

The evolution of immunity: a low-life perspective

Georg Hemmrich¹, David J. Miller² and Thomas C.G. Bosch¹

¹ Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstrasse 40, 24098 Kiel, Germany

² ARC Centre of Excellence in Coral Reef Studies and Comparative Genomics Centre, James Cook University, Townsville, Queensland 4811, Australia

Several of the key genes and pathways of vertebrate immunity appear to have much earlier origins than has been assumed previously and are present in some of the simplest of true animals. Surveys of recently released whole-genome sequences and large EST (expressed sequence tag) datasets imply that both the canonical Toll/Toll-like receptor (TLR) pathway and a prototypic complement-effector pathway, involving C3 and several membrane attack complex–perforin proteins, are present in corals and sea anemones, members of the basal phylum *Cnidaria*. However, both pathways are likely to have degenerated substantially in *Hydra*, leaving open the molecular mechanism by which antimicrobial activities are induced in this cnidarian. Surprisingly, the cnidarian genomes also encode a protein related to deuterostome RAG1 (recombination activation gene 1). The finding that RAG1 is likely to have originated from a Transib transposase implies that it might be possible to use *in silico* approaches to identify its target loci in ‘lower’ animals.

Introduction: the big picture

Comparisons with *Drosophila* and *Caenorhabditis* have yielded some important insights into the origins of mammalian immune functions; however, with the availability of whole-genome sequences for some of the simplest ‘true’ animals, comparative genomics is about to enter a new era. Complete genomic sequences are now available for the cnidarians *Nematostella vectensis* (a sea anemone) and *Hydra magnipapillata* (a fresh water polyp) and for the sponge *Amphimedon* (known formerly as *Reniera*). In addition, EST collections are available for several other basal animals, including the coral *Acropora millepora* – a close relative of *Nematostella* but, unlike either it or *Hydra*, a colonial animal (Figure 1). These datasets have already yielded some remarkable general insights into genome evolution [1–3]. The evolutionary origins and timing of major genetic transitions have generally been inferred based on comparisons among vertebrates and the model invertebrates *Drosophila*, *Anopheles* and *Caenorhabditis*. However, from the cnidarian data, it is now clear that extensive gene loss has occurred in these model ecdysozoans [1–3]. Thus, many previous assumptions concerning the evolution of the immune system and the ancestral immune toolkit need to be re-evaluated.

The canonical Toll/Toll-like receptor (TLR) pathway predates the cnidarian–bilaterian divergence

A Toll/TLR protein closely resembling *Drosophila* Toll in both domain structure and amino acid sequence, as well as likely orthologs of many of the other key components of the pathway, is present in anthozoan cnidarians [4,5]. The Toll/TLR pathway known from higher animals therefore predates the divergence of the simplest of true animals and was part of the basic eumetazoan gene complement.

In *Nematostella*, at least six other Toll–interleukin-1 receptor (TIR) proteins are present, including a MyD88 orthologue [4]. Surprisingly, however, three of the sea-anemone TIRs are in proteins whose domain structure parallels that of the vertebrate interleukin-1 receptor (IL-1R) in having two to three immunoglobulin domains in the predicted extracellular portion. Although structural homologues might exist, interleukins in the strictest sense presumably came much later in evolution and the molecular nature of the ligands for these IL-1R-like proteins remains questionable. In addition to these, fragments of several other TIRs could also be identified in the *Nematostella* genome, implying that additional loci are likely to be present. Several TIR loci appear to be present in corals because unequivocal TIRs are encoded by pilot-sequence data that probably represent only a few percent of the genomes of both *Acropora palmata* and *Porites lobata*. All of the complete coral TIRs identified to date appear to correspond to the *Nematostella* IL-1R-type, rather than Toll/TLR or MyD88 (Figure 2b).

