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Article

Loss of Nucleosomal DNA Condensation Coincides with Appearance of a Novel Nuclear Protein in Dinoflagellates

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Summary

Background: The packaging, expression, and maintenance of nuclear genomes using histone proteins is a ubiquitous and fundamental feature of eukaryotic cells, yet the phylum Dinoflagellata has apparently abandoned this model of nuclear organization. Their nuclei contain permanently condensed, liquid crystalline chromosomes that seemingly lack histone proteins, and contain remarkably large genomes. The molecular basis for this reorganization is poorly understood, as is the sequence of evolutionary events that led to such radical change. We have investigated nuclear organization in the closest relative to dinoflagellates, *Perkinsus marinus*, and an early-branching dinoflagellate, *Hematodinium* sp., to identify early changes that occurred during dinoflagellate nuclear evolution.

Results: We show that *P. marinus* has a typical nuclear organization that is based on the four core histones. By the early divergence of *Hematodinium* sp., however, dinoflagellate genome size is dramatically enlarged, chromosomes are permanently condensed, and histones are scarcely detectable. In place of histones, we identify a novel, dominant family of nuclear proteins that is only found in dinoflagellates and, surprisingly, in a family of large algal viruses, the Phycodnaviridae. These new proteins, which we call DVNPs (*d*inoflagellate/*v*iral *n*ucleo*p*roteins), are highly basic, bind DNA with similar affinity to histones, and occur in multiple posttranslationally modified forms. We find these proteins throughout all dinoflagellates, including early- and late-branching taxa, but not in *P. marinus*.

Conclusions: Gain of a major novel family of nucleoproteins, apparently from an algal virus, occurred early in dinoflagellate evolution and coincides with rapid and dramatic reorganization of the dinoflagellate nucleus.

Introduction

The dinoflagellate nucleus is so unusual that it was once believed to represent an intermediate stage between prokaryotes and eukaryotes—a so-called "mesokaryon" [1]. Dinoflagellates are a successful and prolific lineage of eukaryotes fulfilling diverse environmental roles, including major marine primary producers and essential symbionts of reef-building corals [2]. About half of dinoflagellates have lost photosynthesis and are heterotrophic micropredators, saprophytes, or parasites of marine animals and other organisms. The notion of dinoflagellates as evolutionary intermediates was abandoned when the sister relationship of dinoflagellates with apicomplexan parasites (e.g., the malaria causative agent *Plasmodium*) was recognized, because apicomplexans contain nuclei typical of most eukaryotes. The dinoflagellate nucleus, or "dinokaryon," is therefore clearly a derived state.

While the term dinokaryon lacks a strict definition, it is generally associated with the following characteristics: (1) chromosomes display a persistent condensed state and are conspicuous by light microscopy throughout the cell cycle [3]; (2) histone proteins are absent or below detectable limits [4]; (3) chromosomes have a cholesteric liquid crystalline organization, evident by birefringence under polarized light and an arched fibrillar or banded appearance in electron micrographs [5–7]; and (4) nuclear DNA content is extremely large (up to 200 pg DNA, compared to 3 pg for humans, per haploid nucleus) [8, 9]. Dinoflagellate nuclei are also unusual in that up to 70% of thymine is replaced by the rare base 5-hydroxymethyluracil, they contain high levels of transition metals (Fe, Ni, Cu, and Zn), and chromosome number can be very high (>100) [4, 9]. The loss of histones as dominant nuclear proteins is perhaps the most striking of dinokaryotic characters because these proteins are central to the packing and regulation of eukaryotic nuclear DNA [10–12]. Histone octamers create nucleosome particles around which DNA is wrapped twice and organized into higher-order chromatin fibers. Dynamic modifications to nucleosomal histones or incorporation of specialized variant histone types regulate genomic functions such as gene expression, DNA repair, chromosome segregation, and meiotic recombination. The presence of a highly conserved set of histones throughout eukaryotes is testimony to their importance and suggests a seminal role in the evolution of eukaryotic nuclei. The radical change in dinoflagellates to a dinokaryon therefore presents a clear challenge to the dogma of the eukaryotic nucleus [13].

Most dinoflagellates, so-called "typical" dinoflagellates or "dinokaryotes," are thought to possess all of the derived nuclear characters (although thorough characterization has only been made for a handful of taxa). Lower-branching members, however, often display either variant or limited dinokaryotic characteristics. For example, some possess alternate cell stages that lack permanently condensed chromosomes (e.g., *Amoebophrya*, *Noctiluca*, and *Blastodinium*), and the condensed chromosomes in some taxa lack conspicuous fibrillar banding in electron micrographs (e.g., *Hematodinium* and *Oxyrrhis*) [14–17]. Further, chromosome number can be relatively small (e.g., four to ten in syndinians) [18, 19], and the presence or absence of histones is poorly characterized. Thus, it has been unclear what sequence of nuclear character changes led to the dinokaryotic state.

To determine the early events in the evolution of the dinokaryon, we have investigated the nuclei of two early-branching members of the dinoflagellate lineage. The earliest member is *Perkinsus marinus*, a parasite of marine mollusks. *P. marinus* lacks the conspicuous features of the dinokaryon morphology, and although it is not considered to be a member of the dinoflagellates proper, it represents the closest known lineage

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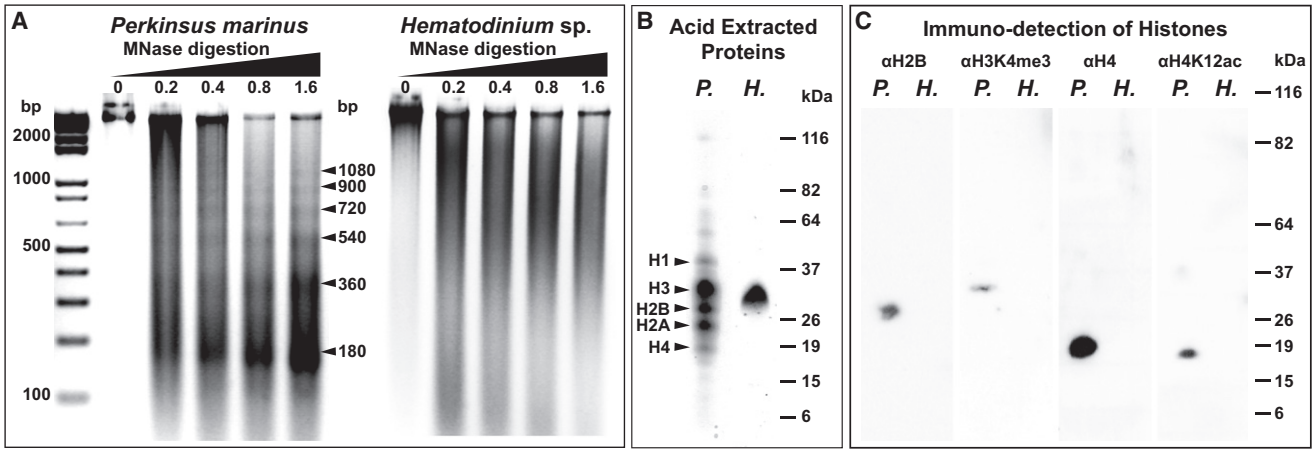

