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The Proto-MHC of Placozoans, a Region Specialized in Cellular Stress and Ubiquitination/Proteasome Pathways

Jaanus Suurväli,* Luc Jouneau,† Dominique Thépot,‡ Simona Grusea,§ Pierre Pontarotti,¶ Louis Du Pasquier,‖ Sirje Rüütel Boudinot,* and Pierre Boudinot†

The MHC is a large genetic region controlling Ag processing and recognition by T lymphocytes in vertebrates. Approximately 40% of its genes are implicated in innate or adaptive immunity. A putative proto-MHC exists in the chordate amphioxus and in the fruit fly, indicating that a core MHC region predated the emergence of the adaptive immune system in vertebrates. In this study, we identify a putative proto-MHC with archetypal markers in the most basal branch of Metazoans—the placozoan Trichoplax adhaerens, indicating that the proto-MHC is much older than previously believed—and present in the common ancestor of bilaterians (contains vertebrates) and placozoans. Our evidence for a T. adhaerens proto-MHC was based on macrosynteny and phylogenetic analyses revealing approximately one third of the multiple marker sets within the human MHC-related paralogy groups have unique counterparts in T. adhaerens, consistent with two successive whole genome duplications during early vertebrate evolution. A genetic ontologic analysis of the proto-MHC markers in T. adhaerens was consistent with its involvement in defense, showing proteins implicated in antiviral immunity, stress response, and ubiquitination/proteasome pathway. Proteasome genes psma, psmb, and psmd are present, whereas the typical markers of adaptive immunity, such as MHC class I and II, are absent. Our results suggest that the proto-MHC was involved in intracellular intrinsic immunity and provide insight into the primordial architecture and functional landscape of this region that later in evolution became associated with numerous genes critical for adaptive immunity in vertebrates. The Journal of Immunology, 2014, 193: 2891–2901.

The human MHC of jawed vertebrates is defined as a large genetic region of ∼4 megabases (Mb) encoding more than 100 genes, approximately half of which are implicated in immunity (1). It is divided into three major regions. Class I and class II regions encode the polymorphic Ag-presenting molecules class I, Hα, and β, factors such as B30.2 proteins, and genes involved in Ag processing pathways such as proteasome genes and TAP. The B30.2 domain (2) mediates defense and other functions in several families of proteins, such as butyrophilins and tripartite motif proteins (TRIM) (2–7). The gene-dense class III region encodes several complement components and other genes involved in inflammation (8). The architecture of vertebrate MHCs vary, from the comparatively small MHC of the chicken to the teleost fish, in which class I and II genes are not linked (1, 9–11).

However, the various genes of the complex and their basic functions have been generally conserved, and the elements of the human MHC represent the archetypal MHC genes found across jawed vertebrates (8).

The origin of the MHC is incompletely understood, but the MHC backbone is considered ancient and linked to innate immunity. Class I and class II MHC genes have been suggested to originate from families of molecules present in what is called a "proto-MHC region" for convenience but is involved in innate immunity (12, 13). This ancient backbone could be inherited from the invertebrate ancestors of deuterostomians and protostomians (14–17) (Fig. 1A), from a proto-MHC region. It has been proposed that cis-duplications and translocations of this single ancestral region occurred during evolution, leading to three original complexes on three different chromosome segments: a primordial MHC that gave rise to the set of genes involved in Ag presentation; a neurotrophin complex that led to genomic regions comprising neurotrophin receptors and the Leukocyte Receptor Complex; and a third genetic complex, the tunicate MHC-related complex JAMNECTIN (JN) that is at the origin of paralogous regions containing many Ig superfamily members. These proteins have cell adhesion properties (JAM, NECTINs, poliovirus receptors’ family members) and are often implicated in the biology of lymphocytes, NK cells, and other leukocytes (CD96, cd155, cd112, JAM B, and CTX family members) (15, 16, 18, 19). Pairs of structurally related molecules can act as receptor–ligand systems and mediate interactions between leukocytes and endothelia, linking this third proto-MHC derived complex to immunity.

According to the hypothesis originally proposed by Susumu Ohno (20), these three genetic regions were duplicated twice at an early stage of vertebrate evolution, leading to three independent tetras of paralogous regions (paralogons; see Fig. 1B for definitions) with some overlaps (e.g., B7 receptors were spread over those three regions) (21) and breaks. Ohno’s hypothesis of two

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Abbreviations used in this article: ACLG, ancestral chordate linkage group; GO, gene ontology; GRB, genomic regulatory block; JN, JAM-NECTIN; Mb, megabase; RBBH, reciprocal best blast hit; TRIM, tripartite motif proteins.

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cycles of whole genome duplications in ancestral vertebrates is supported by the study of hox complexes, and it applies to the MHC-related complexes as well as other regions (22–30). In the human genome, there are three main paralogs of the MHC on chromosomes 1 (1q21-q25/1p13-Ip11-Ip10), 9 (9q32-q34), and 19 (19p13.1-p13.3) (22–24). In addition, there are smaller fragments, such as those located on 15q13-q26 and 5q11-q23, translocated from the MHC paralogs on chromosomes 6 and 9, respectively (25). Four paralogs of neurotrophin are on chromosomes 1 (1q32-44, 1p13), 14 (14q11-q32), and 19 (19q13-14q4) that contains the leukocyte receptor complex, and 11 (11q12-q13, 11q23-q24, 11p12-p15) (18). Additional fragments of the neurotrophin group are on chromosomes 2 (2p12-p23), 20 (20p11-p12), and 12 (12q22-q24, 12p11-p13) with the NK complex. Finally, linkage groups corresponding to the JN complex are on chromosomes 1 (1q22-24), 3 (3q13-21), 11 (11q23-25), 21 (21q21-22), and 19q (again with some leukocyte receptor complex elements) (15, 16, 31) (see Fig. 1C for a map of all human MHC related paralogs).

