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Molecular characterization of the evolution of phagosomes

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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.
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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 (Mushegian 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 linking 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

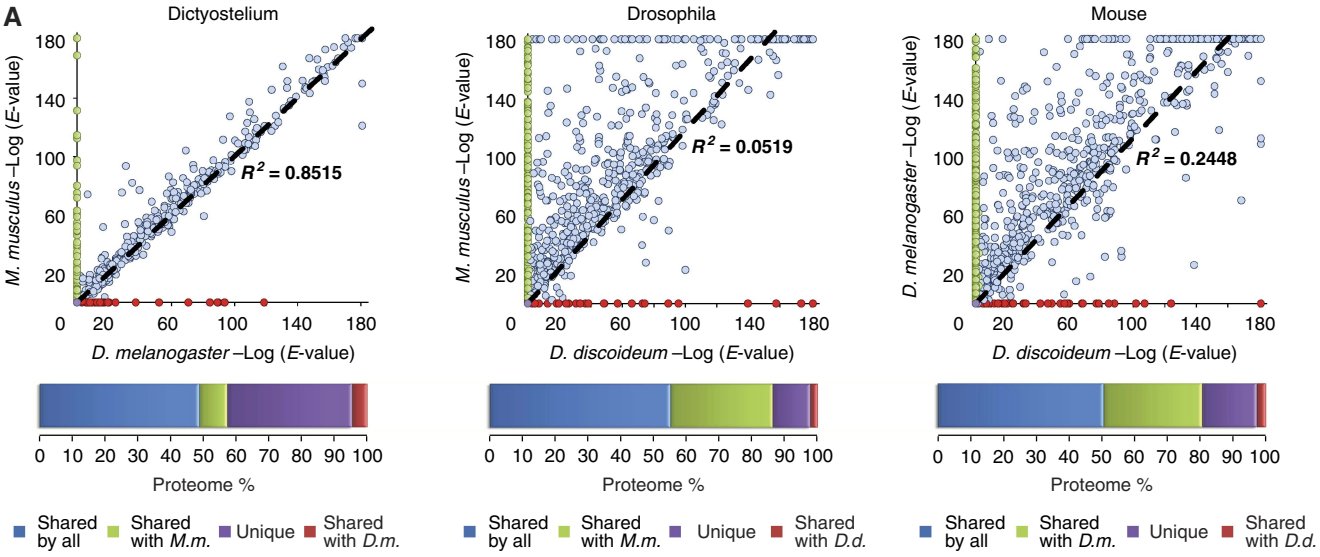
Proteomics analyses of phagosomes

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 $-\mathrm{Log}_{10}$(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.


