

1 **Phosphorylation of the canonical histone H2A marks foci of damaged DNA in malaria**
2 **parasites**

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19 **Abstract**

20 *Plasmodium falciparum* parasites proliferate within circulating red blood cells and are responsible
21 for the deadliest form of human malaria. These parasites are exposed to numerous intrinsic and
22 external sources that could cause DNA damage, therefore, they have evolved efficient mechanisms
23 to protect their genome integrity and allow them to proliferate in such conditions. In higher
24 eukaryotes, double strand breaks rapidly lead to phosphorylation of the core histone variant H2A.X
25 which marks the site of damaged DNA. We show that in *P. falciparum* that lacks the H2A.X
26 variant, the canonical PfH2A is phosphorylated on serine 121 upon exposure to sources of DNA
27 damage in a dose dependent manner. We further demonstrate that phosphorylated PfH2A is
28 recruited to foci of damaged chromatin shortly after exposure to sources of damage, while the non-
29 phosphorylated PfH2A remains spread throughout the nucleoplasm. In addition, we found that
30 PfH2A phosphorylation is dynamic and as the parasite repairs its DNA over time, this
31 phosphorylation is removed. We also demonstrate that these phosphorylation dynamics could be
32 used to establish a novel and direct DNA repair assay in *P. falciparum*.

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34 **Keywords:** Plasmodium falciparum, DNA damage, DNA repair, H2A phosphorylation, double
35 strand break

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38 **Importance:**

39 *Plasmodium falciparum* is the deadliest human parasite that causes malaria when it reaches the
40 blood stream and begins proliferating inside red blood cells where the parasites are particularly
41 prone to DNA damage. The molecular mechanisms that allow these pathogens to maintain their
42 genome integrity under such condition are also the driving force for acquiring genome plasticity
43 that enable them to create antigenic variation and become resistant to essentially all available
44 drugs. However, mechanisms of DNA damage response and repair have not been extensively
45 studied in these parasites. The paper addresses our recent discovery, that *P. falciparum* that lacks
46 the histone variant H2A.X, phosphorylates its canonical core histone PfH2A in response to
47 exposure to DNA damage. The process of DNA repair in Plasmodium was mostly studied
48 indirectly. Our findings enabled us to establish a direct DNA repair assay for *P. falciparum* similar
49 to assays that are widely used in model organisms.

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52 **Introduction:**

53 *Plasmodium falciparum* is the protozoan parasite responsible for the deadliest form of
54 human malaria. This parasite is estimated to infect 200-300 million people worldwide each year,
55 resulting in approximately half a million deaths, primarily of young children [1]. *P. falciparum*
56 replicates within the circulating red blood cells of an infected individual, and its virulence is
57 attributed to its ability to modify the erythrocyte surface and to evade the host immune attack.
58 During their intra-erythrocytic development, *Plasmodium* parasites replicate their haploid
59 genomes multiple times through consecutive mitosis cycles called schizogony, which makes them
60 particularly prone to errors during DNA replication. In addition, blood stage parasites that live in
61 a highly oxygenated environment produce potent DNA damaging agents while digesting
62 hemoglobin and are exposed to oxidative substances released from immune cells [2].

63 Therefore, *Plasmodium* parasites that are exposed to numerous sources that can damage
64 their DNA must have evolved efficient mechanisms to protect their genome integrity. Orthologues
65 to many of the proteins involved in the DNA damage response (DDR) are encoded in *P. falciparum*
66 genome [2] including those involved in homologous recombination (HR), microhomology-
67 mediated end joining (MMEJ) [2] and mismatch repair machineries [3]. However, these
68 mechanisms have not been extensively studied in these parasites. It appears that malaria parasites
69 utilize both HR and an alternative end joining pathway to maintain their genome integrity [4].
70 Thus, in the absence of a homologous sequence in their haploid genome that can serve as a template
71 for HR, blood stage parasites primarily repair double strand breaks (DSB) using the alternative
72 microhomology-mediated end joining mechanism (MMEJ) [4, 5].

73 In mammals, a single double-strand break of the DNA triggers the DDR that rapidly leads
74 to extensive ATM-kinase-dependent phosphorylation of the core histone isoform H2A.X to form

75 a phospho-H2A.X (γ -H2A.X), which marks the site of damaged DNA [6]. However, the *P.*
76 *falciparum* genome lacks an orthologue of the H2A.X variant, and it only encodes two H2A
77 variants, the canonical PfH2A (PF3D7_0617800) and PfH2A.Z (PF3D7_0320900), which was
78 shown to be associated with subset of active promoters [7]. Previous histone phosphorylation
79 analysis suggested that PfH2A could be phosphorylated on serine 121 [8]. We were interested to
80 determine whether in *P. falciparum* phosphorylation of PfH2A might be correlated with DNA
81 damage. We show that these parasites phosphorylate the canonical PfH2A on serine 121 in
82 response to DNA damage and that the phosphorylated PfH2A is recruited to the damaged foci. In
83 addition, the ability to specifically detect the dynamics of this phosphorylation using an anti γ -
84 H2A.X antibody provides a useful marker for studying DNA damage mechanisms which allowed
85 us to establish a direct DNA repair assay in *P. falciparum*.

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88 **Results:**

89 In model systems, phosphorylation of H2A.X is elevated following exposure to DNA
90 damaging agents and is commonly used as a marker for double strand breaks [6]. The
91 phosphorylation of serine 139 found on a conserved SQ (Serine-Glutamine) motif of mammalian
92 H2A.X serves as a differential epitope for detection of the phosphorylated form known as γ -
93 H2A.X. *Plasmodium* parasites do not contain a gene encoding the H2A.X variant in their genome,
94 but instead they express the canonical H2A and the H2A.Z variants (PF3D7_0617800 and
95 PF3D7_0320900 respectively). In the absence of a good marker for DNA damage in *Plasmodium*,
96 we were interested to test whether the antibody that recognizes γ -H2A.X in mammals could be
97 used as a marker for DNA damage in *P. falciparum*. As a first step we aligned the two plasmodium
98 H2A variants with human H2A.X and noted that only the canonical PfH2A has a long C-terminal
99 tail containing the SQ motif, which is conserved among *Plasmodium* species, while no SQ motif
100 is found in PfH2A.Z (Fig. S1). This SQ motif is conserved among the canonical H2A of several
101 protozoan parasites such as *P. falciparum*, *Giardia lamblia* and *Trichomonas vaginalis* that lack
102 H2A.X orthologues. Similarly, the budding yeast *Saccharomyces cerevisiae* is lacking the H2A.X
103 orthologue, and instead its canonical H2A was found to be phosphorylated on serine 129 (SQ
104 motif). This phosphorylation is detected by the anti- γ -H2A.X antibody, and thus, the
105 phosphorylated form of *S. cerevisiae* H2A is often referred to as γ -H2A.X [9]. Interestingly,
106 contrary to *Plasmodium spp.*, the apicomplexan parasite *Toxoplasma gondii* has an H2A.X variant
107 in addition to its canonical H2A (Fig. 1A). As expected, *in silico* structural prediction of PfH2A
108 suggests that the SQ motif is found in its C' terminal tail (Fig. 1B) and that this motif is likely to
109 be an ATM kinase phosphorylation site (Fig. 1C), similar to the serine 139 of the mammalian

110 H2A.X, which is the major residue phosphorylated in response to DNA damage by the ATM
111 kinase [6].

112

113 To test if PfH2A is indeed phosphorylated in response to exposure of the parasite to a
114 source of DNA damage, we exposed tightly synchronized ring stage NF54 parasites to X-ray
115 irradiation. We chose to irradiate early stage parasites that are not replicating their DNA and do
116 not have haemozoin, and therefore the detected DNA damage should be mostly due to the
117 exogenous source. We used TUNEL assays as direct evidence that exposure of the parasite to 6000
118 Rad caused DNA damage, which was detected in most parasite's nuclei (Fig 2A). We then used
119 the γ -H2A.X antibody for immuno-fluorescence assays (IFA) and were able to detect strong
120 signals within the parasites' nuclei after exposure to X-ray irradiation (Fig 2B). This observation
121 was further confirmed using γ -H2A.X antibody on proteins extracted from parasites exposed to
122 increasing levels of irradiation which showed a corresponding elevation in the levels of γ -PfH2A
123 (Fig. 2C, left panel). Similarly, exposing the parasites to H₂O₂, another source of DNA damage,
124 caused an increase in the levels of γ -PfH2A recognition (Fig. 2C, right panel). To ensure that the
125 anti- γ -H2A.X antibody specifically recognized the phosphorylated form of PfH2A and did not
126 cross-react with the non-phosphorylated form, we incubated the extracted proteins with calf
127 intestine phosphatase (CIP) that removes phosphate residues. The CIP treatment specifically
128 abolished immunoblot detection using the anti- γ -H2A.X antibody while the non-phosphorylated
129 PfH2A was detected at similar levels in parasites exposed to increasing X-ray levels (Fig 2D). In
130 addition, when we initially probed with the anti- γ -H2A.X antibody after irradiation, we observed
131 increasing levels of phosphorylation, however, when the blot was stripped, treated with CIP and
132 re-probed, the anti- γ -H2A.X antibody signal disappeared while detection of the canonical PfH2A

133 was unchanged (Fig. 2E). Altogether, these data suggest that PfH2A is phosphorylated in response
134 to DNA damage and that the anti- γ -H2A.X antibody is specific to the phosphorylated form of
135 PfH2A.