The concentration of genes involved in immunity linked to class I and II MHC regions might confer a selective advantage (32). It is unknown whether the conservation of macrosynergy across vertebrates might be partly explained by inheritance independent of constraints imposed by selective advantages of natural selection. However, the conservation of linked MHC/neurotrophin/JN complexes in invertebrates suggests a significant role of such constraints. In chordates, there is a proto-MHC in the amphioxus Branchiostoma floridae (33, 34) (Fig. 1B), and a genetic complex related to JN exists in the tunicate Ciona intestinalis (14–16), whereas a clearly MHC-related region was not identified (14). There is conserved synteny between human MHC paralogs and a genomic region of the protostome Drosophila melanogaster (17). There is scant information on MHC-related regions in other groups of invertebrates, especially in the most ancient ones.

Placozoans constitute one of the most basal branches of the evolutionary tree of Metazoa (35) (Fig. 1A); it is generally accepted that they are a sister group to bilaterians and chordians, such as corals and jellyfish (36, 37), and there is increasing evidence they are the most basal branches of metazoans (38–40).

Since segments of large-scale genome organization are similar in vertebrates and the placozoan Trichoplax adhaerens (35), this species is a useful model to investigate the origin of the MHC and MHC-related genetic complexes in ancestral metazoans. T. adhaerens is the only recognized species of the phylum Placozoa, although a greater degree of diversity has been proposed for this phylum (41–44). T. adhaerens is a small (100–200 μm), disc-shaped marine organism consisting of two epithelial layers enclosing a layer of multinucleate fiber cells. Only four cell types have been identified; it lacks most of the elements associated with multicellular organisms, such as complex organ systems and extracellular matrix (43, 45). The genome of T. adhaerens is 100 megabases long with twelve pairs of chromosomes and ~11,500 predicted protein-coding genes (35), of which 6516 have been found expressed in the first comprehensive proteome analysis (46). The homologs of these genes are often associated with complex developmental and signaling pathways and are highly conserved in metazoans (35, 47). Importantly, T. adhaerens scaffolds have been mapped on the reconstruction of ancestral chordate linkage groups (ACLGs) and extensive phylogenetic analyses performed for conserved regions (35). The ACLGs are defined from conserved synteny between the amphioxus and vertebrates genomes, and they correspond to the gene content of a putative chromosome of their last common ancestor (48). Hence, the ACLGs are a key generic reference for studying the conservation of blocks of synteny among chordates, vertebrates (including humans), and other species.

In this study, we examined T. adhaerens for the presence of genomic regions enriched in archetypal markers of proto-MHC and proto-neurotrophin complexes. Identified markers of the proto-MHC region were evaluated by gene ontology analysis to test whether such markers contained genes suggestive of immune-related functions, such as stress response and ubiquitination/proteasome pathways.

Materials and Methods
Identification and analysis of T. adhaerens counterparts of human genes located in MHC-, neurotrophin-, and JN-related paralogs

Human protein-coding genes located within the genomic regions described as MHC-, neurotrophin-, and JN paralogs in (21, 49), and within the MHC-related region defined by the R4 RGS genes (50) were extracted from Ensembl Genome Browser release 70 (human genome assembly GRCh37; 7957 genes). The sequence of the longest protein isoform encoded by each gene was extracted using Ensembl Biomart. Protein sequences (human queries) were blasted using ∆BLAST (51) against the genome of T. adhaerens (assembly ASM15021v1 from release 19 of Ensembl Genomes). The e-value cutoff was set at 1e-10, and 5,865 human sequences retrieved 2540 unique best blast hits in T. adhaerens scaffolds. To target counterparts of the MHC-related regions, we selected T. adhaerens scaffolds with highest density of best blast hits—that is, scaffolds in which the proportion of hits of at least one human gene was higher than a given threshold. A density threshold was set from the distribution at 25% to minimize the contrast between the groups, selecting the scaffolds 1, 2, 3, 6, 7, 9, 10, 12, 14, 15, 22, 28, 31, 34, 35, 40, and 42 for further analysis. Protein sequences corresponding to the 6721 genes located within these scaffolds were extracted using Ensembl Biomart and blasted back against the human genome using ∆BLAST with an e-value cutoff of 1e-10. This second blast analysis (using T. adhaerens queries) identified 1273 human genes for which the T. adhaerens best blast hit matched the initial query sequence when “back” blasted against the human proteome. Such human and T. adhaerens entries identified by the reciprocal blast analyses were designated as human and T. adhaerens reciprocal best blast hits (RBBH).

Identification of a putative T. adhaerens proto-MHC

To analyze the distribution of RBBH across T. adhaerens scaffolds, the number of RBBH per total number of genes (RBBH density) was calculated within 1 Mb blocks across scaffolds (or per scaffold for scaffolds shorter than 1 Mb). A threshold was set from the distribution at 15% to optimize the contrast between the groups, and identified a set of 1-Mb regions with a high density of RBBHs. Regions connecting blocks with high density of RBBH were included in this set. These RBBH-enriched regions were further analyzed as to which of the three sets of paralogs (MHC-related, neurotrophin-related, or JN) of the RBBH were related. For each block, the percentage of hits of neurotrophin-related and JN-related among all RBBH was calculated in two ways: 1) all RBBH with human counterparts on the respective paralogs were included, including regions where different paralogs overlap in the human genome; and 2) RBBHs with a human counterpart in the ambiguous regions were omitted, and the percentages were calculated based on the number of genes with human counterpart located in nonambiguous neurotrophin-related, MHC-related, and JN-related regions. If the percentage of the dominant set among all RBBH exceeded 75% in a block, this block was considered MHC-related, neurotrophin-related, or JN-related. The putative T. adhaerens proto-MHC was identified as a collection of all RBBH-enriched regions (located on scaffolds 2, 3, 7, 9, 10, and 15) where MHC-related RBBH were dominant. Out of the 1198 genes in these regions, 307 were found to be the T. adhaerens counterparts of genes located in the human MHC-related paralogs.