A
Dictyostelium
Drosophila
Mouse
M. musculus –Log (E-value)
D. melanogaster –Log (E-value)
R² = 0.8515
R² = 0.0519
R² = 0.2448
D. melanogaster –Log (E-value)
D. discoideum –Log (E-value)
D. discoideum –Log (E-value)
Proteome %
Proteome %
Proteome %
Shared by all
Shared with M.m.
Unique
Shared with D.m.
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Shared with D.d.
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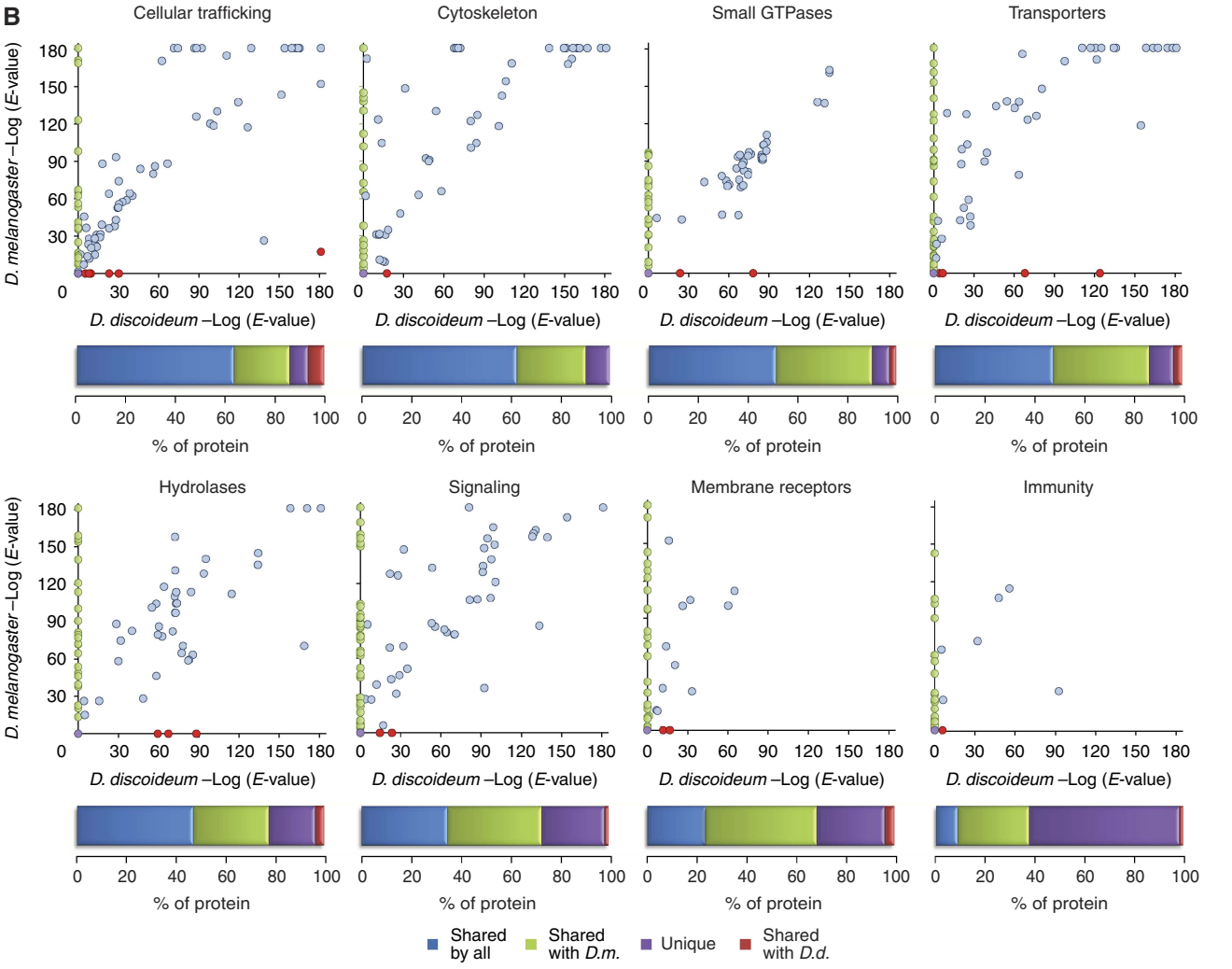
B
Cellular trafficking
Cytoskeleton
Small GTPases
Transporters
Hydrolases
Signaling
Membrane receptors
Immunity
D. melanogaster –Log (E-value)
D. discoideum –Log (E-value)
% of protein
Shared by all
Shared with D.m.
Unique
Shared with D.d.

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'-recognition) (Hatherley *et al*, 2009) and several proteins of unknown functions. Therefore, we used the term 'late adaptive immunity' to highlight the fact that these 50 phagosome proteins emerged when adaptive immunity was already well established.

Refinement of the phagosome functions occurred during two major periods of gene duplication

Gene duplication and the expansion of gene families produced organelle complexity by functional gain during evolution (Cavalier-Smith, 2002; Dacks *et al*, 2008). To determine whether this process had a significant impact on phagosomal properties, we identified paralogs present in the mouse phagosome proteome and determined the origin of their duplication during evolution by using the Ensembl database. Our analyses indicate that 74.5% of the mouse phagosome proteins could be paired with one or more paralogs. Of these, 50.0% have been identified by MS/MS on the mouse phagosome, accounting for a total of 952 paralogs pairs. Comparative analyses reveal that the majority of these paralogs (79.1%) originated from proteins present in a phagotrophic ancestor (Figure 3A). The duplication of a large proportion of these proteins occurred in Bilateria (39.6%) and Euteleostomi (44.1%), coinciding with periods that saw the emergence of innate and adaptive immunity (Figure 3B, Supplementary Dataset 8). This rate of duplication differs markedly from that observed for the whole mouse genome. Interestingly, much less difference was observed when we compared the rate of duplication of the proteins constituting the proteome of the smooth or rough endoplasmic reticulum (sER and rER) (Gilchrist *et al*, 2006) with that of the corresponding rat genome (Supplementary Figure S3A). Duplication in the mouse phagosomal proteome has influenced proteins such as GTPases and SNAREs, regulating membrane fusion events, as well as hydrolases involved in the acquisition of phagosomal lytic properties (Figure 3C). Analysis of the Drosophila phagosome proteome indicated that gene duplication also contributed to the complexification of this organelle in Bilateria (Supplementary Figure S3B). Altogether, these results highlight the importance of gene duplication in the emergence of functional phagosome properties.