Toll/TLR pathway degeneration in *Hydra*

Extracts of the freshwater cnidarian *Hydra* have been known for some time to have potent antimicrobial activities [6] that are probably owing to some of the diverse range of peptides known to be produced by epithelial cells [7]. The data from *Nematostella* summarized earlier imply that a classic Toll/TLR pathway is ancestral within the Cnidaria, so one might reasonably hypothesize that genes encoding antimicrobial peptides are downstream targets of Toll/TLR signalling in *Hydra*. Surprisingly, however, the Toll/TLR pathway appears to have degenerated substantially in *Hydra*; although hidden Markov model (HMM)-based searching indicates that the genome encodes several of the key molecules, intriguingly, it is clear that *Hydra* has no canonical Toll/TLR protein. In fact, the *Hydra* genome encodes only four TIR proteins, two of which are unambiguous homologues of MyD88, which functions downstream of

Corresponding author: Bosch, T.C.G. (tbosch@zoologie.uni-kiel.de). Available online 12 September 2007.

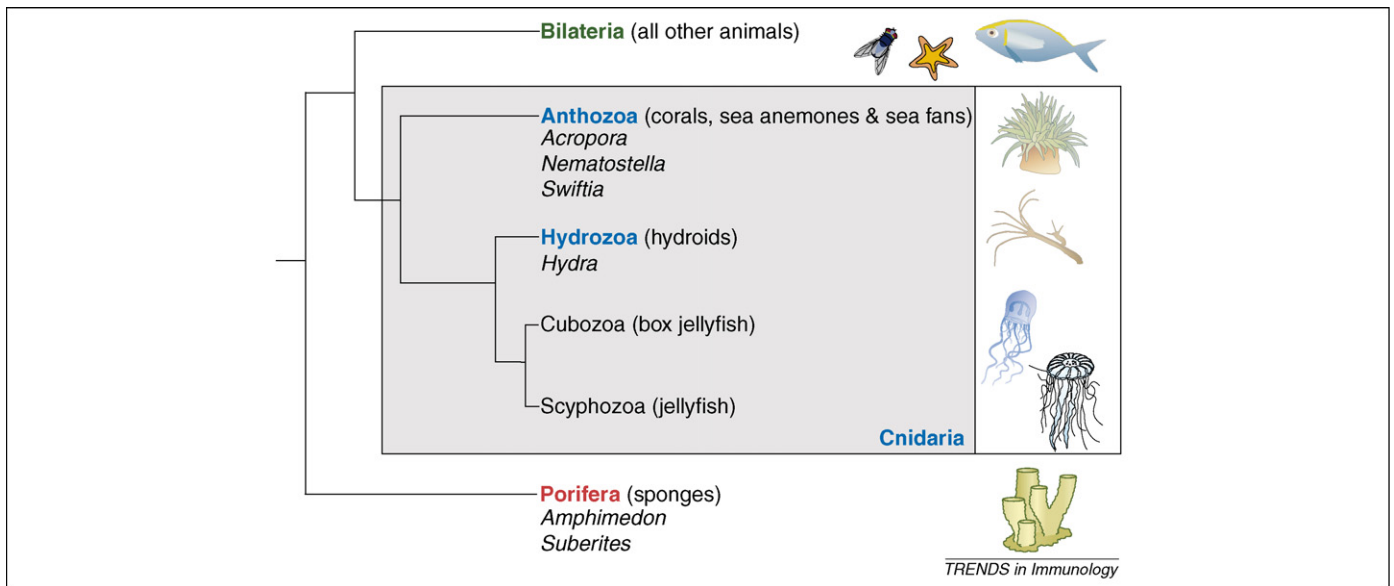


Figure 1. Phylogeny of the 'lower' Metazoa. The Cnidaria are a sister group to all 'higher' animals. Within the Cnidaria, the class to which the coral *Acropora* and the sea anemone *Nematostella* belong (the Anthozoa) is basal. *Hydra* is the textbook representative of the phylum, and remains the best-characterised cnidarian at the cellular level but is a derived member of the Hydrozoa. The illustrations used are courtesy of the Integration and Application Network (ian.umces.edu/symbols), University of Maryland Center for Environmental Science (MD, USA).

Toll/TLRs. Phylogenetic analysis of the TIR domains groups these *Hydra* proteins with fly and mammalian MyD88 (Figure 2b) and each of the *Hydra* MyD88s also contain a death domain that is characteristic of these metazoan-specific proteins. The remaining *Hydra* TIR proteins, HyTRR-1 and HyTRR-2, are clearly related to classic Toll/TLR proteins and have a key role in *Hydra*'s innate immune response (T.B., unpublished) but are atypical in having relatively short extracellular domains lacking any of the leucine-rich repeat (LRR) motifs that are responsible for the pattern-recognition function in typical Toll/TLR proteins [4].

