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1 **Regardless of the deposition pathway, aminoacid 31 in histone**  
2 **variant H3 is essential at gastrulation in Xenopus**

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4

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13

14 ***Abstract***

15 The closely related replicative H3 and non-replicative H3.3 variants show specific  
16 requirement during development in vertebrates. Whether it involves distinct mode of  
17 deposition or unique roles once incorporated into chromatin remains unclear. To disentangle  
18 the two aspects, we took advantage of the Xenopus early development combined with  
19 chromatin assays. Our previous work showed that in Xenopus, depletion of the non-  
20 replicative variant H3.3 impairs development at gastrulation, without compensation through  
21 provision of the replicative variant H3.2. We systematically mutated H3.3 at each four  
22 residues that differ from H3.2 and tested their ability to rescue developmental defects.  
23 Surprisingly, all H3.3 mutated variants functionally complemented endogenous H3.3,  
24 regardless of their incorporation pathways, except for one residue. This particular residue, the  
25 serine at position 31 in H3.3, gets phosphorylated onto chromatin in a cell cycle dependent  
26 manner. While the alanine substitution failed to rescue H3.3 depletion, a phosphomimic  
27 residue sufficed. We conclude that the time of gastrulation reveals a critical importance of the  
28 H3.3S31 residue independently of the variant incorporation pathway. We discuss how this  
29 single evolutionary conserved residue conveys a unique property for this variant in  
30 vertebrates during cell cycle and cell fate commitment.

31

32

### 33 ***Introduction***

34 The organization of DNA into chromatin provides not only a means for compaction but also a  
35 versatile landscape contributing to cell fate and plasticity (Schneider and Grosschedl 2007;  
36 Yadav et al. 2018). The basic unit, the nucleosome core particle, is composed of a histone  
37 tetramer (H3-H4)<sub>2</sub> flanked by two histone dimers H2A-H2B, around which 147bp of DNA  
38 are wrapped as shown by crystal structure (Luger et al. 1997). Importantly, modulation of this  
39 unit exploits the choice of histone variants and reversible PTMs (Post-translational  
40 modification) to impact cell functions (Kouzarides 2007; Gurard-Levin and Almouzni 2014).  
41 Three out of the four-histone families possess histone variants (Franklin and Zweidler 1977;  
42 Sarma and Reinberg 2005; Buschbeck and Hake 2017; Sitbon et al. 2017; Talbert and  
43 Henikoff 2017). In the H3 family, while the centromere-specific histone CENP-A  
44 (Centromere protein A) is very distinct and marks the centromere, the other well-  
45 characterized H3 histone variants are closely related and thought to ensure similar functions  
46 (Maze et al. 2014). Indeed, while they show similar structural features at the core particle  
47 level (Tachiwana et al. 2011), an important difference concerned their cell cycle regulation  
48 and distinct mode of incorporation (Mendiratta et al. 2018). In human, the two replicative  
49 variants H3.1 and H3.2 are incorporated into chromatin in a DSC (DNA-synthesis coupled)  
50 manner via the histone chaperone complex CAF-1 (Chromatin assembly factor 1) (Stillman  
51 1986; Smith and Stillman 1989; Gaillard et al. 1996; Tagami et al. 2004; Polo et al. 2006;  
52 Groth et al. 2007; Latreille et al. 2014). By contrast, the non-replicative form H3.3, which  
53 differs from H3.1 and H3.2 by five and four residues respectively, is incorporated in a DSI  
54 (DNA-synthesis independent) manner (Ahmad and Henikoff 2002b; Ray-Gallet et al. 2002;  
55 Tagami et al. 2004; Ray-Gallet et al. 2011). This incorporation depends on the histone  
56 chaperone complex HIRA (Histone regulator A) in euchromatin regions (Lamour et al. 1995;  
57 Ray-Gallet et al. 2002; Tagami et al. 2004; Goldberg et al. 2010; Ray-Gallet et al. 2011),  
58 while the presence of H3.3 at telomeres and pericentric heterochromatin rather relies on the  
59 histone chaperone complex DAXX (Death domain-associated protein)/ATRX (Alpha  
60 thalassemia/mental retardation syndrome X-linked) (Gibbons et al. 1995; Yang et al. 1997;  
61 Drane et al. 2010; Goldberg et al. 2010; Lewis et al. 2010). Thus, the dynamics of the  
62 different histone variants with their deposition is critically linked to dedicated histone  
63 chaperones (Gurard-Levin et al. 2014; Hammond et al. 2017). Inspired by the quote “nothing  
64 in biology makes sense except in the light of evolution” (Dobzhansky 1973), we considered  
65 H3 variants in light of their conservation in different organisms. In *Saccharomyces cerevisiae*,  
66 the only non-centromeric histone H3 is mostly related to H3.3, and achieves both replicative  
67 and non-replicative variant functions, illustrating the capacity of survival with a single variant  
68 (Dion et al. 2007; Jamai et al. 2007; Rufiange et al. 2007). Intriguingly, however, humanized