Statistical analysis

The significance of marker clustering in T. adhaerens proto-MHC region was tested using a statistical test developed in (52). This test is adapted to approaches in which one starts with a fixed, reference genomic region in the genome of a certain species A (e.g., human) and searches for orthologous regions in the genome of another species B (e.g., T. adhaerens). The test analyzes the probability of finding a number of genes clustered in a given region rather than randomly distributed in the whole genome B.
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given the size of the region and the size of the whole genome B. Importantly, the size is computed as the number of genes not bp. Qualitatively, the statistical significance increases when the size of the region in which markers are clustered decreases and when the size of the whole genome investigated increases. Notably, the total numbers of genes in humans and T. adhaerens differ only by a factor of 2 (≈20,000 versus 10,000, respectively). The test is based on a compound Poisson approximation for computing the p value of an orthologous gene cluster under the null hypothesis of random gene order. A critical feature of the method is that it accounts for the existence of multigene families—that is, the existence of multiple counterparts (co-orthologs) in the genome B for genes of the reference region (e.g., the human MHC paralogs); to do so, the co-orthologs of the target genome B are weighted in inverse proportion to the size of the multigene family to which they belong. If a gene from the starting region has a weight of 1. If a gene from the starting region has k counterparts in the target genome, then each of these co-orthologs will be given a weight of 1/k. The weight of a given region of the genome B (e.g., the T. adhaerens proto-MHC region) is defined as the sum of the weights of the orthologs belonging to it. The p value of this region is the probability, under the null hypothesis of random gene order, of finding somewhere in the genome B a region of higher weight/smaller length, and is computed using a compound Poisson approximation, as explained previously (52).

Gene ontology enrichment analysis

BiNGO, a plugin of Cytoscape (53), was used to look for local enrichment of gene ontology (GO) terms relating to biological processes among human counterparts of the 307 MHC-related genes located in the T. adhaerens proto-MHC region. The list of all 3239 genes located within the human MHC paralogs according to Ensembl 70 was used as a reference set. For every GO term was subjected to statistical test for enrichment, Benjamini and Hochberg false discovery rate p value adjustment was applied to correct for multiple testing (54, 55) and performed by BiNGO.

Microsynteny analysis

Conserved microsynteny between T. adhaerens proto-MHC and human MHC paralogs were defined as sets of RBBH genes that are located in close proximity to each other in both humans and T. adhaerens (i.e., separated by 30 genes or fewer). A linkage was considered a conserved microsynteny only when three or more such genes were linked in such a way in both humans and in T. adhaerens. These microsynteny gene sets were then used to test for the presence of conserved DNA-binding transcription factors using a list of 1988 such genes from humans (56).

Analysis of noncoding regions of the T. adhaerens proto-MHC

To identify putative regulatory regions, we looked for noncoding sequences conserved between T. adhaerens proto-MHC and the human genome. Using the masked sequence of Trichoplax genome (from release 19 of Ensembl Genomes), we first masked all the exons by stretches of N. The masked sequence of the proto-MHC regions was scanned for stretches of poly(N) and all stretches of more than 10 Ns were deleted, leading to sequences corresponding to introns and intergenic DNA. Fragments of <30 bp were discarded, and Blastn and tBlastX analyses were performed for the remaining 18,860 fragments.

Analysis of the Monosiga brevicollis genome

A publicly available list of Mus musculus best blast hits for the genes of M. brevicollis was obtained from the JGI Genome Browser. Human MHC paralogous genes corresponding to these mouse genes were determined using Ensembl Biomart. The list of 295 human-Monosiga gene pairs was compared with our set of human-T. adhaerens RBBH, including those with T. adhaerens counterpart located in the proto-MHC. Human genes with a counterpart in either one or both organisms were then analyzed for gene ontology enrichment as described above. The complete list of M. brevicollis genes with human counterparts located on MHC paralogs was further tested for microsynteny with humans as described above for T. adhaerens.

Results

Identification of putative proto-MHC and proto-neurotrophin genomic regions in T. adhaerens

To determine whether the most basal group of Metazoaans possessed MHC-related paralogs, we examined the genome of T. adhaerens for conserved blocks of synteny corresponding to such genomic segments (Fig. 1).

We recently found that the RGS1/RGS16 region, located next to the MHC paralgon on human chromosome 1, provides useful markers to investigate the origins and the evolution of the MHC in invertebrates (50). The best blast hits of most of these markers were clustered in one 9.7-Mb scaffold (scaffold 2) of T. adhaerens, which also contained homologs of typical MHC markers. We therefore undertook a systematic survey of T. adhaerens homologs of all 7957 human genes located within the 19 genomic regions corresponding to MHC-, neurotrophin-, and JN- paralogs (21, 25, 49, 50). Sequences of the longest protein isoforms encoded by each of these genes were extracted from the human genome assembly and were used as query for reciprocal Δ-BLAST (51) searches against the predicted proteome of T. adhaerens. Forward and reverse blast analyses identified 1273 pairs of markers for which the T. adhaerens gene was the best hit of the forward analysis, and the human sequence used as an initial query in the forward blast was retrieved as the best blast hit of the reverse analysis. Human and T. adhaerens entries of such pairs identified by reciprocal blast will be designated below as human and T. adhaerens RBBH, respectively.