Figure 1. Histone-Based Nucleosomal DNA Packing Is Present in *Perkinsus* but Absent in *Hematodinium*

(A) MNase digestion assay of *P. marinus* and *Hematodinium* sp. chromatin testing for nucleosomal protection of DNA. For each taxon, increasing MNase concentration (from 0–1.6 units) is indicated by the wedge.

(B) Acid-extracted proteins from isolated nuclei from *P. marinus* (*P.*) and *Hematodinium* sp. (*H.*) stained with Coomassie blue.

(C) Western detection of histones in acid-extracted nuclear proteins from *P. marinus* (*P.*) and *Hematodinium* sp. (*H.*) (equal protein loading is used for each lane). Antibodies are directed against H2B, H3 trimethylated at lysine 4, H4, and H4 acetylated at lysine 12.

[20–23]. The second member is *Hematodinium* sp., which is grouped within the true dinoflagellates, but at the base of this clade [14, 24]. *Hematodinium* sp. is also a marine parasite that causes significant economic damage to decapod crustacean fisheries [25, 26]. Its nuclei contain a small number of permanently condensed chromosomes but are otherwise uncharacterized [17]. In this study, we show that histone function appears to be normal in *P. marinus*, consistent with typical genome packing and regulation, as known for apicomplexans relatives and other eukaryotes. However, some time prior to the divergence of *Hematodinium* sp., major changes to the nucleus have taken place. Massive genome expansion has occurred, and histone function is either lost or severely limited. In place of histones, we identify a novel DNA-binding protein as the major basic protein of this nucleus. This novel protein is apparently of viral origin and is present throughout dinoflagellates but absent in *P. marinus*. These data suggest that conversion to the dinokaryotic state may have occurred rapidly and implicate acquisition of a new nucleoprotein as a key early change.

Results

Dinoflagellate Genome Enlargement and Loss of Nucleosomal DNA Packing Occurred prior to Divergence of *Hematodinium*

Perkinsus marinus displays a typical nuclear morphology with noncondensed chromatin for the majority of the cell cycle [23]. In contrast, *Hematodinium* sp. has a small number (four to five) of permanently condensed V-shaped chromosomes throughout its cell cycle [17]. The genome size of this early-branching dinoflagellate has previously not been measured. We applied quantitative propidium iodide DNA staining and fluorescence-activated cell sorting [27] to estimate the *P. marinus* and *Hematodinium* sp. genome sizes by comparison to two standards: human (3101 Mb) and *Toxoplasma gondii* (60.9 Mb). The *P. marinus* nuclear DNA content is estimated at 58 Mb (9 Mb SD), whereas that for *Hematodinium* sp. is 4,800 Mb (500 Mb SD). Although the ploidy of *Hematodinium* sp. is currently unknown (most dinoflagellate cells are haploid), this measurement is approximately 80-fold larger than the diploid nucleus of *P. marinus*, whose genome size is comparable with that of its apicomplexan relatives (~9–60 Mb).

We tested for nucleosomal packing of nuclear DNA for *P. marinus* and *Hematodinium* sp. using a *Micrococcus* nuclease (MNase) protection assay on isolated nuclear material. MNase preferentially cuts the linker DNA between nucleosomes before it cuts the DNA that is wrapped around the nucleosomal histone octamer units [28]. This typically produces a ladder of DNA bands corresponding to single or multiples of the nucleosomal DNA unit (~180 bp) when digested DNA is extracted and separated by gel electrophoresis. For *P. marinus*, we obtained DNA bands corresponding to as many as six nucleosomal DNA units with the majority of the DNA being present at sizes corresponding to mono- and dinucleosomes (Figure 1A). For *Hematodinium* sp., no such pattern was observed, and gross degradation of DNA suggested that nucleosome-mediated MNase protection was entirely absent (Figure 1A). Thus, while *P. marinus* DNA exhibits canonical nucleosomal packing and protection of its nuclear DNA, the bulk of the *Hematodinium* sp. DNA is apparently not organized in this fashion.

Perkinsus Nuclei Contain Typical Histones

P. marinus genomic data contains coding sequences for the four core histone proteins (H2A, H2B, H3, and H4) [29], as well as the linker histone H1 and classic histone variant species (e.g., H2A.Z and H3.3). These sequences all show typical high sequence identity with their homologs in other eukaryotes, consistent with histone primary sequences being strongly conserved throughout eukaryotes (Figure S1 available online). Via acid extraction of proteins from isolated *P. marinus* nuclei—a standard procedure for histone purification—five dominant protein species were resolved by SDS-PAGE with apparent masses expected for the four core histones and the linker histone H1 (Figure 1B). Western blots verify the identities of the H2A, H3, and H4 bands (Figure 1C),

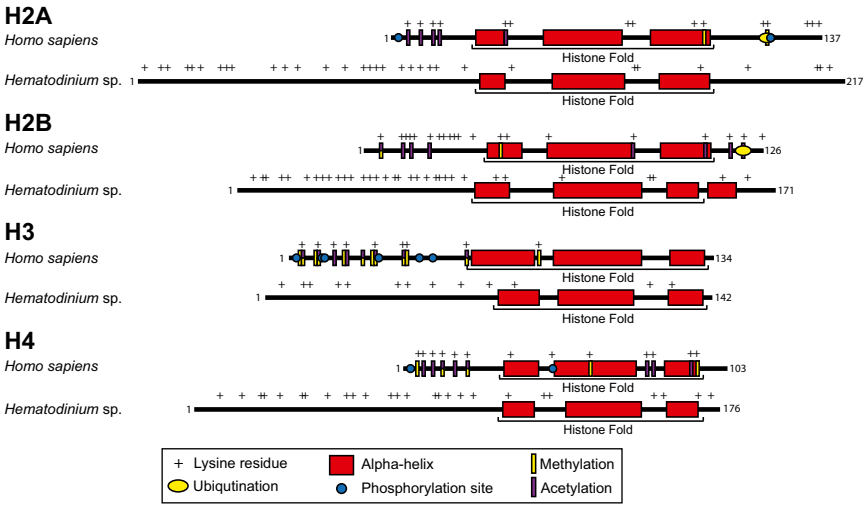

Figure 2. Schematics of Core Histones from Humans and Representative Homologs from *Hematodinium* sp.

