Molecular characterization of the evolution of phagosomes

BOULAIS, Jonathan, et al.

Abstract

Amoeba use phagocytosis to internalize bacteria as a source of nutrients, whereas multicellular organisms utilize this process as a defense mechanism to kill microbes and, in vertebrates, initiate a sustained immune response. By using a large-scale approach to identify and compare the proteome and phosphoproteome of phagosomes isolated from distant organisms, and by comparative analysis over 39 taxa, we identified an 'ancient' core of phagosomal proteins around which the immune functions of this organelle have likely organized. Our data indicate that a larger proportion of the phagosome proteome, compared with the whole cell proteome, has been acquired through gene duplication at a period coinciding with the emergence of innate and adaptive immunity. Our study also characterizes in detail the acquisition of novel proteins and the significant remodeling of the phagosome phosphoproteome that contributed to modify the core constituents of this organelle in evolution. Our work thus provides the first thorough analysis of the changes that enabled the transformation of the phagosome from a phagotrophic compartment into an [...]
Molecular characterization of the evolution of phagosomes

Jonathan Boulais1,9, Matthias Trost2,3,9, Christian R Landry4, Régis Dieckmann5, Emmanuel D Levy6, Thierry Soldati5, Stephen W Michnick2, Pierre Thibault3,6,8,* and Michel Desjardins1,7,8,*

1 Département de Pathologie et Biologie Cellulaire, Université de Montréal, Montréal, Québec, Canada, 2 MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee, Scotland, 3 Unité protéomique et spectrométrie de masse bioanalytique, Institut de Recherche en Immunologie et Cancérologie (IRIC), Université de Montréal, Montréal, Québec, Canada, 4 Département de Biologie, PROTEO et Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec, Canada, 5 Department of Biochemistry, University of Geneva, Geneva, Switzerland, 6 Département de Biochimie, Université de Montréal, Montréal, Québec, Canada, 7 Département de Microbiologie et Immunologie, Université de Montréal, Montréal, Québec, Canada and 8 Département de Chimie, Université de Montréal, Montréal, Québec, Canada

These authors contributed equally to this work

* Corresponding authors. P Thibault, Institut de Recherche en Immunologie et Cancérologie (IRIC), Université de Montréal, C.P. 6128, Succ Centre Ville, Montréal, Québec, Canada

Received 25.5.10; accepted 15.9.10

Amoeba use phagocytosis to internalize bacteria as a source of nutrients, whereas multicellular organisms utilize this process as a defense mechanism to kill microbes and, in vertebrates, initiate a sustained immune response. By using a large-scale approach to identify and compare the proteome and phosphoproteome of phagosomes isolated from distant organisms, and by comparative analysis over 39 taxa, we identified an ‘ancient’ core of phagosomal proteins around which the immune functions of this organelle have likely organized. Our data indicate that a larger proportion of the phagosome proteome, compared with the whole cell proteome, has been acquired through gene duplication at a period coinciding with the emergence of innate and adaptive immunity. Our study also characterizes in detail the acquisition of novel proteins and the significant remodeling of the phagosome phosphoproteome that contributed to modify the core constituents of this organelle in evolution. Our work thus provides the first thorough analysis of the changes that enabled the transformation of the phagosome from a phagotrophic compartment into an organelle fully competent for antigen presentation.

Molecular Systems Biology 6:423; published online 19 October 2010; doi:10.1038/msb.2010.80

Subject Categories: proteomics; immunology

Keywords: evolution; immunity; phosphoproteomics; phylogeny; proteomics

This is an open-access article distributed under the terms of the Creative Commons Attribution Noncommercial Share Alike 3.0 Unported License, which allows readers to alter, transform, or build upon the article and then distribute the resulting work under the same or similar license to this one. The work must be attributed back to the original author and commercial use is not permitted without specific permission.

Introduction

Phagocytosis is the process by which multiple cell types internalize large particulate material from the external milieu. In mammals, this receptor-mediated function has important functions in embryogenesis and tissue remodeling (through the clearance of apoptotic cells), as well as in the elimination of a variety of microbial pathogens causing important diseases such as salmonellosis, chlamydia infection, and tuberculosis. The functional properties of phagosomes are acquired through a complex maturation process, referred to as phagolysosome biogenesis. This pathway involves a series of interactions with other intracellular organelles, enabling the delivery of hydrolytic enzymes and the generation of other molecules, such as nitric oxides and superoxides, involved in the killing and degradation of the phagosome content.

Phagocytosis has been maintained during evolution and was shown to have important functions in organisms such as amoeba and paramecium. For example, the degradative environment encountered in the phagosome lumen has enabled the use of phagocytosis as a predation mechanism for feeding (phagotrophy) in amoeba (Desjardins et al., 2005; Jutras and Desjardins, 2005; Gotthardt et al., 2006). The degradative properties of phagosomes were exploited for the control of pathogen invasion in multicellular organisms, through the introduction of molecules involved in the recognition of microbial determinants such as the Toll-like receptors (TLRs), with one representative in Caenorhabditis elegans, and 9 and 10 in Drosophila melanogaster and human, respectively (Mushgeian and Medzhitov, 2001). Killing of microorganisms in phagosomes is a key feature of innate immunity, the part of our immune system that defends the host from infection in a non-specific manner. The emergence of...
genes associated to the MHC locus in mammals that appeared originally in the genome of jawed fishes, contributed to the development of complex molecular mechanisms linking innate and adaptive immunity (the part of the immune system triggered specifically after antigen recognition) (Kasahara et al., 2004). Several of the genes of this locus encode proteins known to have important functions in antigen presentation, such as subunits of the immunoproteasome (LMP2 and LMP7), MHC class I and class II molecules, as well as tapasin and the transporter associated with antigen processing (TAP1 and TAP2), involved in the transport and loading of peptides on MHC class I molecules. Remarkably, all of these proteins have been identified on phagosomes of different organisms by various biochemical and morphological approaches (Dermine et al., 2001; Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Grotzke et al., 2009), suggesting that their advent during evolution might have contributed to the pivotal role played by phagosomes in innate and adaptive immunity. Nevertheless, the molecular mechanisms that enabled the emergence of novel phagosomal functions during evolution are poorly understood. Here, we present the first large-scale comparative proteomics/phosphoproteomics study characterizing some of the key steps that contributed to the remodeling of phagosomes that occurred during evolution.