Evolution of the phagosome phosphoproteome

Phosphorylation has important roles in the regulation of phagosome functions (Trost *et al*, 2009). To determine the extent to which the phagosome phosphoproteome has been modified during evolution, we performed comparative analyses to determine the level of phosphosite conservation among a group of 10 organisms ranging from Drosophila to mouse. To do so, we used the mouse phagosome phosphoproteome data published recently, where 2949 phosphosites were precisely identified and mapped on 1166 proteins (Trost *et al*, 2009). The alignment of these proteins with their respective orthologs in the 10 chosen organisms allowed us to align 534 phosphosites from 238 phosphoproteins (Figure 4A). These alignments reveal that a small proportion of the phosphosites were conserved prior to the emergence of

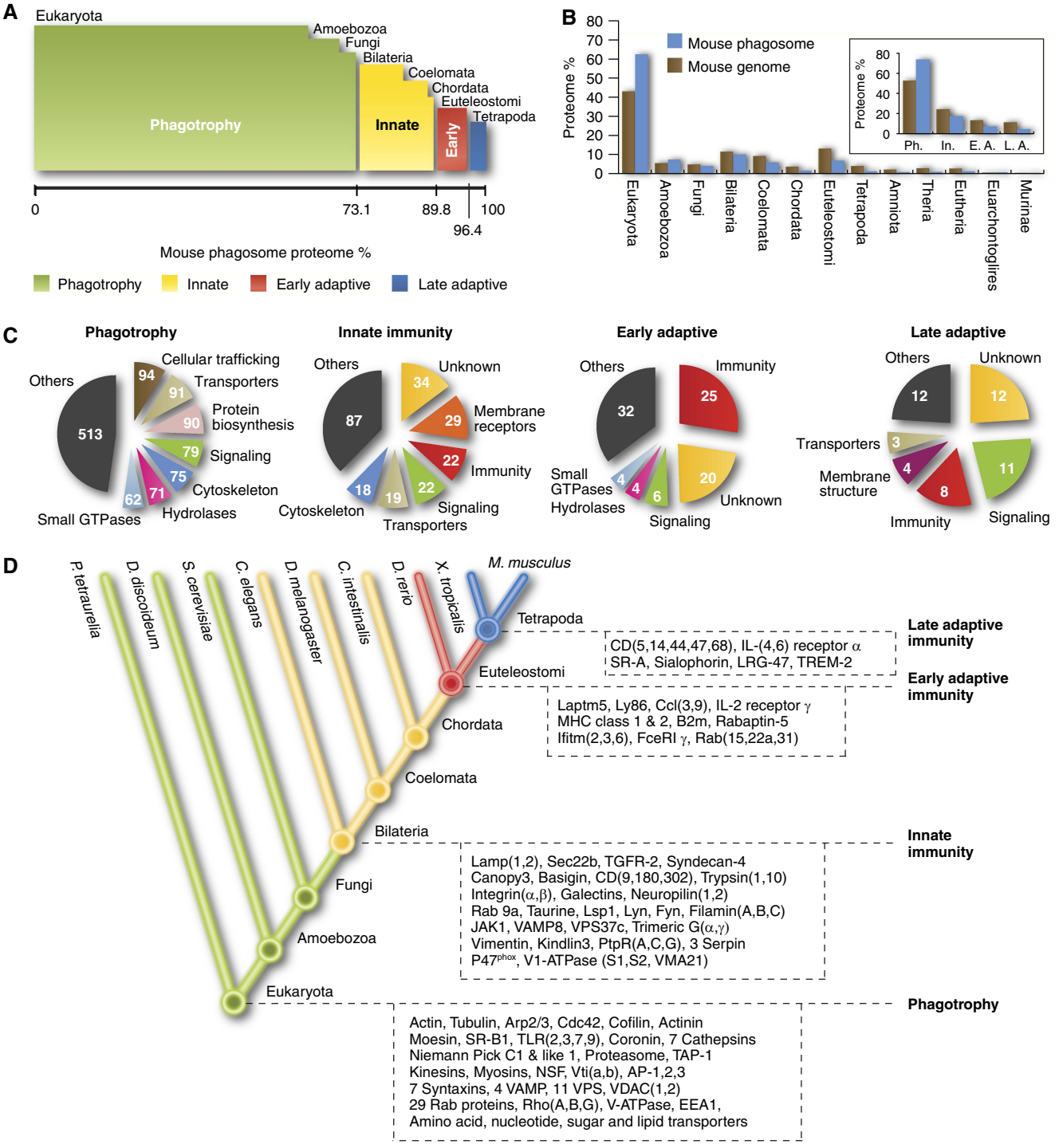

Figure 2 Origin of the mouse phagosome proteome. Comparative analyses of the mouse phagosome proteome among 39 taxa identified the origin of each protein. (**A**) Proportions (in %) of the evolutionary origin of the mouse phagosome proteome are reported through four major evolutionary groups of proteins: 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.

