has yet to be tested functionally in vivo.

For *Alr2*, this homophilic binding can be very specific. Amino acid differences in just the N-terminal Ig domain are sufficient to prevent binding (Karadge et al. 2015). In one clade of six closely related sequences for this domain, three distinct binding specificities could be observed (Huene et al. 2021). The likely evolutionary history of how these binding specificities arose was then traced using ancestral sequence reconstructions. This revealed that one specificity evolved with a single amino acid change, while a second likely evolved via two sequential mutations that created an intermediate that had a broader binding specificity. Aside from demonstrating that point mutations could lead to the evolution of new binding specificities, this study also indicated that it is not straightforward to equate sequence diversity to allotype diversity. Several Alr2 variants had different sequences but the same binding specificity. Moreover, we still lack a structural model for how amino acid changes alter binding specificities, making it difficult to predict how different sequence variants will bind. Therefore, we still do not know how many distinct Alr2 specificities might exist in the field. The available data suggest that it will be less than the nearly 200 sequence variants we observe.

Although the two-locus model of allorecognition reliably predicts allorecognition responses in laboratory strains, it is probably incomplete. Some field-collected colonies—and even a few, rare inbred colonies—fail to fuse even if they share identical or nearly identical alleles at *Alr1* and *Alr2* (Cadavid et al. 2004; Powell et al. 2007; Nicotra et al. 2009; Rosa et al. 2010). This could be explained if additional, polymorphic allodeterminants exist in nature, but became homozygous for the same alleles in laboratory strains due to inbreeding. Without variation at these loci, they would have no effect on the phenotypes of inbred strains, leaving the impression that there were only two allodeterminants.

Genetic studies have shown that any additional dominant or codominant allodeterminants must be linked to *Alr1* and *Alr2* (Powell et al. 2011). We call this genomic region the allorecognition complex (ARC) (Cadavid et al. 2004; Nicotra et al. 2009; Rosa et al. 2010). One byproduct of the work to clone *Alr1* and *Alr2* was a combined total of 4.3-Mb additional DNA sequence surrounding both genes (Fig. 2a). This revealed that the *Alr1* was surrounded by a family of *Alr*-like sequences, and *Alr2* was downstream of two *Alr2* pseudogenes. Work to sequence and annotate this region is ongoing, but it appears that the ARC contains a large family of *Alr*-like genes (Aidan Huene & Matthew Nicotra, unpublished results). These uncharacterized *Alr* genes appear to have the same extracellular domain architecture as *Alr1* and *Alr2* but distinct cytoplasmic tails. This raises the possibility that they might have different functions, both related and unrelated to allorecognition.

Possible functions for additional *Alr* genes

Allodeterminants or sources of sequence variation

An obvious function for new *Alr* genes would be that they are allodeterminants similar to *Alr1* and *Alr2*. This is particularly true for the *Alr*-like genes that surround *Alr1* because some of these appear to be protein coding genes. Even if these genes do not turn out to be allodeterminants themselves, they may play an indirect role in allorecognition by acting as sequence donors. In fact, when assessing natural allelic variation at *Alr1*, many alleles code for extracellular Ig domains that are more similar to alleles of the surrounding *Alr*-like genes than they are to other *Alr1* alleles (Rosa et al. 2010). This suggests that the exons encoding the extracellular Ig domains may frequently recombine between *Alr1* and nearby *Alr*-like loci. A similar pattern has also been detected at *Alr2*; many naturally occurring *Alr2* alleles are more similar to the *Alr2* pseudogenes than they are to protein coding *Alr2* alleles (Gloria-Soria et al. 2012). Thus, in addition to point mutations, *Alr1* and *Alr2* alleles encoding new binding specificities might evolve via exon shuffling between genes and/or pseudogenes. The fact that the *Alr* Ig domains are all encoded by single exons could facilitate this recombination.

Activation of rejection responses

Although the most obvious potential function for the *Alr*-like genes would be allodeterminants, there are several other possibilities. One is that some *Alrs* might be important for the recognition of *non*-self, rather than self. In our current model of allorecognition, colonies reject if they do not have Alr1 or Alr2 proteins that bind to one another. Yet, rejection involves both the failure to fuse and the launch of a dramatic effector response. It seems unlikely for this effector response to be triggered simply by a lack of binding. A more plausible scenario might be that rejection is elicited by signals that tell a colony it has contacted a conspecific. Two seemingly disparate observations support this idea. First, *Hydractinia* colonies only mount allorecognition responses in response to other *Hydractinia*, but rarely to other hydroids and never against other organisms on the shell (Lange et al. 1992; Matthew Nicotra, unpublished data). This indicates that they can identify the type of tissue they encounter. Second, the initial stages of rejection and fusion are indistinguishable morphologically (Lange et al. 1989). In both responses, nematocytes migrate to the point of contact and arrange their nematocysts as batteries pointing at their opponent. In rejection, these batteries fire, but in fusion, the nematocytes migrate away from the zone of contact as the tissues merge. This suggests that rejection is the default allorecognition response, which is inhibited if colonies have compatible allotypes.

