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Genomic Analysis of Organismal Complexity in the Multicellular Green Alga *Volvox carteri*

Simon E. Prochnik, *et al.*

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the FANCD2 exonuclease (3′ to 5′) is predicted to generate 5′ ssDNA tails, contrary to a general view that only 3′ ssDNA tails stimulate recombination. However, recent biochemical work shows that the fungal ortholog of FANCD1/BRCA2 is able to promote HR by using 5′ ssDNA tails (*17*–*19*). We therefore speculate that FANCD2 may function with BRCA2 in a similar manner to repair DSBs generated during cross-link repair.

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Genomic Analysis of Organismal Complexity in the Multicellular Green Alga *Volvox carteri*

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The multicellular green alga *Volvox carteri* and its morphologically diverse close relatives (the volvocine algae) are well suited for the investigation of the evolution of multicellularity and development. We sequenced the 138–mega–base pair genome of *V. carteri* and compared its ~14,500 predicted proteins to those of its unicellular relative *Chlamydomonas reinhardtii*. Despite fundamental differences in organismal complexity and life history, the two species have similar protein-coding potentials and few species-specific protein-coding gene predictions. *Volvox* is enriched in volvocine-algal–specific proteins, including those associated with an expanded and highly compartmentalized extracellular matrix. Our analysis shows that increases in organismal complexity can be associated with modifications of lineage-specific proteins rather than large-scale invention of protein-coding capacity.

Multicellularity and cellular differentiation evolved independently in diverse lineages, including green and red algae, animals, fungi, plants, Amoebozoa, and Chromalveolates (*1*) (fig. S1A), yet the genetic changes that underlie these transitions remain poorly understood. The volvocine algae, which include both unicellular and multicellular species with various levels of morphological and developmental complexity, are an appealing model for studying such an evolutionary transition (*2*) [fig. S2 and supporting online material (SOM) text]. Multicellular *Volvox carteri* (hereafter *Volvox*) has two cell types: ~2000 small biflagellate somatic cells that are embedded in the surface of a transparent sphere of glycoprotein-rich extracellular matrix (ECM), and ~16 large reproductive cells (termed gonidia) that lie just below the somatic cell monolayer (*2*) (Fig. 1A and figs. S1B and S3). The somatic cells resemble those of *Chlamydomonas reinhardtii*, a model unicellular volvocine alga (*3*) (Fig. 1B). The changes that are associated with the evolution of *Volvox* from a *Chlamydomonas*-like unicellular ancestor have clear parallels in other multicellular lineages but took place more recently than in land plants and animals (*4*).

To begin to characterize the genomic features that are associated with volvocine multicellularity, we sequenced the 138–mega–base pair (Mbp) *Volvox* genome to ~11.1× redundant coverage (~2.9 million reads) using a whole-genome shotgun strategy (*5*). The assembly captures over 98% of known mRNA sequences and expressed sequence tags (ESTs) (*5*). The *Volvox* nuclear genome is 19.6 Mbp (17%) larger than the *Chlamydomonas* genome (Table 1), primarily because of increased repeat content in *Volvox* relative to *Chlamydomonas* (*5*) (table S1). Whereas a few repeat families show bursts of expansion in the *Volvox* and *Chlamydomonas* lineages, most have changed gradually (fig. S4) (*5*).

The sequence divergence between *Volvox* and *Chlamydomonas* is comparable to that between human and chicken [which diverged ~310 million years ago (Ma)], human and frog (~350 Ma), and *Arabidopsis* and poplar (~110 Ma), based on the frequency of synonymous transversions at fourfold-degenerate sites (4DTV distance) (*5*, *6*) (table S2). Although conserved synteny between *Volvox* and *Chlamydomonas* genomes is evident, these volvocine algae show higher rates of genomic rearrangement than vertebrates and eudicots do (tables S2 to S4 and fig. S5).