136 To further confirm that the phosphorylation detected by the anti- γ -H2A.X antibody,
137 following parasite's exposure to DNA damage, is indeed phosphorylation of the canonical PfH2A,
138 we extracted and purified histones from parasites that were either exposed or not exposed to X-ray
139 irradiation. Immunoblot analysis of the total histone extract shows an increase in the level of the
140 phosphorylated form of PfH2A following irradiation while the total levels of PfH2A are similar
141 (Fig. 3A & B). We further exposed parasites to X-ray irradiation and performed immuno-
142 precipitation (IP) of total PfH2A using an anti-H2A antibody. The IP fractions were subjected to
143 immunoblot with the anti- γ -H2A.X antibody, which demonstrated significant enrichment of the
144 phosphorylated form of PfH2A in the elution (Fig. 3C). This fraction was subjected to trypsin
145 digestion followed by mass spectrometry analysis, which identified phosphorylation on serine 121
146 of PfH2A (Fig. 3D).

147 The specificity of the anti γ -H2A.X antibody to the phosphorylated form of PfH2A allowed
148 us to image its nuclear distribution compared with the non-phosphorylated PfH2A. Immuno
149 fluorescence assay using anti H2A and anti γ -H2A.X antibodies indicated that while the canonical
150 PfH2A is spread throughout the nucleoplasm, its phosphorylated form is found at distinct foci (Fig.
151 4A). To further validate this observation, we performed super resolution STORM imaging that
152 enabled us to image the nuclear distribution of the two forms of PfH2A in detail at the nanoscale
153 level. This analysis clearly demonstrates the differential distribution of the two PfH2A forms in
154 the nucleoplasm. The non-phosphorylated PfH2A is indeed spread throughout the nucleoplasm

155 while the phosphorylated form is much less abundant, and is found at distinct nuclear foci (Fig.
156 4B).

157 Thus far, the process of DNA repair in Plasmodium was mostly studied indirectly by
158 measuring the recovery of parasites in culture after exposure to a source of DNA damage [10]. In
159 addition, repair mechanisms were studied directly by creating a transgenic inducible DSB system
160 by integrating an *I SceI* cleavage site into the *P. falciparum* genome and sequencing of the repaired
161 locus after induction of the *I SceI* endonuclease [4]. Our data strongly suggest that phosphorylation
162 of PfH2A could be used as a specific and immediate marker for damaged DNA in *P. falciparum*.
163 Therefore, we were interested to examine the dynamics of this phosphorylation over time after
164 exposure to X-ray irradiation, hypothesizing that it could be exploited to establish a direct DNA
165 repair assay for *P. falciparum* similar to assays that are widely used in model organisms. We
166 exposed parasite cultures to different levels of X-ray irradiation and measured the levels of PfH2A
167 phosphorylation over time. We observed that the levels of PfH2A phosphorylation, which had
168 increased immediately after irradiation, decreased already 3 hours after irradiation to levels that
169 are similar to those prior to irradiation (Fig. 5). These data imply that during this period of time,
170 the parasites were able to repair their damaged DNA, and thus, these dynamics could be used as a
171 valuable tool to study DNA repair in malaria parasites.

172

173 **Discussion**

174 In any living organism, the ability to repair damaged DNA is key for maintaining genome integrity.
175 This DNA damage repair (DDR) machinery should be extremely efficient in organisms such as
176 *Plasmodium* parasites that are continuously exposed to numerous intrinsic and exogenous sources
177 that may damage their DNA. In addition to its crucial role for the parasite's basic biological
178 functions under these conditions, efficient DDR machinery contributes to the parasite's ability to
179 expand its antigenic repertoire and to maintain mutations that enable it to resist drug treatment.
180 However, although many regulators of DDR were identified encoded in the *Plasmodium* genome,
181 the mechanisms for DDR in these parasites remained understudied and poorly understood. A major
182 obstacle for advancing our knowledge on DDR machinery in *Plasmodium* is the lack of good
183 molecular markers for damaged DNA and the inability to perform an accurate assay that directly
184 measures the kinetics of DNA repair. Here we show that exposure of *P. falciparum* parasites to X-
185 ray irradiation and H₂O₂, which cause double-strand breaks (DSB), leads to phosphorylation of
186 the canonical PfH2A in a dose dependent manner. We found that although *Plasmodium* has no
187 H2A.X variant, the canonical PfH2A is phosphorylated on the SQ motif found in its C'-terminal-
188 tail and that the phosphorylated PfH2A could be differentiated from the non-phosphorylated form
189 of this core histone protein. Since PfH2A is phosphorylated in a dose-dependent manner, the
190 quantitative measurement of phosphorylated PfH2A can act as a sensitive molecular marker for
191 DNA damage in *P. falciparum*. Most of the approaches employed to date to study DNA damage
192 in *Plasmodium* rely on measuring the relative instability of DNA damage products under alkaline
193 conditions (comet assay) [11, 12] or on the relative expression of DNA damage and repair genes
194 (qRT-PCR) [11, 13, 14]. In addition, thus far, the ability of *P. falciparum* parasites to repair DNA
195 damage was estimated by the rate of recovery of parasite populations exposed to DNA damaging

196 agents i.e. the time it takes for these populations to reach approximately 5% parasitemia (usually
197 10-20 days) [10]. Any delay in recovery was then interpreted as a malfunction of the repair
198 machinery, which is of course indirect evidence reflected two weeks after the actual repair has
199 happened. In this manner, the analysis of PfH2A phosphorylation kinetics can fulfill the need for
200 a direct, simple, sensitive/quantitative and reproducible way of measuring DNA damage and repair
201 kinetics in Plasmodium in a time scale of minutes to hours, which better represents the velocity of
202 the repair machinery.

203 In higher organisms, phosphorylation of histone variant H2A.X is a highly specific and
204 sensitive molecular marker for monitoring DNA damage and repair [15, 16]. However, in some
205 organisms, other histone H2A variants undergo phosphorylation in response to exposure to DNA
206 damage. For example, in *Drosophila melanogaster* H2A.Z is phosphorylated in response to DNA
207 damage instead of H2A.X [17], and in the budding yeast *Saccharomyces cerevisiae* that do not
208 encode an H2A.X variant, the canonical H2A is phosphorylated at the serine found near its C-
209 terminus at an SQ motif [18], similar to what we report here in *P. falciparum*. This also appears to
210 be the case in protozoan species where histone H2A.X is either missing or replaced by other
211 histone variants. A marked example is in *Trypanosoma brucei* and other trypanosomatids as well,
212 in which histone H2A undergoes phosphorylation at a threonine residue (Thr 130) instead of serine
213 in response to DNA damage [19]. Interestingly, in the apicomplexan parasite *Toxoplasma gondii*,
214 which does contain an H2A.X variant, the canonical H2A (also named H2A1) was also proposed
215 to be phosphorylated at a C-terminal SQ motif as a response to DSBs [20]. This may suggest the
216 possibility of functional redundancy among these variants that could be exploited through
217 evolution for functional replacement by the canonical H2A when the H2A.X variant is missing.
218 This is somehow supported by the high level of conservation of the SQ motif in the canonical H2A

219 of other protozoan and in particular in other *Plasmodium* species that face similar exposure to
220 sources of DNA damage such as *P. falciparum*. Interestingly, lower eukaryotes prefer high fidelity
221 HR as the mechanisms to repair DSB while higher eukaryotes prefer NHEJ [21]. We noted that
222 both *P. falciparum* and *S. cerevisiae* that phosphorylate their canonical H2A in response to DSB
223 use HR for repair, while *T. gondii* that encode and phosphorylate TgH2A.X variant use mainly
224 NHEJ similar to higher eukaryotes [21, 22]. One can speculate whether the preference for NHEJ
225 might have evolved in association with the H2A.X variant.

226 In higher eukaryotes, histone H2A.X is known to be phosphorylated by members of
227 phosphatidylinositol 3-kinase family (PI3K) namely Ataxia Telangiectasia Mutated (ATM)
228 kinase, ATM Rad-3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK) [23].
229 However, to the best of our knowledge only one PI3K was identified in Plasmodium [24] while
230 other members of this family are not well characterized. Interestingly, in the apicomplexan parasite
231 *T. gondii*, an ATM kinase orthologue (TGME49_248530) was proposed to be involved in
232 TgH2A.X phosphorylation [25]. Incubation of cultured parasites with a known ATM kinase
233 inhibitor (KU-55933), which was tested as a potential anti *T. gondii* agent, caused cell cycle arrest
234 and was able to inhibit phosphorylation of TgH2A.X [26]. The single PI3K which was previously
235 identified in *P. falciparum* (PF3D7_0515300), shows some sequence conservation with *T. gondii*
236 (TGME49_248530) and Human (AAB65827) ATM kinases (Fig. S2), and incubation of parasites
237 with KU-55933 was able to reduce the levels of PfH2A phosphorylation after irradiation (Fig S3).
238 However, this *P. falciparum* PI3K kinase (PF3D7_0515300), which was shown to be important
239 for hemoglobin digestion was localized to the parasite Plasma Membrane (PM), Parasitophorous
240 Vacuole Membrane (PVM) and the Food Vacuole (FV) but did not appear to be localized to the

241 nucleus [24]. Thus, the plasmodium ATM kinase homologue that phosphorylates PfH2A is yet to
242 be identified.