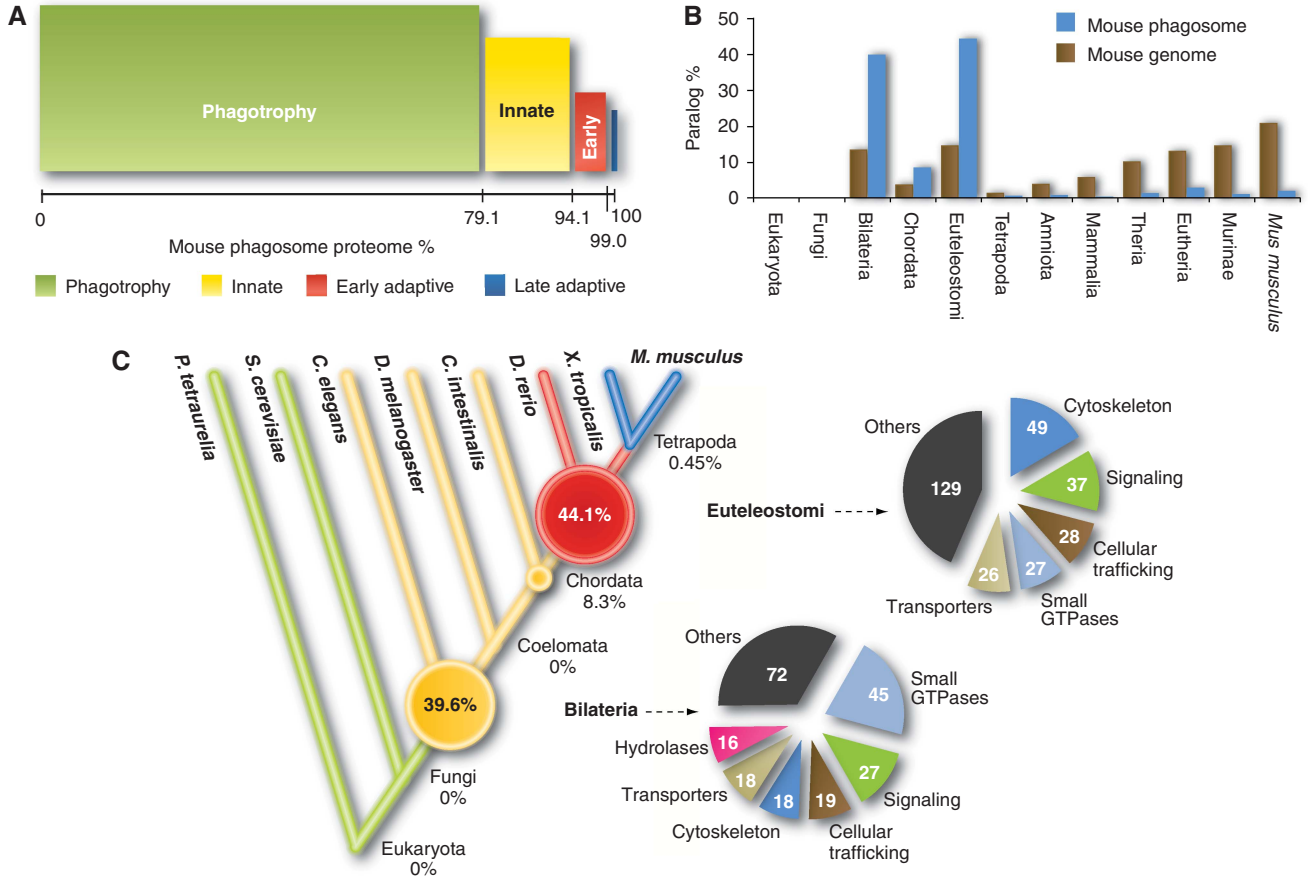

Figure 3 Novel components of the mouse phagosome emerged through two major periods of gene duplication. (**A**) Of 952 pairs of duplicated genes encoding for mouse phagosomal proteins, the majority of these genes have their origin in the phagotrophy stage of evolution. (**B**) Proportional representation of the origin of gene duplication events on the phagosome and the whole mouse genome shows that duplication of genes encoding mouse phagosomal proteins occurred mainly in Bilateria (emergence of innate immunity) and Euteleostomi (emergence of adaptive immunity), whereas gene duplication in the whole murine genome occurred more evenly throughout evolution. (**C**) Functional analysis of phagosomal proteins duplicated originally in bilaterians and euteleosts reveals a preference for small GTPases, signaling, and proteins involved in cellular trafficking. The function 'Others' contains the merging of remaining functions. See also Supplementary Figure S3 and Supplementary Dataset 8.