Alpha helices in *Hematodinium* sp. proteins are predicted according to the Garnier Osguthorpe Robson algorithm (GOR I) provided by the EMBOSS suite in Geneious Pro 5.6.3. See also Figure S1 and Table S2 for protein alignments and for histone peptides detected by ESI-MS, respectively.

and electrospray ionization-mass spectrometry (ESI-MS) analyses of tryptic peptides confirm the presence of all four core histones (plus histone H1 and variant H2A.Z) in this nuclear sample (Figure S1 and Table S1). Typical posttranslational modification, by methylation and acetylation, is detected in *P. marinus* histones by antibodies specific to H3 trimethylation at lysine 4 (H3K4me3) and H4 acetylation at lysine 12 (H4K12ac) (Figure 1C). ESI-MS further identifies peptide masses consistent with acetylation of H2A at lysine 121 (H2AK121ac) and H2B at lysine 108 (H2BK108ac), as well as methylation of H4 at lysine 79 (H4K79me) (Figure S1 and Table S1). Collectively these data are consistent with the expected presence and behaviors of histones in *Perkinsus*, as for other typical eukaryotes.

Hematodinium Histones Show Dramatic Sequence Divergence and Reduced Abundance

In the absence of genome sequence data, we have identified coding sequence for histone proteins in *Hematodinium* sp. by deep sequencing transcriptomes at multiple growth stages [30, 31]. From these data, we identified sequences for all four of the core histones (H2A, H2B, H3, and H4) and generated complete coding sequences of each transcript, making use of a conserved splice-leader sequence that is appended to the 5′ end of messenger RNAs (mRNAs) in dinoflagellates [32]. Although all core histones are represented, these sequences are exceptionally divergent compared to other eukaryotes (Figures 2 and S1). The "histone-fold" region of *Hematodinium* sp. histones is reasonably conserved with eukaryotic homologs, but the N-terminal region of each protein shows uncharacteristic sequence divergence and is often substantially longer, although always enriched for positive residues, notably lysine. In *Hematodinium* sp., multiple gene transcript species were found for H2A (H2A.1 and H2A.2), H3 (H3.1 and H3.2), and H4 (H4.1, H4.2, and H4.3), and these all show further variability at the N terminus when compared to each other. The divergence of the *Hematodinium* histones from other eukaryotes is substantially greater than that seen across broad eukaryotic diversity (Figure S1).

To test for expression of *Hematodinium* sp. histones in the nucleus, we performed enrichment of basic nuclear proteins as for *P. marinus* (predicted *Hematodinium* sp. histone isoelectric points [pIs] of 11.02–11.83 are similarly basic to classical histones pIs of ~11.00–11.90). The *Hematodinium* sp. basic protein profile was very different to *P. marinus*, with only a single dominant Coomassie-stained band of apparent molecular mass ~30 kDa (Figure 1B). Western blot analysis was unable to detect reactive bands equivalent to *P. marinus* histones (Figure 1C; epitopes for antibodies used are conserved in *Hematodinium* sequences). Further, ESI-MS analysis identified the major protein band as a nonhistone protein (discussed below), and analysis of either the acid-extracted nuclear proteins or total cell proteins was only able to detect peptides from *Hematodinium* H2A (Figure S1 and Table S1). While this is the first detection of an expressed histone protein in a dinoflagellate, compared to the readily observed histones in *P. marinus* it appears that *Hematodinium* histones are much less abundant.

DVNPs Are Novel Major Nuclear Proteins Exclusively Found in Dinoflagellates and Algal Viruses

The dominant 30 kDa acid-extracted protein band of the *Hematodinium* sp. nucleus (Figure 1B) was identified by ESI-MS tryptic peptide matching to multiple *Hematodinium* sp. transcriptomes [30, 31]. This band corresponds to a family of 13 highly similar but novel protein genes (Figure S2 and Table S1). The mass spectrometry data showed that at least four of these proteins were expressed in our samples (DVNP.5, 10, 12, and 13). The complementary DNAs for these proteins were the most abundant transcripts in the 454 sequenced *Hematodinium* sp. transcriptomes. Searching public molecular databases, we found homologous gene sequences for this protein present in a wide range of dinoflagellate taxa (Figure 3A) but no evidence of an equivalent gene in any other eukaryotes or prokaryotes, not even in the closely related *P. marinus*. We did, however, find a highly conserved sequence restricted to a group of algal viruses of the Phycodnaviridae family (Figure 3A). The function of this viral protein is unknown. Due to the restricted occurrence of this protein in dinoflagellates and a family of viruses, and its nuclear location in dinoflagellates (see below), we have given this protein group the name *d*inoflagellate/*v*iral *n*ucleo*p*roteins (DVNPs).

Hematodinium sp. DVNPs range in size from 124 to 186 amino acids. While their predicted molecular mass (13.4–20.2 kDa) is less than the apparent mass on SDS-PAGE (~30 kDa), recombinantly expressed DVNP.6 shows the same retarded mobility, likely due to its high positive charge (Figure 4A). DVNPs have a variable N-terminal region that accounts for most of the length variation, but the remainder of the protein shows high sequence identity (52%–98%, Figure S2). DVNPs from throughout dinoflagellates also show this strong conservation of the core region of the protein