## Results

### Proteomics analyses of phagosomes

To study how the phagosome has been remodeled during evolution, we isolated this organelle from three distant organisms that use phagocytosis for different purposes, and performed detailed proteomics and phosphoproteomics analyses. These original data were analyzed and compared with a wide variety of organisms using comparative genomics to characterize the nature of the modifications that enabled phagosomes to have an important function in innate and adaptive immunity during evolution. This approach proved to be efficient for the comparative study of complex cellular structures like synapses (Emes et al., 2008). Tandem mass spectrometry (MS/MS) analyses led to the identification of 818 Dictyostelium, 1132 Drosophila and 1391 mouse phagosome proteins (Supplementary Datasets 1–3). Compared with previous studies (Garin et al., 2001; Gotthardt et al., 2006; Rogers and Foster, 2007; Stuart et al., 2007; Jutras et al., 2008), we obtained a two- to four-fold enhancement in the number of proteins identified, with unparallel protein coverage for this organelle. Based on the proteome of each organism, we identified orthologs present in the genome of the two other organisms using the established Ensembl (to compare mouse with Drosophila) and Inparanoid (to compare mouse and Drosophila with Dictyostelium) databases (Supplementary Datasets 4–6), and mapped them accordingly to their BLAST E-value (Figure 1A). These analyses identified proteins unique to a given organism (point of origin in purple), proteins sharing orthologs with one of the two other organisms (data points on x and y axes in green or red), or proteins sharing orthologs with the two other organisms (data points out of the axes in blue). The proportion for each group of proteins is highlighted in the bar graph under each scatter plot with respective colors. As expected, the mouse and Drosophila phagosomes are more related to each other than to Dictyostelium phagosomes. Nevertheless, a large proportion of proteins are maintained in phagosomes from Dictyostelium to mouse, highlighting a subset of molecules likely to have been present in the phagosome core of their common ancestors.

Next, we annotated each of the mouse proteins (based on literature searches and the curated Uniprot database) to determine the distribution of orthologs among established phagosome structural and functional properties. Our data indicate that cytoskeletal elements, proteins associated with cellular trafficking, and small GTPases were highly maintained in the three organisms (Figure 1B, Supplementary Dataset 7). The presence of these elements could be explained by their involvement in the advent of phagocytosis in pre-eukaryotic cells (Cavalier-Smith, 2009; Yutin et al., 2009). Conversely, functional groups such as membrane receptors, signaling, and immunity are predominantly represented in the mouse phagosomes, or in both the mouse and Drosophila, highlighting the emergence of novel phagosomal properties in multicellular organisms (Figure 1B).

So far, our data indicate that a large proportion of the mouse phagosomal proteins have orthologs in the Drosophila and/or Dictyostelium genome. Thus, a related question is whether these proteins are also present on the phagosome of these organisms, or expressed elsewhere in the cell. Comparison of the mouse phagosome proteome with the proteomics analyses of phagosomes isolated from Drosophila and Dictyostelium performed in this study, as well as compiled data published by our groups previously (Gotthardt et al., 2006; Stuart et al., 2007) indicate that 61.7 and 51.2% of the mouse orthologs were identified by MS/MS in Drosophila and Dictyostelium phagosomes, respectively (41.7% of the mouse phagosome proteome is shared by the three species) (Supplementary Figure S1A). Although a certain proportion of these differences might be due to the fact that some of the proteins present in Drosophila and Dictyostelium phagosomes were not

**Figure 1.** Shared components define the ‘ancient’ phagosome. (A) Predicted orthologs of phagosome proteins of Dictyostelium, Drosophila, and mouse were analyzed by BLAST against the two other species and mapped according to $-\log_{10}$(E-value), where 0 indicates the absence of an ortholog and 181 a perfect alignment. Four distinct groups of proteins are highlighted for each organism: (1) a set of orthologs shared by the three organisms defining the ‘ancient’ phagosome (blue data points outside the x and y axes), (2 and 3) groups of conserved proteins shared only between the plotted organism and one of the two others found on the x or y axis (green or red data points), and (4) a set of proteins unique to the plotted organism (purple data points at the origin of the graph). As several data points may overlay in the scatter plot, a histogram below each plot reports the relative distribution of proteins among the four distinct groups of proteins. (B) Annotation of a function to each protein of the mouse proteome highlights the level of conservation of relevant phagosome functions among the three organisms. Although a large proportion of the proteins associated with functions such as ‘membrane trafficking,’ ‘small GTPases,’ and ‘cytoskeleton’ are majorly shared by the three organisms, some like ‘membrane receptors’ and ‘immunity’ are more specific to mouse and Drosophila phagosomes. See also Supplementary Figure S1 and Supplementary Datasets 4–6.
Molecular characterization of the evolution of phagosomes

J Boulais et al

© 2010 EMBO and Macmillan Publishers Limited

Molecular Systems Biology 2010 3
sampled during the mass spectrometer analyses (sampling limitation), it is also arguable that a path to the complexification of the phagosome proteome arose, for example, from the possibility that proteins localized in the cytoplasm of basal organisms would be eventually recruited to phagosomes during evolution (co-option). We argue that a sampling limitation would potentially affect most of the proteins, irrespective of their functional properties. On the other hand, differences related to biological diversification during evolution is more likely to be related to changes for proteins of specific functional properties. Our data support the proposal that proteins associated with specific functional properties have accumulated on phagosomes during evolution. Indeed, significant differences were observed in the functional properties of the mouse orthologs that were effectively identified on Drosophila and Dictyostelium phagosomes. For example, we observe that predicted orthologs of proteins such as GTPases and cellular trafficking components were highly represented on the Drosophila and Dictyostelium phagosomes, compared with proteins such as transporters and membrane receptors (Supplementary Figure S1B and C). Further quantitative studies would be required to confirm that certain proteins present on the mouse phagosome are expressed in the other organisms (present in the cell) but not recruited to the phagosome.

Origin of the mouse phagosome proteome

We performed comparative analyses among 39 taxa to identify the origin of 1385 mouse phagosome proteins, by using gene phylogeny web databases (PhylomeDB (phylomeDB.org) and TreeFam (treefam.org)) (Figure 2A, Supplementary Dataset 7). Interestingly, 73.1% of this proteome consists of proteins already present in phagotrophic single-celled eukaryotes and in Amoebozoa and Fungi that had lost phagotrophy. Around 16.7% of the phagosome proteins appeared in organisms that use phagocytosis for innate immunity (Bilateria to Chordata), whereas 10.2% appeared in Euteleostomi or Tetrapoda where phagosomes have an important function in linking innate and adaptive immunity. The phagosome is an organelle formed following the internalization of large particles. Hence, it is made of molecules taken from a variety of sources within the cell, including the cytoplasm, the cytoskeleton and membrane organelles. Despite the evolution and diversification of these various cellular systems (Erickson, 2007; Dacks et al., 2008; Fritz-Laylin et al., 2010; Wickstead et al., 2010), the mammalian phagosome proteome is made preferentially of ancient proteins (Figure 2B). Functional annotation highlighted the emergence of specific phagosomal properties at various steps during evolution (Figure 2C). Some of these proteins and their point of origin during evolution are highlighted in Figure 2D. Strikingly, we identified in Tetrapods a set of 50 proteins that arose around 450 million years (Hedges, 2009) after the emergence of adaptive immunity, including IRG47/Irgm1 (a strong resistance factor induced by interferons (IFNs)), CD5 (a scavenger receptor that has an important function in B- and T-cell selection as well as generation and maintenance of tolerance) (Raman, 2002), CD14 (a co-receptor along with TLR-4 and MD-2 for the detection of bacterial LPS) (Sepulcre et al., 2009), CD47 (a protein that interacts for ‘self-
Figure 2  Origin of the mouse phagosome proteome. Comparative analyses of the mouse phagosome proteome among 39 taxa identified the origin of each protein. 