243 Overall, the identification of PfH2A phosphorylation as a marker for DNA damage and the
244 ability to quantify and time the appearance and disappearance of this marker in response to
245 exposure to sources of DNA damage, opens new possibilities for understanding the mechanisms
246 of DNA damage and repair that contribute to the persistence and pathogenicity of these important
247 pathogens.

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249

250 **Materials and Methods:**

251 **Parasite culture**

252 All experiments were conducted on the human malaria NF54 parasite line. The parasites were
253 cultivated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen at 5%
254 hematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate, and
255 0.1 mg/ml gentamicin. The parasites were synchronized using percoll/sorbitol gradient method in
256 which infected RBCs were layered on a step gradient of 40/70 % percoll containing 6% sorbitol.
257 The gradient was subsequently centrifuged at 12,000g for 20 minutes at room temperature. The
258 late stage synchronized parasites were recovered from the interphase, washed twice with complete
259 culture media and placed back in culture. The percentage of parasitemia was calculated by
260 SYBRGreen I DNA stain (Life Technologies) using CytoFLEX (Beckman Coulter) Flow
261 Cytometer.

262 **Bioinformatics analyses**

263 The full-length sequence of histone H2A and its variants were obtained from different species
264 based on sequence similarity. Multiple sequence alignment of PfH2A (PF3D7_0617800) and other
265 histone H2A variants from different species were performed using CLUSTALW and further
266 analyzed using ESPript3 program. A homology model of the PfH2A was built by comparative
267 modeling using crystal structure of histone H2A (Protein Data Bank entry 1eqz, chain A) by using
268 SWISS-MODEL server. The structure visualization of PfH2A 3D-model was performed using
269 Pymol program.

270 **DNA damage of parasites by X-ray irradiation and H₂O₂**

271 DNA damage in the parasites was performed by X-ray irradiation using a PXi precision X-ray
272 irradiator set at 225 kV, 13.28 mA. In brief, 2 % ring stage NF54 parasites were exposed to
273 different doses of X-ray irradiation (1000, 3000 and 6000 Rad). After irradiation parasites were
274 either collected immediately (i.e 15 minute after irradiation) or put back in culture with fresh media
275 for further analysis at different time points as mentioned elsewhere. The level of DNA damage
276 was measured by In Situ DNA Fragmentation (TUNEL) Assay and phosphorylated H2A by
277 western blot as described. To check the hydrogen peroxide (H₂O₂) mediated DNA damage, ring
278 stage infected RBCs (~2 %) were treated with different concentrations of H₂O₂ (0-10, 50, 100 and
279 400 µM) for 1 hour at 37°C. Parasites were collected from RBCs by saponin lysis and the level of
280 DNA damage was measured by phosphorylated H2A following western blot as described.

281 **In Situ DNA Fragmentation (TUNEL) Assay:**

282 Tightly synchronized ring stages parasites (NF54) were fixed for 30 minutes in freshly prepared
283 fixative (4% paraformaldehyde and 0.005 % glutaraldehyde). After fixation cells were rinsed three
284 times with PBS and incubated with permeabilization solution (0.1% Triton X-100 in PBS) for 10
285 min on ice. The cells were washed twice with PBS, and one time with wash buffer supplied with
286 TUNEL Assay Kit-BrdU Red (Abcam cat # ab6610). TUNEL assay was performed as per
287 manufacturer guidelines. Briefly, following washing 50 µl of TUNEL reaction mixture (DNA
288 labeling solution) was added to each sample. The cells were incubated for 60 min at 37°C with
289 intermittent shaking. Cells were then washed three times with rinse buffer (5 min each time) and
290 re-suspended in 100 µl of antibody solution for 30 minutes at room temperature. Cells were then
291 washed three times with PBS and mounted using Invitrogen™ Molecular Probes™ ProLong™
292 Gold Antifade reagent with DAPI, and imaged using Nikon Eclipse Ti-E microscope equipped with
293 a CoolSNAPMyo CCD camera..

294 **Western immunoblotting**

295 Infected RBCs were lysed with saponin and parasites were pelleted down by centrifugation. The
296 parasite pellet was subsequently washed twice with PBS and lysed in 2x Laemmli sample buffer.
297 The protein lysates were centrifuged and the supernatants were subjected to SDS-PAGE (gradient
298 4-20%, Bio-Rad) and electroblotted to a nitrocellulose membrane. Immunodetection was carried
299 out by using rabbit anti- γ -H2A.X (S^PQ) primary antibody (generated using peptide containing the
300 S^PQ motif, Cell signaling cat # 9718S, 1:1000), anti-H2A antibody (Abcam cat# ab88770, 1:1000)
301 and rabbit polyclonal anti-aldolase antibody (1:3000) [27]. The secondary antibodies used were
302 antibodies conjugated to Horseradish Peroxidase (HRP), goat anti-rabbit (Jackson Immuno
303 Research Laboratories, 1:10000). The immunoblots were developed in EZ/ECL solution (Israel
304 Biological Industries).

305 **Immunofluorescence assay**

306 Immunofluorescence assay (IFA) was performed as described previously with minor
307 modifications[28]. In brief, iRBCs were washed twice with PBS and re-suspended in a freshly
308 prepared fixative solution (4% Paraformaldehyde (EMS) and 0.0075% glutaraldehyde (EMS) in
309 PBS) for 30 minutes at room temperature. Following fixation iRBCs were permeabilized with
310 0.1% Triton-X 100 (Sigma) in PBS, and then blocked with 3% BSA (Sigma) in PBS. Cells were
311 then incubated with primary rabbit anti- γ -H2A.X (S^PQ) (Cell signaling, cat # 9718S, 1:300) and
312 anti H2A (Abcam cat# ab8870, 1:100) antibodies for 1.5 h at room temperature and washed three
313 times in PBS. Following this, cells were incubated with Alexa Fluor 488 goat anti-rabbit (Life
314 Technologies, 1:500) or Alexa Fluor 568 goat anti-rabbit (Life Technologies, 1:500) antibodies
315 for 1h at room temperature. Cells were washed three times in PBS and laid on “PTFE” printed
316 slides (EMS) and mounted in ProLong Gold antifade reagent with DAPI (Molecular Probes).

317 Fluorescent images were obtained using a Plan Apo λ 100x oil NA=1.5 WD=130 μ m lens on a
318 Nikon Eclipse Ti-E microscope equipped with a CoolSNAPMyo CCD camera. Images were
319 processed using the NIS-Elements AR (4.40 version) software.

320 **Stochastic Optical Reconstruction Microscopy (STORM) imaging and analysis**

321 STORM imaging was performed as described recently [29] using anti-H2A (Abcam cat# ab88770,
322 1:150) and rabbit anti- γ -H2A.X (S^PQ) (Cell signaling, cat # 9718S, 1:300) as primary antibodies.
323 Alexa Fluor 594 goat anti-rabbit (Life Technologies, 1:500) was used as a secondary antibody.
324 Parasite nuclei were labeled with YOYO-1 (1:300, life technologies) for orientation and were not
325 subjected to STORM. STORM was performed by a Nikon Eclipse Ti-E microscope with a CFI
326 Apo TIRF \times 100 DIC N2 oil objective (NA 1.49, WD 0.12 mm) as described. For each acquisition,
327 10000 frames were recorded onto a 256 x 256 pixel region (pixel size 160nm) of an Andor iXon-
328 897 EMCCD camera. Super-resolution images were reconstructed from a series of the least 5000
329 images per channel using the N-STORM analysis module, version 1.1.21 of NIS Elements AR v.
330 4.40 (Laboratory imaging s.r.o.).

331 **Total Histone Extraction**

332 Total histones were extracted using an acid extraction method as described previously with minor
333 modifications [30]. All steps were performed at 4°C in buffers containing protease and
334 phosphatase inhibitors to protect the enzymatic interference with PTMs. In brief, 200 ml of parasite
335 cultures (~10% parasitemia) were saponin lysed and washed with PBS containing protease and
336 phosphatase inhibitors. To prepare the intact nuclei, the cell pellet was re-suspended in 1 ml lysis
337 buffer (20 mM HEPES pH 7.8, 10 mM KCl, 1mM EDTA, 1% Triton X-100 and 1mM DTT) and
338 incubated for 30 min on rotator at 4 °C. Following cell lysis, the intact nuclei were washed and

339 pelleted by centrifugation at 10,000g, for 10 min at 4 °C. The nuclei were re-suspended in 400 μ l
340 0.4 N H₂SO₄ or 0.25 N HCl. The nuclei were incubated on a rotator overnight and supernatant
341 containing the acid soluble histone fraction was collected after centrifugation at 16,000g for 10
342 min.