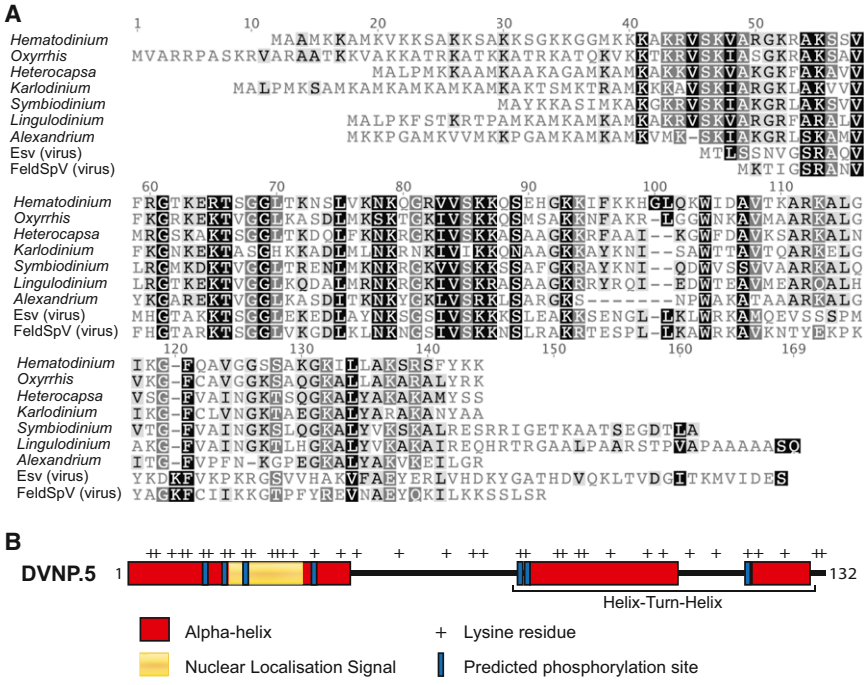

Figure 3. Dinoflagellate/Viral Nucleoproteins Are Restricted to Dinoflagellates and Phycodnaviridae Viruses

(A) Alignment of DVNPs from diverse dinoflagellate genera and two viruses: Esv, *Ectocarpus siliculosus* virus; FeldSpV, *Feldmannia* sp. virus.
(B) Schematic of representative DVNP from *Hematodinium* sp. showing predicted features.
See also Figure S2 and Table S1 for alignment of all *Hematodinium* sp. DVNPs and for DVNP peptides detected by ESI-MS, respectively.

with a variable N-terminal segment, while the viral homologs of DVNP lack the variable N-terminal region (Figure 3). This region in dinoflagellates is predicted to contain nuclear localization signals (using the Motif Scan tool [33] at MyHits, http://myhits.isb-sib.ch). DVNPs are highly basic proteins (mean pI of 12.11 in *Hematodinium* sp.), largely due to their high lysine content. Structural predictions of DVNP suggest several helical regions, including a potential helix-turn-helix conformation in the conserved region (Figure 3B).

DVNP Locates to Chromatin

Immunofluorescent localization of DVNPs with an antibody to the recombinantly expressed DVNP shows they occur exclusively in the *Hematodinium* sp. nucleus and colocate with the condensed chromosomes clearly evident by DNA staining (Figures 4A and 4B). Strong staining with alkaline fast green, a classic stain for basic nuclear proteins [34], confirms the abundance of basic proteins in the *Hematodinium* sp. nucleus (Figure 4C) (later-branching dinoflagellate nuclei are alkaline fast green negative [9]). To test whether the putative nucleus localization signal (NLS) in the N-terminal extension of dinoflagellate DVNPs is involved in targeting this protein to the nucleus, we expressed this portion alone (first 47 residues of *Hematodinium* sp. DVNP.5) fused to the reporter protein GFP in the apicomplexan parasite *Toxoplasma gondii*. A heterologous system was used because dinoflagellates remain recalcitrant to transformation technologies. This fusion protein (NLS-GFP) targeted exclusively to the *T. gondii* nucleus, predominantly locating to the conspicuous nucleolus in these cells (Figure 4D). We also expressed a GFP fusion with the complete DVNP sequence (DVNP-GFP) and this fusion protein also located only to the *T. gondii* nucleus (Figure 4E). Interestingly, the pattern of nuclear localization of this protein was different to NLS-GFP, with little protein in the nucleolus and most located to the chromatin regions of this nucleus. This indicates that the conserved core of DVNP is required for chromatin localization. The nuclei in the DVNP-GFP cells were also enlarged (Figure 4D), and these cells showed poor growth compared to the NLS-GFP or wild-type cells (data not shown).

DVNPs Bind to DNA and Are Posttranslationally Modified

To determine whether DVNP localization to chromatin might be explained by direct DNA-binding activity, we tested the affinity of recombinantly expressed DVNP for double-stranded DNA attached to a solid medium. DVNP bound strongly to DNA, requiring salt concentrations of 0.8–1.0 M for dissociation (Figure 5A). This high affinity is similar to that of histones H2A and H2B [35].

We have also tested for possible posttranslational modifications of DVNPs using two dimensional (2D) SDS-PAGE. The predicted pIs for *Hematodinium* sp. DVNPs range from 11.72–12.53, and the recombinantly expressed DVNP resolves on 2D gels, consistent with its predicted pI of 11.98 (data not shown). Western blots of whole-cell lysates for DVNPs show that their pI values are reduced in vivo and appear as a trail of approximately eight protein spots ranging in pI from ~10.5–11.8 (Figure 5B). Identification of these DVNP species by mass spectrometry of ten gel slices across this window (increments of approximately 0.2 pH units) showed that a single DVNP (DVNP.5, predicted pI = 12.46) is found across this entire range of different pIs. Together, these data suggest that modifications that alter the charge state of DVNPs occur after translation and that multiple isoforms coexist.

One type of posttranslational modification that reduces protein pI is phosphorylation of serine/threonine residues, and several potential phosphorylation sites are predicted for the DVNPs. With Motif Scan, for example, of 14 serine residues in DVNP.5, two offer potential cAMP- and cGMP-dependent protein kinase phosphorylation sites and five are putative protein kinase C phosphorylation sites (Figure 3B). Using a phosphorylation-sensitive stain (ProQ), we tested for phosphorylated proteins from whole-cell *Hematodinium* sp. lysates separated by 2D SDS-PAGE. This method detected a trail of putatively phosphorylated proteins consistent in size and pI with DVNPs (Figures 5B and S3). ProQ treatment of acid-extracted nuclear proteins confirmed that the DVNP band is strongly stained (Figure 5C).

Discussion

This study has provided insight into the evolution of dinoflagellate nuclear characters by defining the nuclear state of early-branching members of the dinoflagellate lineage, *Perkinsus*