(A) Proportions (in %) of the evolutionary origin of the mouse phagosome proteome are reported through four major evolutionary groups of proteins: phagotrophy (Eukaryota, Amoebozoa, and Fungi), innate immunity (Bilateria, Coelomata, and Chordata), early (Euteleostomi), and late adaptive immunity (Tetrapoda and beyond).

(B) Comparison between the evolutionary origin of the mouse phagosome proteins and the entire mouse proteome (reported by their relative proteome proportion in %) through a cladistic distribution (x axis) reveals that phagosomes are of ancient origin. The inbound graph shows the same proteome proportion in % through a cladistic distribution under the four major evolutionary groups of proteins reported in a: phagotrophy (Ph.), innate immunity (In.), early adaptive (E.A.), and late adaptive immunity (L.A.).

(C) Comparative functional analysis of the mouse phagosome proteins reveals that specific phagosomal functions originated from different stages of evolution. The function ‘Others’ contains the merging of remaining functions, and numbers indicate the amount of proteins found in each function.

(D) Specific examples of proteins originating at the four major evolutionary groups are found in dash boxes. See also Supplementary Dataset 7.

© 2010 EMBO and Macmillan Publishers Limited
Tetrapods. This feature was especially observed for phosphosites present in disordered regions of proteins, as described previously (Dafforn and Smith, 2004; Landry et al., 2009). These results indicate that the phagosomal phosphoproteome has been extensively modified between coelomates and mammals. We showed recently that treatment of macrophages with IFN-γ induces significant changes in the level of expression of various proteins and the state of phosphorylation of several of their potential phosphosites (Jutras et al., 2008; Trost et al., 2009). This cytokine affects the relative abundance of at least 386 mouse phagosomal proteins. Our analyses reveal that 81.9% (316) of these proteins originated before the emergence of IFN-γ in teleosts, indicating that the introduction of this cytokine during evolution enabled the modulation of ancient phagosome proteins in ways not possible before its emergence (Supplementary Figure S4B).

We observe a higher level of conservation of the IFN-γ-modulated phosphosites among all vertebrates, compared with tunicates and coelomates (Drosophila) (Figure 4B). Interestingly, this difference coincides with the emergence of IFN-γ at the vertebrates-tunicates split (Savan et al., 2009), suggesting that this cytokine might have introduced functional gains, creating selective pressure to stabilize a part of the phagosomal phosphoproteome in vertebrates.

To evaluate more directly the extent of the reorganization of the phagosome phosphoproteome during evolution, we characterized the phosphoproteome of phagosomes isolated from Drosophila and Dictyostelium. Our analyses led to the identification of 968 phosphosites in 420 Dictyostelium phagosome phosphoproteins, and 2919 phosphosites in 910 Drosophila phagosome phosphoproteins, with a false-discovery rate (FDR) below 1% (Supplementary Datasets 9 and 10). Although the alignment of these phosphoproteins with the mouse orthologs predicted that a similar proportion (~33%) of the murine phosphosites aligned with phosphorylatable residues in Drosophila or Dictyostelium, a relative small proportion of these sites was, in fact, effectively phosphorylated. Indeed, our phosphoproteomics data show that 12.8% (n=88) and 5.0% (n=20) of the mouse phosphosites are also phosphorylated in Drosophila and Dictyostelium phagosome proteins, respectively. It should be emphasized that although these numbers appear to be low, they are, in fact, 8- and 12-fold higher.
Evolution of phagosomal protein networks

Thus far, our results indicate that the emergence of novel proteins, series of duplication events, and an extensive remodeling of the phagosome proteome are elements that contributed to the acquisition of new phagosomal functions during evolution. How emerging proteins have been integrated into existing cellular pathways throughout evolution is poorly understood. It has been proposed that the integration of novel components into protein networks tends to occur through association with ‘hub’ proteins that are already interacting with a high number of partners. This feature of complex networks is favored by gene duplication (Barabasi and Oltvai, 2004). As duplication had a profound effect on the actual phagosome proteome, we studied how phagosome components of various evolutionary origins interact to assemble the molecular machines enabling the functional properties of this organelle in mammals. To circumvent the fact that a limited set of experimental interactions have been reported for mouse proteins, we used our mouse phagosome data to retrieve orthologous human protein–protein interaction data from the Intact (Kerrien et al, 2007) and UniProt databases (Consortium, 2009). This approach led to the characterization of 2637 interactions (edges) involving 1258 proteins (nodes) of the three main evolutionary groups (phagotrophy 864; innate immunity 243; and adaptive immunity 151 nodes). Although proteins of each groups have a similar average number of interactions (edges), proteins acquired later in evolution interact considerably more often with proteins of ancient origin (Table I), consistent with the evolutive architecture of a scale-free network (Eisenberg and Levanon, 2003). From the total network, we generated two subnetworks highlighting proteins involved in vesicle trafficking, and interaction with the cytoskeleton (Figure 5A) and immunity (Figure 5B).

Remarkably, most of the functional modules present on phagosomes are constituted of proteins that have appeared at various stages of evolution, often through a duplication process, indicating a high degree of integration and a diversification of pre-existing functional units (e.g. Cdc42
and Rabs and their effectors in Figure 5A). However, certain functional modules such as the Ena/Vasp complex, receptor signaling, the NADPH oxidase complex, as well as the antigen presentation machinery appeared later during evolution, promoting the direct emergence of novel functional properties. Particularly, a complex process like antigen processing and presentation requires the concerted action of a number of molecular machines. A model of the various steps performed in the phagosome to enable the processing of proteins into peptides, and their loading on MHC molecules is presented (Figure 6). This model highlights the fact that although this process is unique to evolutionarily recent phagosomes (starting in jawed fishes, about 450 million years ago) (Hedges, 2009), it uses and integrates molecular machines composed of proteins that emerged throughout evolution.