343 **Immunoprecipitation**

344 Immunoprecipitation of PfH2A was performed as described (23) with slight modification. In brief,
345 200 ml of parasite cultures (~10% parasitemia) were saponin lysed and washed with PBS
346 containing protease and phosphatase inhibitors. Subsequently, the parasite pellet was dissolved in
347 chilled lysis buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.01% SDS
348 and 1% NP40 supplemented with protease and phosphatase inhibitors (Roche) and sonicated for
349 4-8 cycles of 10-15 sec at 45% output using Hielscher UP200S sonicator. The sonicated pellet was
350 incubated for 30 minutes on ice. The lysate was purified by a few rounds of centrifugations at
351 10000g for 10 min. and incubated with primary antibody (anti H2A antibody (Abcam cat#
352 ab88770) for 10-12 h at 4°C with continuous swirling. The supernatant was further incubated for
353 4–6 h with Protein A/G agarose beads (Pierce) at 4°C and beads were pelleted by centrifugation at
354 4°C. Beads were then washed with ice chilled washing buffer. Immunoprecipitated proteins were
355 eluted with SDS Lamelli buffer and used for detection by SDS–PAGE and western blot analysis.

356 **Mass spectrophotometry (LC-MS/MS Analysis)**

357 To identify the phosphorylated serine of PfH2A, the extracted histones were digested by trypsin
358 and analyzed by LC-MS/MS on Q Exactive plus (Thermo). The peptides were identified by
359 Discoverer software version 1.4 against the Plasmodium NCBI-NR database and against decoy
360 databases (to determine the false discovery rate (FDR) using the sequest and mascot search

361 engines. Semi quantitation was done by calculating the peak area of each peptide. The area of the
362 protein is the average of the three most intense peptides from each protein. The results were filtered
363 for proteins identified with at least 2 peptides with 1% FDR.

364 **KU-55933 inhibition assay**

365 To check the effect of ATM kinase inhibitor (KU-55933) on intra erythrocytic parasite growth,
366 and to calculate the IC₅₀ (50% inhibitory concentration), a SYBR Green I based parasite growth
367 assay was performed. KU-55933 was dissolved in DMSO to make a stock concentration of 10
368 mM and stored at (-20°C). For growth assay, sorbitol synchronized ring stage iRBCs were seeded
369 in a 96 well plate at a final parasitemia of approximately 0.2 % and a hematocrit of 5 %. ATM
370 kinase inhibitor (KU-55933) stock solution was diluted to 2X final concentration in complete
371 RPMI medium and added to the prepared parasites at a volumetric ratio of 1:1. Each concentration
372 of inhibitor was plated in triplicate and each well contained a total volume of 200 µl, with or
373 without an appropriate concentration of KU-55933 (0-50 µM). In order to check the effects of
374 DMSO, 1 µl of DMSO was added to all control wells. The plate was kept at 37°C in modular
375 incubators that was gassed every 24 hours with 5% oxygen, 5% carbon dioxide and 90% nitrogen.
376 After 72 hours, the media was discarded, and cells were washed twice with PBS. Parasite growth
377 was determined by counting proportions of infected cells by SYBR Green I DNA stain (Life
378 Technologies) using CytoFLEX (Beckman Coulter) Flow Cytometer. IC₅₀ value was calculated
379 by plotting % survival vs log inhibitor concentration using GraphPad Prism 6.0. Values were
380 normalized and then curve fitted by non-linear regression. To check the effect of ATM kinase
381 inhibitor (KU-55933) on histone PfH2A phosphorylation, Percoll/sorbitol synchronized late stage
382 parasite were incubated with or without the inhibitor (20µM). After 24 hours, parasites were
383 subjected to different dosages of X-ray irradiation (1000, 3000 Rad). After irradiation parasites

384 were collected immediately (i.e 15 minute after irradiation) and subjected to western
385 immunoblotting to check the level of phosphorylated H2A as described.

386

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394

395

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475 **Figure legends:**

476 **Figure 1.** *In silico* analyses of putative ATM kinase-specific phosphorylation site (conserved SQ-
477 motif) in PfH2A. **(A).** Multiple sequence alignment of amino acid sequences of PfH2A and some
478 of H2A variants from human, budding yeast, and protozoan parasites using ESPript 3. Similar and
479 identical amino acids are boxed and marked with a red background. The conserved C'-terminal
480 SQ motif is underlined with asterisks. The species names and corresponding uniprot accession
481 number are as follows: PfH2A; *P. falciparum* histone H2A, HsH2AX; *Homo sapiens* Histone
482 H2AX, ScH2A1; *Saccharomyces cerevisiae* Histone H2A1, TgH2A1; *Toxoplasma gondii* Histone
483 H2A1, TgH2AX; *Toxoplasma gondii* Histone H2A.X, TvH2A; *Trichomonas vaginalis* Histone
484 H2A, and GiH2A; *Giardia intestinalis* Histone H2A. **(B).** 3D-Homology model of PfH2A
485 (developed using structure homology-modeling server SWISS-MODEL) showing core histone
486 domain and extended C-terminal tail contains the conserved S¹²¹Q¹²² motif. **(C).** *In silico*
487 prediction of ATM kinase-specific phosphorylation sites in PfH2A (using KinasePhos, version
488 2.0). The sequence-based amino acid coupling-pattern analysis and solvent accessibility of PfH2A
489 suggest Serine 121 as the most prominent ATM kinase specific phosphorylation site.

490

491 **Figure 2.** DNA damage in *P. falciparum* causes histone H2A phosphorylation in a dose-dependent
492 manner. **(A).** DNA fragmentation imaging by TUNEL assay of RBCs infected with NF54 *P.*
493 *falciparum* parasites exposed to X-ray irradiation (6000 rad) showing nuclear foci of damaged
494 DNA. **(B).** Immunofluorescence analysis of X-ray irradiated parasites (6000 rad), using anti- γ -
495 H2A.X(S^PQ) antibody, shows foci of phosphorylation signal in the nucleus. **(C).** Western blot
496 analysis, using anti- γ -H2A.X(S^PQ) antibody, of protein extracts from parasites exposed to
497 increasing levels of X-ray irradiations (left) and from parasites treated with increasing

498 concentrations of H₂O₂ (right). **(D-E)**. The anti- γ -H2A.X (S^PQ) antibody specifically recognizes
499 the phosphorylated PfH2A and does not cross-react with non-phosphorylated PfH2A. Protein from
500 parasites, which were irradiated with increasing doses of X-ray radiation (control (no irradiation),
501 1000, and 6000 Rad respectively) were subjected to WB analysis using either anti- γ -H2A.X (S^PQ)
502 antibody or anti-H2A antibody. The membrane was either incubated with calf intestine
503 phosphatase (CIP) before incubation with the antibodies (D) or incubated with the antibodies,
504 stripped, treated with (CIP) and re-incubated with the antibodies (E). anti-Aldolase antibody was
505 used as a loading control. The anti- γ -H2A.X (S^PQ) antibody detected increasing levels of protein
506 associated with the increasing levels of irradiation only without CIP treatments while the anti-H2A
507 antibody detected constant protein levels even after CIP treatment.

508

509 **Figure 3.** Histone extraction followed by Mass spectrometry shows that Serine 121 of PfH2A is
510 phosphorylated upon exposure to X-ray irradiation. **(A)**. SDS page analysis of histone extraction
511 and purification from X-ray irradiated (6000 rad) and untreated parasites. **(B)**. Western blot
512 analysis of total histone extracted from untreated and X-ray treated (6000 Rad) parasite using anti-
513 γ -histone H2AX (S^PQ) and anti-PfH2A antibodies. **(C)**. X-ray irradiated parasites (6000 Rad) were
514 subjected to Immunoprecipitation (IP), using anti-H2A antibody, followed with WB analysis using
515 both anti- γ -H2A.X (S^PQ) and anti-H2A antibodies. **(D)**. Trypsin digestion followed by mass
516 spectrometry analysis identified phosphorylation of Serine 121 of PfH2A in the irradiated
517 parasites.

518

519 **Figure 4.** Phosphorylated PfH2A is located at distinct nuclear foci while the non-phosphorylated
520 PfH2A is spread throughout the nucleoplasm. **(A).** Immunofluorescence (red, α - γ -H2A; green, α -
521 γ -H2A.X; blue, DAPI. Scale bar 2 μ m) and **(B)** Super resolution STORM imaging of PfH2A and
522 phosphorylated PfH2A (green, Alexa 647 staining each of the PfH2A isoforms; blue, YOYO1
523 staining of DNA at low resolution for orientation. Scale bar 0.5 μ m) in *P. falciparum* nuclei
524 following X-ray irradiation (6000 Rad).