The receptor is not the only Toll/TLR-pathway component to have been lost or diverged beyond recognition in *Hydra*; although anthozoan NF- κ B molecules have been identified [4,5], no convincing counterpart is present in *Hydra*. These facts suggest that *Hydra* might lack a functional Toll/TLR pathway but an apparent inconsistency is the persistence of some pathway components and the roles of the HyTRR-1 and HyTRR-2 are unknown. One possibility is that these TIR proteins might function in conjunction with an as yet unidentified pattern-recognition molecule, effectively reconstituting a receptor.

Earlier origins of the Toll/TLR pathway?

Given that the Toll/TLR pathway predates the cnidarian–bilaterian divergence (approximately 550 million years ago), it will be necessary to look deeper for its origins. The availability of the whole-genome sequence of the sponge *Amphimedon* and the imminent genome sequence of the simplest extant animal, *Trichoplax* (a placozoan), should be informative, however, it is as yet unclear how faithfully either organism has maintained ancestral character states.

The Müller group have identified proteins resembling some Toll/TLR-pathway components in another sponge, *Suberites domuncula*, and have put forward several models

for sponge immunity [8]. A sponge Toll/TLR-related (TIR-containing) receptor has been reported recently [8], however, as in the case of *Hydra* TRR proteins, the predicted extracellular domain is atypically short and lacks true LRRs. Several TIR domains are encoded by the *Amphimedon* genome. Although preliminary analyses indicate that most sponge TIRs are likely to have been duplicated independently since divergence from the common ancestor (Figure 2b), scanning the available genomic data identified a probable Toll/TLR locus in the *Amphimedon* genome; a continuous open-reading frame (ORF) encoded by a genomic contig encodes both LRRs and an incomplete TIR (because the TIR is incomplete – presumably interrupted by an intron – the TIR is not included in the phylogeny shown in Figure 2b). Hence, a Toll/TLR precedes even tissue-level organization in the animal kingdom.

Some other Toll/TLR pathway components are demonstrably present in sponges; for example, a clear orthologue of LBP (LPS-binding protein), which facilitates the interaction of lipopolysaccharide (LPS) and TLRs is present in *Amphimedon*. Others are divergent or have so far eluded detection. A MyD88-like protein has been reported in *Suberites* [9] but this differs significantly in that it lacks a clear death domain. Many aspects of Toll/TLR-pathway function in sponges therefore remain to be elucidated.

A prototypic complement system in the common ancestor?

The complement effector system has a central role in innate immunity in vertebrates but, until recently, was assumed to be confined to the deuterostome lineage (chordates and echinoderms, such as starfish and sea urchins) because none of the central components (C2, C3, C4, C5) are encoded by the genomes of insects, such as *Drosophila* and *Anopheles*, or the nematode *Caenorhabditis*. However, recent reports of unequivocal complement C3 proteins in a