**Discussion**

Over more than a billion years, the phagosome has evolved from a digesting organelle, where bacteria are degraded as a source of nutrients, into a complex compartment involved in the killing of pathogens and the regulated processing of their proteins for antigen presentation. In the present study, we performed the first comparative analysis of an organelle isolated from distant organisms using a protocol allowing high levels of purification. Previous characterization of isolated latex bead-containing phagosomes demonstrated the low levels of contamination of these preparations due to the isolation procedure (Gotthardt et al., 2002; Stuart et al., 2007). The recent finding that phagosomes interact with autophagosomes (Sanjuan et al., 2007) would certainly provide a possible explanation for the presence of proteins in phagosomes that could be considered as contaminants. A good example of this is the identification of ribosomal proteins in our samples. Interestingly, phagosome-autophagosome interaction during mycobacterial infection has been shown to enable the delivery and degradation of ribosomal proteins in the lumen of these organelles, a process generating bactericidal molecules (Ponpuak et al., 2010). The three ribosomal subunits involved, L30, S19, and S30 have been identified in the mouse phagosome preparations. This led us to consider all the proteins identified in our preparations as ‘potential’ phagosomal proteins; these being either structural proteins or proteins present in the phagosome lumen as cargo.

Our data indicate that a large proportion of the phagosome proteome is of ancient origin (73.1% of the proteome is conserved in the genome of most eukaryotic organisms). This number is somehow misleading as one has to consider that analyses of whole genomes will include large groups of proteins that are parts of well-conserved machineries involved in basic cellular functions. Nevertheless, this stresses the fact that phagocytosis is a very ancient process, as shown by its possible involvement in the emergence of eukaryotic cells (eukaryogenesis) (Cavalier-Smith, 2002). Of the 1391 proteins found on the mouse phagosome, 290 were effectively identified by MS/MS in phagosomes of the two other studied organisms, defining a protein core from which the immune functions of phagosomes likely evolved. A clearer image of the ancient phagosome core and the early steps in the evolution of this organelle is likely to emerge once more free-living amoebozoan genomes will be sequenced.

Our study highlights the fact that the functional properties of phagosomes emerged by the remodeling of ancient molecules, the addition of novel components, and the duplication of existing proteins leading to the formation of molecular machines of mixed origin. Gene duplication is a process that contributed continuously to the complexification of the mouse proteome during evolution. In sharp contrast, the phagosome proteome was mainly reorganized through two periods of gene duplication, in Bilateria and Euteleostomi, coinciding with the emergence of adaptive immunity (in jawed fish), and what might have been the emergence of innate immunity. These results strongly suggest that selective constraints may have favored the maintenance of the phagosome paralogs to ensure the establishment of the novel functional gain associated with this organelle. For instance, the duplication of TLRs, hydrolases, and sets of novel SNARE and Rab proteins have contributed to the specialization of cell lineages and the establishment of innate immunity (Stuart and Ezekowitz, 2008).

The emergence of novel proteins is not the only way by which phagosomal functions have been modified during evolution. Several of the phagosome proteins shared among distant organisms have been modified by a significant remodeling of their phosphosites, indicating that phagosome proteins of ancient origin are far from being fixed entities. The reorganization of phosphosites, occurring at a much faster pace than the introduction of novel proteins, is likely to have endowed proteins with additional functional properties, and/or introduced finer ways to regulate their activity and/or the nature of their interacting partners. This is particularly the case in disordered regions of proteins, known to be fast evolving sequences that are often involved in protein interactions (Brown et al., 2002; Dafforn and Smith, 2004; Tompa, 2005). The impact of the phosphoproteome plasticity on phagosome functional properties is currently unknown. We have shown previously that IFN-γ alters the expression and the level of phosphorylation of a large number of proteins on phagosomes of activated macrophages (Jutras et al., 2008; Trost et al., 2009). Remarkably, several of these proteins were present in the...
MHC class I presentation machinery, thereby enhancing antigen presentation efficiency. The duplication of PSME1, coinciding with the emergence of adaptive immunity, gained a KEK E-motif.

During this duplication event, duplicated proteins of which both paralogs have been identified by MS/MS on mouse phagosomes are circled in blue. From the total network, subnetworks of cytoskeleton and vesicle trafficking proteins were extracted, showing the evolutionary mixed origin of most protein complexes and the addition of novel modules such as the cytoplasm and vesicle trafficking complexes.

**Figure 5** Evolution of the phagosome proteins network. Experimental data from the Intact database and curated entries of the UniProt database were used to generate a network from protein–protein interactions of identified mouse phagosomal proteins. From the total network, subnetworks of cytoskeleton and vesicle trafficking proteins (A) and immunity-related proteins (B) were extracted, showing the evolutionary mixed origin of most protein complexes and the addition of novel modules such as the MHC class I and II presentation machinery, the receptor signaling, the NADPH oxidase complex or the Ena/VASP complex to the phagosome in evolutionary steps of adaptive and innate immunity, respectively. Duplicated proteins of which both paralogs have been identified by MS/MS on mouse phagosomes are circled in blue. (C) Example how duplication might affect phagosome function: (immuno-)proteasome activator complex subunits PSME1 (PA28α) and PSME2 (PA28β) were duplicated with the appearance of jawed fishes, coinciding with the emergence of adaptive immunity. During this duplication event, PSME1 gained a KEKE-motif that was not present in the common ancestor. KEKE-motifs have been described to interact with each other and are also present in several chaperones including Calnexin (Li and Rechsteiner, 2001; Rechsteiner and Hill, 2005). It is likely that introduction of the KEKE-motif in PSME1 might locate the immunoproteasome to Calnexin and the MHC class I presentation machinery, thereby enhancing antigen presentation efficiency.
common eukaryotic ancestor, > 1.2 billion years prior to the emergence of IFN-γ in teleosts (Savan et al., 2009; Bhattacharya et al., 2009). Thus, the emergence of a variety of cytokines appears to have been an important event that conferred novel functional properties to vertebrate phagosomes by fine-tuning the expression and/or phosphorylation of several proteins of this organelle, including proteins of ancient origin. Furthermore, our data indicate that despite its overall recent origin, the mouse phagosome phosphoproteome also contains ancestral phosphosites, maintained for more than a billion years, highlighting their potential importance in the functional properties of this organelle.