525

526 **Figure 5.** DNA damage and repair assay in *P. falciparum* iRBCs. **(A).** Parasites were treated with
527 different doses of X-ray radiation (i.e. 500, 1000 and 6000 Rad) and put back in culture (3h and
528 6h) to allow them to repair their damaged DNA. Protein extracts from these parasites were then
529 used for WB analysis with anti- γ -H2A.X (S^PQ) antibody and anti-aldolase antibody as a loading
530 control. The ability of the parasites to repair their damaged DNA is demonstrated by the rapid
531 reduction in the levels of phosphorylated PfH2A found 3h after irradiation. **(B).** Semi-quantitative
532 densitometry analysis of the WB presented in (A). Quantification of the changes in the ratio
533 between the signal detected by the anti- γ -H2A.X and anti-aldolase antibody before irradiation,
534 immediately after irradiation, 3h and 6h after irradiation. The density of each band is presented as
535 a proportion of the total signal obtained. C, parasites that were not exposed to irradiation and used
536 as control.

537 .

538 **Figure S1:** **(A).** Multiple sequence alignment of amino acid sequences of histone H2A variants of
539 *P. falciparum* and the human H2A.X, indicating that PfH2A.Z does not contain an SQ motif in its
540 C' tail. **(B).** Multiple sequence alignment of amino acid sequences of histone H2A from different

541 *Plasmodium* spp., indicating that the SQ motif in their C' tail is highly conserved. The similar and
542 identical amino acids are boxed and marked with a red background respectively. The conserved
543 C-terminal SQ motif is marked with an asterisk. The species names and corresponding accession
544 number are as follows: PfH2A; *Plasmodium falciparum* histone H2A (PF3D7_0617800),
545 PfH2AZ; *Plasmodium falciparum* histone H2AZ (PF3D7_0320900) HsH2AX; *Homo sapiens*
546 Histone H2A (NP_002096.1), PvxH2A; *Plasmodium vivax* histone H2A (PVX_114015), PvH2A;
547 *Plasmodium vinckei* histone H2A (YYE_02539), PyH2A;, *Plasmodium yoelli* histone H2A
548 (PY05076), PkH2A; *Plasmodium knowlesi* histone H2A (PKNH_1132600), Pch2A; *Plasmodium*
549 *chabaudi* histone H2A (PCHAS_1116500), and PbH2A, *Plasmodium berghei* histone
550 H2A(PBANKA_1117000), respectively.

551

552 **Figure S2:** Multiple sequence alignment of amino acid sequences of putative ATM kinase from
553 *Toxoplasma* and *Plasmodium*. The species names and corresponding uniprot accession number
554 are as follows: PfATM1/ATR; *Plasmodium falciparum* PF3D7_0515300, TgATM; *Toxoplasma*
555 *gondii* TGME49_248530. The similar and identical amino acids are boxed and marked with a red
556 background respectively.

557

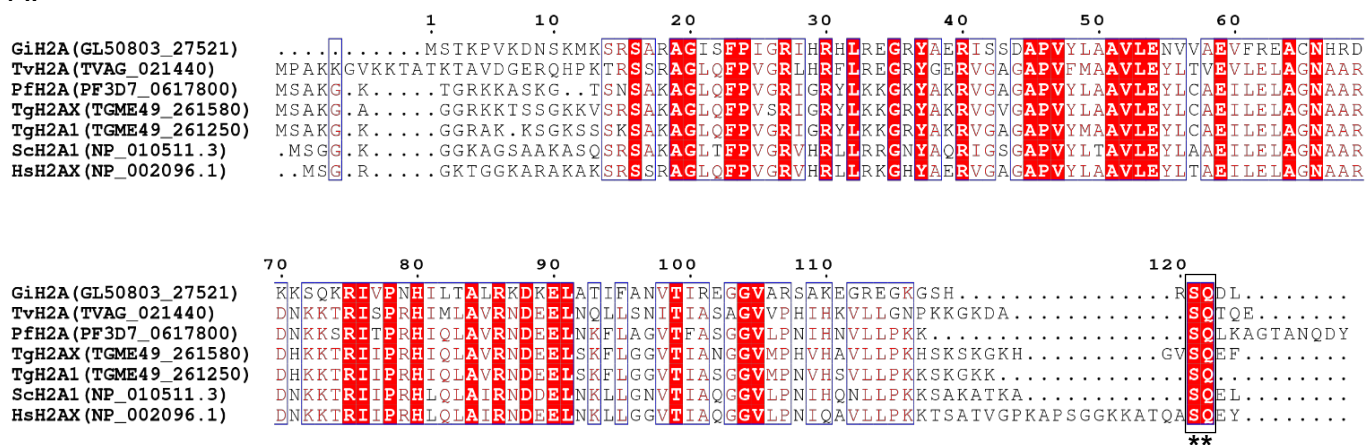
558 **Figure S3.** Effect of ATM kinase inhibitor KU-55933 on *Plasmodium falciparum* growth and
559 PfH2A phosphorylation. (A). *P. falciparum* iRBCs were treated with different doses of KU-55933
560 inhibitor (0-50 μ M) for 72 h. After that, the iRBCs were washed with PBS, and the parasitemia
561 was measured using SYBR Green. The IC50 value was calculated by plotting % survival vs log
562 inhibitor concentration, normalized and curve fitted by non-linear regression. Data presented are

563 mean of three biological replicates \pm SD. The graph is representative of three independent
564 experiments with similar results. **(B)**. Giemsa staining of parasites treated with KU-55933 inhibitor
565 (25 μ M). **(C)**. *P. falciparum* iRBCs (Schizonts stage) were grown in the presence and absence of
566 KU-55933 inhibitor (20 μ M) for 24 hours prior to exposure of iRBCs (ring stage) to different doses
567 of X-ray radiation (i.e. 1000 and 3000 Rad or none). The levels of PfH2A phosphorylation with or
568 without the inhibitor was measured by WB using anti- γ -H2A.X (S^PQ) antibody and indicated a
569 reduction when parasites were irradiated by 3000 Rad.

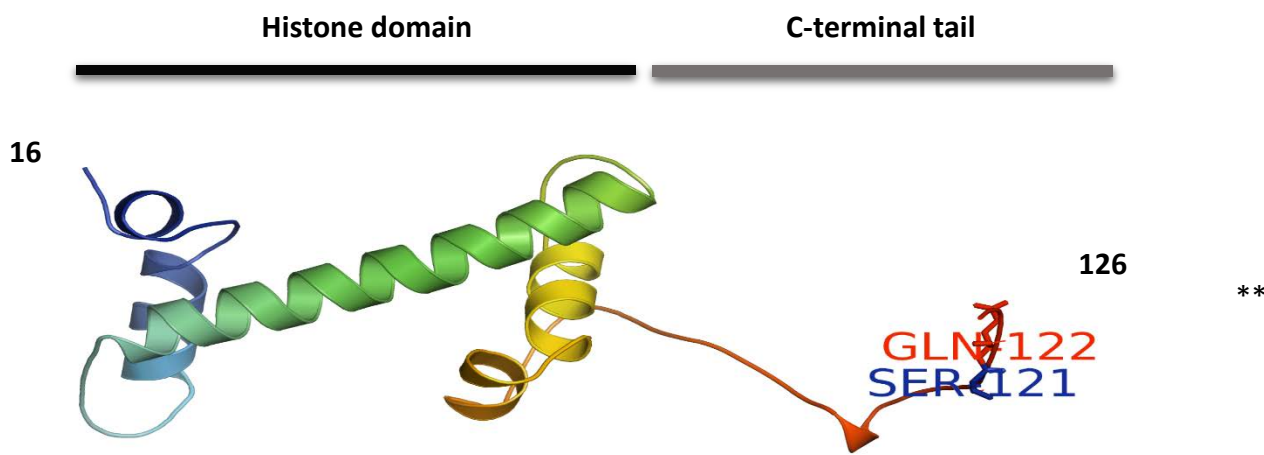
570

Figure 1

A.



B.



C.