We predicted 14,566 proteins (at 14,520 loci) in *Volvox* (*5*) (Table 1 and tables S5 to S7). *Volvox* and *Chlamydomonas* have similar numbers of genes (Table 1) (*3*) and more genes than most unicellular organisms (table S8). Genes in both algae are intron-rich (Table 1), like those of most multicellular organisms (table S8), and introns are longer, on average, in *Volvox* (fig. S6) (*5*). Novel protein domains and/or combinations are proposed to have contributed to multicellularity in metazoans (*7*), and such expansions are evident in both the plant and animal lineages (Fig. 2A and table S9). In contrast, the numbers of domains and combinations in *Volvox* are very similar

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to those in *Chlamydomonas* and other unicellular species (Fig. 2A and table S9) (*5*). microRNAs (miRNAs) have been identified in *Chlamydomonas*, most of which have no homologs in *Volvox* (*8*, *9*). It is likely that *Volvox* also has miRNAs, but these have yet to be characterized.

To investigate protein evolution in *Chlamydomonas* and *Volvox*, we constructed families containing both orthologs and paralogs from 20 diverse species, including animals, plants, fungi, protists, and bacteria (*5*) (table S10). We assigned 9311 (64%) *Volvox* and 9189 (63%) *Chlamydomonas* protein sequences to 7780 families (Fig. 2B), of which 80% (5423) contain one ortholog from each alga (table S11). 1835 families (26%) contain orthologs only from *Volvox* and *Chlamydomonas* (that is, they are volvocine-specific) (Fig. 2B). Only 32 EST-supported *Volvox* gene models lack detectable homologs in *Chlamydomonas* or other species (*5*) (tables S12 and S13), suggesting that limited protein-coding innovation occurred in the *Volvox* lineage.

Gene-family expansion or contraction is an important source of adaptive variation (*10*, *11*). In a density plot of proteins per family in *Volvox* versus *Chlamydomonas* (Fig. 2C), most points lie on or near the diagonal, showing that the majority of families have approximately equal membership from each alga. Exceptions include the gametolysin/VMP (*Volvox* matrix metalloprotease) family, whose substrates are cell-wall/ECM proteins (*12*) (Fig. 2C, g), and a family containing leucine-rich repeat proteins (LRRs), whose functions in green algae have not been well defined (Fig. 2C, L). Conversely, families containing core histones and ankyrin repeats have more members in *Chlamydomonas* (Fig. 2C, a). In contrast, the subset of 1835 volvocine algae–specific families (*5*) shows a strikingly different distribution (Fig. 2D), with a significant bias toward more members in *Volvox* ($P = 2 \times$

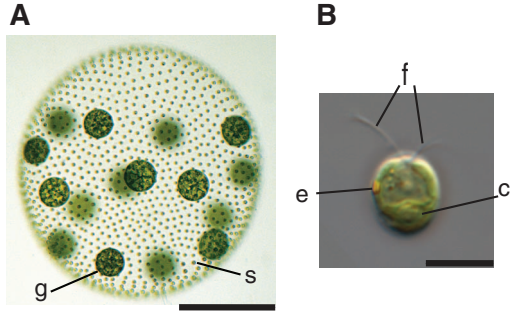

Fig. 1. *Volvox* and *Chlamydomonas*. (**A**) Adult *Volvox* is composed of ~2000 *Chlamydomonas*-like somatic cells (s) and ~16 large germline gonidia (g) (scale bar, 200 μm) (fig. S1B). (**B**) *Chlamydomonas* cell showing apical flagella (f), chloroplast (c), and eyespot (e) (scale bar, 10 μm). The microscopy used is described in (*5*).

Table 1. Comparison of the *Volvox* and *Chlamydomonas* genomes.

Species	Genome size (Mbp)	Number of chromosomes	% G and C	Protein-coding loci	% coding	% of genes with introns	Introns per gene	Median intron length (bp)
V. carteri	138	14*	56	14,520	18.0	92	7.05	358
C. reinhardtii	118	17	64	14,516	16.3	91	7.4	174

*See (*15*).