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

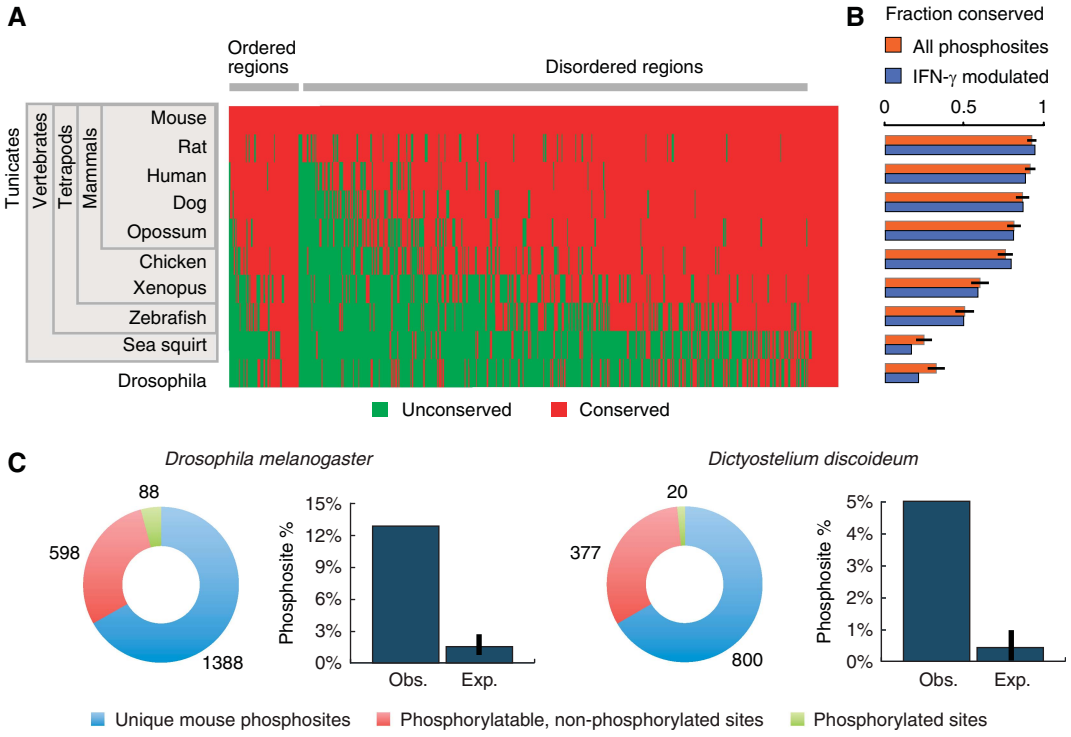

Figure 4 Evolution of the phagosome phosphoproteome. (**A**) Alignment of mouse phagosomal phosphoproteins revealed strong conservation of phosphosites within mammals, but fewer phosphosites are conserved across vertebrates, chordates, and tetrapods. In proportion, a larger fraction of conserved phosphosites (in red) is observed in ordered regions compared with disordered regions. (**B**) Phosphosites modulated by IFN-γ are on average as conserved as other phosphorylated residues in vertebrates but not in tunicates or Drosophila. (**C**) Comparative alignment of conserved mouse, Drosophila, and Dictyostelium phagosomal phosphoproteins identified by MS revealed that the majority (near 66%—doughnut plot) of phosphosites are not conserved (blue), indicating that the mouse phagosome phosphoproteome is globally recent in evolution. Still, around 33% of mouse phosphosites are phosphorylatable (red) in Drosophila or Dictyostelium. Of these, 12.8 and 5.0% phosphosites were observed to be phosphorylated (green) in Drosophila and Dictyostelium phagosomes, respectively (Obs.). These phosphosites are 8 and 12 times more conserved compared with random S/T/Y (expected, Exp.) of the same phosphoproteins in Drosophila and Dictyostelium, respectively. Bars indicate 95% confidence interval.

higher than what would be expected if an equivalent ratio of phosphorylatable residues sampled randomly among S/T/Y of the same set of mouse phosphoproteins would align by chance with the phosphorylated residues of Drosophila and Dictyostelium (Figure 4C; Supplementary Figure S4C). Altogether, our results indicate that although the phagosome phosphoproteome has been extensively modified during evolution, some phosphosites have been maintained for >1.2 billion years (Bhattacharya *et al*, 2009), highlighting their particular significance in the regulation of key phagosomal functions.