Figure 6 Role of molecular machines of mixed origin in phagosome functions. Many of the functional properties of mammalian phagosomes involve molecular machines made of proteins that emerged at different periods during evolution. For example, in the context of antigen cross-presentation, key steps such as phagosome/endosome fusion, the killing of microbes and their degradation into peptides, as well as their loading on MHC class I molecules are made possible by proteins that appeared in organisms where the phagosome has its main role in phagotrophy (green proteins), innate immunity (yellow proteins), and adaptive immunity (red proteins). Remarkably, the emergence of a cytokine such as IFN-γ inteleosts, > 1.2 billion years after the emergence of phagotrophy (Bhattacharya et al., 2009), allowed the fine-tuning of the expression and/or phosphorylation of proteins of each of these groups (red shadow). Early endosome (EE), late endosome (LE), lysosome (Ly), and endoplasmic reticulum (ER).
In addition to their ability to present peptides on MHC class II molecules, phagosomes of vertebrates have been shown to be competent for the presentation of exogenous peptides on MHC class I molecules, a process referred to as cross-presentation (Desjardins et al., 2005). From a functional point of view, the involvement of phagosomes in antigen cross-presentation is the outcome of the successful integration of a wide range of multimolecular components that emerged throughout evolution. The digestion of exogenous proteins into small peptides that can be loaded onto MHC class I molecules is inherited from the phagotrophic properties of unicellular organisms, where internalized bacteria are degraded into basic molecules and used as a source of nutrients. Ancient processes have therefore been co-opted for new functionalities. The complete degradation of proteins in higher organisms is, however, restricted to favor the generation of antigenic peptides, notably through the action of IFN-γ on phagosome acidification and protease activity (Yates et al., 2007; Jutras et al., 2008; Trost et al., 2009). Cross-presentation in phagosomes is believed to be facilitated by the presence of molecular machines acquired through interactions with the ER (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Grotzeke et al., 2009). Interestingly, the presence of ER components has been shown in proteomics and morphological analyses of phagosomes from distant organisms, including Dicyostelium (Gotthardt et al., 2006; Dieckmann et al., 2008), Drosophila (Stuart et al., 2007), mouse (Garin et al., 2001; Trost et al., 2009), and human (Burlak et al., 2008). These studies clearly indicated that ER components were present on phagosomes before the advent of innate and adaptive immunity. In basal organisms, the ER could serve as an alternative source of membrane providing part of the material needed for the formation of a large number of phagosomes, or trigger spikes of localized Ca^{2+} concentration needed for phagocytosis (Cuttell et al., 2008). This concept is supported by the finding that downregulation of two ER proteins, calnexin and calreticulin, strongly inhibited phagocytosis in Dicyostelium (Muller-Taubenberger et al., 2001). Arguably, the presence of ER on phagosomes found a novel usage in ‘jawed’ vertebrates with the advent of the MHC locus, where several proteins involved in antigen presentation are encoded. Thus, peptides generated in the phagosome lumen potentially gained direct access to MHC class I molecules and the loading complex expressed in the ER, a process maximizing the ability of ER on phagosomes found a novel usage in ‘jawed’ vertebrates with the advent of MHC class I molecules and the loading complex expressed in the ER, a process maximizing the ability of ER to present exogenous peptides and stimulate CD8+ T cells (Bertholet et al., 2006). This alternative usage of molecular machines is often observed during evolution (True and Carroll, 2002). Another example of co-option is the contribution of the proteasome in antigen cross-presentation in mammals (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). Indeed, this complex, which we identified on phagosomes of J774 mouse macrophages, S2 Drosophila cells and AxA Dicyostelium cells was prepared according to previous methods (Desjardins et al., 1994; Stuart et al., 2007; Dieckmann et al., 2008). In order to maximize the proteomic identification coverage, early and late phagosomes were isolated for each organism. For Dicyostelium, three different preparations (5’/0’, 15’/0’, and 15’/15’) were mixed for early phagosomes, and late phagosomes (15’/45’, 15’/105’, and 15’/165’). For Drosophila and mouse, one early (30’/0’) and one late phagosome (30’/120’) preparation were analyzed. These samples were selected on the basis that they were sufficient for the identification of proteins largely exceeding the number identified on these organelles in previous studies.

Materials and methods

Phagosomes preparation

Phagosomes from 1774 mouse macrophages, S2 Drosophila cells and Ax2 Dicyostelium cells were prepared according to previous methods (Desjardins et al., 1994; Stuart et al., 2007; Dieckmann et al., 2008). In order to maximize the proteomic identification coverage, early and late phagosomes were isolated for each organism. For Dicyostelium, three different preparations (5’/0’, 15’/0’, and 15’/15’) were mixed for early phagosomes, and late phagosomes (15’/45’, 15’/105’, and 15’/165’).

Phagosomal protein identification by SDS–PAGE and MS

A sample of 20 μg of phagosomal proteins from Dicyostelium discoideum, D. melanogaster, and Mus musculus were reduced with tris-[2-carboxyethyl]phosphine (Pierce), alkylated with iodoacetamide (Sigma-Aldrich) and separated on a 4–12% pre-cast NuPAGE gel (Invitrogen). The gel was stained by colloidal Coomassie, and lanes were cut into 12 equal pieces using an in-house cutting device. The gel pieces were digested by trypsin (Promega, Madison, WI) and peptides extracted three times with 90% acetonitrile (ACN)/0.5M urea. Combined extracts were dried and re-suspended in 5% ACN, 0.1% trifluoro acetic acid (TFA) prior to MS analyses. Peptides were separated on a 150-μm ID, 10 cm reversed phase nano-LC column (Jupiter C18, 3 μm, 300 Å, Phenomenex) with a loading buffer of 0.2% formic acid (FA). Peptide elution was achieved by a gradient of 5–40% ACN in 70 min on an Eksigent 2D-nanoLC (Dublin, CA) operating at a flow rate of 600 nl/min. The nano-LC was coupled to an LTQ-Orbitrap mass spectrometer (Thermo- Electron, Bremen, Germany), and samples were injected in an interleaved manner. The mass spectrometer was operated in a data-dependent acquisition mode with a 1-s survey scan at 60 000 resolution, followed by three product ion scans (MS/MS) of the most abundant precursors above a threshold of 10 000 counts in the LTQ part of the instrument.

Phosphopeptide enrichment and MS

Phosphopeptide sample of 1.2 mg/replicate of early phagosomal proteins of Drosophila (four replicates) and Dictyostelium (two replicates) were reduced, cysteines blocked by carbamidomethylation and digested with trypsin. Subsequently, phosphopeptides were enriched on house-made TiO2 microcolumns (GL Science, Japan) as published before (Thingholm et al., 2006; Trost et al., 2009) and eluted with 30 μl 1% NH4OH. Eluates were acidified by adding TFA to a final concentration of 3%, dried down, re-suspended in 5% ACN, 0.1% TFA and subjected to mass spectrometric analysis. Peptides were separated on a self-packed 45 mm x 300 μm Polysulfoethyl column (Next Step, Southborough, MA) and online eluted in six fractions with 0 mM, 50 mM, 75 mM, 100 mM, 500 mM, and 2M ammonium formate, 2% ACN, 0.2% FA, pH 3.0 on a 150 μm ID, 10 cm reversed phase nano-LC column (Jupiter C18, 3 μm, 300 Å, Phenomenex) coupled to an LTQ-Orbitrap mass spectrometer using the same settings as described above.