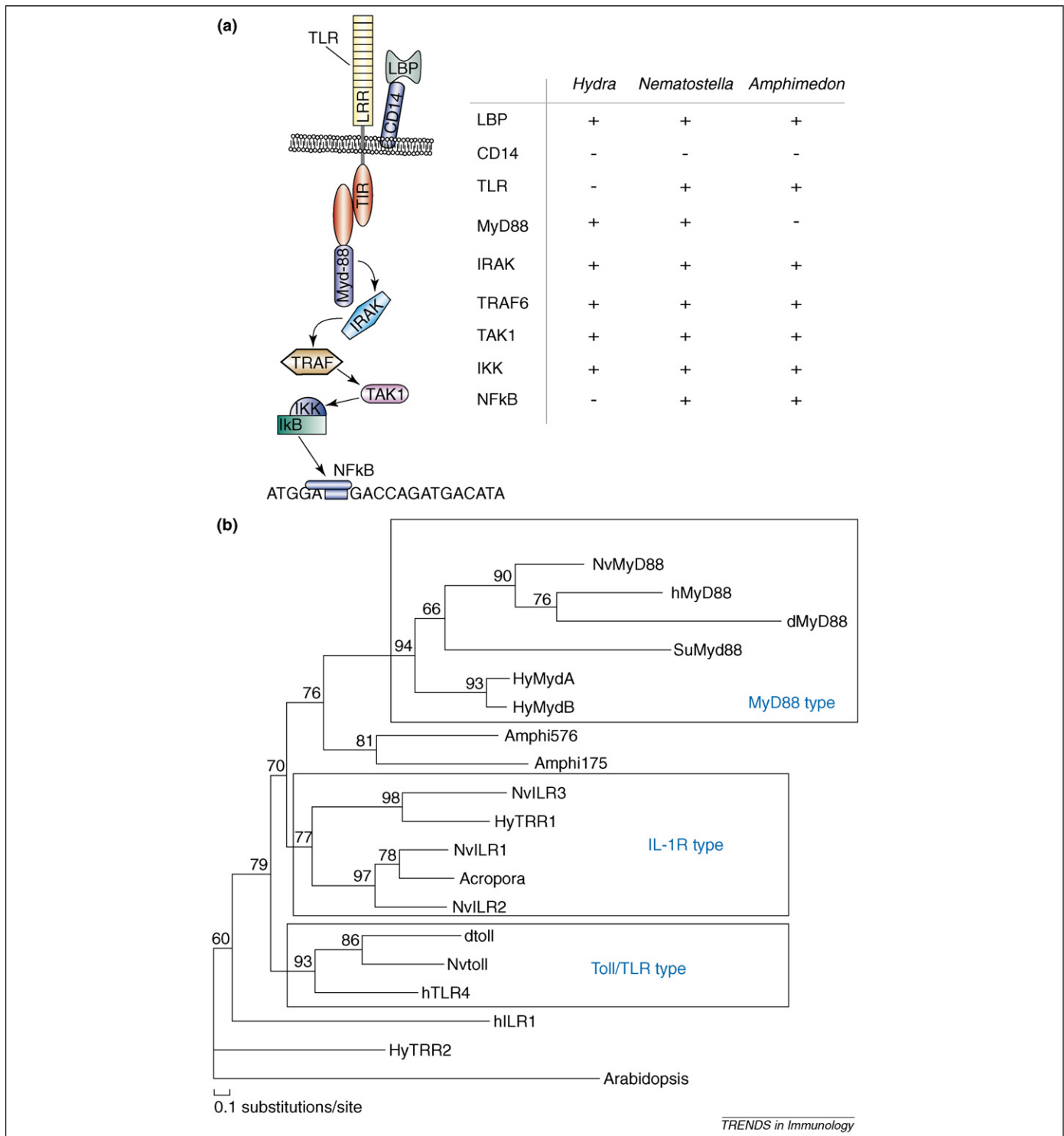


Figure 2. (a) The Toll/TLR pathway and its components present in basal metazoans, such as the cnidarians *Hydra* (freshwater polyp) and *Nematostella* (sea anemone), and the poriferan *Amphimedon* (sponge). (b) Phylogenetic tree of Toll/interleukin-1 receptor (TIR) domains identified in basal animals, based on data in [4]. The tree is rooted with a plant TIR from the *Arabidopsis* sequence AAN28912. The numbers on nodes indicate bootstrap support (percentage of 1000 replicates) in maximum-likelihood analyses (using MolPhy v.2.3; see [4] for details). Only four TIR proteins are present in *Hydra*, which lacks a classic Toll/TLR. By contrast, *Nematostella* has more TIR proteins, including a clear homolog of *Drosophila* Toll and several interleukin-1 receptor-like proteins. Most sponge TIRs do not correspond to the major types present in cnidarians and higher animals. Nv, *Nematostella vectensis*; Hy, *Hydra magnipapillata*; h, human; d, *Drosophila*; Su, *Suberites* (sponge); Amphi, *Amphimedon* (sponge).

chelicerate, the horseshoe crab *Carcinoscorpius* [10], and in the cnidarian octocoral *Swiftia* [11] indicate that, as in other cases [1,2], the absence of corresponding genes in the fly, worm and mosquito reflect secondary losses and that some form of complement system might predate even the cnidarian–bilaterian divergence.