To analyze the distribution of T. adhaerens RBBH across genomic scaffolds, the density (ratio between the number of RBBH and the total number of genes) was calculated within 1 Mb blocks across scaffolds, or per scaffold for scaffolds shorter than 1 Mb. Among blocks containing more than 15% RHHB, we identified those specifically enriched in counterparts of human MHC, neurotrophin, and JN tetras (Fig. 2), for which we hypothesized these regions represented T. adhaerens counterparts (Fig. 2). Many human genes from the MHC-related paralogs had a counterpart on the T. adhaerens scaffold 2 that we identified in our initial screen. However, a high density of such genes was also found on parts of scaffolds 3, 7, 9, 10, and 15 (Fig. 2). Human genes from the neurotrophin-related paralogs had counterparts in other regions of the T. adhaerens genome mostly scattered across scaffold 1, but also located on scaffolds 6, 9, 12, 22, 31, 34, 40, and 42. In contrast, we did not find any region enriched mostly in markers from the third set of paralogs (JN) identified by Du Pasquier et al. (15, 16), with the exception of the very small scaffold 35. This observation suggested that this tetrad might have been produced by a later duplication during the evolution of bilaterians. However, alternative scenarios cannot be excluded; these could not be properly tested in this study because our approach would miss markers that evolve very fast or do not fall into enriched regions.

To test the validity of our approach, we applied it to search for the counterparts of MHC, neurotrophin, and JN paralogs in a vertebrate species. A similar procedure as described above for T. adhaerens was followed for chicken (Gallus gallus), and it successfully identified MHC paralogs on chromosomes 8, 10, 17, 25, 28, and Z and putative neurotrophin and JN regions (Supplemental Fig. 1).

These data show that a method that successfully maps MHC paralogs in the chicken also identified candidate counterparts of proto-MHC and proto-neurotrophin regions in the T. adhaerens genome.

Confirmation of a proto-MHC in T. adhaerens by macrosynteny, phylogenetic analyses, and a statistical test of gene distribution

The T. adhaerens genome shows extensive large-scale conservation in genomic organization between placozoans (T. adhaerens) and chordates (35). One of the most prominent conserved segments is located on scaffold 2, matching the region identified in this study as the main counterpart of the MHC and MHC orthologs. For further analysis, we therefore selected each ACLG corresponding to the putative T. adhaerens proto-MHC regions from reference (35). The segments of human chromosomes matching
these ACLG are shown in Table I. Our candidate regions matched ACLG 8, 10, and 11, which contain essentially all segments of human MHC or MHC ohnologs as described in (49), namely 1p21.1-34.2, 1q23.3-32.1, 5p15.33-q13.2, 5q13.2-31.1, 6p21.2-22.2, 9q22.3-24.3, 9q22.31-34.3, 15q15.3-26.3, 19p13.1-13.2 (Table I). Hence, the common origin of the proto-MHC region identified in this study, and of the MHC set of paralogons described in vertebrates, is confirmed by the conserved macrosynteny mapping and the extensive phylogenetic analysis reported by Srivastava et al. (35).

In addition, we independently assessed the validity of the correspondence between T. adhaerens proto-MHC and human MHC paralogons using a statistical test for the genomic distribution of...
FIGURE 2. T. adhaerens RBBH distribution on genomic scaffolds identifies putative counterparts of MHC and MHC-related human paralogs. Scaffolds containing RBBH genes are divided into 1-Mb blocks. Blocks where >15% of all genes were RBBH are highlighted. Different tones or motifs (consistent with Fig. 1) distinguish blocks with best affinity to MHC, neurotrophin, or JN sets. Comparable results were obtained by excluding the human genes located in regions were paralogs overlap (ambiguous regions). Asterisks indicate differences observed when ambiguous regions are taken into account. The number of RBBH genes corresponding to the three sets of paralogs is indicated below each scaffold. NT, neurotrophin.

RBBH (16). Based on a compound Poisson approximation for computing its \( p \) value under the null hypothesis of random gene order, this statistical evaluation tested the clustering of the T. adhaerens RBBH for MHC and MHC ohnologs within selected regions from scaffolds 2, 3, 7, 9, 10, and 15. Our results showed that the enrichment in the T. adhaerens proto-MHC is highly significant (\( p < 10^{-50} \)). The distributions of conserved markers on T. adhaerens scaffolds were also assessed, including the genes that putatively belong to MHC or neurotrophin (MHC or JN, respectively) sets of ohnologs (Supplemental Table I), and similar statistical results were found.

These results demonstrated that the candidate T. adhaerens regions identified by reciprocal blast analysis from the human MHC paralogs constitute an ancestral proto-MHC. The conservation of macrosynteny between human and T. adhaerens MHC-related regions were supported by orthology relationships, and statistical testing unambiguously rejected a random enrichment of MHC related markers in the T. adhaerens proto-MHC region.

Many human MHC markers with paralogous versions on different ohnologs have a unique counterpart in the T. adhaerens proto-MHC region

Ohno’s theory of whole genome duplications (20) predicts that human sets of paralogs represented in two, three, or four MHC paralogs would generally have a unique counterpart in the genome of a prototypic nonpolyploid invertebrate located in a proto-MHC region. We evaluated whether our results were consistent with this prediction by listing T. adhaerens RBBH of at least one gene from human sets of ohnologs with representatives in three or four paralogs. There were three unique T. adhaerens genes corresponding to sets of paralogs represented on four paralogs among the 33 such sets present in the human genome and 10 corresponding to sets of paralogs present on three paralogs, among the 106 such sets found in human (Fig. 3). These markers were mainly located in T. adhaerens scaffold 2 and include the homologs of several sets of MHC markers such as RXR, PBX, and VAV (25, 29, 49, 57, 58). Among 443 sets of paralogs with representatives on at least two of the MHC paralogs in human, 129 (~30%) had a unique counterpart in the conserved regions of T. adhaerens. Thirteen additional sets of paralogs had multiple targets in T. adhaerens due to secondary duplications. Overall, the observations indicated that approximately one third of the multiple marker sets have a unique counterpart in T. adhaerens, as predicted by Ohno’s model of whole genome duplications during early vertebrate evolution.