69 *S. cerevisiae* with all histones replaced by human orthologs survived better with hH3.1 than  
70 hH3.3 (Truong and Boeke 2017). The better adaptation to H3.1 could argue for non-essential  
71 roles of H3.3 in *S. cerevisiae*, a unicellular organism. However, in metazoans like *Drosophila*  
72 *melanogaster*, the replicative variant can compensate for the loss of H3.3 during development  
73 in somatic tissues, although the adults are sterile (Loppin et al. 2005; Bonnefoy et al. 2007;  
74 Hodl and Basler 2009; Sakai et al. 2009; Orsi et al. 2013). Since the sterility could simply  
75 reflect a shortage of maternal H3.3 to replace protamine from sperm chromatin after  
76 fertilization, the most parsimonious hypothesis suggests that the nature of the variant itself  
77 might not necessarily be essential. Similarly, H3.3 is dispensable in *Caenorhabditis elegans*,  
78 since its removal does not result in lethality but rather to reduced fertility and viability in  
79 response to stress (Delaney et al. 2018). In *Arabidopsis thaliana*, in contrast, replicative and  
80 non-replicative H3 variants are clearly essential. The absence of H3.3 leads to embryonic  
81 lethality and also partial sterility due to defective male gametogenesis (Wollmann et al.  
82 2017). In vertebrate models like *Mus musculus*, the deletion of one of the two copies of the  
83 H3.3 gene leads not only to sterility but also to developmental defects at E12.5 (Couldrey et  
84 al. 1999; Bush et al. 2013; Tang et al. 2015). Finally in human, while a complete absence is  
85 not reported, dominant effects with substitutions in H3.3, like H3.3 K27M and H3.3 G34R/V,  
86 along with mutations affecting their chaperones like DAXX/ATRX, have been involved in  
87 distinct aggressive cancers (Heaphy et al. 2011; Jiao et al. 2011; Schwartzenuber et al. 2012;  
88 Wu et al. 2012; Behjati et al. 2013; Lewis et al. 2013; Lindroth and Plass 2013; Fontebasso et  
89 al. 2014; Taylor et al. 2014; Wu et al. 2014). Furthermore, not only mutation but also  
90 expression of histone chaperones is often found altered in aggressive cancer (Corpet et al.  
91 2011; Montes de Oca et al. 2015; Gurard-Levin et al. 2016). Thus, the developmental defects,  
92 along with situation encountered in particular cancers, underline the importance of each H3  
93 variant and their chaperones in vertebrates. Given the limited sequence difference between  
94 H3 variants, a major puzzle is whether the need for a particular histone variant could reflect  
95 either (i) a unique mode of incorporation and provision or (ii) a distinct identity once  
96 incorporated into chromatin to drive their functions. While, the first hypothesis had been  
97 largely favored, based on previous work including ours, the issue has never been formally  
98 addressed. To disentangle these two possibilities, we decided to use *Xenopus laevis* as it is an  
99 ideal model system to tackle this issue. Indeed, extensively characterized both in  
100 developmental biology and chromatin studies (Laskey et al. 1977; Almouzni and Mechali  
101 1988; Almouzni et al. 1990; Almouzni and Wolffe 1993), its external development permits  
102 direct access to embryos for observation and manipulation (Nieuwkoop and Faber 1994).  
103 With retention of H3 variants in sperm (Katagiri and Ohsumi 1994) and only one replicative  
104 histone H3.2, it offers a simpler situation while retaining amino acid sequence conservation  
105 with human variants for both H3.2 and H3.3. Furthermore, following fertilization,

106 development starts by twelve rapid embryonic cell divisions, which include only S and M  
107 phases (Newport and Kirschner 1982; Etkin 1988; Masui and Wang 1998). At the MBT  
108 (midblastula transition), zygotic activation occurs concomitantly with a progressive  
109 lengthening of the cell cycle, to reach, at gastrulation, a typical cell cycle with two gap  
110 phases. In addition, cells begin to engage into defining different types with acquisition of  
111 migration properties. Importantly, previous work in our laboratory revealed a specific  
112 requirement of H3.3 during *Xenopus laevis* early development at the time of gastrulation  
113 (Szenker et al. 2012). This work showed that depletion of endogenous H3.3 (further referred  
114 to as H3.3) leads to severe gastrulation defects that cannot be rescued by providing the  
115 replicative counterpart H3.2 but only by H3.3 itself. Interestingly, there are only four residues  
116 that diverge between these two forms. A first region of divergence now known as the AIG  
117 motif, in the globular domain of H3.3, accounts for histone variant recognition by dedicated  
118 histone chaperones (Ahmad and Henikoff 2002a; Elsasser et al. 2012; Liu et al. 2012;  
119 Ricketts et al. 2015). This first region is thus key for the choice of deposition pathways. The  
120 other distinctive residue, a serine only present in H3.3, is located on the histone N-terminus,  
121 at position 31, and can be phosphorylated (Hake et al. 2005; Wong et al. 2009; Hinchcliffe et  
122 al. 2016). In our study, we systematically mutated the H3.3 histone variant in its distinct  
123 residues to assess their ability to rescue the gastrulation defects and examined their  
124 incorporation mode. In our assays, we find that mutations affecting the incorporation pathway  
125 proved neutral to set specific H3.3 functions at the time of gastrulation. By contrast, the serine  
126 at position 31 is critical in the rescue experiments after H3.3 depletion. In *Xenopus*, this  
127 residue, which is notably conserved in human cells, gets phosphorylated during the cell cycle  
128 with a peak in mitosis. Remarkably, in our rescue experiments, the phosphomimic form,  
129 which cannot be dynamically modified, fully rescued. We discuss how this residue and the  
130 charge at this site can contribute to establish particular chromatin states important in the  
131 context of somatic cell cycle and development.

132

## 133 ***Results***

### 134 **Conservation of histone H3 variants and their chaperones to assay H3.3 defects at** 135 **gastrulation in *Xenopus*.**

136 While *Homo sapiens* presents two replicative H3 variants, H3.1 and H3.2, *Xenopus laevis*  
137 only possesses one replicative variant, H3.2. Both H3.2 and H3.3 are conserved with their  
138 human orthologs (Figure 1 A). Interestingly, the two H3 variants are almost identical and  
139 conserved through evolution (Waterborg 2012) (Figure 1 B). Two regions show differences in  
140 H3.2 and H3.3. The first one encompasses positions 87, 89 and 90 with a serine, a valine, and  
141 a methionine, known as the SVM motif in H3.2. Instead, these positions correspond to an