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 phosphoproteome are elements that contributed to the acquisition of new phagosome 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 (interactions/protein: phagotrophy, 2.8; innate immunity, 3.0; adaptive immunity, 2.8), 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*

Table I Interaction levels of the phagosome network within different phagocytosis purpose

	Nodes	Edges (degree)	Versus phagotrophy	Versus innate	Versus adaptive
Phagotrophy	864	2387 (2.76)	1589 (1.84)	511 (0.59)	287 (0.33)
Innate immunity	243	723 (2.98)	511 (2.10)	118 (0.49)	94 (0.39)
Adaptive immunity	151	419 (2.77)	287 (1.90)	104 (0.62)	38 (0.25)
Total network	1258	2637 (2.10)			

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

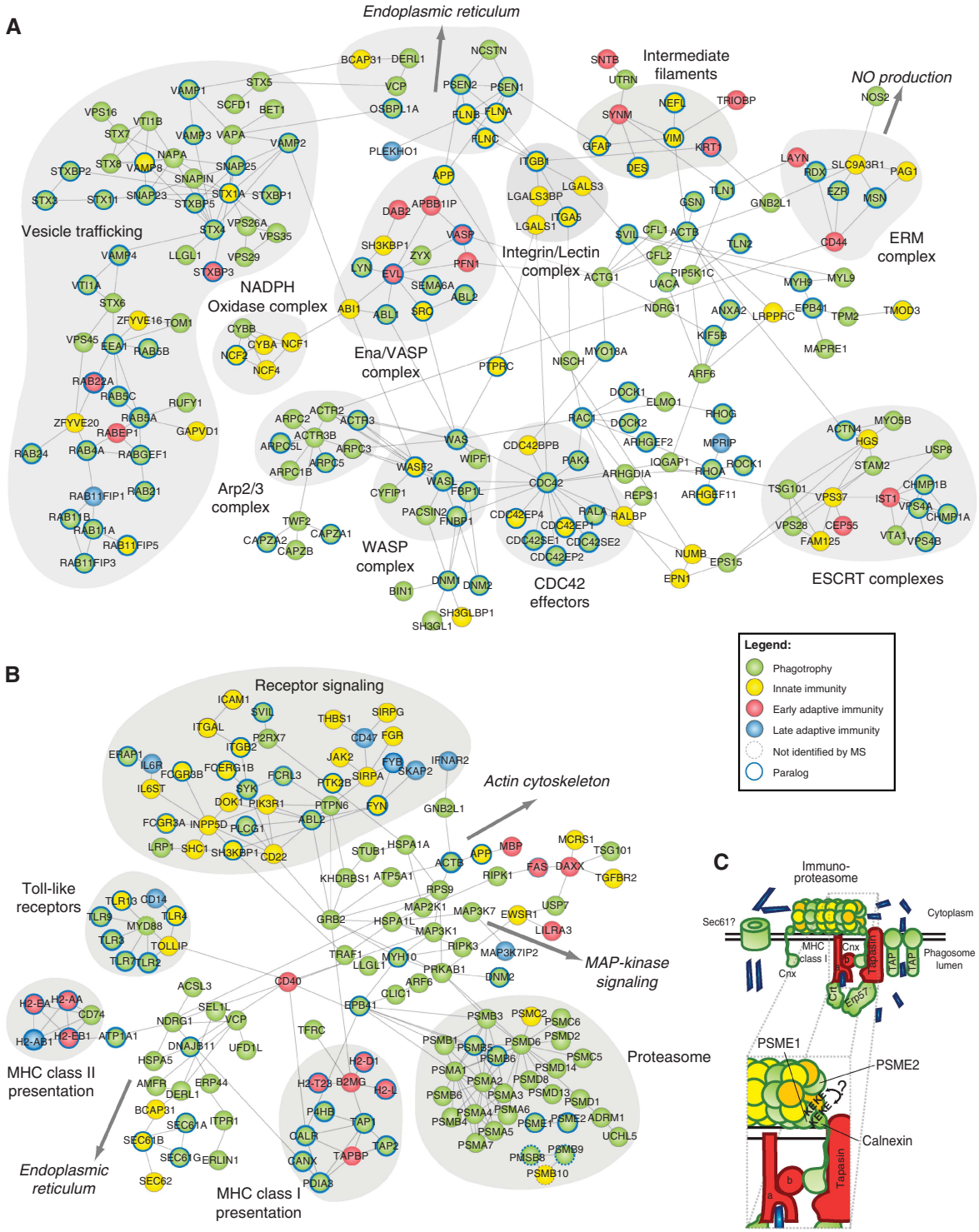

Figure 5 Evolution of the phagosome proteins network. Experimental data from the Intact database and curated entries of the UniProt database were used to generate a network from protein–protein interactions of identified mouse phagosomal proteins. From the total network, subnetworks of cytoskeleton and 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.