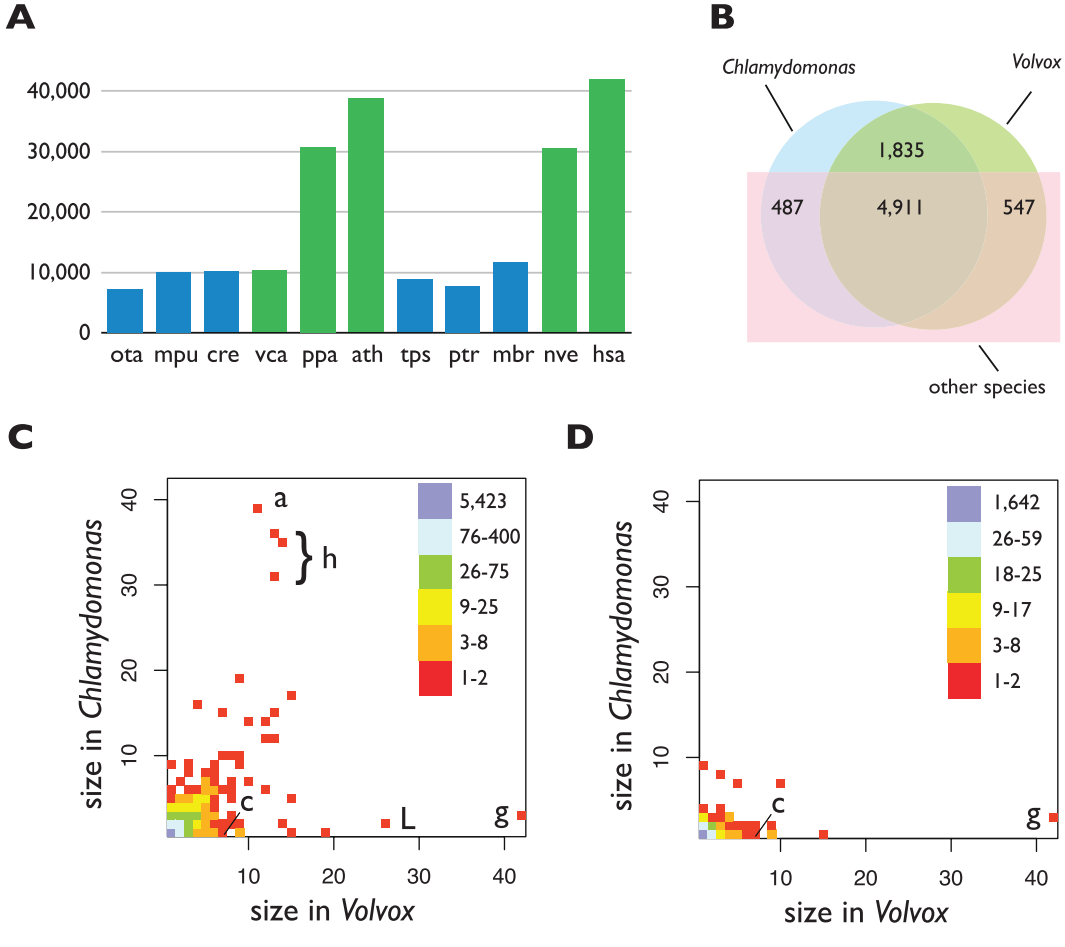

Fig. 2. Comparisons of protein domains and families. (**A**) Total number of Pfam domains in the multicellular (green) and unicellular (blue) species: *Ostreococcus tauri* (ota); *Micromonas pusilla* (mpu); *Chlamydomonas reinhardtii* (cre); *Volvox carteri* (vca); *Physcomitrella patens* (ppa); *Arabidopsis thaliana* (ath); *Thalassiosira pseudonana* (tps); *Phaeodactylum tricornutum* (ptr); *Monosiga brevicollis* (mbr); *Nematostella vectensis* (nve); and *Homo sapiens* (hsa). (**B**) The numbers of protein families from *Volvox*, *Chlamydomonas*, and other species (*5*) are shown in a Venn diagram. The numbers of *Volvox* and *Chlamydomonas* members per protein family are plotted for all families (**C**) and for the volvocine algae–specific subset (**D**). In these density plots, the position of each square represents the number of family members in *Volvox* (*x* axis) and *Chlamydomonas* (*y* axis), with coloring to indicate the total number of families plotted at each position. The Pfam domains for outlier families are abbreviated as follows: a, ankyrin repeat; c, cysteine protease; g, gametolysin; h, histone; L, LRR.