142 alanine, an isoleucine and a glycine, known as the AIG motif in H3.3. The second difference  
143 lies at position 31 where H3.2 shows an alanine and H3.3 a serine. Considering sequences for  
144 H3.2 and H3.3 histone variants from five different model organisms in which the function  
145 along with the deposition pathways of H3 variants have been studied, the replicative variant  
146 H3.2 shows around 3% (four variable residues out of 136). In the case of the non-replicative  
147 variant H3.3, it varies of 4% differences, with six variable residues. Remarkably, the region  
148 responsible for histone chaperone recognition shows the highest variations, in line with  
149 possible coevolution with their respective histone chaperones that are not closely conserved  
150 (Supplemental figure 1 A). To examine how deposition pathways and the role for histone  
151 variants can be related, targeting these regions could thus be considered. Using *Xenopus*  
152 *laevis* embryos, we had previously found that a morpholino against H3.3 leads to defects  
153 during late gastrulation (Szenker et al. 2012) (Figure 1 C and Supplemental figure 1 B and  
154 Film 1). While the blastopore forms and invaginates during gastrulation, depletion of H3.3  
155 leads to an arrest of the blastopore closure. Exogenous HA-tagged H3.3, but not HA-tagged  
156 H3.2 mRNAs (further referred as eH3s) when co-injected with the morpholino, can rescue the  
157 phenotype (Szenker et al. 2012). To optimize the assay conditions, we decided to titrate the  
158 concentration of morpholino (Supplemental figure 1 C). The lowest concentration enabled the  
159 blastopore to start to invaginate without complete closure and lead to late gastrulation defects,  
160 consistent with our previous findings (Szenker et al. 2012). By increasing by two to fourfold  
161 morpholino concentrations, gastrulation defects coincide with an even earlier phenotype,  
162 where the blastopore closure does not happen at all. These data are in line with a titration  
163 effect, whereby gastrulation time allows to readily reveal H3.3 requirements. We could thus  
164 use it as a read out to assess the role for a distinct mode of incorporation for H3 variants or a  
165 need of the histone variant itself once incorporated.

166

167 **Permuting individually each amino acid of the H3.3 AIG motif towards H3.2 still**  
168 **ensures H3.3 functions during *Xenopus laevis* early development.**

169 To investigate the importance of the deposition pathway, we first look at the H3.3 histone  
170 chaperone recognition motif (Figure 2 A). Incorporation into chromatin of the non-replicative  
171 variant H3.3 occurs at any time of the cell cycle (DSI), and involves the HIRA complex in  
172 gene-rich regions (Ahmad and Henikoff 2002b; Ray-Gallet et al. 2002; Tagami et al. 2004;  
173 Ray-Gallet et al. 2011) and the DAXX/ATR complex in heterochromatin regions (Gibbons  
174 et al. 1995; Yang et al. 1997; Drane et al. 2010; Goldberg et al. 2010; Lewis et al. 2010). In  
175 contrast, incorporation of the replicative variants is coupled to DNA synthesis (DSC) and is  
176 mediated by the CAF-1 complex (Stillman 1986; Smith and Stillman 1989; Gaillard et al.  
177 1996; Tagami et al. 2004; Polo et al. 2006; Groth et al. 2007; Latreille et al. 2014).  
178 Importantly, structural studies enabled the identification of how histone chaperones

179 discriminate the distinct histone variants through a motif located in the globular domain of  
180 histones (Elsasser et al. 2012; Liu et al. 2012; Ricketts et al. 2015). Both DAXX and HIRA  
181 complexes bind to the AIG motif of H3.3, with a particular affinity for the glycine at position  
182 90. Therefore, we mutated H3.3 in the histone chaperone recognition motif to test their ability  
183 to rescue the loss of H3.3. We first designed single mutants for each residue of the AIG motif  
184 (Figure 2 B). To our surprise, every single mutant for the motif, *i.e.* eH3.3 A87S, eH3.3 I89V  
185 and eH3.3 G90M could rescue loss of H3.3, and embryo development occurred with the same  
186 efficiency. We verified that all constructs expressed at similar levels in the embryo  
187 (Supplemental figure 2 A) and incorporated into chromatin (Supplemental figure 2 B). Since  
188 it was possible that early development may allow looser interactions with the dedicated  
189 chaperones, we decided to completely change the recognition motif and assess both  
190 development and incorporation means.

191

192 **Swapping the H3.3 histone chaperone recognition motif with the one from H3.2 still**  
193 **enables *Xenopus laevis* early development yet switching the mode of incorporation.**

194 Since the individual substitution in the H3.3 motif did not affect the developmental rescue *in*  
195 *vivo*, we assessed whether mutation of all three residues of the motif would then affect H3.3  
196 functions (Figure 3). To our surprise, in this context, this eH3.3 SVM hybrid form proved still  
197 able to rescue the loss of H3.3 as eH3.3 WT (wild-type), while eH3.2 WT cannot. In addition,  
198 such mutant form is expressed as well as incorporated into chromatin at similar levels in the  
199 embryo (Supplemental figure 3 A and B). Thus, by substituting the H3.3 recognition motif for  
200 its histone chaperone with the one from H3.2, we could ensure the rescue *in vivo*. Thus, the  
201 next question was whether in this context, the histone chaperone recognition motif actually  
202 determined the respective deposition pathways for each histone variant. The H3.3 dedicated  
203 chaperone complex HIRA recognized best H3 carrying the H3.3 AIG motif (Figure 4 A).  
204 However, p60, one subunit of the CAF-1 complex dedicated to H3.2, could perfectly  
205 recognize eH3.3 SVM, *i.e.* when the whole H3.3 motif is substituted to the one of H3.2. This  
206 shows that eH3.3 SVM can be recognized by CAF-1, arguing for a possible swap in the  
207 means for incorporation. We confirmed this finding *in vivo* by immunoprecipitation of eH3  
208 forms directly from embryos at gastrulation stage (Supplemental figure 4 A). We found that  
209 p60 again only interacted with eH3 carrying the H3.2 SVM motif. Therefore, changing the  
210 motif alters the chaperone interactions. We thus explored the potential impact on the mode of  
211 histone variant incorporation. To test this aim, we performed chromatin assembly assays  
212 using extracts derived from *Xenopus laevis* eggs (Smythe and Newport 1991; Almouzni and  
213 Wolffe 1993). We supplemented *Xenopus* egg extracts with eH3.3 WT, eH3.2 WT or eH3.3  
214 SVM and monitored their incorporation into chromatin using sperm nuclei under conditions  
215 allowing or not DNA synthesis (Figure 4 B). In interphase extracts, sperm DNA forms nuclei,