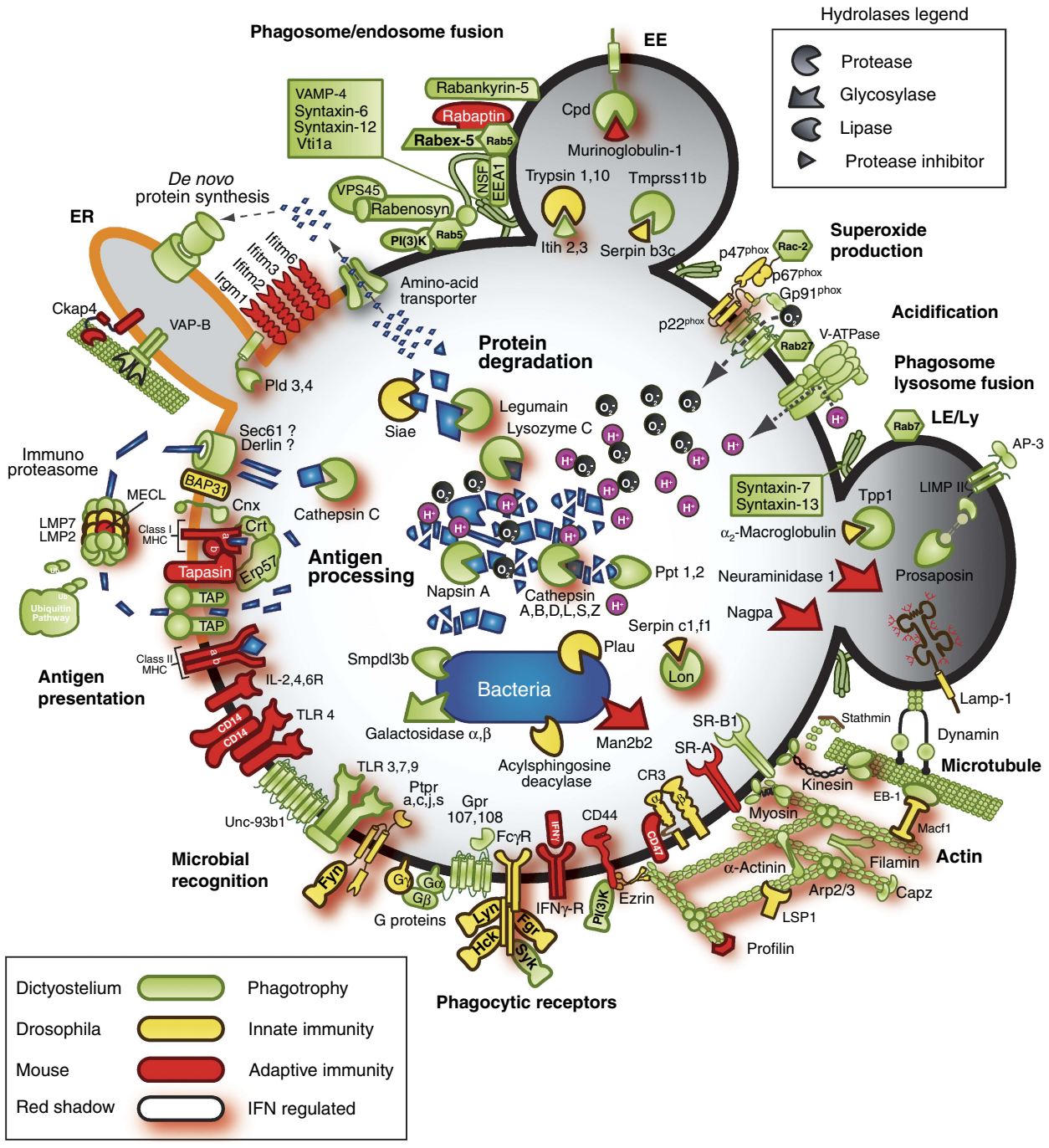

Figure 6 Role of molecular machines of mixed origin in phagosome functions. Many of the functional properties of mammalian phagosomes involve molecular machines made of proteins that emerged at different periods during evolution. For example, in the context of antigen cross-presentation, key steps such 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).

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.

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; Grotzke *et al*, 2009). Interestingly, the presence of ER components has been shown in proteomics and morphological analyses of phagosomes from distant organisms, including Dictyostelium (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*, 2006). 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 Dictyostelium (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 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 all three species, was proposed to have a function in the degradation of endogenous proteins for phagotrophy in the ancestral eukaryotes (Cavalier-Smith, 2009), and contributes to the recycling of self-components from apoptotic cells internalized by phagocytosis in Drosophila (Silva *et al*, 2007). Interestingly, remodeling of the phagosome proteome has continued to occur after the establishment of adaptive immunity in teleosts, with the integration of at least 50 proteins in tetrapods, including several transmembrane proteins of unknown function. Detailed analyses of these proteins should provide further understanding of the molecular mechanisms conferring specialized functions to mammalian phagosomes linking innate and adaptive immunity.