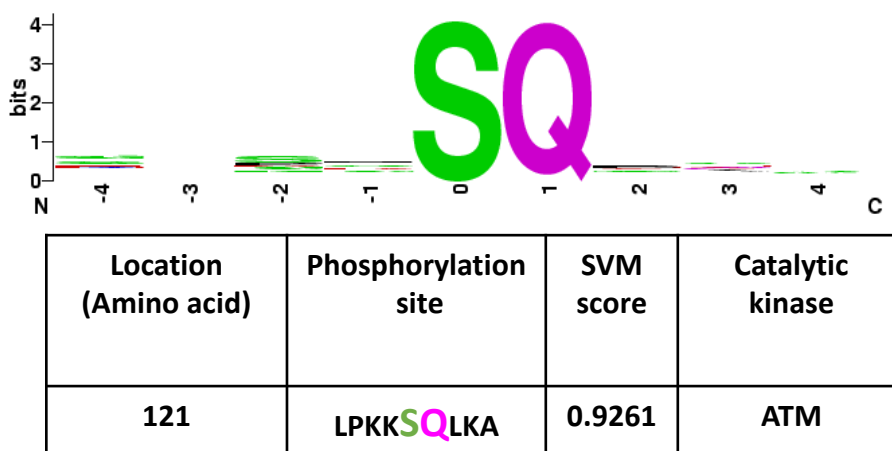


Figure 2

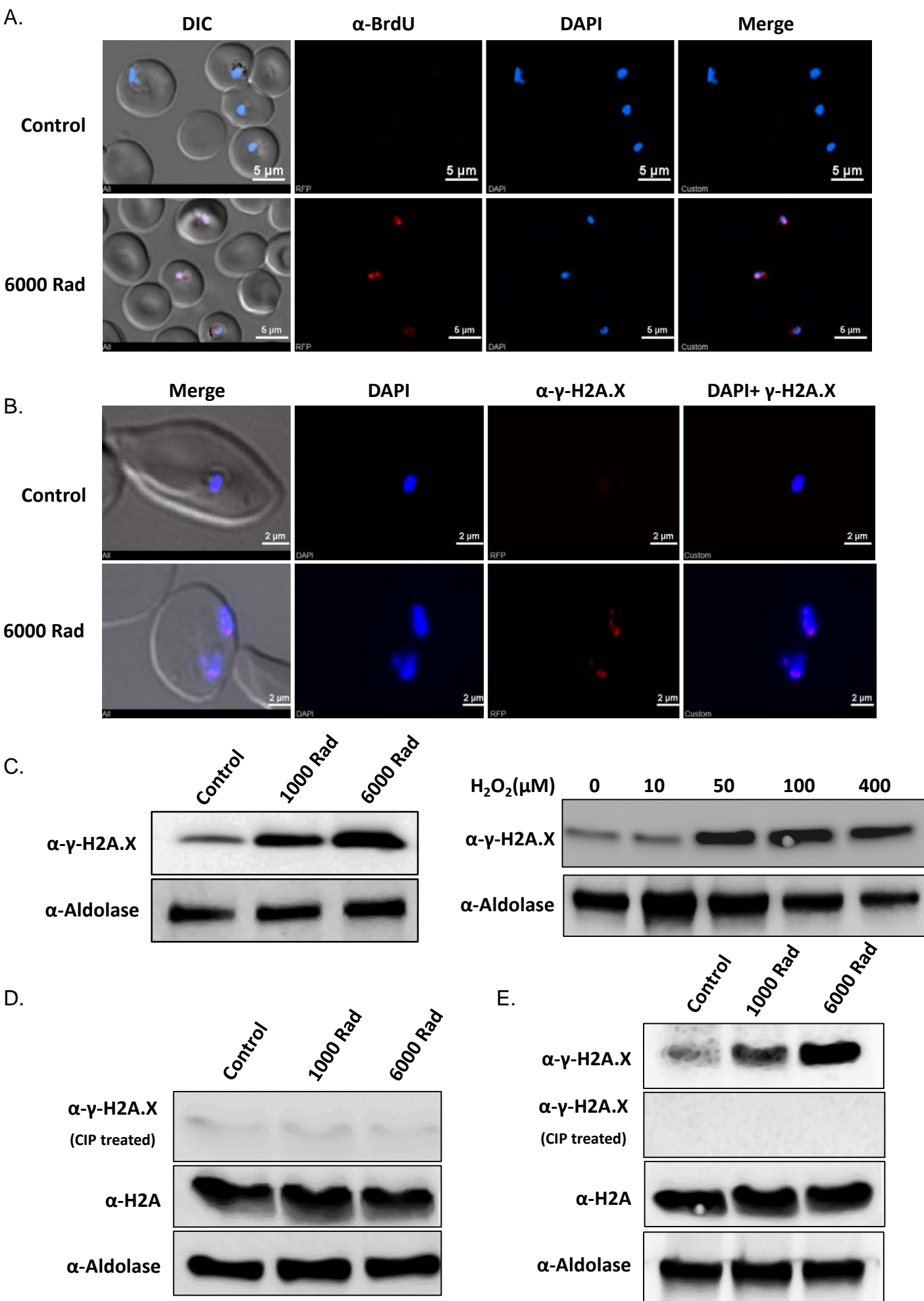
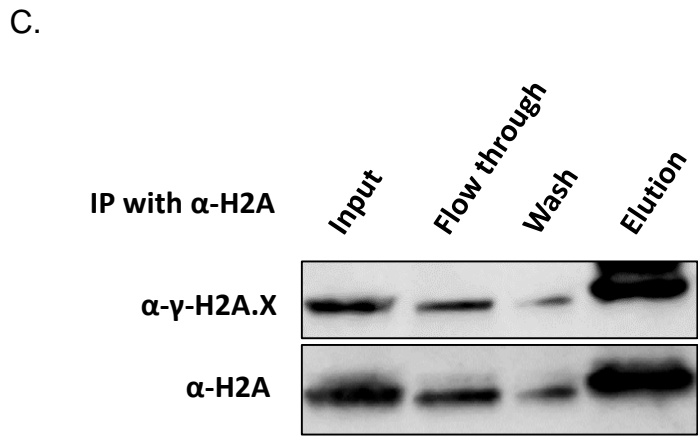
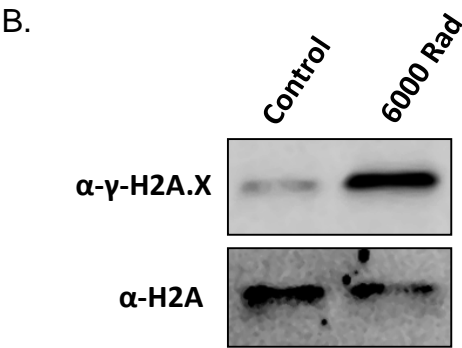
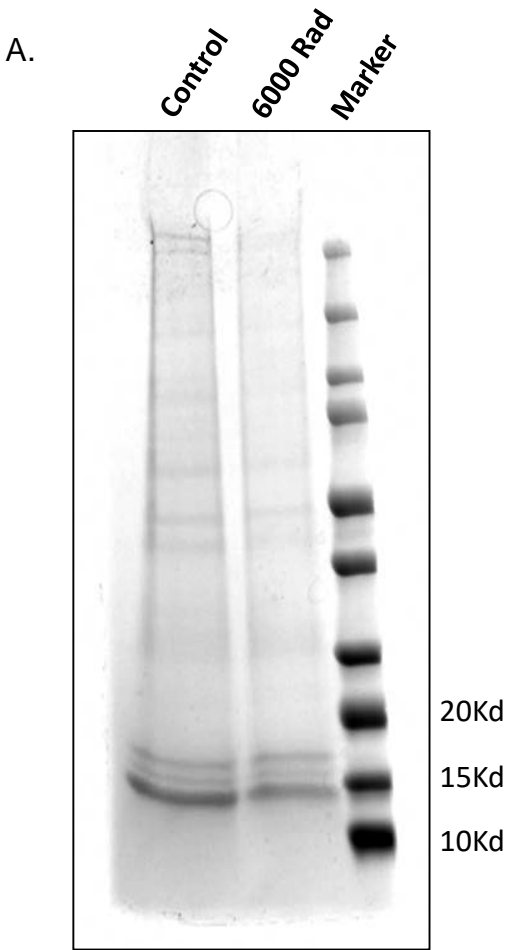


Figure 3



D.

NVLLPKK**S**^PQLKAGTANQDY

Peptide Summary

Sequence: NVLLPKKSQLKAGTANQDY, S8-Phospho (79.96633 Da)
Charge: +3, Monoisotopic m/z: 723.37524 Da (0 mmu/-0.01 ppm), MH+: 2168.11118 Da, RT: 41.89 min,
Identified with: Sequest HT (v1.3); XCorr:2.66, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.05 Da

Fragment Matches

Fragment Spectrum

Extracted from: D:\Discoverer_projects\62000-63000\62092\Seq62092_prot_QE3.raw #20247 RT: 41.89
FTMS, HCD@25.00, z=+3, Mono m/z=723.37524 Da, MH+=2168.11118 Da, Match Tol.=0.05 Da