216 can replicate and reassemble chromatin whereas mitotic extracts lack this DNA replication  
217 capacity. We isolated and analyzed sperm chromatin nuclei from interphase extracts, in  
218 presence or absence of the DNA synthesis inhibitor aphidicolin. Regardless of DNA  
219 synthesis, the incorporation of eH3.3 WT using sperm nuclei occurred with a similar  
220 efficiency in the presence or absence of DNA synthesis. By contrast, eH3.2 WT incorporation  
221 was severely affected by the presence of aphidicolin. Importantly, eH3.3 SVM incorporation  
222 shows the same dependency on DNA replication, arguing that its incorporation mode is  
223 switched toward a DNA-synthesis coupled mechanism. Consistently, p60 recruitment to  
224 chromatin is, as expected, highly reduced when DNA synthesis is inhibited. We further  
225 confirmed in mitotic extracts that only variant with the AIG motif could get incorporated  
226 independently of DNA synthesis (Supplemental figure 4 B). Based on these data, we  
227 conclude that the H3.3 histone variant could be efficiently provided regardless of the pathway  
228 for incorporation. Thus, defects at the time of gastrulation do not arise from a need for a  
229 distinct incorporation pathway outside S phase independently of DNA synthesis. It rather  
230 reflects an inherent feature of the variant when incorporated into chromatin. We thus  
231 examined more closely if the only remaining specific residue of H3.3, aminoacid 31, could  
232 account for this unique feature.

233

### 234 **H3.3S31 residue is critical for *Xenopus laevis* early development and is phosphorylated** 235 **during the cell cycle.**

236 To address the role of the H3.3 residue at position 31, we first constructed a new H3.3  
237 mutant, H3.3 S31A, containing an alanine instead of a serine at position 31 while keeping its  
238 original AIG motif (Figure 5 A and Supplemental figure 5 A). Remarkably, eH3.3 S31A was  
239 not able to rescue H3.3 depletion during *Xenopus* early development, indicating that the  
240 specific serine of eH3.3 at position 31 cannot be substituted by an alanine, which is notably  
241 the corresponding residue of H3.2, to fulfill H3.3 dedicated functions at this window of time  
242 during development. Interestingly, this particular residue has first been found phosphorylated  
243 in human cell line during mitosis (Hake et al. 2005). Furthermore, a threonine replaces this  
244 serine in *Arabidopsis*, a residue that can possibly also undergo phosphorylation, albeit this has  
245 not yet been documented. A key question was thus whether the actual need for H3.3 was  
246 linked to the capacity of H3.3S31 to be phosphorylated. To this end, we examined H3.3S31  
247 phosphorylation in the *Xenopus* system using a *Xenopus* A6 cell line derived from kidney.  
248 By immunofluorescence, we detected a strong enrichment of H3.3S31p during mitosis  
249 (Figure 5 B). Interestingly, when compared to a mitotic modification, H3S10p, common to  
250 both H3 variant forms, its pattern was different, indicating a possible distinct function. While  
251 H3S10p covers the edges of mitotic chromosomes, H3.3S31p is enriched at centric and



252 pericentric heterochromatin. This pattern, similar to previous observations in HeLa B cells  
253 (Supplemental figure 5 B) as in (Hake et al. 2005), is consistent with observations in mouse  
254 and monkey cell lines (Wong et al. 2009; Hinchcliffe et al. 2016). Kinases responsible for  
255 H3.3S31 phosphorylation are still unclear, currently implicating either CHK1 (Chang et al.  
256 2015) or Aurora B (Li et al. 2017). Using *Xenopus* sperm chromatin, we further characterized  
257 the acquisition of this mark prior to or after replication in interphase extract or in extracts  
258 pushed into mitosis (Figure 5 C). We did not detect any significant signal for H3.3S31p in the  
259 soluble pool of H3.3 in either mitotic or interphase extracts, indicating that the modification is  
260 likely acquired once the variant is incorporated into chromatin. In addition, we could not  
261 detect any signal for this mark on non-remodeled sperm chromatin or on chromatin  
262 assembled in interphase extracts. This mark was most enriched in the fraction corresponding  
263 to isolated mitotic chromatin, in a profile similar to the H3S10p mark. We therefore conclude  
264 that H3.3S31p showed predominantly a mitotic chromatin-dependent mark in both somatic  
265 cells and reconstituted chromatin in early embryonic extracts, a mark imposed within  
266 chromatin and not prior to deposition. The next question was whether this modification or  
267 charge was also critical at the time of gastrulation in our rescue experiments.

268

269 **The negative charge of H3.3S31 is essential during *Xenopus laevis* early development at**  
270 **gastrulation.**

271 In order to explore a potential need for the phosphorylation of H3.3S31, we designed another  
272 H3.3 mutant for the serine carrying an aspartic acid, eH3.3 S31D, which acts as a  
273 phosphomimic version of this residue and carries a constitutive negative charge at position 31  
274 that cannot be dynamically regulated by kinases and phosphatases (Figure 6 A). Remarkably,  
275 this mutant form could rescue the depletion of H3.3 comparable to the eH3.3 WT. Notably,  
276 all mutants were expressed and incorporated into chromatin in the same proportion  
277 (Supplemental Figure 6 A and B) and we verified that these mutations on the H3.3 tail did not  
278 indirectly alter the ability to interact with specific histone chaperones (Figure 6 B).  
279 Especially, neither H3.3 S31A nor S31D mutations affected the mode of incorporation of  
280 H3.3 in the *Xenopus* egg extracts-base chromatin assembly assays (Figure 6 C and  
281 Supplemental figure 6 C). This enabled us to discard any defects that could be related to  
282 inefficient incorporation. We can thus conclude that the residue at position 31 in H3.3, either  
283 as a serine or as a negatively charged residue (phosphomimic), is specifically needed for the  
284 function of the H3.3 variant once incorporated into chromatin as revealed at the time of  
285 gastrulation during early development in the vertebrate *Xenopus*.