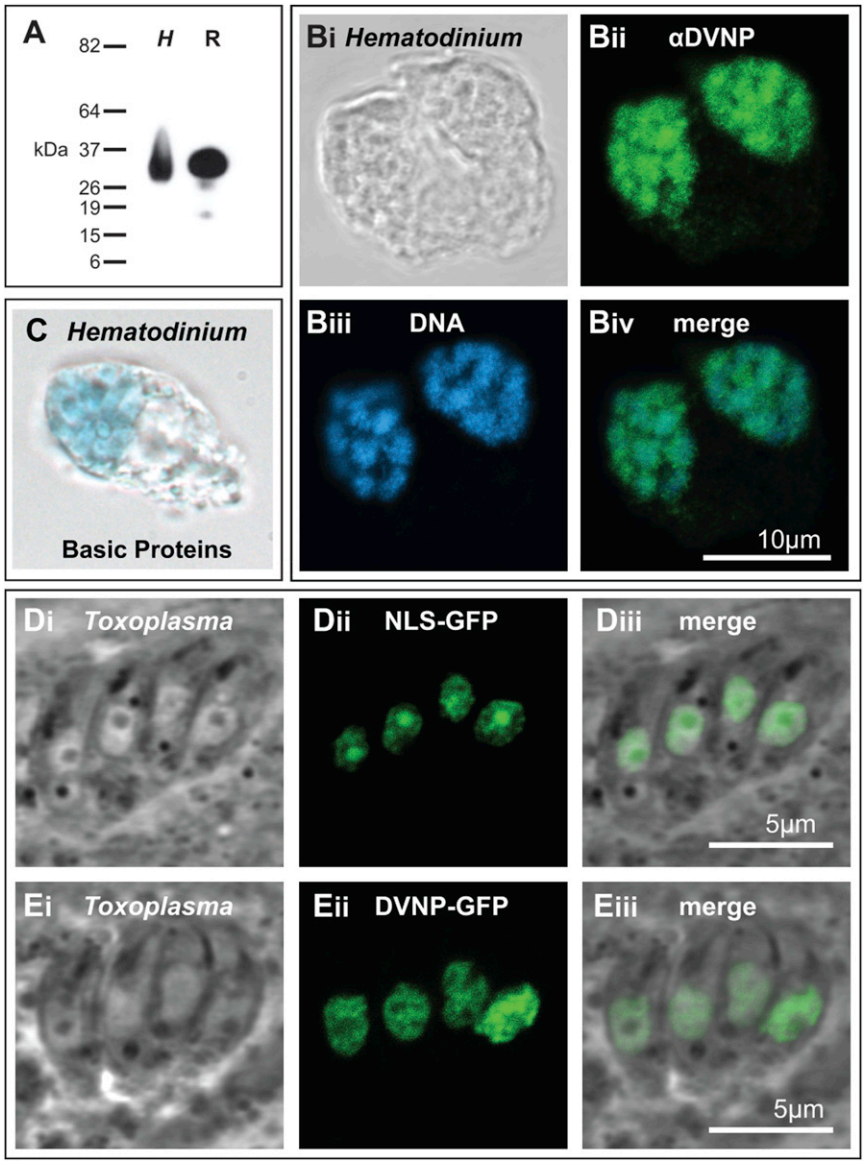

Figure 4. DVNP Locates to Chromatin

(A) DVNP antibodies raised to recombinant DVNP.6 (R) are specific and strongly reactive for the ~30 kDa protein band on western blots of *Hematodinium* sp. total protein extract (*H*).

(B) Immunolabeling of DVNP (ii) is confined to the cell nuclei and colocates with condensed chromosomes stained with DAPI (iii and iv).

(C) Alkaline fast green stains basic proteins of the nucleus.

(D and E) Transiently expressed GFP-fusions of the DVNP.5 N-terminal nucleus localization signal (NLS, 47 residues) or full protein locate to the nucleus of *Toxoplasma gondii* cells. Parasite vacuoles shown each contain four parasites.

marinus and *Hematodinium* sp. *Perkinsus* is classified within "Dinozoa" but separately from the phylum Dinoflagellata [22, 29]. Both molecular phylogenies and the presence of certain dinoflagellate-specific molecular characters (e.g., splice leader RNAs) place *Perkinsus* on the earliest branch of the dinoflagellate lineage, but it exhibits a canonical eukaryotic nucleus with dispersed DNA, not a dinokaryon [20, 21, 23, 24, 29]. The phylogeny of true dinoflagellates has been notoriously difficult to resolve; however, *Hematodinium* consistently groups with early-diverging taxa [14, 24, 36–38]. The nucleus of *Hematodinium* was previously shown to contain few chromosomes (four to five) that are typically permanently condensed (although variable in morphology in different life cycle stages) and lack conspicuous arched banding patterns in electron micrographs [17]. Here, we have shown that although *P. marinus* fulfills all of the expectations of a typical eukaryotic nucleus, *Hematodinium* sp. has expanded its genome size, lacks nucleosomal DNA packing, possesses lowly expressed divergent histones, and contains an abundant novel DNA-binding protein, DVNP, that is present throughout dinoflagellate diversity but absent from *P. marinus*. Therefore, the evolution of the dinokaryotic state was well advanced early in dinoflagellate evolution, and DVNPs might be key to this extraordinary change.

The long-accepted paradigm that dinoflagellate nuclei lack histones has been bolstered by a trail of biochemical, histochemical, and microscopic observations across a broad spread of dinoflagellate taxa, namely: (1) low ratios of basic protein to DNA, (2) poor staining with alkaline fast green for basic nuclear proteins, (3) lack of typical histone bands in acid extracted nuclear proteins, (4) absence of nucleosomal protection of DNA in nuclease digestion assays, and (5) lack of evidence of nucleosomal beads in DNA spreads for electron microscopy (reviewed in [4, 9]). This paradigm has slowly been challenged in recent years by gradual revelation of genes for histones in dinoflagellate transcriptome data, and complete sets of core histone genes have recently been reported for the later-branching taxa *Lingulodinium polyedrum* and *Symbiodinium* sp. [39–44]. To date, however, no detection of expressed histone proteins has been made.

Our detection of *Hematodinium* sp. H2A peptides by mass spectrometry is the first confirmation that histone proteins are present in dinoflagellates, yet their difficulty of detection (by SDS-PAGE, mass spectrometry, or immune detection) is consistent with very low protein abundance, and lack of evidence of a role in bulk packing of nuclear DNA. The exceptional sequence divergence of *Hematodinium* sp. histone gene sequences compared to the conservation of histone sequences seen across eukaryotic diversity is consistent with divergent histone genes found also in later-branching dinoflagellates [39, 40] (Figure S1). Considerable relaxation of histone sequence thus occurred prior to the divergence of *Hematodinium* sp. In addition to the core histones, most eukaryotes contain variant histone forms with dedicated regulatory functions, but these vary less from the core histone forms than do the dinoflagellate histones [10]. By contrast, *P. marinus* histone sequences are much more similar to those