Putting this together, we hypothesize that allorecognition could involve a default rejection response that is triggered by a *Hydractinia* signal. This signal is expected to be relatively invariant, and probably detected by a relatively invariant receptor, in much the same way that pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) in many immune systems. Rejection proceeds unless colonies have Alr1 and Alr2 proteins that can bind to each other. Alr1, Alr2, and other allodeterminants might directly inhibit rejection via signaling events that depend on their cytoplasmic tails. Alternatively, or perhaps simultaneously, rejection might be inhibited by other fusion-dependent processes that do not directly involve an allodeterminant.

Cell adhesion

Proteins with tandem Ig domains often function as cell adhesion molecules. The allorecognition function of Alr1 and Alr2 appears to depend on their adhesive properties (Karadge et al. 2015; Huene et al. 2021). Moreover, *Alr1* and *Alr2* are expressed in all tissue types, including feeding polyps and reproductive polyps, which do not take part in allorecognition responses (Nicotra et al. 2009; Rosa et al. 2010). Both genes are also expressed as maternal transcripts in eggs and at all stages of embryonic development. These life stages do not possess a functional allorecognition system (Lange et al. 1992; Poudyal et al. 2007). *Alr1* and *Alr2* might therefore be essential for cell adhesion in all *Hydractinia* tissues.

Cell adhesion might actually be the ancestral function of the *Alr* gene family. Allorecognition might have evolved if natural selection favored variation in cell adhesion molecules that prevented adhesion between tissues with "unmatched" sequences, thus preventing fusion with unrelated colonies. The *Hydractinia* allorecognition system may have thus evolved by co-opting a fundamental cellular process, much like what appears to have happened in fungal allorecognition systems (Gonçalves et al. 2020).

Immunity

Most of what we know about the *Hydractinia* immune system comes from surveys of its genome and transcriptome. Transcriptomic studies have shown that *Hydractinia* has an array of canonical and non-canonical NOD-like receptors, Ig domain containing receptors, six families of lectins, scavenger receptors, and lipopolysaccharide-binding proteins,

NF-kappa B, and a polydom protein (Schwarz et al. 2007, 2008; López et al. 2011; Zárate-Potes et al. 2019). Curiously, the *Hydractinia* genome appears to lack toll-like receptors or any gene with a Toll/interlukine-1 receptor (TIR) domain (Zárate-Potes et al. 2019). It remains unclear whether *Alr1* or *Alr2* play a role in immunity. The sequence diversity of their extracellular domains would seem to make it unlikely that they recognize a common pathogen. Immune functions for additional *Alr* genes seem plausible, particularly those with less allelic polymorphism.

Conclusion

Although much has been learned about the *Hydractinia* allorecognition system, its origin remains a mystery. It is difficult to identify homologs for the *Hydractinia* allorecognition genes in other cnidarian genomes and more distantly related taxa. This includes the allorecognition genes that have been identified to date in the sponge *Amphimedon queenslandica* (Grice et al. 2017) and the protochordate *Botryllus schlosseri* (De Tomaso et al. 2005; Nyholm et al. 2006; McKitrick et al. 2011; Voskoboynik et al. 2013). It seems, therefore, that the molecules responsible for self/non-self discrimination have evolved independently in different metazoan lineages. Having said that, a common feature of these systems appears to be a reliance on domains involved in cell adhesion or cellular interactions (Grice and Degnan 2015).

Allodeterminants, however, are only one element of an allorecognition system. In *Hydractinia*, the sequences of *Alr1* and *Alr2*—and probably other *Alr*-like genes—encode a colony's identity in the sequence of their extracellular domains. That information must be conveyed into cells such that it initiates an appropriate response. The proteins that carry out these intracellular processes are more likely to be conserved than allodeterminants. Thus, elucidating the allorecognition signal transduction pathway in *Hydractinia* may prove to be the key to unraveling its evolutionary history.

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