286

## 287 **Discussion**

288 By exploiting depletion/complementation assays in *Xenopus laevis* and monitoring the  
289 capacity to undergo gastrulation, we disentangle the critical role of H3.3 within chromatin  
290 through its amino acid 31 independently of its mode of incorporation.

291

292 **H3.3 incorporation into chromatin is important during *Xenopus* early development**  
293 **regardless of its mode of incorporation.**

294 Replicative and non-replicative H3 variants do show distinct genome wide distributions  
295 (Clement et al. 2018), with enrichment of H3.3 at enhancers, or proximal to telomeres and  
296 centromeres as observed in several models in somatic cells and embryonic cells (Filipescu et  
297 al. 2014). How these distinct patterns arise and evolve dynamically during development and  
298 are then maintained in given lineages remains to be established. Moreover, how the unique  
299 properties of H3.3 influence cell cycle related functions and/or cell fate programming is still  
300 an open question. We first examined whether the mutations of the residues assigned to a key  
301 role in the choice of the deposition pathway (Ahmad and Henikoff 2002a; Elsasser et al.  
302 2012; Liu et al. 2012; Ricketts et al. 2015) affect the capacity to rescue the depletion of H3.3  
303 (Figure 2 and Figure 3). Surprisingly, an eH3.3 form containing H3.2 recognition motif still  
304 complements H3.3 functions in our developmental assay (Figure 3) even though this swap  
305 between motifs does effectively alter the chaperone interactions and the incorporation  
306 pathway (Figure 4). These data underline the fact that neither the chaperone interaction nor  
307 the mode of incorporation of the variant is critical to enable H3.3 specific roles at this time of  
308 development. Therefore, in the context of *Xenopus* early development, in sharp contrast with  
309 what one would have anticipated, it is the presence *per se* of H3.3 into chromatin that proves  
310 most important *in vivo*, regardless of the means to get incorporated, (Figure 7). This discovery  
311 shed light on unique roles performed by the single amino acid S31 on H3.3, highlighting the  
312 idea that *every amino acid matters* (Maze et al. 2014)!

313

314 **H3.3S31 and a constitutive negative charge are essential for H3.3 functions during**  
315 ***Xenopus* early development.**

316 We showed by replacing the residue at position 31 in the N-terminal tail of H3.3 with the  
317 alanine found in H3.2 that such eH3.3 S31A mutant could not replace H3.3 (Figure 5 A). This  
318 proves that H3.3S31 is essential in our complementation assay after H3.3 depletion.  
319 Moreover, this serine on H3.3 can undergo phosphorylation while the alanine on H3.2 cannot.  
320 This specific phosphorylation of H3.3 regulated during the cell cycle shows enrichment  
321 during metaphase (Hake et al. 2005) (Figure 5 B). In our experiments, remarkably a  
322 phosphomimic form with a negative charge on the serine at position 31 could readily rescue

323 the depletion of H3.3 (Figure 6 A). However, the exact role of H3.3S31 remains enigmatic.  
324 Although, we (Figure 5 and Supplemental figure 5) and others (Hake et al. 2005; Wong et al.  
325 2009; Hinchcliffe et al. 2016) detected strong H3.3S31p signals primarily in mitosis, we  
326 cannot exclude that discrete sites of transcription could be marked but undetected in our  
327 assays. Indeed, this mark has been associated with particular transcribed regions in activated  
328 macrophages (Thorne et al. 2012). Furthermore, in mammals, a function for H3.3S31p has  
329 been linked to intron retention and pre-mRNA processing by preventing binding of the tumor  
330 suppressor BS69, also known as ZMYND11 (Zinc finger MYND domain-containing protein  
331 11), an H3.3K36me3-specific reader (Guo et al. 2014; Wen et al. 2014). Our assays  
332 demonstrate the importance of H3.3S31 at gastrulation (Figure 5 and Figure 6), a critical time  
333 for lineage commitment accompanied by dramatic cell cycle changes and modifications in  
334 gene expression patterns. Taking into account the specific importance given to H3.3 that  
335 ranges from transcription to reprogramming (Ahmad and Henikoff 2002b; Schwartz and  
336 Ahmad 2005; Ng and Gurdon 2008; Jullien et al. 2012), the observed developmental defects  
337 could arise from a role for H3.3S31 and its phosphorylation in transcription initiation or  
338 maintenance. Importantly, MBT in *Xenopus* also leads to changes in the cell cycle  
339 progression: establishment of the longer cell cycle "somatic-type" with the gap phases and  
340 acquisition of checkpoints (Etkin 1988; Masui and Wang 1998). Moreover, histones are key  
341 component in regulating the start of the MBT, possibly through titration mechanisms  
342 (Almouzni and Wolffe 1995; Amodeo et al. 2015). It should be valuable to explore the role of  
343 specific variants into this context. Thus, H3.3S31 function could equally depend on a specific  
344 role of H3.3 during the cell cycle. Indeed, H3.3 has been involved in chromosome  
345 segregation as shown in double H3.3 KO embryonic stem cells that leads to an increase in  
346 anaphase bridges and lagging chromosomes (Jang et al. 2015). Furthermore, this mark can  
347 coat lagging chromosomes in parallel to p53 activation in order to prevent aneuploidy  
348 (Hinchcliffe et al. 2016). Considering H3.3S31p accumulation at centromeres in mitosis  
349 (Figure 5 B) (Hake et al. 2005), it is thus tempting to speculate that H3.3S31p could crosstalk  
350 with CENP-A incorporation at late mitosis and the beginning of G1. Interestingly, CENP-A  
351 incorporation at the centromere has been shown to be dependent on both the presence of H3.3  
352 as a placeholder (Dunleavy et al. 2011) and on transcription (Ohkuni and Kitagawa 2011;  
353 Bobkov et al. 2018). To reconcile both aspects, we could envision that H3.3S31p may act as a  
354 phosphoswitch in mitosis to regulate transcription at critical chromosomal landmarks and in  
355 interphase to control/maintain a transcription program. Alternatively, one could also consider  
356 that the alanine present on H3.2 could prevent particular interactions specific to H3.3S31 and  
357 permitted by an aspartic acid. Notably in *Arabidopsis*, the replicative form cannot be rescued  
358 by H3.3 (*i.e.* A31T mutation) (Jiang and Berger 2017). Indeed, ATXR5 (*Arabidopsis*  
359 trithorax-related protein 5), a plant specific H3K27 methyltransferase, specifically recognizes