10^{-120}, heterogeneity chi-squared test). These families include ECM proteins such as VMPs and pherophorins that both participate in ECM biogenesis (*12*), and an algal subgroup of cysteine proteases (Fig. 2D, c).

Although some of the genomic differences between *Volvox* and *Chlamydomonas* may reflect environmental adaptations that have not been extensively investigated (*5*), we expected many of the changes to be in protein families that are associated with the large differences in organismal complexity. Therefore, we investigated in detail pathways related to key developmental processes that are either novel or qualitatively different in *Volvox* relative to *Chlamydomonas* (*2*). These include the following: protein secretion and membrane trafficking [potentially involved in cytoplasmic bridge formation via incomplete cytokinesis (*13*, *14*)]; the cytoskeleton [potentially involved in *Volvox*-specific basal body rotation, inversion, and asymmetric cell division (*15*)]; ECM and cell-wall proteins [involved in ECM expansion, sexual differentiation, and morphogenesis (*12*) (fig. S2)]; and cell-cycle regulation (potentially involved in cell division patterning or asymmetric cell division). The components of these pathways are nearly identical in *Volvox* and *Chlamydomonas* (table S14). Transcription-related proteins also have highly similar repertoires in the two species (fig. S7 and table S15) (*5*). Thus, with three exceptions (see below), we found little difference in the complements of proteins that might underlie developmental complexity in *Volvox*.

The ECM composes up to 99% of an adult *Volvox* spheroid and is larger and more structurally complex than the ancestral *Chlamydomonas*-like cell wall from which it was derived (*12*) (fig. S3 and SOM text). These changes are mirrored by at least two dramatic changes in ECM protein family size in *Volvox* as compared with *Chlamydomonas*: pherophorins (49 versus 27 members) and VMPs (42 versus 8 members) (Fig. 3A, fig. S8, and table S14). We found expanded *Volvox*-specific clades of pherophorins and VMPs as well as species-specific duplications in both algae (Fig. 3A and fig. S8). Besides their role in ECM structure, *Volvox* pherophorins have evolved into a diffusible sex-inducer glycoprotein that has replaced nitrogen deprivation (used in *Chlamydomonas* and other volvocine algae) as the trigger for sexual differentiation (*16*). The co-option of an ECM protein for sexual signaling shows parallels in the sexual agglutinins of *Chlamydomonas* that are themselves related to cell-wall/ECM proteins (*17*). The *Volvox* ECM proteins, pherophorins, and VMPs diversified and then presumably were recruited to novel developmental roles in *Volvox*, thus representing a source of adaptive plasticity that is specific to the volvocine algae.

The *Volvox* and *Chlamydomonas* cell cycles are fundamentally similar, but *Volvox* has evolved additional regulation of timing, number, and types of cell divisions (symmetric and asymmetric) among different subsets of embryonic cells (*2*). The division program of males and females is further modified during sexual development to produce sperm and eggs (*18*). Whereas most of the core cell-cycle proteins of *Volvox* and *Chlamydomonas* have a 1:1 orthology relationship, the cyclin D family is notably larger in *Volvox*. In addition to three D cyclins that have *Chlamydomonas* orthologs (Cycd2, Cycd3, and Cycd4), *Volvox* has four D1-related cyclins (Cycd1.1 to Cycd1.4), whereas *Chlamydomonas* has only one (Fig. 3B). D cyclins bind cyclin-dependent kinases and target them to phosphorylate retinoblastoma (RB)–related proteins (*19*). In *Chlamydomonas*, the RB-related protein MAT3 controls the timing and extent of cell division (*20*), so it is plausible that the expanded D-type cyclin family in *Volvox* plays a role in regulating its cell division program during development.