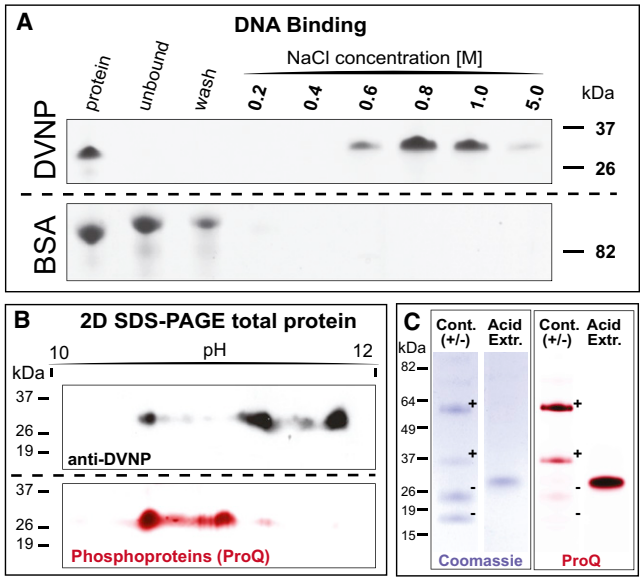

Figure 5. *Hematodinium* sp. DVNP Binds DNA and Is Posttranslationally Modified

(A) Protein binding to immobilized double-stranded DNA shows that DVNP binds DNA and requires high NaCl concentrations to be dissociated, while control protein bovine serum albumin (BSA) does not.

(B) 2D SDS-PAGE resolution of total protein according to size and isoelectric point (pI). Immunodetection of DVNP shows a trail of protein spots which stain positive with phosphoprotein stain ProQ in the lower pI range.

(C) ProQ staining of acid-extracted nuclear proteins (Acid Extr.). Control sample (Cont.) contains two phosophoproteins (+) and two nonphosphorylated proteins (–). All protein bands are ~0.5 μg.

See also Figure S3 for a full gel image of (B).

of other eukaryotes, and their expression levels appear normal. *P. marinus* nuclear DNA displays classic nucleosomal packing and conserved histone posttranslational modifications, which indicates expected roles in chromatin regulation.

Together, these data suggest that major change in histone function and nuclear organization has occurred after *Perkinsus* but before *Hematodinium* in dinoflagellate evolution. Despite the very low abundance and divergence of dinoflagellate histone sequences, many conserved sites of histone posttranslational modification are preserved (although not all; see Figure S1). In *Hematodinium* sp., we also find transcripts of several histone maturation and -modifying proteins (e.g., nucleosome assembly protein 1 NAP1, the dpy-30 core subunit of the SET1/MLL histone methyltransferase complex, structure-specific recognition protein 1 SSRP1, and histone acetyltransferase NAT10) as have been found in other dinoflagellates [39–41]. It is therefore likely that a reduced role for histones, restricted to select chromatin regulatory functions, has been preserved in the dinokaryon.

The second major change seen in the *Hematodinium* sp. nucleus is the presence of a single major basic protein type, DVNP. We have shown that DVNPs are small basic proteins that bind DNA with affinity similar to histones H2A and H2B (H3 and H4 have even higher binding affinities) [35]. DVNPs are located to the condensed chromatin in *Hematodinium* nuclei. Like histones, DVNPs have N-terminal nuclear localization signals rich in lysine residues, a conserved core region predicted to form helix-turn-helix structures, multiple posttranslational modifications, and multiple coexpressed isoforms, but there is no obvious primary sequence similarity between DVNPs and histones. DVNPs undergo phosphorylation, generating pI shifts, but other modifications such as acetylation could also account for the multiple charged species observed. While no known modification types were identified in the DVNP peptides detected in mass spectrometry profiles, the observed peptides were all limited to a small region of the protein (see Figure S2). Multiple modification sites and types occur on canonical histones, and individual changes can have dramatic effect on the histone behavior and function, such as either relaxing chromatin packing structure or recruiting other proteins involved in genome expression or maintenance [45, 46]. It is possible that expression of different DVNPs with their variable N-termini, and combinations of different posttranslational modifications, could contribute to similar dynamic functions in the absence of abundant histones.

DVNP genes do not occur in *Perkinsus* or any other eukaryote, but they are found in all dinoflagellates for which broad molecular data exists. This includes wide representation in typical "dinokaryotes," but also another early-branching dinoflagellate, *Oxyrrhis marina*. Similar to *Hematodinium* sp., *O. marina* nuclei contain a single dominant basic nuclear protein species (called NP23 for its approximate gel mobility of ~23 kDa) [47]. While the identity NP23 has not been confirmed, it now seems likely that NP23 is a DVNP and that these proteins had become a dominant feature of the dinokaryon prior to the divergences of *O. marina* and *Hematodinium* sp. In *O. marina*, the ratio of basic protein to DNA is ~1:2 (in histone-containing eukaryotes, it is ~1:1), but in later-branching dinoflagellates it is even less (~1:10), suggesting further change to the dinokaryotic state [9, 48, 49]. At least six basic nuclear protein species are found in the later-branching dinoflagellate *Crypthecodinium cohnii* [50], and the sequence of three of these have been identified as a family of "histone-like proteins" (HLPs) (HCc1, HCc2, HCc3) [51]. Similar genes have also been reported in *Lingulodinium polyedrum* (HLp) and *Alexandrium fundyense* (HAp) [52, 53], and we find evidence of these genes in some further EST data sets (from *Karlodinium*, *Pfiesteria*, and *Pyrocystis*). These HLPs share limited sequence similarity with bacterial DNA-binding protein HU, show weak sequence-specific DNA binding, are posttranslationally modified by acetylation, and can induce ordered bending of DNA in vitro [52, 54, 55]—activities all consistent with chromatin function. Genes for the HLPs, however, are not found in current data for the early-diverging taxa *Hematodinium* sp. and *O. marina*. Moreover, presence of HLPs does not exclude DVNPs, with both protein types co-occurring in at least *Lingulodinium*, *Alexandrium*, and *Karlodinium* (their presence in *C. cohnii* or the identity of the remaining three basic nuclear proteins is currently unknown). Therefore, the presence of HLPs suggests that either development or recruitment of further basic nuclear proteins occurred later in dinoflagellate radiation, but apparently after the appearance of DVNPs.