Materials and methods

Phagosomes preparation

Phagosomes from J774 mouse macrophages, S2 Drosophila cells and Ax2 Dictyostelium 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 Dictyostelium, 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.

Phagosomal protein identification by SDS–PAGE and MS

A sample of 20 μg of phagosomal proteins from *Dictyostelium 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)/0.5 M 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, Phenomex) 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 TiO_2 microcolumns (GL Science, Japan) as published before (Thingholm *et al*, 2006; Trost *et al*, 2009) and eluted with 30 μl 1% NH_4OH. 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 × 300 μm Polysulfoethyl column (Nest Group, 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, Phenomex) coupled to an LTQ-Orbitrap mass spectrometer using the same settings as described above.

Peptide identification

Peak detection of raw MS^2 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 v2.20 (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) v(22.12.2008) containing 52 326, 13 522, and respective 13 391 forward protein sequences. Search conditions included trypsin set 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 p.p.m. and 0.5 Da, respectively. For protein identification, all assigned peptides with a MOWSE score >15 were considered. Proteins 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 $-\mathrm{Log}_{10}$ (e-value) and assigned an e-value of 1 ($-\mathrm{Log}_{10}$(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 zeae, Neurospora crassa, Bos taurus, Arabidopsis thaliana, Leishmania major, Plasmodium falciparum, Schizosaccharomyces pombe, Yarrowia lipolytica, Tetraodon nigroviridis, Xenopus tropicalis, Plasmodium yoelii, Caenorhabditis briggsae, Saccharomyces cerevisiae, Chlamydomonas reinhardtii, Ashbya gossyppii, D. discoideum, Candida glabrata, Candida albicans, Fugu rubripes, C. elegans, Paramecium tetraurelia, Pan troglodytes, Gallus gallus, Debaryomyces hansenii, Gillardia theta, Homo sapiens, Ciona intestinalis, Encephalitozoon cuniculi, D. melanogaster, Danio rerio, 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 (phylomes) (Huerta-Cepas *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 $-10\times\log_{10}(P)$, 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 dictyostelium, drosophila, and mouse phagosomal phosphoproteins

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) at 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 the phosphosites 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

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 v.2.51 (cytoscape.org) for visualization. Subnetworks of proteins involved in immunity and the cytoskeleton 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).

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Conflict of interest

The authors declare that they have no conflict of interest.

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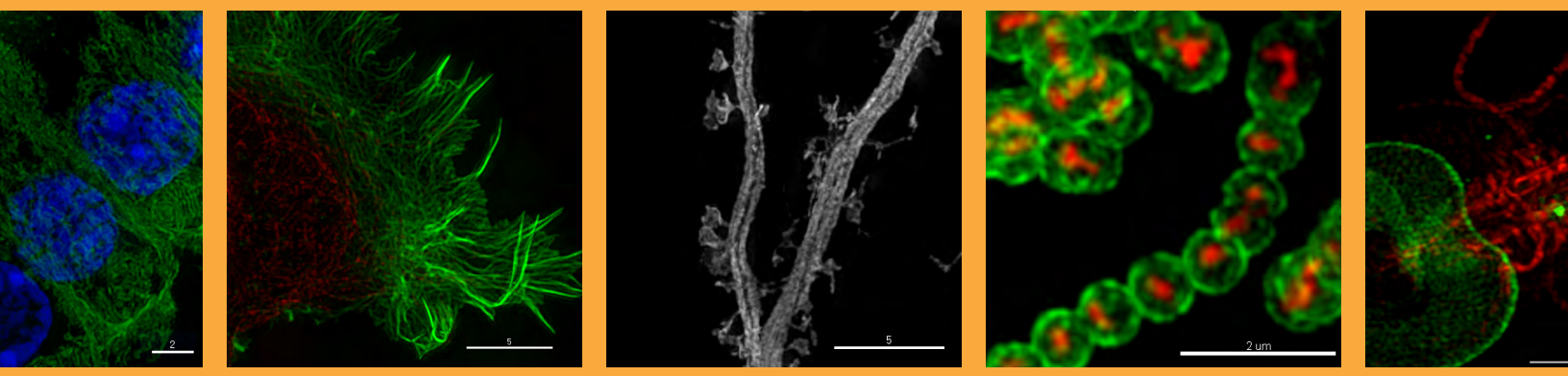

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