Complement C3 is a large protein with a characteristic domain structure; each of these domains is present in the anthozoan complement C3, whereas several are missing in the case of *Hydra*, implying that the C3-related protein in *Hydra* has a different function (Figure 3). Searching the sponge genome identified only a few of these

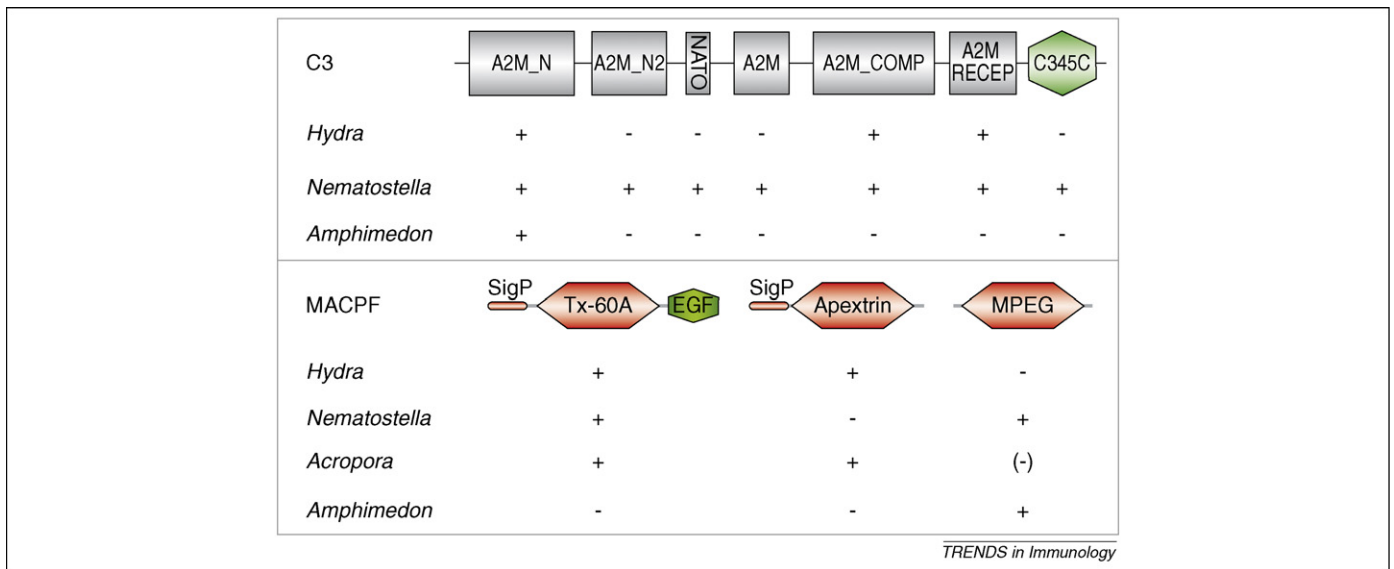


Figure 3. (Upper) Complement C3-related domains in basal metazoans. A clear homolog of deuterostome (chordate and echinoderm), complement C3 is present in anthozoan cnidarians, including *Nematostella*. The anthozoan and deuterostome C3 proteins have the same complex domain structure, whereas *Hydra* has lost the corresponding gene and also completely lacks several of the domains. (Lower) Major groups of membrane-attack complex–perforin (MACPF) proteins identified in lower metazoans. Tx-60A is a component of nematocyst venom in anthozoans [13]; a structurally similar protein is present in *Hydra* but their relationship is not clear. The macrophage-expressed gene (MPEG) protein type was identified in the sponge *Suberites* [9] and corresponds to a conserved but uncharacterised protein family defined by overall similarity to the human macrophage protein XP_166227. The apextrin type is defined by overall similarity to the echinoderm apextrins [14,15] and *Plasmodium* (a protozoan) membrane-attack ookinete protein (MAOP) [16].

domains, probably corresponding to another TEP (thioester-containing protein) superfamily member rather than a C3 (G.H., unpublished); however, the presence of the latter cannot be ruled out at this stage.

Complement C3 occupies a pivotal position in the vertebrate complement cascade – the three complement activation pathways converge on it and its cleavage triggers a cascade of events that lead, ultimately, to the formation of a membrane-attack complex (MAC). Although a strictly opsonic role for C3 (as reported for horseshoe crabs [10]) cannot be ruled out, a prototypic complement system could be constructed around complement C3 by the addition of two other components – an activation system and an effector – and the cnidarian datasets provide candidates for both of these roles. Lectins are well represented in the available anthozoan datasets, leading us to hypothesize that a lectin-based system underlies C3 activation. Several potential effectors exist in the form of various membrane-attack complex–perforin (MACPF) domain proteins.