The occurrence of a highly similar protein to dinoflagellate DVNPs in viruses of the family Phycodnaviridae suggests a lateral exchange of the *dvnp* gene between these groups. Phycodnaviruses have huge double-stranded DNA genomes (160–560 kb) encoding up to 600 proteins and infect a wide range of algae [56–58]. They belong to the monophyletic class of nucleocytoplasmic large DNA viruses (NCLDVs), which encode many of the proteins necessary for their own genome replication and maintenance [59, 60]. The viral homologs of

DVNPs lack the N-terminal extension (implicated in nuclear localization) but are otherwise highly conserved with dinoflagellate homologs (Figure 3A). Most Phycodnaviridae have DVNP homologs. Given that this viral family is thought to be as old as eukaryotes themselves (2.0–2.7 billion years old) [56, 57], the viral homolog of DVNP likely predates the emergence of dinoflagellates some 800–900 million years ago [61]. This suggests that dinoflagellates gained the gene for DVNPs from the virus rather than vice versa, and acquisition of the gene by dinoflagellates as a consequence of viral infection is a possible scenario. It is highly unlikely that dinoflagellate DVNPs are the product of a contemporary viral infection because (1) *dvnp* mRNAs possess a dinoflagellate-specific splice leader; (2) DVNPs possess a nuclear localization signal, whereas the viral homologs do not; (3) multiple (over ten) different *dvnp* paralogues are found in dinoflagellates, but only a single gene copy in phycodnavirsuses; and (4) no further phycodnavirus genes could be found in the *Hematodinium* sp. transcriptome data, despite searches with over 140 viral genes. Given the occurrence of this common DNA-binding protein in both dinoflagellates and viruses, it is intriguing that both are possessed of far greater amounts of DNA than their close relatives. The role of the DVNP homologs in phycodnaviruses remains to be determined, and it will be interesting to explore whether it has a function in DNA packing for these viruses with extraordinarily large genomes.

Regardless of whether DVNPs first evolved in viruses or dinoflagellates, their appearance as major nucleoproteins in dinoflagellates coincides with the dramatic changes associated with the dinokaryotic state. The nuclear genome size of *Hematodinium* sp. (4.8 Gb) is at least 80-fold that of *P. marinus*, and *O. marina* has also greatly expanded its genome at 55 Gb [62]. This massive expansion of DNA content coincides with the loss of canonical, histone-based nucleosomal packing and the appearance of DVNPs and persistently condensed chromosomes. Although the arched banding pattern seen in chromosome electron micrographs of late-branching dinoflagellates is not evident in *Hematodinium* sp., this is also the case in *O. marina* and vegetative cells of the Noctilucales and Blastodiniales. But the chromosomes of these cells do display limited birefringent properties, suggesting that cholesteric liquid crystal organization of chromosomes does occur in these taxa [16]. The nuclei of early-branching taxa are also distinct in containing a greater basic protein content compared to later-branching dinoflagellates, evident both biochemically and by positive alkaline fast green staining of nuclei seen only in the basal groups (e.g., *Hematodinium*, *Oxyrrhis*, *Syndinium*, *Amoebophyra*, *Noctiluca*, and *Blastodinium*) [9, 14, 49]. Therefore, there is evidence of further development of the dinokaryotic state during dinoflagellate radiation, although it appears that the most substantial biochemical changes occurred very early. No dinoflagellates with intermediate nuclear forms have been reported (i.e., DVNPs combined with nucleosomal DNA packing, or abundant histones with liquid crystal chromosomes). Therefore, at present it is not possible to identify which change to the dinoflagellate nucleus came first and whether subsequent changes were dependent on early ones. However, it seems plausible that reduction in the role of histone proteins required an alternative protein to be present, and DVNPs offer reasonable candidates for this exchange. Whether DVNPs also dictated an alternative chromosome packing structure and expanded genome sizes or whether other factors contributed to these radical changes is currently unknown.

Experimental Procedures

Cell Culture

Hematodinium sp. (ex *Nephrops norvegicus*) was cultured in the dark at 10°C in *Nephrops* saline media, supplemented with 10% fetal calf serum, penicillin, and streptomycin [17]. *Perkinsus marinus* strain TXsc (ATCC50983; American Type Culture Collection, VA) was cultured in the dark at 26°C in ATCC1886 medium with penicillin and streptomycin. The apicomplexan parasite *Toxoplasma gondii* and human-derived Jurkat cells were cultured at 37°C as previously described [63, 64].

Genome Size Estimation

Propidium iodine (PI) FACS estimation of the cellular DNA content was adapted from standard protocols [27] with stained permeablized cells. Cultured cells (1–2 × 10^6) were washed in phosphate-buffered saline (PBS) and fixed in 1 ml cold 70% ethanol for 1 hr at 4°C, washed twice in PBS, and incubated at 4°C over night in 500 μl, 50 μg ml^{-1} PI in PBS with 50 μg ml^{-1} RNase A (Invitrogen). Approximately 10,000 cells per sample were analyzed in a BD LSRII flow cytometer (Becton Dickinson & Company, North Ryde, NSW, Australia) at a flow rate of ~50 cells per second and at constant instrument settings between samples. Nuclear DNA content was calculated according to relative PI peak staining area with the standards (Jurkat cells, *T. gondii*).

Micrococcus Nuclease Protection Assay

MNase assays were performed according to established protocols [65]. In brief, 5 × 10^6 *Hematodinium* sp. or *P. marinus* cells were washed in *Nephrops* saline or artificial seawater, respectively. Cells were lysed by 30 min incubation in hypotonic cell lysis buffer (10 mM Tris-Cl [pH 8.0], 1 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT in 1× complete protease inhibitor cocktail [Roche]), and nuclei were enriched by centrifugation at 10,000 g for 10 min at 4°C. Pelleted nuclei were then resuspended in 1.2 ml chromatin digestion buffer (20 mM Tris [pH 7.5], 15 mM NaCl, 60 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 300 mM sucrose, and 0.4% NP40) aliquoted to 6 × 200 μl and warmed to 37°C for 1 min. To each aliquot, 0, 0.2, 0.4, 0.8, or 1.6 units MNase (New England Biolabs) was added, incubated at 37°C for 3 min, and then stopped with 0.2× volumes 100 mM EDTA and 4% SDS. Samples were proteinase K digested and DNA phenol-chloroform was extracted before agarose gel electrophoresis and ethidium bromide staining.

Acid Extraction, Electrophoresis, and Staining of Nuclear Proteins

Acid-soluble proteins were extracted from nuclei-enriched fractions of *Hematodinium* sp. and *P. marinus* cells as for MNase assay (above). Nuclei were resuspended in 400 μl 0.4 N H_2SO_4 and incubated with slow rotation overnight at 4°C. Insoluble material was pelleted at 16,000 g for 10 min at 4°C, and soluble proteins in the supernatants were trichloroacetic acid (TCA) precipitated, washed twice in ice-cold acetone, air dried, and then dissolved in protease-free water. SDS-PAGE was performed in either 1D (on 4%–12% gradient gels) or 2D with a linear pH range of 9–12 in the first dimension. Western blots were performed with commercial rabbit polyclonal antibodies for histones (Abcam) H2B (ab1790), H3K4me3 (ab8580), H4 (ab10158), and H4K12ac (ab 46983) or rabbit polyclonal antiserum to recombinantly expressed DVNP (see below) according to standard protocols. Staining of phosphorylated proteins was performed with the ProQ Diamond stain according to manufacturer's protocols (Molecular Probes).