Table I. Phylogenetic analyses of the T. adhaerens proto-MHC support its relationship with human MHC and MHC paralogs

<table>
<thead>
<tr>
<th>Regions of the Putative T. adhaerens Proto-MHC (Scaffold:Location in Mb)</th>
<th>Corresponding ACLGs (11)</th>
<th>Location of the ACLG Blocks in Homo Sapiens (11)</th>
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<td>2:2,000,000–2,3,500,000</td>
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<td>1p22; 1q23-32; 6p21-22; 9q32-34(*)</td>
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<td>1p22; 1q23-32; 6p21-22; 9q32-34(*)</td>
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<tr>
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<td>4q12; 4q35; 5q31; 10q11</td>
</tr>
<tr>
<td>10:1,500,000–2,000,000</td>
<td>8 (4.85E-08)(*)</td>
<td>1p21-34; 4p15-q13; 15q22-24; 9q22-32; 19p13(*)</td>
</tr>
<tr>
<td>10:2,000,000–2,435,506</td>
<td>8 (3.07E-08)(*)</td>
<td>1p21-34; 4p15-q13; 15q22-24; 9q22-32; 19p13(*)</td>
</tr>
<tr>
<td>15:1–500,000</td>
<td>3 (18.71E-11)(*)</td>
<td>1p21-34; 4p15-q13; 15q22-24; 9q22-32; 19p13(*)</td>
</tr>
<tr>
<td>15:500,000–1,000,000</td>
<td>16 (4.06E-05)</td>
<td>2q13; 3p21; 7p11; 7q11; 7q36; 10p13; 12q13; 17q12; 17q23</td>
</tr>
</tbody>
</table>

*Linkage groups corresponding to MHC paralogons in human.

The \( p \) values for significant numbers of ancestral genes are shared between the segments of T. adhaerens putative proto-MHC and corresponding ACLGs (11).
Microsynteny groups conserved between the human MHC ohnologs and the T. adhaerens proto-MHC do not systematically colocalize with key developmental master genes

In addition to large-scale synteny conservation between human MHC-related paralogons and T. adhaerens proto-MHC, there were nineteen conserved microsynteny gene sets implicating three or more genes with five having five or more genes. Marker genes involved in such sets were located in the same region, but the colinearity between humans and T. adhaerens was generally not conserved. A striking example was found on human chromosome 9q32-q34.3 region where 65 markers had a counterpart in T. adhaerens scaffold 2 (between 1.9 and 5.2 Mb, see Supplemental Table II), consistent with the MHC paralogon on human chromosome 9 having retained most of the ancestral configuration (i.e., the plesiomorphic organization) (33, 59). In addition, scaffold 2 has four microsyntenies with the RGS1/RGS16 region located on human chromosome 1 (50).

Large gene deserts with enhancers acting over long distances are mostly found in vertebrate genomes, whereas invertebrate metazoans generally have local regulatory controls of expression (60). However, it has been proposed that locked genomic regulatory blocks (GRBs) defined by key developmental transcription factors and their distal enhancers provide an explanation for the maintenance of long-range conserved synteny across vertebrate and invertebrate genomes (61, 62). It is proposed that bystander genes be trapped in the GRBs and thus form conserved syntenic blocks of genes (61, 62). These bystander genes are unrelated to the developmental transcription factor gene defining the GRBs in terms of function, regulation and phylogenetics. According to this hypothesis, observed syntenic regions of the genome are expected containing key developmental transcription factor genes. To test this hypothesis, we looked for such genes in the microsyntenic regions we identified and found no significant enrichment in transcription factors in the microsyntenies of T. adhaerens. Six transcription factor genes (PBX3 [pre-B-cell leukemia homeobox 3], RXRA [retinoid X receptor α], PRDM12 [PR domain-containing protein 12], GTF3C5 [general transcription factor 3C polypeptide 5], ED1F1 [endothelial differentiation-related factor 1], and COBRA1 [cofactor of BRCA1]) were found in the large conserved gene set (60 genes) from chromosome 9. However, among the other 18 gene sets from conserved microsyntenies, only 3 encompass a transcription factor (DMRT7A2 = DMRT75 [double sex- and mab-3–related transcription factor 5], GTF2B [general transcription factor 2B],...
and CHAF1A [chromatin assembly factor 1, subunit A]). With the exception of the large microsynteny group from chromosome 9q34, none of the five longest gene sets (five genes or more) encoded transcription factors.

We also looked for noncoding sequences conserved between the T. adhaerens proto-MHC and the human genome to identify putative regulatory regions. We did not find significant conserved noncoding sequence with obvious regulatory potential. Only two of the 94 sequences found with Blastn and tblastX showed significant conservation in at least one of several genomes from representative metazoans: Amphimedon queenslandica (Porifera), Nemastotella vectensis (Cnidaria), Capitella teleta and Helobdella robusta (Annelida), Crassostrea gigas and Lottia gigantea (Mollusca), and Strongylocentrotus purpuratus (Echinodermata). The first one, PCNCS53, a 68 bp sequence from T. adhaerens scaffold 7, matched an exon of the human WDR65 gene located on human chromosome 1 in an MHC paralogon (e value = $8 \times 10^{-10}$ with exon 12 of transcript ENST00000372492) and the WDR65 gene in A. queenslandica (Aqu1.205499; Aqu1.217579), N. vectensis (NEMVEDRAFT_v1g213283), C. teleta (CapteG108742), L. gigantea (LotgG118162), and S. purpuratus (SPU_010131). A detailed analysis revealed that this motif represents a non-annotated exon flanking the gene TriadG27693, one of the two WDR65 genes found in T. adhaerens close to each other on Scaffold7. The second hit was PCNCS59, a 38- bp sequence from T. adhaerens scaffold 3 that was found in H. robusta, C. gigas, and L. gigantea. This sequence appeared to be located in a non-annotated exon at the 5' end of a well-conserved gene, the sodium channel scn.