Peptide identification

Peak detection of raw MS² spectra was performed using Mascot Distiller v2.2.2 (Matrix Science, UK) using the default Orbitrap
parameters. The centroided data were merged into single peak-list files and searched with the Mascot search engine v2.2.0 (Matrix Science, UK) against the combined forward and reversed mouse IPI protein database v3.37 (Kersey et al., 2004), the Uniprot Drosophila database v11.3, and the Dictybase database (Eichinger et al., 2005) v12.12.2008 (123,326, 13,522, and respective 13,391 forward protein sequences. Search conditions included trypsin as enzyme, one missed cleavage site, carbamidomethylation (C) as fixed modification and deamidation (N, Q), oxidation (M), phosphorylation (S, T, Y) as variable modifications. Precursor and fragment ion tolerances were set to 10 ppm and 0.5 Da, respectively. For protein identification, all assigned peptides with a MOWSE score > 15 were considered. Protein identification required at least two different peptides with combined score for unique peptide identification exceeding the score of the first reversed-database hit reaching 1%. This resulted in an FDR of <1% at the protein level. For the identification of phosphopeptides, all assigned peptides with a MOWSE score > 17 were considered. This resulted in an FDR of <1% on the peptide level.

Bioinformatics

All proteomics data, bioinformatics analyses, and cited databases of this paper were imported in a local MySQL database, and queried accordingly for specific requests. Custom Python scripts were written in order to parse and analyze the data and databases. To remove proteomics redundancy, identified proteins in early and late time points of each organism were merged together and clustered by gene names where the longest sequence of clustered proteins was kept as a cluster representative. Mouse proteins were annotated manually with a set of predefined 22 functions using Uniprot annotations and searching the literature. In order to perform proteomics cross-species comparison, the predicted orthologs for Dictyostelium (versus Drosophila and mouse) were extracted from the InParanoid database version 6.0 (Berglund et al., 2008), whereas predicted orthologs for Drosophila (versus mouse) and mouse (versus Drosophila) were extracted from the Ensembl database version 52.0 (Hubbard et al., 2009). Proteomics cross-species comparison of mouse phagosome proteins was performed against Drosophila and Dictyostelium’s phagosome proteins identified in this paper, but also against a second set of Drosophila and Dictyostelium phagosomes proteins already published (Gotthardt et al., 2006; Stuart et al., 2007). E-values of mouse orthologs (versus Drosophila and Dictyostelium) were determined by performing BLAST alignment (default parameters) using mouse sequences from the Uniprot mouse version 14.5. (Altschul et al., 1997) against the two other organisms sequence databases (Dictybase version 22.12.2008 and Flybase version 5.13) (Tweedie et al., 2009). Predicted mouse orthologs versus Drosophila and Dictyostelium (from InParanoid and Ensembl) were retrieved among the BLAST hits and the best relative E-value was retained. To plot E-values, we applied – Log10 (x) and plotted the log10(e-value) – Log10(e-value) of 1 (–Log10(e-value) = 0, point of origin) if proteins were devoid of any ortholog, and an e-value of 181 to the proteins that displayed a perfect alignment (the highest e-value).

To identify the origin of the mouse phagosome proteins, comparative analyses of 1324 phagosomal proteins were performed among 39 taxa (Rattus norvegicus, Cryptococcus neoformans, Monodelphis domestica, Giberella zonae, Neospora crassa, Bos taurus, Arabidopsis thaliana, Leitshmania major, Plasmodium falciparum, Schizosaccharomyces pombe, Yarrowia lipolytica, Tetraodon nigroviridis, Xenopus tropicalis, Plasmodium yoelii, Caenorhabditis briggsae, Saccharomyces cereseviae, Chlamydomonas reinhardtii, Ashbya gossypii, D. discoideum, Candida glabrata, Candida albicans, Fugu rubripes, C. elegans, Paramoecium tetraurelia, Pan troglodytes, Gallus gallus, Debaryomyces hansenii, Gillardia theta, Homo sapiens, Ciona intestinalis, Encephalitozoon cuniculi, D. melanogaster, Dario ranio, Kluyveromyces lactis, Anopheles gambiae, Canis familiaris, M. musculus, Macaca mulatta, Apis mellifera), by using the human phylome of PhylomeDB (PhylomeDB.org), a complete database of gene phylogenies (phylogenetic trees) (Storm et al., 2007, 2008). A set of 61 proteins from the mouse phagosome proteome that were not found in PhylomeDB were analyzed using Treefam, a second gene phylogeny database (Treefam.org) (Ruan et al., 2008). For each mouse proteins, orthologs were retrieved from their respective phylogenetic tree in order to identify the most basal species to assign a cladistic origin.

The extent of the effect of gene duplication in the remodeling of phagosomes during evolution was also addressed by identifying all the paralogs linked to the proteins constituting the mouse phagosome proteome, and their cladistic origin, using BioMart Ensembl version 56.0. Among all of these mouse paralogs, only the pairs for which both proteins were identified by MS/MS were retained in order to focus on the proteins that were effectively observed in our phagosome preparations. The same paralog analysis was performed from the Drosophila phagosome proteome and from rough and smooth reticulum endoplasmic proteomics data (Gilchrist et al., 2006).

Phosphorylation site localization

The nature of the MS/MS experiments does not always allow the identification of the exact site of phosphorylation within a phosphopeptide. We used a probability-based approach to identify the exact location using post-translational modification (PTM) scores by Mascot (Trost et al., 2009). In brief, the PTM score is 10 log(P0/P), where P is the probability. The inverted probabilities of all possible phosphorylations are summed up and set equal to one. Then, a proportional probability is assigned to each site and all probabilities for each site are summed up. Probabilities of sites are separated into three classes with class 1 (P > 0.75) being high-confidence identifications, class 2 (0.75 > P > 0.50) medium-confidence and class 3 (P < 0.50) low-confidence site identifications. However, it should be noted that even if the confidence level for a specific site is low, the peptides presented in Supplementary Datasets 7 and 8 are with a certainty of >99% phosphopeptides.

Conservation of phosphosites in drosophila, dicyostelium, and mouse phagosomal proteophosphoproteins

Orthology relationships among these three organisms were settled by InParanoid v6.0 (Berglund et al., 2008) (for Dictyostelium) and Ensembl v52.0 (Hubbard et al., 2009) (for Drosophila and mouse) databases. Orthologous groups were aligned using MUSCLE with default settings (Edgar, 2004). In these comparisons, a conserved phosphosite corresponded to a phosphorylated site in Mm that has a phosphorylatable residue (S/T/Y) in the homologous alignment position in the Dm or Dd protein. These alignments are available in HTML format in the Supplementary information. Finally, disordered regions of proteins were predicted using Disopred (Ward et al., 2004).

Conservation of phosphosites among chordates

In order to compare the evolution of phosphosites among chordates, we obtained orthologs of mouse phagosomal phosphoproteins from rat (R. norvegicus), human (H. sapiens), dog (C. familiaris), opposum (M. domestica), chicken (G. gallus), xenopus (X. tropicalis), zebrafish (D. rerio), sea squirt (C. intestinalis), and drosophila (D. melanogaster) from Ensembl (ensembl.org). Mouse phosphoproteins that have an ortholog in each of these species were aligned (MUSCLE, as above), resulting in a total of 230 orthologous groups (620 phosphosites). Conservation of mouse phosphosites was then analyzed by examining the conservation of the phosphorylatable residues at the orthologous positions of the other species. Finally, we compared the extent of conservation of a phosphosite regulated by IFN-γ to that of non-IFN-γ-regulated phosphosites. We measured the extent of conservation of a phosphosite by the number of species sharing a serine or threonine at that position in the multiple alignments. We then calculated an average conservation for all IFN-γ-regulated sites, and compared it to that of a hundred samples of non-IFN-γ-regulated phosphosites. All statistical analyses were performed in R (r-project.org).