360 H3.1 and not H3.3 (Jacob et al. 2014). Therefore, the alanine at position 31 on replicative  
361 histone H3.1 prevents the heterochromatinization of H3.3-rich regions during replication.  
362 Thus, in plants, the replicative histone H3 variant avoids the presence of a negative charge at  
363 position 31. In our work, we show that there is potentially no need for the dynamics of the  
364 mark. Rather, we demonstrate a need to provide a negative charge or to avoid an alanine  
365 during early development. Indeed, our data indicate that the dynamics of the H3.3S31p may  
366 be dispensable, since the phosphomimic mutant is able to fully rescue H3.3 depletion (Figure  
367 6). We can foresee several possible explanations. On the one hand, the negative charge at  
368 position 31 could be an absolute requirement for H3.3 functions regardless of any dynamics.  
369 On the other hand, the negative charge on this residue could be associated with mechanisms  
370 that occur solely in mitosis and the dynamics may simply be ensured by removal of the  
371 variant without invoking a particular phosphatase. More work, specifically exploring eviction  
372 mechanisms is needed to disentangle these two possibilities. Thus, the residue at position 31  
373 of the replicative and non-replicative H3 variant may be key to promote/allow or exclude the  
374 presence of a negative charge with different outcomes for critical binding partners.  
375 Conversely, other modifications on H3 tail may impact S31 and in this respect the existence  
376 of mutations affecting neighboring residues will be interesting to explore. Notably, H3.3S31  
377 is close to residues often found substituted in aggressive cancers like pediatric glioblastoma  
378 (H3 K27M and G34R) (Heaphy et al. 2011; Jiao et al. 2011; Schwartzenuber et al. 2012;  
379 Wu et al. 2014). Thus, it will be very interesting to evaluate the connection between these  
380 particular cancers involving oncohistones with this H3.3 specific phosphorylation. Altogether,  
381 we show that H3.3S31 is the key residue that confers specific functions to H3.3 within  
382 chromatin. It establishes for the first time the importance of a distinct histone variant residue  
383 for the proper development of a vertebrate during gastrulation. Future work should explore  
384 whether a similar requirement also occurs in mammals to provide a comprehensive view of  
385 the importance of the non-replicative variant H3.3 and its role during vertebrate development  
386 and in disease states.

387

388 *"Qu'importe le flacon, pourvu qu'on ait l'ivresse"*, Alfred de Musset

## 390 **Material & Methods**

### 391 ***Xenopus laevis* embryo manipulation**

392 We used *Xenopus laevis* adults (2 years old) from the Centre de Ressource Biologie Xenope.  
393 We prepared embryos at 18°C as in (Almouzni et al. 1994) and staged them according to  
394 (Nieuwkoop and Faber 1994). We acquired embryos during gastrulation with a MZFLIII  
395 magnifier (Leica) and the SPOT software. Animal care and use for this study were performed  
396 in accordance with the recommendations of the European Community (2010/63/UE) for the  
397 care and use of laboratory animals. Experimental procedures were specifically approved by  
398 the ethics committee of the Institut Curie CEEA-IC #118 (Authorization APAFIS#11226-  
399 2017091116031353-v1 given by National Authority) in compliance with the international  
400 guidelines. David Sitbon, Ekaterina Boyarchuk and Geneviève Almouzni possess the  
401 Authorization for vertebrates' experimental use.

402

### 403 **Plasmid cloning and mRNA transcription**

404 We cloned all H3 cDNAs in the pβRN3P vector (Zernicka-Goetz et al. 1996). This vector  
405 stabilizes RNA and improves their translation efficiency while injected into *Xenopus* eggs. In  
406 addition, an HA tag is inserted in the C-terminal of H3. We obtained mRNAs by *in vitro*  
407 transcription of PCR-amplified fragments of the different pβRN3P vectors (Forward: 5'-  
408 gtaaaacgacggccagt-3' and Reverse: 5'-ggaaacagctatgaccatga-3'). We transcribed mRNAs  
409 starting with 5ng of PCR-amplified fragment, 10μL Buffer 5X, 5μL of DTT 100mM, 0.25μL  
410 bovine serum albumin (BSA) 10mg/mL (NEB), 5μL of ATP, CTP, UTP 10mM, 1.65μL GTP  
411 10mM (Sigma-Aldrich), 3.35μL Me7GTP 10mM (NEB), 2μL of RNasin Plus RNase  
412 Inhibitor (Promega), and 50μL H2O qsp. After 10min on ice, we added 2μL of T3 RNA  
413 Polymerase (Promega) to each sample as well as after 30min at 37°C for another 1h30. After  
414 DNA digestion, we extracted mRNAs with phenol-chloroform and purified them through  
415 Sephadex G-50 Quick Spin Column for radiolabeled RNA purification (Sigma-Aldrich),  
416 previously equilibrated 6 times with 1mL of TE 10mM.