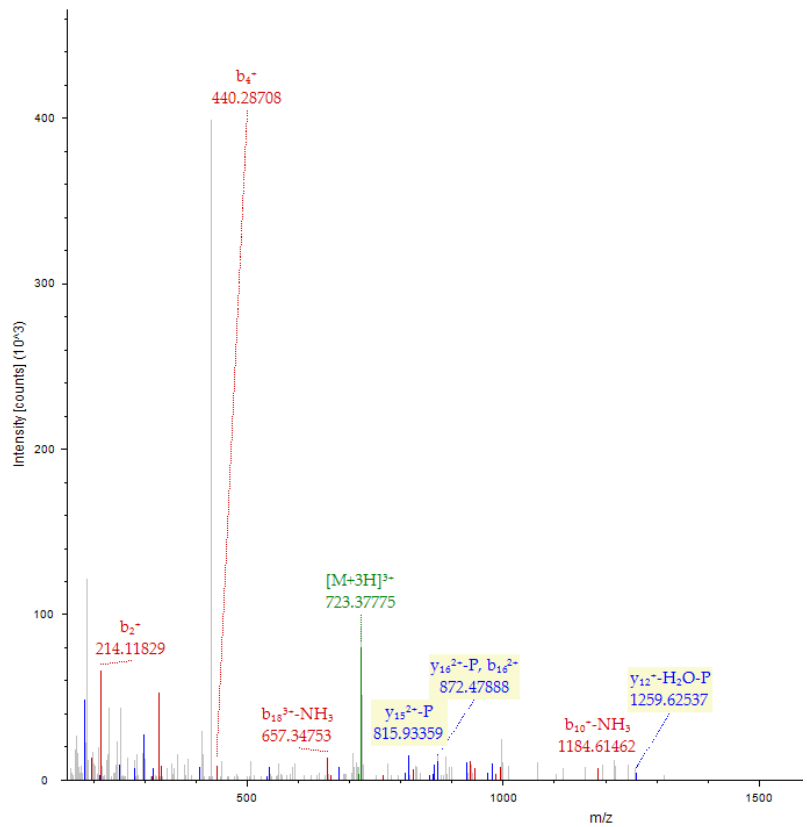
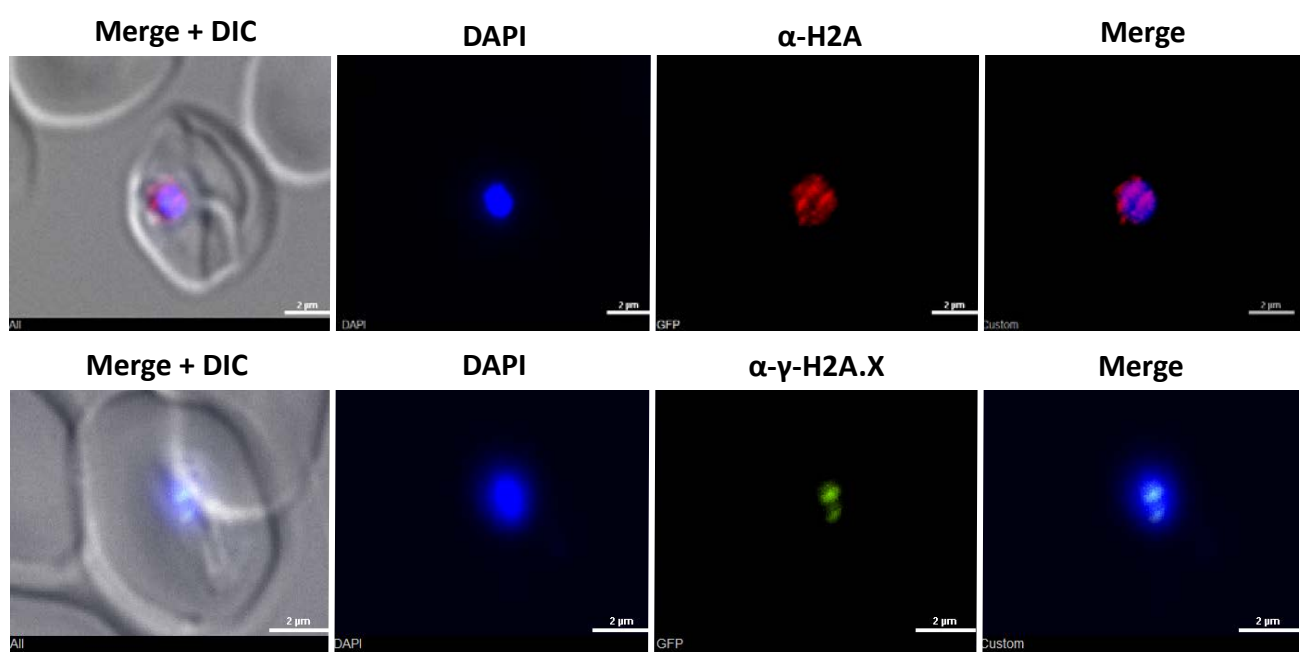


Figure 4

A.



B.

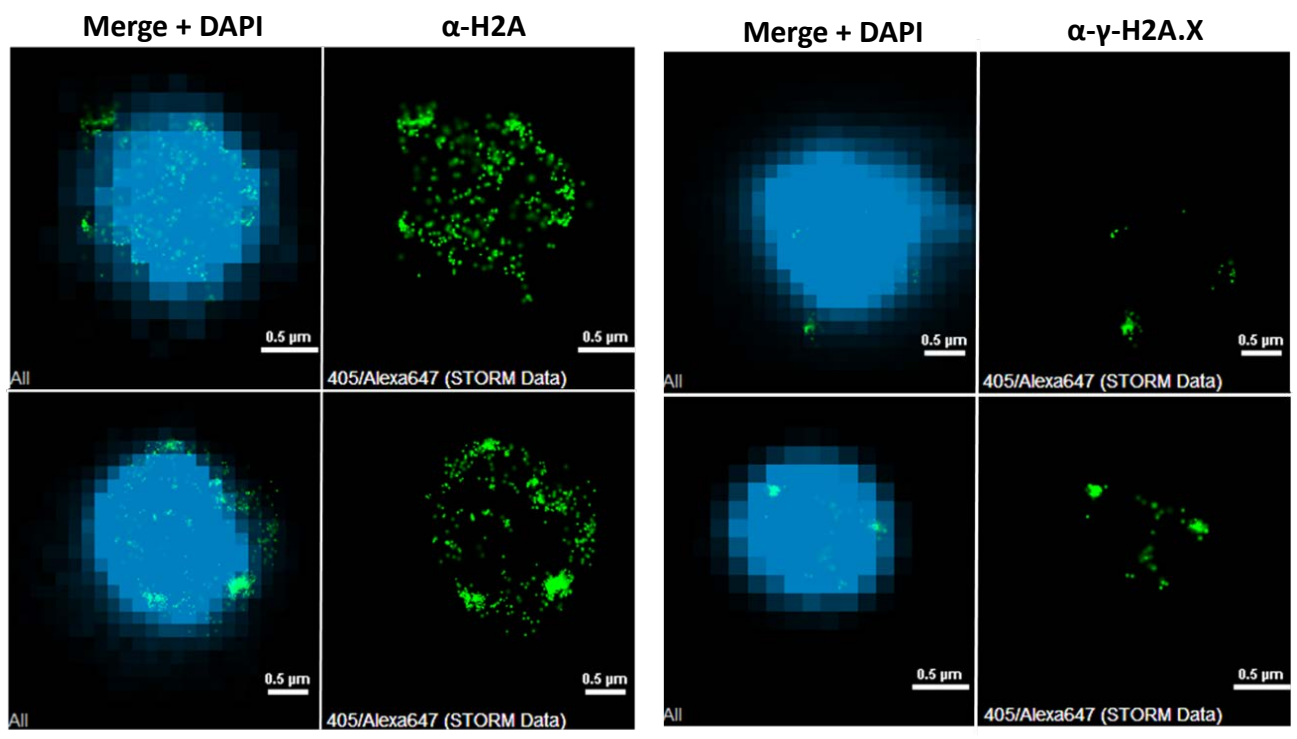
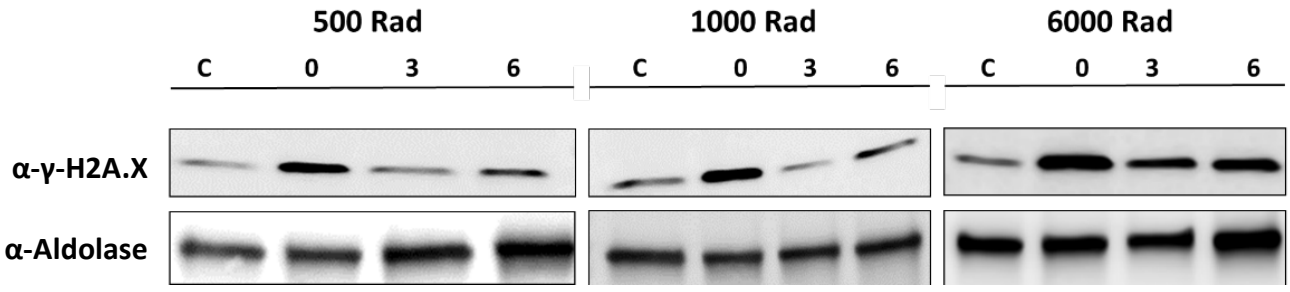


Figure 5

A.



B.

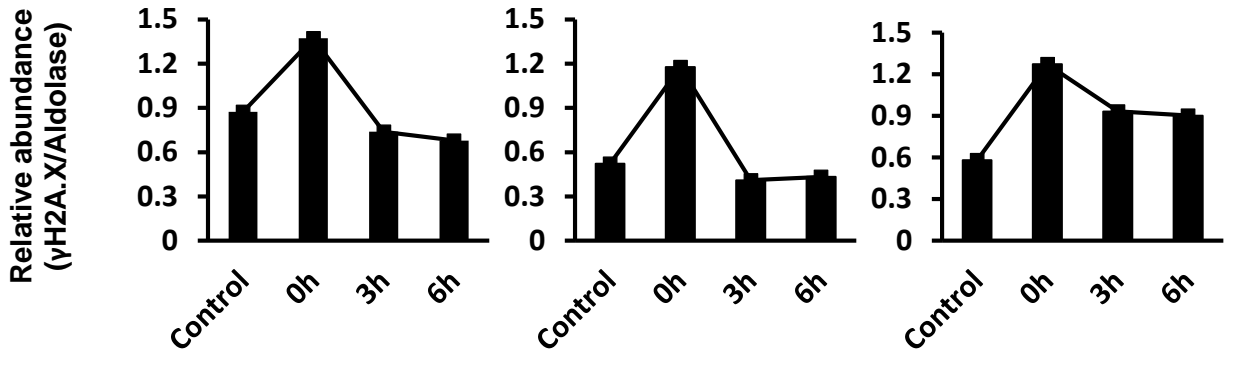


Figure S1

A.