Mass Spectrometry

In-gel and in-solution digests with trypsin were performed as described in [66]. RP-HPLC separation followed by SDS-PAGE was performed as described in [67]. For the thermolysin digest, proteins were resuspended in 8 M guanidine and diluted to 1 M guanidine with 100 mM tris (pH 8.0) and 0.5 mM $CaCl_2$, enzyme was added at 1:100, and digest was performed at 70°C for 3 hr and prepared for MS as described in [66]. Peptides from digests were loaded onto a reversed-phase precolumn (300 μm × 5 mm Zorbax sc300SB-C18; Agilent Technologies, Palo Alto, CA) attached to a Shimadzu Prominence nano LC system (Shimadzu Corporation, Kyoto, Japan). The precolumn was washed with 0.1% formic acid and 5% acetonitrile at a flow rate of 40 μl $minute^{-1}$ for 15 min before placement in line with a 75 μm i.d. × 150 mm Zorbax 300SB-C18 (Agilent Technologies) reversed-phase column. Peptides were eluted with a gradient of 5% to 65% (v/v) acetonitrile in 0.1% formic acid over 60 min at a flow rate of 0.25 μl $minute^{-1}$. Peptides were analyzed via ESI on a QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (AB Sciex, Foster City, CA).

The mass spectrometer was operated in the positive-ion mode, ion source voltage of 2,200 V, with 10 μm uncoated SilicaTips (New Objective, Woburn, MA). Analyst QS 2.0 software (AB Sciex) was used to collect data in a data-dependent acquisition mode for the three most intense ions fulfilling the following criteria: m/z between 450 and 2,000, ion intensity 40 counts, and charge state between +2 and +5. After tandem mass spectrometry (MS/MS) analysis, these ions were dynamically excluded for 18 s, using a mass tolerance of 50 mDa. MS scans were accumulated for 0.5 s, and MS/MS scans were collected in automatic accumulation mode for a maximum of 2 s. Mass and charge state-dependent rolling collision energy was used, and the mass spectrometer instrument was calibrated daily with [Glu]-fibrinopeptide B (Sigma-Aldrich, St. Louis, MO).

Peak lists from the MS/MS spectra were made with ProteinPilot software version 2.0.1 (AB Sciex). The peak lists were searched against the UniProt version 2012_7 database for *P. marinus* and in-house transcriptomes for *Hematodinium* sp. [30, 31] with MASCOT 2.4 [68]. The MASCOT parameters for trypsin digest samples were as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, methyl (K), dimethyl (K), acetylation (K); MS peptide tolerance, 0.15 Da; MS/MS tolerance, 0.1 Da; and number of missed cleavages, up to two. The MASCOT parameters for the thermolysin digest were as follows: enzyme, none; variable modifications, methyl (K), dimethyl (K), acetylation (K); MS peptide tolerance, 0.15 Da; and MS/MS tolerance, 0.1 Da. Only proteins with two or more peptides with a $p < 0.05$ were reported, after satisfying manual inspection.

DVNP Expression in Bacteria and *Toxoplasma gondii*

The coding sequence of DVNP.6 (GenBank accession number JX839699) was cloned as an N-terminal 6 × His fusion in expression vector TOPO pET100/D and overexpressed in DE3 *E. coli* cells according to the manufacturer's protocol (Invitrogen). Protein expressed into insoluble inclusion bodies was isolated with BugBuster (Novagen) and purified on a Nickel-agarose column under denaturing conditions according to the manufacturer's protocol (QIAGEN). Protein was refolded in refolding buffer (50 mM NaH_2PO_4 [pH 8.0]), concentrated with Amicon Ultra 3k Centrifugal filters (Millipore), and stored at −20°C in 20% glycerol or lyophilized. DVNP antisera were generated in rabbits (Walter and Eliza Hall Institute, Bundoora, Australia). DVNP-GFP proteins were made either with the full DVNP.5 (GenBank accession number JX839700) coding sequence (DVNP-GFP) or the coding sequence for the first 47 residues (NLS-GFP) cloned in frame with GFP in the *T. gondii* expression vector pCTG [69]. Fusion proteins vectors were transformed and expressed in *T. gondii* according to standard protocols [63], and expression was observed ~48 hr after transfection.

Microscopy

For immunofluorescence assays, *Hematodinium* sp. was fixed for 20 min in cold acetone, washed in PBS, and then blocked in PBS + 1% bovine serum albumin (BSA) at room temperature. Anti-DVNP antisera was applied at 1/500 diluted in PBS + 1% BSA for 1 hr, washed, then labeled with anti-rabbit Alexa-Flour 488 (Invitrogen). Cells were counter stained with DAPI. Alkaline fast green staining was performed by acid hydrolysis (in 5% trichloroacetic acid) of 4% paraformaldehyde-fixed *Hematodinium* cells followed by 30 min incubation in 0.1% Fast Green FCF (Sigma F7252) at pH 8.1 [34]. Cells were then washed with water, transferred to 96% ethanol, and observed on an Olympus CKX41 microscope. All other fixed *Hematodinium* sp. cells, and live *T. gondii* expressing GFP fusion proteins, were observed and recorded on a Leica TCS2 confocal laser-scanning microscope (Wetzlar, Germany).

DNA Binding Assay

DNA binding of bacterially expressed DVNP.6 was assayed based on previous methods [54]. A 10 mg matrix of cellulose beads bound to ~88 μg double-stranded calf thymus DNA (Sigma-Aldrich D8515) was equilibrated with 1 ml DNA-binding buffer (10 mM Tris-HCl [pH 8.0], 5 mM β-mercaptoethanol, 50 mM NaCl). In fresh buffer, 40 μg recombinant DVNP was added to DNA matrix and incubated with rotation for 5 min at 4°C. Slurry was transferred to a gravity column, unbound protein was collected, and then the column was washed with 500 μl DNA-binding buffer (fraction collected). Bound protein was then eluted by a series of increasing NaCl concentrations (0.2, 0.4, 0.6, 0.8, 1.0, and 5.0 M). All fractions were TCA precipitated and protein separated by SDS-PAGE stained with Coomassie blue. BSA was used as a control.

Accession Numbers

The *Hematodinium* sp. histone and DVNP sequences are deposited at GenBank with accession numbers JX839685–JX839705.

Supplemental Information

Supplemental Information includes three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.10.036.

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