Taken together these results argue against a predominant role of transcription factor genes in the maintenance of the microsynteny gene sets conserved between humans and T. adhaerens.

The gene content of the T. adhaerens proto-MHC reveals that this region was involved in immunity in the placozoan-bilaterian ancestor

A tentative set of primordial MHC markers was inferred based on gene conservation between the proto-MHC of T. adhaerens and one or several human MHC paralogs. A functional ontology analysis of this gene set was then performed using BiNGO, a plugin of Cytoscape that maps the predominant functional analysis of this gene set was then performed using BiNGO, first one,


Table II. GO analysis identifies overrepresentation of terms related to proteasome/ubiquitination and stress response within genes conserved between T. adhaerens proto-MHC and human MHC paralogs

<table>
<thead>
<tr>
<th>Pathway</th>
<th>GO Category Accession</th>
<th>GO Category Description</th>
<th>Genes with a Counterpart in the Trichoplax Proto-MHC, mapped within these GO categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteasome / ubiquitination</td>
<td>6511</td>
<td>Ubiquitin-dependent protein catabolic process</td>
<td>ARIH1, CDC34, CLPX, EDEM3, FAF1, FZRI1, HSPA5, LONP1, PSMA4, PSMB7, PSMD5, RNF11, TOPORS, USP3, USP33</td>
</tr>
<tr>
<td></td>
<td>10498</td>
<td>Proteasomal protein catabolic process</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19941*</td>
<td>Modification-dependent protein catabolic process</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43161</td>
<td>Proteasomal ubiquitin-dependent protein catabolic process</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43632*</td>
<td>Modification-dependent macromolecule catabolic process</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51603*</td>
<td>Proteolysis involved in cellular protein catabolic process</td>
<td></td>
</tr>
<tr>
<td>Stress response</td>
<td>6281</td>
<td>DNA repair</td>
<td>ARNT, ATF6, ATG10, BLM, CCNH, CDK7, CHAF1A, COL4A3BP, DCLRE1B, DHX9, FAN1, FZRI, GNL1, HSPA5, INTS3, LONP1, MAP2K7, MORG4LI, POLG, PRDX6, RXRA, TOPORS, UPF1, USP3, XAB2</td>
</tr>
<tr>
<td></td>
<td>33554*</td>
<td>Cellular response to stress</td>
<td></td>
</tr>
</tbody>
</table>

*GO categories that are also relatively underrepresented among MHC-related human markers without a counterpart in T. adhaerens proto-MHC.
similar to the choanocytes of sponges, and are considered the closest known relatives of metazoans (65–67). Among MHC-related markers with RBBH in *T. adhaerens*, approximately one third had a counterpart in *M. brevicollis* (Fig. 5A). Using BiNGO as above, we looked for enriched GO terms within the different gene subsets and found only markers for cellular response to stress for RBBH present in *T. adhaerens*, whereas markers for proteasome and ubiquitination were enriched both in *T. adhaerens* and *M. brevicollis*. We then examined whether these genes involved in proteasome and ubiquitination might be grouped in a single genomic region within *M. brevicollis*. Fig. 5B shows that these genes are largely dispersed among *M. brevicollis* scaffolds, resulting in the absence of a clustering of genes involved in proteolysis and ubiquitination in this species. However, a subset of MHC-related genes present on the human chromosome region 9q32-34 (59)—the most conserved region among MHC paralogons across vertebrates—appears to be located in the same synteny group in *humans*, *T. adhaerens* and *M. brevicollis* (Fig. 5C). These markers include two markers of human MHC tetrads, *VAV* and *RABGAP1*, but are not obviously related to immunity. A more extensive analysis of the genome of *M. brevicollis* will be required for a comprehensive picture of the distribution of the markers linked to the genes of metazoan MHC and proto-MHC related markers. However, our observations provided no evidence for a proto-MHC region containing genes involved in innate immunity, proteasome function, and stress responses in this species.

**Discussion**

The MHC of vertebrates is a large genetic region that determines Ag recognition by T lymphocytes, graft compatibility, and contains genes encoding receptors, cytokines, and effectors of innate immunity. One approach to understand the functional significance of the components of the vertebrate MHC is to reconstruct its evolutionary history. We report a proto-MHC with archetypal markers in one representative of the most basal branch of metazoans, the placozoan *T. adhaerens*. The presence of a proto-MHC exists in the common ancestor of deuterostomes and protostomes as revealed by studies in *Drosophila*. Our results show that it also exists in more primitive branches of the animal kingdom. Placozoan proto-MHC markers include good homologs of genes key to antiviral immunity, stress response, and ubiquitination/proteasome, suggesting that the appearance of class I and II molecules and Ag presentation pathway in vertebrates took advantage of the molecules encoded in this region. In contrast, we did not find evidence of a proto-MHC in the genome of a choanoflagellate, one of the closest known relatives of metazoans among unicellular organisms; it is therefore tempting to speculate that the proto-MHC as a genetic region is an innovation of metazoans, like other key features and pathways such as the TBCEL/coel-1 dependent microtubule function during development and neuronal differentiation (68). However, because the presence of a proto-MHC has not been evaluated in other opisthokonts (e.g., fungi), the apparent lack of proto-MHC in *M. brevicollis* could be due to the loss of the primordial linkage in this species and does not constitute definitive evidence for the absence of proto-MHC in unicellular organisms. A comprehensive survey of protozoan genomes will be necessary to clarify this issue.