Most of the MACPF proteins identified in lower metazoans can be classified into three types: MPEG, Tx-60A and Apextrin (Figure 2). MPEG (so called because of its similarity to an uncharacterised mammalian macrophage-expressed gene product) is a perforin-like protein [9] encoded by the *Nematostella* genome. The *MPEG* gene cannot be detected in *Hydra* but has sponge [9], vertebrate and mollusc [12] counterparts. In the sponge, *MPEG* expression is up-regulated after treatment of *Suberites* with LPS and a model has been proposed in which *MPEG* is a response gene downstream of a sponge-specific pattern-recognition molecule known as SLIP (sponge LPS-interacting protein), with the MyD88-like protein acting as an intermediate [9]. The recombinant *Suberites* MPEG has antibacterial activity against Gram-negative bacteria but is not active against the Gram-positive bacterium *Staphylococcus aureus* in the range 0.01–1.00 mg/ml [9].

One of only two *Hydra* MACPF proteins, known as Hy-Mac, has the same domain structure as a nematocyst-venom component from sea anemones. Anemone TX-60A [13], its homologues in both *Acropora* and *Nematostella* and Hy-Mac all contain EGF (epidermal growth factor) repeats in the C-terminal of the MACPF, although the relationship between the *Hydra* and anthozoan sequences is unclear because overall identity is low. By contrast, the other *Hydra* MACPF-domain protein, Hy-Apextrin (Figure 3), not only has a clear orthologue in *Acropora* but belongs to a protein family that includes the echinoderm apextrins [14,15] and *Plasmodium* membrane-attack ookinete protein (MAOP) and its relatives in other apicomplexan protozoans [16]. This group of proteins have a wide range of functions and the role of the Hy-Apextrin is unknown. Surprisingly, no related protein is present in *Nematostella*; loss of this gene might be explained in terms of the very different modes of development of *Acropora* and *Nematostella* [4].

The ancient origins of the RAG system

Classical V(D)J recombination of immunoglobulin and T-cell receptor (TCR) genes is a feature of jawed vertebrates (gnathostomes) and is brought about by a complex of the lymphocyte-restricted proteins RAG1 (recombination activation gene 1) and RAG2. Until recently, the consensus view has been that the precursors of these genes entered the ancestral gnathostome genome through one or more lateral genome transfer events and were derived from a transposase. Intriguingly, the emergence of a hot contender for the ancestor of RAG1 [17] appears to push the acquisition of this gene back at least to Ureumetazoa (the common ancestor of all true animals) and possibly earlier – searching of the available genomic data has enabled the identification of sequences clearly related to the RAG1 core and N-terminal regions in the genomes of some invertebrate deuterostomes

(but not protostomes, such as worms and arthropods) and in both *Hydra* and *Nematostella* (G.H., unpublished).

The idea that a transposase gave rise to the RAG system is not new but, until recently, there were no obvious candidates. Now, a clear contender has emerged in the shape of the Transib transposases [17]. Transib elements are 'cut and paste' DNA transposons identified originally in *Drosophila* and *Anopheles* [18] but now recognised as being distributed across the Metazoa. Of the 10 or so known superfamilies of transposons, Transib elements are unique in that their transposition results frequently in the same kinds of short (≤ 5 bp) target-site duplications [17] that are also characteristic of RAG1 [19,20]. Other similarities to the RAG system include target-site composition and spacing of catalytic triad (DDE) residues – of all known transposase families, the Transib type is one of only two with D–E spacing in the same range (206–214 aa) as in RAG1 (253 aa). Kapitanov and Jurka [17] demonstrate a convincing level of amino acid sequence identity between the transposase and the catalytic core of RAG1 and conclude that only the RAG1 core is derived from a Transib element; they consider the N-terminal domain, which is not required for V(D)J recombination and has ubiquitin-ligase activity, to have probably been recruited later in vertebrate evolution by a gene-fusion event. Consistent with this, sequences related to the RAG1 N-terminal domain (NTD) are present in several copies in the sea urchin and amphioxus genomes but are not adjacent to the RAG1 core. However, *Nematostella* represents an exception because, in this case, the predicted RAG1-like protein carries a RING finger motif resembling that in the RAG1 NTD; this is either a remarkable coincidence or reflects the ancestral state. Perhaps an early transposition event resulted in a Transib element inserting into (or adjacent to) a RING-finger locus; this organisation might have been maintained in *Nematostella* (and, ultimately, given rise to vertebrate RAG1) but lost in both sea urchin and amphioxus (a primitive chordate).