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                1      10      20      30      40      50
PfH2A      .....MSAKGKTG.RKKASKGTSNSAKAGLOFPVGRIGRYLK.KKGKYAKRVGAGAPVYLAAVLEYL
HsH2A.X    .....MSGRGKTG.GKARAKAKSRSRAGLOFPVGRVHRLLR.KGHYAERVGAGAPVYLAAVLEYL
PfH2A.Z    MEVPGKVIGGKVGGKVGGKVLGLGKGGKGKTGSGKTKKAPLSRASRAGLOFPVGRVHRLLKSRISSDGRVGSTAVYAAALEYL

                60      70      80      90      100     110     120     130
PfH2A      CAEILELAGNAARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKAG.....TANQDY
HsH2A.X    TAEILELAGNAARDNKKTRITPRHLQLAIRNDEELNKLLGGVTFAQGGVLPNIQAVLLPKKTSATVGPKAPSGGKKATQASQEY
PfH2A.Z    TAEVLELAGNATKDLKVKRITPRHLQLAIRGDEELDTLK.ATAGGGVIEHIHKALMNKVPLPPTAQKKPKN.....**..

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B.

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                1      10      20      30      40      50      60      70
PvxH2A      MSAKGKTGRKKAVKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA
PvH2A       MSAKGKTGRKKAVKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA
PyH2A       MSAKGKTGRKKAVKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA
PkH2A       MSAKGKTGRKKAVKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA
PbH2A       MSAKGKTGRKKAVKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA
PcH2A       MSAKGKTGRKKAVKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA
PfH2A       MSAKGKTGRKKASKGTSNSAKAGLOFPVGRIGRYLKKGKYAKRVGAGAPVYLAAVLEYLCAEILELAGNA

                80      90      100     110     120     130
PvxH2A      ARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKSGATANQDY
PvH2A       ARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKSGATANQDY
PyH2A       ARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKSGATANQDY
PkH2A       ARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKSGATANQDY
PbH2A       ARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKSGATANQDY
PcH2A       ARDNKKSRITPRHIQLAVRNDEELNKFLAGVTFASGGVLPNIHNVLLPKKSQLKSGATANQDY
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                **

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Figure S2

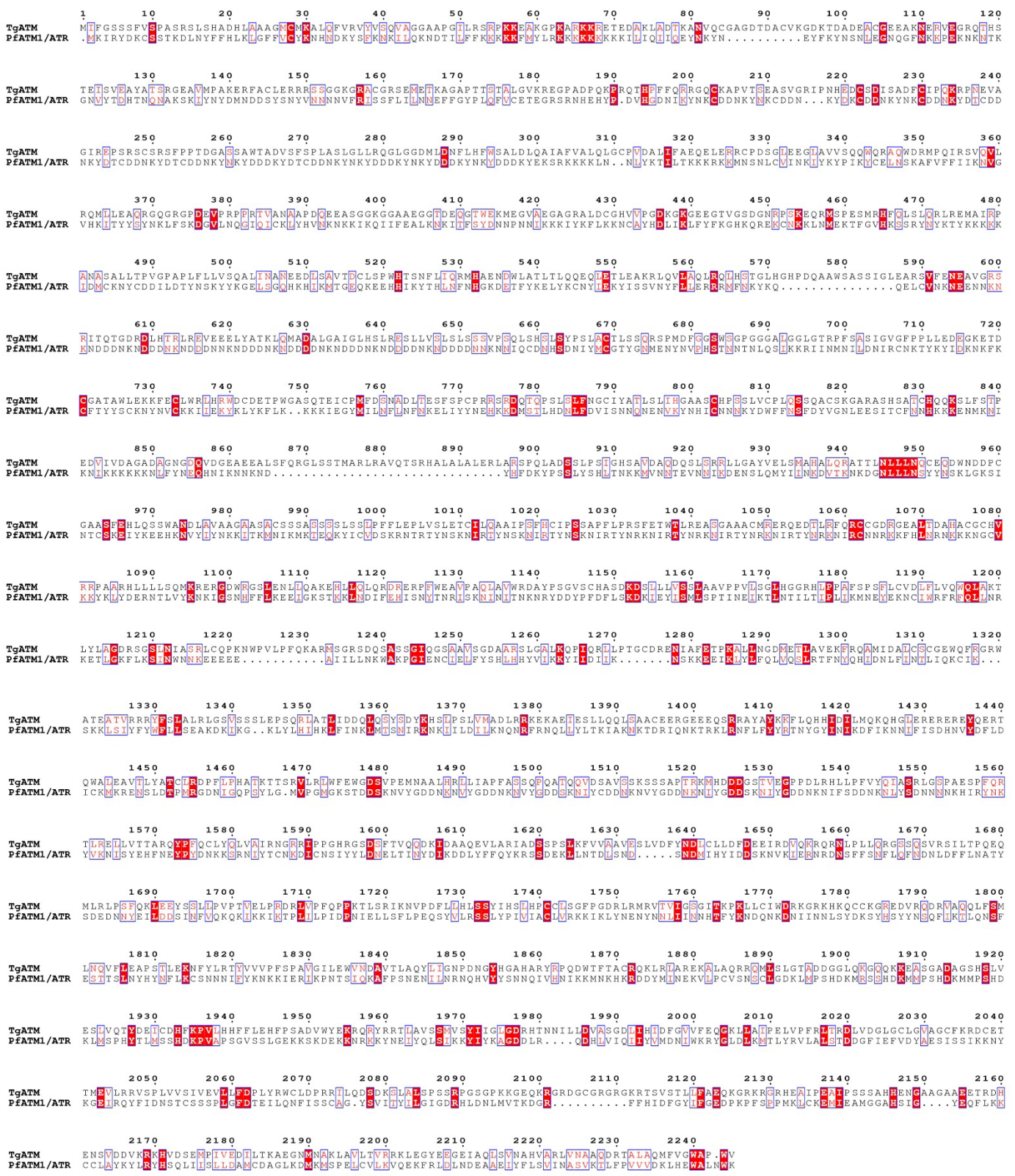
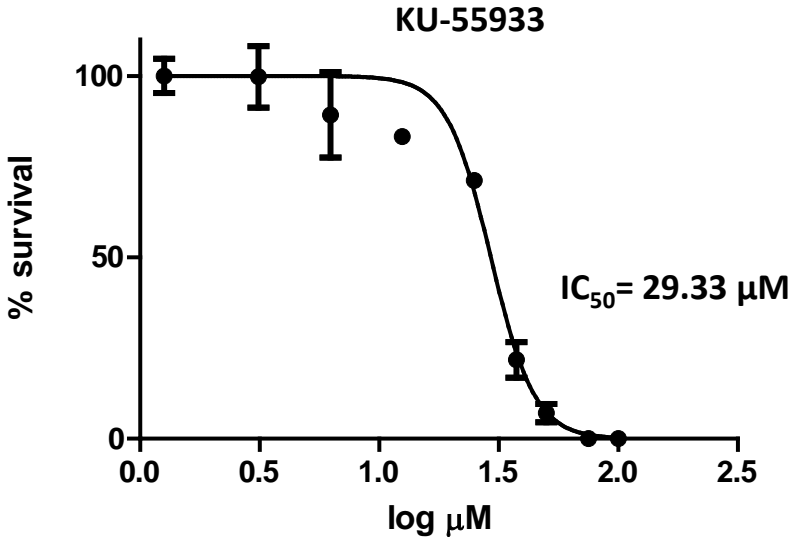
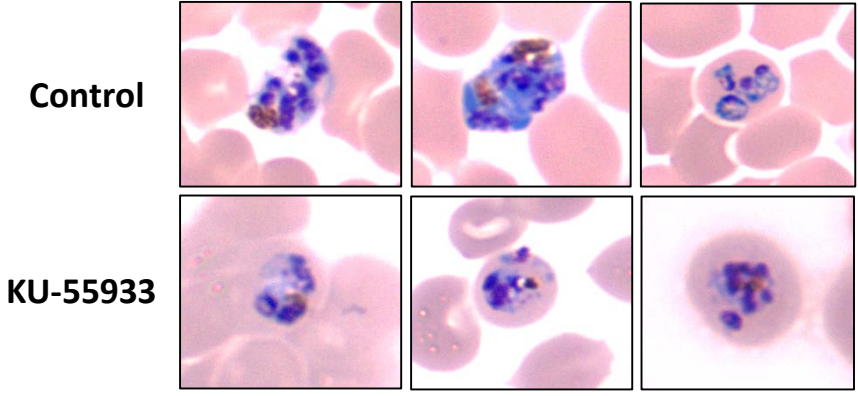


Figure S3

A.



B.



C.