The hypothesis of the presence of proteasomes—ancient components involved in the cellular stress response—in the primordial MHC (23) is consistent with our genetic analysis of *T. adhaerens*. The proteasome genes located in *T. adhaerens* are not the functional counterparts of the specialized immunoproteasomes found in vertebrates. Nonetheless, we speculate they are coregulated with genes involved in stress response, pathogen binding, and
ubiquitination, and their presence in the region offers the possibility they have been co-opted and selected in the bona fide MHC during vertebrate evolution. In fact, the *T. adhaerens psmb*-like corresponds to the subset from which immunoproteasomes were derived. In addition, the early peptide presentation system may have lacked specialized and inducible proteasomes, as constitutive proteasomes can generate peptides that are presented by MHC I, although with lower efficiency (69, 70).

The association of genes of stress response, ubiquitination, and protein catabolism (proteasome) within the proto-MHC—possibly as a coregulated unit—in *T. adhaerens* is consistent with an ancient functional link between these pathways; stress response induces ubiquitination of pathogens or cellular proteins that are either redirected to new compartments or to degradation by the proteasome. Such a stress/ubiquitination/proteasome cascade has been described in *C. elegans*; DNA damage to germ cells induces a response that elicits resistance to stress as well as activation of the ubiquitination–proteasome system in somatic cells in various tissues (71). This fundamental inflammatory response can be involved in several processes, including aging, adaptation, and defense against pathogens. A specific implication of the proto-MHC–neurotrophin in immunity is further supported by the presence of several B30.2 proteins in these regions. We previously proposed that the association of B30.2 domains with key proteins of immunity (e.g., TRIM, butyrophilin) found in the MHC and MHC-related regions of vertebrates could have an ancient origin (63). The B30.2 domain structurally resembles a β-barrel (2, 3) and allows specific recognition of ligands via the loops at the top of the domain. Of note, several B30.2 domains found in *T. adhaerens* are associated with domains that possess E3 ubiquitin ligase activity, such as the RING in TRIMs. In particular, the *T. adhaerens* TRIM has multiple human co-orthologs within paralogs of the MHC, neurotrophin or JN sets, and all of them share the same domain structure (72). Among them are TRIM1 and TRIM9, two key modulators of the IFN pathway that strongly inhibit viral growth (73). In the absence of IFN, an antiviral activity of the *T. adhaerens* TRIM might proceed via direct binding (and ubiquitination) of viral proteins as for TRIM5 in primates, or via modulation of expression of other antiviral factors. Although TRIM genes and B30.2 domains are conservatively associated with vertebrate MHCs (and paralogs) (7, 63), it is striking that they are also present in the MHC/neurotrophin regions in the ancestor of placozoans and bilaterians. More generally, our data show a preferential location of B30.2 proteins within the *T. adhaerens* proto-MHC and proto-neurotrophin, supporting an ancestral and strongly conservative association of this domain associated with viral sensing or defense with the MHC–neurotrophin region as suggested previously (63).

In addition to the stress/ubiquitination/proteasome system and B30.2 domains, we also found the *T. adhaerens* proto-MHC has several genes whose human counterparts are located on chromosome 1 in the “RGS1/RGS16 region,” including a typical rs-r4–like gene. In humans, comparative and responsiveness quantitative trait loci analyses show that this region is critical for antiviral defense (50, 74). The markers from the human RGS1/RGS16 region that are conserved in the *T. adhaerens* scaffold might be components of an ancient antiviral pathway.

Our observations do not reveal the evolutionary mechanisms responsible for the linkage conservation of genes involved in immunity within MHC related regions during evolution. However, it is interesting to compare the evolution of the genes found conserved in the proto-MHC linkage to the one of *Hox/paraHox*
genes. Hox/parahox are clustered in most metazoans because they derive from old successive duplications and their sequential expression is highly regulated (75). In contrast, the constituents of the proto-MHC linkage are multiple, do not derive from the same ancestor gene, and are not found in the same order in different species, suggesting that they are subjected to different constraints. In conclusion, T. adhaerens has retained a simple and primitive organization at both genetic (genomic) and organism levels, and its proto-MHC may reflect the primordial architecture and the functional landscape of this region, which later in evolution became associated with a large number of genes critical for the adaptive immunity in vertebrates.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


### Supplemental Figure 1

**Chicken (Gallus gallus), assembly WASHUC2 (chromosomes)**

<table>
<thead>
<tr>
<th>Mb</th>
<th>0 Mb</th>
<th>20 Mb</th>
<th>40 Mb</th>
<th>60 Mb</th>
<th>80 Mb</th>
<th>100 Mb</th>
<th>120 Mb</th>
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<th>160 Mb</th>
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</thead>
<tbody>
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<td>3</td>
<td>5</td>
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<td>10</td>
<td>15</td>
<td>17</td>
<td>24</td>
<td>25</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>MHC</td>
<td>23</td>
<td>50</td>
<td>68</td>
<td>226</td>
<td>284</td>
<td>208</td>
<td>39</td>
<td>46</td>
<td>116</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>271</td>
<td>375</td>
<td>562</td>
<td>11</td>
<td>2</td>
<td>161</td>
<td></td>
<td>72</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JN</td>
<td>153</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **MHC / NT**: 11
- **MHC / JN**: 22
- **NT / JN**: 3

**Legend**
- Blue: Most blast results from NT (>75% of target genes)
- Red: Most blast results from MHC (>75% of target genes)
- Yellow: Most blast results from JN (>75% of target genes)
- Orange: Mixed regions, without clear dominance of MHC, NT or JN

**Supplemental Figure 1.** Gallus gallus RBBH distribution on genomic scaffolds identifies putative counterparts of MHC and MHC-related human paralogons. Scaffolds containing RBBH genes are divided in 10 Mb blocks. Blocks where >15 % of the genes were RBBH are colored. Orange arrows indicate Chromosomes where known MHC-related markers have been mapped previously.
Supplemental Table I. MHC markers are significantly clustered in the T. adhaerens proto-MHC.