The genetic changes that brought about the evolution of multicellular life from unicellular progenitors remain obscure (*2*, *21*, *22*). For example, many proteins associated with animal multicellularity, such as cadherins and receptor tyrosine kinases (*23*), evolved in the unicellular ancestor of animals and are specific to its descendants. Other critical components of metazoan multicellularity, including key transcription factors and signaling molecules, are absent from the closest unicellular relatives of animals (*22*), suggesting that animal multicellularity also involved protein-coding innovation. Our comparisons of *Volvox* and *Chlamydomonas* indicate that, with the interesting exceptions of pherophorins, VMPs, and D cyclins, the developmental innovations in the *Volvox* lineage did not involve major changes in the ancestral protein repertoire. This is consistent with previous observations indicating co-option of ancestral genes into new developmental processes without changes in copy number or function (*24*–*26*). However, our analyses do suggest that the expansion of lineage-specific proteins occurred preferentially in *Volvox* and provided a key source of developmental innovation and adaptation. Further studies of gene regulation (*27*) and the role of noncoding RNAs (*28*) will be

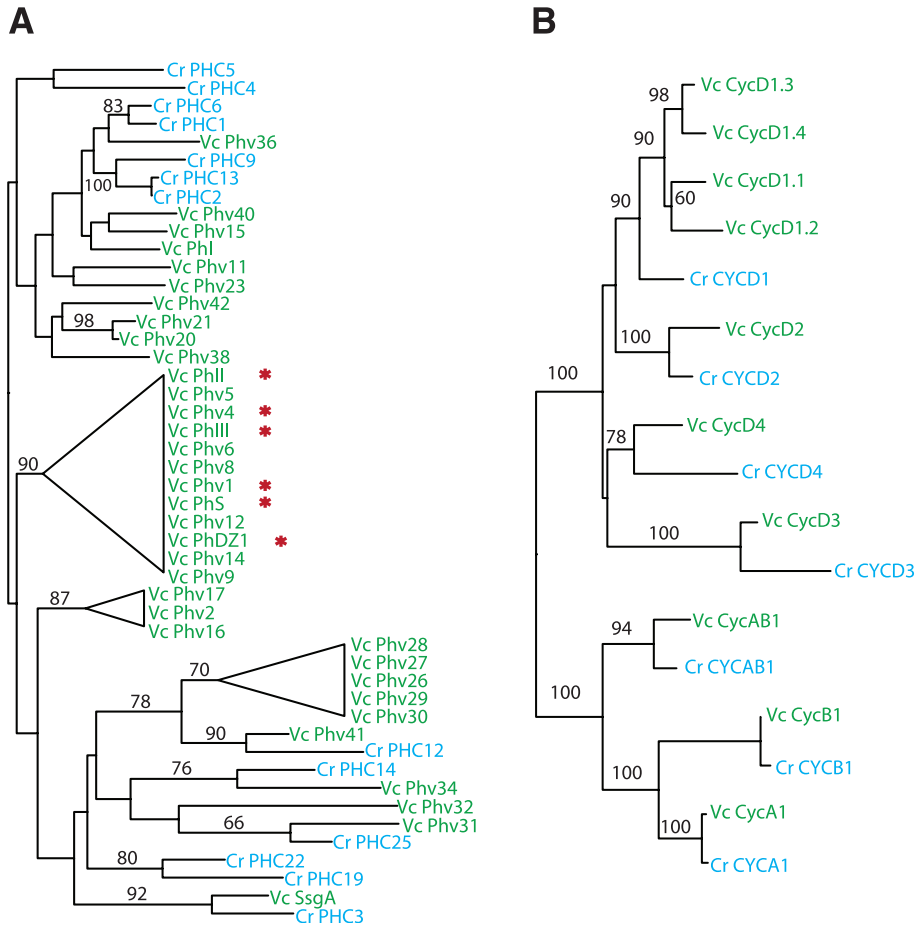

Fig. 3. Diversification of key protein families with known or predicted roles in *Volvox* development. Unrooted maximum likelihood trees (*5*) are shown for pherophorins (**A**) and cyclins (**B**). Protein sequences are from *Volvox* (Vc, green) and *Chlamydomonas* (Cr, blue). Incomplete gene models were not included; *Volvox*-specific clades with poorly resolved branches are collapsed into triangles; bootstrap support ≥50% is indicated on branches. Red asterisks indicate pherophorins whose mRNA levels are up-regulated by a sex inducer (*16*).

enabled by the *Volvox* genome sequence, allowing a more complete understanding of the transformation from a cellularly complex *Chlamydomonas*-like ancestor to a morphologically and developmentally complex "fierce roller."