417

### 418 **Morpholino and mRNA microinjection into *Xenopus laevis* embryo**

419 We microinjected 2-cell embryos using a Brinkmann micromanipulator and a Drumond  
420 microinjector on two-cell stage eggs with an injection volume set to 9.2nL to deliver the  
421 appropriate quantity of morpholino and mRNAs (1X=4.6ng). Between 20 and 30 embryos are  
422 injected per condition, with at least 3 biological replicates. We knocked down H3.3 with  
423 morpholino designed to bind to the initiation region of *Xenopus laevis* H3.3 mRNAs (Szenker  
424 et al. 2012).

425

426 ***Xenopus laevis* embryo protein extract preparations and Western blotting**

427 We prepared total protein extracts from *Xenopus laevis* embryos using the CellLytic Express  
428 reagent (Sigma-Aldrich) and centrifuging at full speed the extracts after 30min incubation.  
429 We analyzed protein samples by electrophoresis on 4%–12% NuPAGE SDS-PAGE gels with  
430 MES SDS Running buffer (Life Technologies) and corresponding LDS buffer NuPage  
431 (Invitrogen) with DTT. Primary antibodies were detected using horse-radish-peroxidase-  
432 conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and ECL2 kit  
433 (Pierce).

434

435 ***Xenopus laevis* embryo immunoprecipitation**

436 We collected embryos at stage 12 and centrifuged them at 25000g after 3 washes in TE buffer  
437 (10mM Hepes, 75mM KCl and 50mM Sucrose). After ultracentrifugation at 150000g of the  
438 supernatant, we used 100µg of each condition for every immunoprecipitation overnight at  
439 4°C in 400µL of IP buffer (10mM Hepes, 150mM KCl, 0;05% NP-40, 5mM DTT and  
440 protease inhibitors). After 3 washes, we eluted proteins and analyzed samples by  
441 electrophoresis on 4%–12% NuPAGE SDS-PAGE gels with MOPS SDS Running buffer  
442 (Life Technologies) and corresponding LDS buffer NuPage (Invitrogen) with DTT. Primary  
443 antibodies were detected using horse-radish-peroxidase-conjugated secondary antibodies  
444 (Jackson ImmunoResearch Laboratories) and ECL2 kit (Pierce).

445

446 ***Xenopus laevis* embryo fractionation**

447 We collected 40 embryos at stage 12 and performed fractionation as in (Szenker et al. 2012).  
448 We lysed the embryos in 200µL of Lysis Buffer 1 (10mM Tris-HCl pH 7.5, 200mM NaCl,  
449 5mM MgCl<sub>2</sub>, 0.5% NP40, protease inhibitors) or Lysis Buffer 2 (10mM Tris-HCl pH 7.5,  
450 10mM NaCl, 5mM MgCl<sub>2</sub>, 0;5% NP40, protease inhibitors) and centrifuged them at 1000g  
451 for 2min at 4°C. We obtained the clear soluble fraction after ultracentrifugation of the  
452 supernatant at 160000g for 1 hour at 4°C from Lysis Buffer 1. We washed the chromatin  
453 fraction from Lysis Buffer 2 after centrifugation in Buffer A (10mM Tris-HCl pH 7.5, 15mM  
454 NaCl, 60mM KCl, 0.34M sucrose, 1mM DTT, protease inhibitors). We performed MNase  
455 (Roche Diagnostics, #1010792100) digestion during 10min with 11.25U/mL final  
456 concentration, after addition of CaCl<sub>2</sub> (2mM final) and RNase A (Roche Diagnostics,  
457 #10109142001) treatment (75µg/ml final, 5min at 37°C). We stopped the reaction by EDTA  
458 (50mM final) and recovered solubilized chromatin fraction after a centrifugation at 1000 g for  
459 2min.

460

461

462

#### 463 ***Xenopus laevis* egg extract preparation**

464 We prepared *Xenopus laevis* sperm nuclei and low-speed extracts arrested by CSF (cytostatic  
465 factor) of *Xenopus laevis* eggs as previously described (Kornbluth et al. 2001). Briefly, we  
466 collected eggs freshly and centrifuged them at low speed (16000g) to conserve the mitotic  
467 phase, below lipids. We induced interphase by the addition of CaCl<sub>2</sub> at the final concentration  
468 0.06mM to CSF-arrested egg extracts. We added sperm chromatin at a concentration 1000–  
469 4000 nuclei/μl. After DNA replication, a 2/3 volume of the corresponding CSF-arrested  
470 extract was added to induce mitosis.

471

#### 472 ***Xenopus laevis* egg extract immunoprecipitation**

473 Briefly, we produced recombinant H3 mutant proteins from mRNAs using rabbit reticulocyte  
474 lysate (Promega L4600). After 3h of incubation at 4°C in interphase extracts followed by  
475 another 3h incubation with anti-HA beads, we pulled-down and washed the proteins in 0.8x  
476 CSF-XB buffer (10mM Hepes-KOH, pH 7.7, 100mM KCl, 2mM MgCl<sub>2</sub>, 5mM EGTA)  
477 containing 5% glycerol, 0.5% Triton X-100 and protease and phosphatase inhibitors.

478

#### 479 ***Xenopus laevis* sperm chromatin purification**

480 We diluted fivefold 100μl aliquots of each reaction with 0.8× CSF-XB buffer containing  
481 20mM β-glycerophosphate, 5% glycerol and 0.5% Triton X-100, which we incubated for 1  
482 min at RT. We then layered the samples onto a 35% glycerol-containing CSF-XB cushion  
483 and centrifuged them at 10000g for 5 min at 4°C. We resuspended the pellets in the same  
484 buffer, and repeated the centrifugation step. For purification of interphase chromatin, we  
485 diluted 100μl aliquots of extract with 0.8× CSF-XB buffer containing 20 mM β-  
486 glycerophosphate and 5% glycerol, which we incubated for 1 min at RT, followed by  
487 centrifugation through the cushion at 10000g for 5 min at 4°C. We analyzed protein samples  
488 by electrophoresis on 4%–12% NuPAGE SDS-PAGE gels with MES SDS Running buffer  
489 (Life Technologies) and corresponding LDS buffer NuPage (Invitrogen) NuPage reducing  
490 agent (Invitrogen). Primary antibodies were detected using horse-radish-peroxidase-  
491 conjugated secondary antibodies (Jackson Immunoresearch Laboratories). We revealed the  
492 signal by chemiluminescence with SuperSignal West Dura Extended Duration Substrate  
493 (ThermoFisher). The signal was acquired using ChemiDoc Imager (Biorad).