Total Nb of genes in the relevant scaffolds of the *T. adhaerens* genome (reference): 6721
Total Nb of genes in the proto-MHC regions in the *T. adhaerens* genome (as defined in Figure 2 and in Table I): 1198

<table>
<thead>
<tr>
<th>Excluding genes that may belong either to MHC, neurotrophin or JAM-NECTIN set of paralogs in human:</th>
<th>Including genes that may belong either to MHC, neurotrophin or JAM-NECTIN set of paralogs in human (ie “with ambiguous markers”):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of <em>T. adhaerens</em> counterparts of MHC paralogon markers, located within the “proto-MHC” = 282</td>
<td>Number of <em>T. adhaerens</em> counterparts of MHC paralogon markers, located within the “proto-MHC” = 307</td>
</tr>
<tr>
<td>Total number of <em>T. adhaerens</em> counterparts of MHC paralogon markers, located within the relevant scaffolds = 528</td>
<td>Total number of <em>T. adhaerens</em> counterparts of MHC paralogon markers, located within the relevant scaffolds = 579</td>
</tr>
<tr>
<td>Number of <em>T. adhaerens</em> counterparts (single or multiple) of MHC paralogon markers, located within the relevant scaffolds:</td>
<td>Number of <em>T. adhaerens</em> counterparts (single or multiple) of MHC paralogon markers, located within the relevant scaffolds:</td>
</tr>
<tr>
<td>- 491 single counterparts</td>
<td>- 537 single counterparts</td>
</tr>
<tr>
<td>- 14 pairs of co-orthologs (14 multigene families of size 2)</td>
<td>- 15 pairs of co-orthologs (15 multigene families of size 2)</td>
</tr>
<tr>
<td>- 3 triples of co-orthologs (3 multigene families of size 3).</td>
<td>- 4 triples of co-orthologs (4 multigene families of size 3).</td>
</tr>
<tr>
<td>Number of <em>T. adhaerens</em> counterparts (single or multiple) of MHC paralogon markers, located within the “proto-MHC”:</td>
<td>Number of <em>T. adhaerens</em> counterparts (single or multiple) of MHC paralogon markers, located within the “proto-MHC”:</td>
</tr>
<tr>
<td>- 261 single counterparts among the 491.</td>
<td>- 284 single counterparts among the 537.</td>
</tr>
<tr>
<td>- Among the 14 multigene families of size 2:</td>
<td>- Among the 15 multigene families of size 2:</td>
</tr>
<tr>
<td>6 families have only one of the two genes in this region;</td>
<td>6 families have only one of the two genes in this region;</td>
</tr>
<tr>
<td>5 families have both genes present in this region.</td>
<td>5 families have both genes present in this region.</td>
</tr>
<tr>
<td>- Among the 3 multigene families of size 3:</td>
<td>- Among the 4 multigene families of size 3:</td>
</tr>
<tr>
<td>1 family has one gene and 2 familys have two genes present in this region.</td>
<td>1 family has one gene and 3 familys have two genes present in this region.</td>
</tr>
</tbody>
</table>

As explained in the Experimental procedures, the weight of the “proto-MHC” region is computed as: 261 + 6 x 1/2 + 5 x 2 x 1/2 + 1/3 + 2 x 2/3 = 270 + 2/3.

As explained in the Experimental procedures, the weight of the “proto-MHC” region is computed as: 284 + 6 x 1/2 + 5 x 2 x 1/2 + 1/3 + 3 x 2/3 = 294 + 1/3.

In both cases, the statistical test described in (52) gives a p-value which is extremely low (less than 10^-50), hence the clustering of MHC markers in *T. adhaerens* proto-MHC region is extremely significant.
### Supplemental Table II. Microsynteny region between human chromosome 9 and *T. adhaerens* scaffold 2

<table>
<thead>
<tr>
<th>Human gene ID</th>
<th>Human gene name</th>
<th>Human gene location</th>
<th>TriadG1</th>
<th>TriadG1 rank on human chromosome</th>
<th>Human coding gene rank on scaffold 2</th>
<th>Human coding gene end</th>
<th>Scaffold 2</th>
<th>Tadrahes <em>T. adhaerens</em> scaffold Tadrahes coding gene scaffold 2</th>
<th>Tadrahes coding gene end</th>
<th>Tadrahes coding gene blast e value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSG00000130653</td>
<td>9q34.3</td>
<td>Tadrahes</td>
<td>821</td>
<td>1.27e-42</td>
<td>3.21e-35</td>
<td>7.78e-26</td>
<td>5.00e-17</td>
<td>500</td>
<td>568</td>
<td></td>
</tr>
<tr>
<td>ENSG00000148356</td>
<td>9q34.11</td>
<td>TriadG47130</td>
<td>136,228,325</td>
<td>5.16e-108</td>
<td>4.72e-107</td>
<td>3.35e-148</td>
<td>7.32e-168</td>
<td>1.26e-131</td>
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</tr>
<tr>
<td>ENSG00000165699</td>
<td>9q34.2</td>
<td>Tadrahes</td>
<td>614</td>
<td>3.69e-86</td>
<td>1.10e-21</td>
<td>5.42e-87</td>
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<td>ENSG00000165695</td>
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<td>TriadG16229</td>
<td>135,285,430</td>
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<td>9.35e-85</td>
<td>1.26e-49</td>
<td>1.89e-79</td>
<td>4.97e-18</td>
</tr>
</tbody>
</table>

**Notes:**
- The table lists the human and *T. adhaerens* genes present in the microsynteny region between human chromosome 9 and *T. adhaerens* scaffold 2.
- The human gene IDs and names are listed along with their locations on chromosome 9.
- The *T. adhaerens* gene IDs and names are listed along with their locations on scaffold 2.
- The table includes the blast e-values for the alignments between the human and *T. adhaerens* genes.

**References:**
- The table is referenced in the main text. Further details can be found in the supplementary material.