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Supporting Online Material

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A Molecular Clock for Malaria Parasites

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The evolutionary origins of new lineages of pathogens are fundamental to understanding emerging diseases. Phylogenetic reconstruction based on DNA sequences has revealed the sister taxa of human pathogens, but the timing of host-switching events, including the human malaria pathogen *Plasmodium falciparum*, remains controversial. Here, we establish a rate for cytochrome b evolution in avian malaria parasites relative to its rate in birds. We found that the parasite cytochrome b gene evolves about 60% as rapidly as that of host cytochrome b, corresponding to ~1.2% sequence divergence per million years. This calibration puts the origin of *P. falciparum* at 2.5 million years ago (Ma), the initial radiation of mammalian *Plasmodium* at 12.8 Ma, and the contemporary global diversity of the Haemosporida across terrestrial vertebrates at 16.2 Ma.

The rate of nucleotide substitution in DNA sequences can provide a molecular clock useful for inferring absolute times in phylogenetic trees (*1*). This rate can be estimated by direct observation over reasonable time periods, as with several viral parasites of humans (*2*, *3*) and experimental populations of *Drosophila* (*4*). Relatively slow nucleotide substitution precludes this approach for malaria parasites, for which calibration is indirect. For some specialized parasites, phylogenetic analyses have revealed codivergence of host and parasite evolutionary lineages, which permits calibration of genetic distance in one relative to the other (*5*–*7*). In contrast, *Plasmodium* and other haemosporidian parasites of terrestrial vertebrates exhibit widespread host switching, often across considerable host taxonomic distance (*8*–*11*). Cospeciation cannot, therefore, provide a means of clock calibration.

In spite of evident host switching, biologists have used the ages of host phylogenetic ancestral nodes to calibrate the rate of nucleotide substitution in *Plasmodium* and to estimate the ages of *Plasmodium* lineages. For example, Ollomo *et al.* (*12*) suggested that a *Plasmodium* lineage newly discovered in chimpanzees diverged from another chimpanzee pathogen, *Plasmodium reichenowi*, 21 ± 9 million years ago (Ma) on the basis of placing the *P. reichenowi*–*P. falciparum* divergence coincident with the human-chimp divergence 4 to 7 Ma. In another analysis, Hayakawa *et al.* (*13*) calibrated amino acid substitutions in three mitochondrial genes based on host-parasite codivergence of *P. gonderi* (a parasite of African primates) and a clade of malaria parasites of southeast Asian primates, including humans. This calibration yielded a divergence time of either 2.5 ± 0.6 million years (My) or 4.0 ± 0.9 My for *P. falciparum* and *P. reichenowi*, depending on the dating of the split between lineages of Asian and African macaques, on one hand, and Asian and African colobine monkeys, on the other hand. However, as Rich *et al.* (*14*) point out, humans could have acquired *P. falciparum* any time after the split of the human-chimpanzee lineage "by a single host transfer, which may have

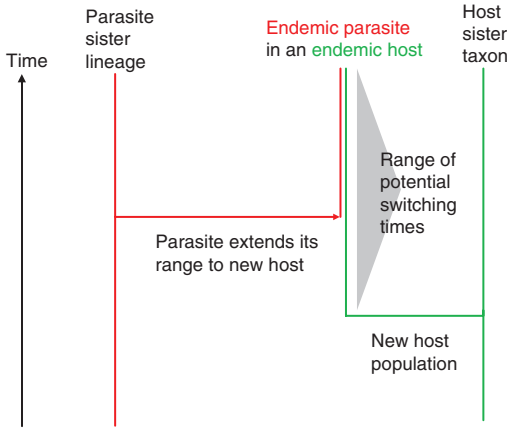

Fig. 1. An approach to estimating a calibration for the rate of haemosporidian nucleotide substitution. We assume that a parasite can switch to a new host at any point with equal probability during the host's independent evolutionary history. Although the range of switching times corresponds to the age of the contemporary host taxon, the range of genetic distances relative to the host is equal to the ratio of the parasite-to-host nucleotide substitution rate. Endemic parasites limited to a single host are suitable for analysis because their divergence from their sister taxon in a different host represents the historical event of host switching (for alternatives, see appendix S1).

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