An early origin for RAG1 begs two related questions. First, where did RAG2 come from? Because the *RAG1* and *RAG2* loci are adjacent in all jawed vertebrates so far examined, the consensus has been that both genes entered an ancestral genome simultaneously. Until very recently, no matches to *RAG2* have been found in any invertebrate genome (including *Nematostella* and *Hydra*) and none of the known Transib-type transposases encodes more than a single protein (the recombinase). A complicating issue is that *RAG2* is significantly more divergent than *RAG1* and hence more difficult to identify. However, a putative (albeit divergent) *RAG2*-like locus is linked tightly to the '*RAG1*' gene in the sea urchin *Strongylocentrotus* [21]. Like vertebrate RAG1 and RAG2, the urchin proteins form stable complexes with each other and with their counterparts from various vertebrates and are co-expressed both during development and in adult tissues. These similarities, in terms of structure, genomic organization and properties, argue strongly for simultaneous acquisition of the precursors of RAG1 and RAG2 very early in animal evolution and justify more careful scrutiny of the genomic regions around the RAG1-like loci in cnidarians.

The second open question is whether the cnidarian RAG1-like proteins are capable of executing RAG-like functions – driving recombination at specific loci. The similarity between Transib terminal-inverted repeats and RAG recombination-signal sequences (RSS) suggests that, potentially, RAG1-mediated recombination events analogous to those at the vertebrate immunoglobulin and TCR loci might occur at any locus encoding a surface protein if it contained terminal inverted-repeat or RSS-like sequences, which could perhaps be derived from non-autonomous Transib elements. The availability of complete-genome sequences for *Nematostella* and other RAG1-containing invertebrates suggests the possibility of *in silico* screening for candidate target loci.

Synthesis – a sophisticated ancestral immune toolkit?

One clear implication of these genomic and functional analyses is that the common ancestor of all higher animals contained many of the genes that constitute the mammalian immune system, including a Toll/TLR system, a prototypic complement effector pathway and perhaps even a RAG1-related recombinase. Functional analyses implicate a Toll/TLR system in antimicrobial activity in cnidarians and the pathway might also be present in sponges, pushing back the origins of this system to the base of the animal radiation, some 550 MYA (million years ago) or perhaps beyond. A second important implication of preliminary comparative analyses of basal metazoan genome sequences is that the ancestral immune toolkit seems to be best preserved in anthozoan cnidarians. In *Hydra*, some ancestral genes cannot be detected using the resources available presently and are likely to have either diverged beyond recognition or been lost throughout evolution. The apparent reduction of MACPF complexity and loss of C3 in *Hydra* suggests that some ancestral complement-related functions might have been assumed by pathways leading to the production of antimicrobial peptides [6,7]. Although a classic Toll/TLR receptor was present in Ureumetazoa (and perhaps earlier), this gene appears to have been lost from the *Hydra* genome. However, several Toll/TLR-pathway components remain in *Hydra*, leaving open the possibility that a derived version of the pathway still exists, perhaps using a novel pattern-recognition mechanism.

In terms of mining the basal metazoan datasets for hints about the origins of vertebrate immunity, these are yet early days. There is much to learn, particularly concerning immediate responses to immunological challenge and self-non-self discrimination. Nevertheless, it is clear that these apparently simple animals will have important roles in understanding the evolution of the immune system and, undoubtedly, their genomes have many more surprises in store.

Update

Since submission it has been pointed out to us that the Amphimedon trace archive genomic sequence (id 930677611) has a perfect match with the assembled genome of another animal, the limpet *Lottia gigantea*. This sequence, which encodes both LRRs and a TIR, therefore apparently represents contamination. As a result, there is no evidence for the presence of a canonical Toll/TLR in the sponge genome. We are grateful to B. Degnan and M. Gauthier for alerting us to this.

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