Network analyses

Proteins from this and former (Trost et al., 2009) experiments were mapped against the Uniprot v15.6 to obtain human orthologs. This was necessary as there are very few mouse protein–protein

Molecular characterization of the evolution of phagosomes

J Boulais et al
interactions in the Intact database (Kerrien et al., 2007). Experimental protein–protein interaction data were extracted from the Intact database v(31.07.2009). We then added manually and through automated parsing known interactions from the curated ‘subunit’ comment field of UniProt v15.6. The network was loaded into Cytoscape v2.2.1 (cytoscape.org) for visualization. Subnetworks of proteins involved in immunity and the cytoketoskeleton were generated using a GO-term analysis described elsewhere (Trost et al., 2009) and manually arranged in Cytoscape.

Supplementary information

Supplementary information is available at the Molecular Systems Biology website (http://www.nature.com/msb).

Acknowledgements

We thank Christiane Rondeau, Annie Laplante for technical assistance, and Sébastien Lemieux and Manuel Santos for insightful bioinformatic discussions. This work was supported by research grants from the Natural Sciences and Engineering Research Council of Canada (JB), the Canadian Institute for Health Research (MD), and the Human Frontier Science Program (MD). CRL is a CIHR new investigator. EDL acknowledges financial support from the HFSP post-doctoral program.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Ackerman AL, Kyritsis C, Tampe R, Cresswell P (2003) Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proc Natl Acad Sci USA 100: 12889–12894

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Barabasi AL, Oltvai ZN (2004) Network biology: understanding the cell’s functional organization. Nat Rev Genet 5: 101–113

Bertrand S, Sijlrand E, Ostlund G, Sonnhammer EL (2008) InParanoid 6: eukaryotic ortholog clusters with inparalogs. Nucleic Acids Res 36: D263–D266

Bertholet S, Goldszmid R, Morrot A, Collazo-Custodio C, Houdé M, Desjardins M, Sher A, Sacks D (2006) Leishmania antigens are presented to CD8+ T cells by a transporter associated with antigen processing-independent pathway in vitro and in vivo. J Immunol 177: 3525–3533

Bhattacharya D, Yoon HS, Hedges SB, Hacket JD (2009) Eukaryotes (Eukarya). In The Timetree of Life, Hedges SBAK, S (ed), pp 116–120. New York: Oxford University Press

Brown CJ, Takayama S, Campen AM, Vise P, Marshall TW, Oldfield CJ, Williams CJ, Dunker AK (2002) Evolutionary rate heterogeneity in the secretory pathway. J Cell Biol 152: 165–180

Cavaleri-Smith T (2002) The phagotrophic origin of eukaryotes and the cytoketoskeleton were generated using a GO-term analysis described elsewhere (Trost et al., 2009) and manually arranged in Cytoscape.

Cuttell L, Vaughan A, Silva E, Escarón CJ, Lavine M, Van Goethem E, Eid JP, Quirin M, Franc NC (2008) Undertaker, a Drosophila junctionophilin, links Draper-mediated phagocytosis and calcium homeostasis. Cell 135: 524–534

Dacks JB, Poon PP, Field MC (2008) Phylogeny of endocytic components yields insight into the process of nonendosymbiotic organelle evolution. Proc Natl Acad Sci USA 105: 588–593

Dafourn TR, Smith CJ (2004) Natively unfolded domains in endocytosis: hooks, lines and linkers. EMBO Rep 5: 1046–1052

Dermitie JF, Duclos S, Garin J, St-Louis F, Rea S, Parton RG, Desjardins M (2001) Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. J Biol Chem 276: 18507–18512

Desjardins M, Celis JE, van Meer G, Dieckmann R, Lehmann R, Hamlin N, Davies R, Gaudet P et al (2005) The genome of the social amoeba Dictyostelium discoideum. Nature 435: 43–57

Eisenberg E, Levanon E (2005) Phagocytosis: the convoluted way from nutrition to adaptive immunity. Immunol Rev 207: 158–165

Dieckmann R, Gopalass N, Escalera C, Soldati T (2008) Monitoring time-dependent maturation changes in purified phagosomes from Dictyostelium discoideum. Methods Mol Biol 445: 327–337

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5: 113

Eichinger L, Pacheco JA, Glenckier G, Rajandream MA, Sugasag R, Berriman M, Song J, Olsen R, Szafirski K, Xu Q, Tunggal B, Kumfermel S, Madera M, Konfortov BA, Rivero F, Bankier AT, Lehmann R, Hamlin N, Davies R, Gaudet P et al (2005) The genome of the social amoeba Dictyostelium discoideum. Nature 435: 43–57

Eisenberg E, Levanon E (2005) Phagocytosis: the convoluted way from nutrition to adaptive immunity. Immunol Rev 207: 158–165

Emes RD, Pocklington AJ, Anderson CN, Bayes A, Collins MO, Vickers CA, Croning MD, Malik BR, Choudhary JS, Armstrong JD, Grant SG (2008) Evolutionary expansion and anatomical specialization of synapse proteome complexity. Nat Neurosci 11: 799–806

Erkisson HP (2007) Evolution of the cytoktoskeleton. Bioessays 29: 668–677

Fritz-Laylin LK, Prochnik SE, Ginger ML, Dacks JB, Carpenter ML, Field MC, Kuo A, Paredes A, Chapman J, Pham J, Shu S, Neupane R, Cipriano M, Mancuso J, Tu H, Salamon A, Lindquist E, Shapiro H, Lucas S, Grigoriev IV et al (2010) The genome of Naegleria gruberi illuminates early eukaryotic versatility. Cell 140: 631–642

Garin J, Diez R, Kieffer S, Derminie JD, Duclos S, Gagneron E, Sadoul R, Rondeau C, Desjardins M (2001) The phagosome proteome: insight into phagosome functions. J Cell Biol 152: 165–180

Gilchrist A, Au CE, Hiding J, Bell A W, Fernandez-Rodriguez J, Lesimple N, Nilsson T, Bergeron JJ (2006) Quantitative proteomics analysis of the secretory pathway. Cell 127: 1265–1281

Gotthardt D, Blanchetou V, Bossierhoff A, Ruppert T, Delorenzi M, Soldati T (2006) Proteomics fingerprinting of phagosome maturation and evaluation for the role of a Galpha during uptake. Mol Cell Proteomics 5: 2228–2243

Grotzke JE, Harriff MJ, Siler AC, Noit D, Delepine J, Lewinsohn DA, Lewinsohn DM (2009) The Mycobacterium tuberculosis phagosome is a HLA-I processing competent organelle. PLoS Pathog 5: e1000374

Guemondrez P, Saveau L, Kleijmeer M, Davoust J, Van Endert P, Amorgenasa S (2003) ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425: 397–402

Hatherley D, Graham SC, Harlos K, Stuart DI, Barclay AN (2009) Structure of signal-regulatory protein alpha: a link to antigen receptor evolution. J Biol Chem 284: 26613–26619

Hedges SB (2009) Vertebrates (Vertebrata). In The Timetree of Life, Hedges SBAK, S (ed), pp 309–314. New York: Oxford University Press
Molecular characterization of the evolution of phagosomes
J Boulaïs et al

Houde M, Berthrolet S, Gagnon E, Brunet S, Goyette G, Laplante A, Princotta MF, Thibault P, Sacks D, Desjardins M (2003) Phagosomes are competent organelles for antigen cross-presentation. Nature 425: 402–406

Hubbard TJ, Aken BL, Ayling S, Ballester B, Beal K, Bragan E, Brent S, Chen Y, Clapham P, Clarke L, Coates G, Fairley S, Fitzgerald S, Fernandez-Banet J, Gordon L, Graf S, Haider S, Hammond M, Holland R, Howe K et al (2009) Ensembl 2009. Nucleic Acids Res 37: D690–D697

Huerta-Cepas J, Bueno A, Dopazo J, Gabaldon T (2008) PhylomeDB: a database for genome-wide collections of gene phylogenies. Nucleic Acids Res 36: D491–D496

Huerta-Cepas J, Dopazo H, Dopazo J, Gabaldon T (2007) The human phylome. Genome Biol 8: R109

Jutras I, Desjardins M (2005) Phagocytosis: at the crossroads of innate and adaptive immunity. Anna Rev Cell Dev Biol 21: 511–527

Jutras I, Houde M, Currier N, Boulais J, Duclos S, LaBoissiere S, Bonnell E, Kearney P, Thibault P, Paramithiotis E, Hugo P, Desjardins M (2008) Modulation of the phagosome proteome by interferon-gamma. Mol Cell Proteomics 7: 697–715

Kasahara M, Suzuki T, Pasquier LD (2004) On the origins of the adaptive immune system: novel insights from invertebrates and cold-blooded vertebrates. Trends Immunol 25: 105–111

Kerrien S, Alam-Faruque Y, Aranda B, Bancarz I, Bridge A, Derow C, Dimmer E, Feuermann M, Friedrichsen A, Hunley R, Kohler C, Khadake J, Leroy C, Liban A, Liefitck C, Montecchi-Palazzi L, Orchard S, Risse J, Robbe K, Roechert B et al (2007) IntAct–open source resource for molecular interaction data. Nucleic Acids Res 35: D561–D565

Kersey PJ, Duarte J, Williams A, Karavidopoulou Y, Birney E, Apweiler R (2004) The International Protein Index: an integrated database for proteomics experiments. Proteomics 4: 1985–1988

Landry CR, Levy ED, Michnick SW (2009) Weak functional constraints on phosphoproteomes. Trends Genet 25: 193–197

Li J, Rechsteiner M (2001) Molecular dissection of the 11S REG (PA28) proteasome activators. Biochimie 83: 373–388

Muller-Taubenberger A, Lupas AN, Li H, Ecke M, Simmeth E, Gerisch G (2001) Calreticulin and calnexin in the endoplasmic reticulum are proteasome activators. Biochimie 83: 373–388

 requirements for the endoplasmic reticulum. Nature 450: 1253–1257

Sanjuan MA, Dillon CP, Tait SW, Moshiachi S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S, Green DR (2007) Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature 450: 1253–1257

Savan R, Ravichandran S, Collins JR, Sakai M, Young HA (2009) Structural conservation of interferon gamma among vertebrates. Cytokine Growth Factor Rev 20: 115–124

Sepulcre MP, Alcaraz-Perez F, Lopez-Munoz A, Roca FJ, Meseguer J, Cayuela ML, Mulero V (2009) Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. J Immunol 182: 1836–1845

Silva E, Au-Teung HW, Van Goethem E, Burden J, Franc NC (2007) Requirement for a Drosophila E3-ubiquitin ligase in phagocytosis of apoptotic cells. Immunity 27: 585–596

Stuart LM, Boulais J, Charriere GM, Hennessy EJ, Brunet S, Jutras I, Goyette G, Rondeau C, Letarte S, Huang H, Ye P, Morales F, Kocks C, Bader JS, Desjardins M, Ezekowitz RA (2007) A systems biology analysis of the Drosophila phagosome. Nature 445: 95–101

Stuart LM, Ezekowitz RA (2008) Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol 8: 131–141

Thingholm TE, Jorgensen TJ, Jensen ON, Larsen MR (2006) Highly selective enrichment of phosphorylated peptides using titanium dioxide. Nat Protoc 1: 1929–1935

Tompa P (2005) The interplay between structure and function in intrinsically unstructured proteins. FEBS Lett 579: 3346–3354

Trost M, English L, Lemieux S, Courcelles M, Desjardins M, Thibault P (2009) The phagosomal proteome in interferon-gamma-activated macrophages. Immunity 30: 143–154

True JR, Carroll SB (2002) Gene co-option in physiological and morphological evolution. Anna Rev Cell Dev Biol 18: 53–80

Tweddle S, Ashburner M, Falis K, Leyland P, McQuilton P, Marxgold S, Millburn G, Osumi-Sutherland D, Schroader A, Seal R, Zhang H (2009) FlyBase: enhancing Drosophila Gene Ontology annotations. Nucleic Acids Res 37: D555–D559

Ward JJ, Siddhi JS, McCuffin LJ, Buxton BF, Jones DT (2004) Prediction and functional analysis of native disorder in proteins from the three kingdoms of life. J Mol Biol 337: 635–645

Wickstead B, Gull K, Richards TA (2010) Patterns of kinesin evolution reveal a complex ancestral eukaryote with a multifunctional cytoskeleton. BMC Evol Biol 10: 110

Yates RM, Hermetter A, Taylor GA, Russell DG (2007) Macrophage activation downregulates the degradative capacity of the phagosome. Traffic 8: 241–250

Yutin N, Wolf MY, Wolf YI, Koonin EV (2009) The origins of phagocytosis and eukaryogenesis. Biol Direct 4: 9

Molecular Systems Biology is an open-access journal published by European Molecular Biology Organization and Nature Publishing Group. This work is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License.
Real data.
Real installations.
Real super-resolution imaging.

Learn more about the DeltaVision OMX super-resolution imaging system at www.superresolution.com.

Really.