494

#### 495 **Chromatin assembly assays**

496 We added 100000 sperm nuclei to each 150μL of corresponding extracts with or without  
497 aphidicolin (50μg/mL). We then supplied 15μL of the rabbit reticulocyte lysate used to  
498 produce recombinant H3 mutant proteins from mRNAs. After 40min of incubation at room

499 temperature, we purified chromatin and we analyzed protein samples by electrophoresis as  
500 described above.

501

### 502 **WebLogo sequence alignment**

503 After performing multiple sequence alignment using MUSCLE (Edgar 2004), we displayed  
504 the alignments using WebLogo3 (Crooks et al. 2004) with probability units.

505

### 506 **Antibody list**

Antibody List	WB	IF
HA (3F10, Roche)	1/1000	/
H3.3S10p (Mabi0312, Wako)	1/1000	1/1000
H3.3S31p (ab92628, Abcam)	1/1000	1/200
H3.3 (M01, Abnova)	1/200	/
H4 (05-858, Upstate)	1/1000	/
Tubulin (T9026, Sigma)	1/1000	/
H3 (ab1791, Abcam)	1/5000	/
xEmi2 (From Mary Dasso)	1/1000	/
xHIRA (Agro Bio)	1/1000	/
xDAXX (From Mary Dasso)	1/500	/
xp60 (Agro Bio)	1/1000	/
xCENP-A (From Aaron Straight)	1/1000	/

507

### 508 **Immunofluorescence and epifluorescence microscopy**

509 We first fixed A6 and HeLa B cells on coverslips for 20 min in 4% and 2%  
510 paraformaldehyde, respectively, before to permeabilize them with 0.2% Triton X-100. We  
511 blocked them for 45min with 5% bovine serum albumin. We then incubated coverslips with  
512 primary and secondary antibodies and stained them with DAPI. We mounted the coverslips in  
513 Vectashield medium. We used a Confocal Zeiss LSM780, and we acquired images using  
514 63×/1.4NA under Zen blue software (Zeiss Germany).

515



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691

## 692 ***Author contributions***

693 D.S., E.B. and G.A. conceived the overall strategy and wrote the paper. G.A. supervised the  
694 work. D.S. performed *Xenopus laevis* and cellular experiments and analyzed data. E.B.  
695 performed *Xenopus laevis* egg extract experiments and analyzed data. Critical reading and  
696 discussion of data involved all authors.

697

## 698 ***Competing interests***

699 The authors declare no competing interests.

700

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- 992

## 516 **Figure Legends**

517 **Figure 1: H3.3 is essential for gastrulation of *Xenopus laevis*.** **A)** Best-studied non-  
518 centromeric H3 histone variants in *Homo sapiens* and *Xenopus laevis*. The two well-  
519 characterized forms of non-centromeric H3 variants correspond to the replicative histones  
520 H3.1 and H3.2 and the non-replicative histone H3.3, depicted in purple and green  
521 respectively. In human, the replicative H3 form is comprised of H3.1 and H3.2 that differ by  
522 only one residue at position 96, a cysteine and a serine respectively. The non-replicative form  
523 H3.3 shares more than 96% of identity with replicative forms, with five and four residue  
524 differences with H3.1 and H3.2 respectively. Additionally, *Xenopus laevis* embryos possess  
525 only one replicative histone variant, H3.2. Finally, histone sequences are conserved between  
526 *Homo sapiens* and *Xenopus laevis*. **B)** Logo for H3 variant sequence differences of *Homo*  
527 *sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Xenopus laevis*, and *Arabidopsis thaliana*  
528 after performing multiple sequence alignment using MUSCLE. We display this alignment  
529 using WebLogo3. Specific histone variants are highlighted in green and purple. Histone  
530 variants show an extreme conservation between species. **C)** Developmental assay to monitor  
531 H3.3 functions. Morpholino and eH3.3 mRNA are injected at the 2-cell stage and associated  
532 defects can be observed at the gastrulation stage if there is no rescue. Scale bar corresponds to  
533 500 $\mu$ m. See film 1.

534

535 **Figure 2: eH3.3 AIG single mutants rescue depletion of H3.3 during *Xenopus laevis***  
536 **early development.** **A)** Highlights of the histone chaperone recognition motif residues of H3  
537 variants. Dedicated histone chaperones recognize histone variants by the H3.2 SVM and H3.3  
538 AIG motifs. Although both motifs are structurally similar, the main difference appears for the  
539 residue 90 that is critical for histone chaperone binding. Crystal structure adapted from PDB  
540 ID codes: 5B0Z (Suzuki et al. 2016) and 3AV2 (Tachiwana et al. 2011). **B)** Rescue assays  
541 with H3.3 AIG single mutants. Injections are performed at 2-cell stage. The morpholino  
542 against H3.3 induces gastrulation defects that are rescued by all eH3.3 with single mutations  
543 of the AIG motif. Scale bar corresponds to 500 $\mu$ m. Quantification of properly developed  
544 embryos after injections of the different H3.3 AIG mutant forms shows no difference of  
545 rescue efficiency after H3.3 depletion. Each experiment has been reproduced at least 3 times  
546 with a minimum of 30 embryos.

547

548 **Figure 3: eH3.3 AIG triple mutant rescues depletion of H3.3 during *Xenopus laevis* early**  
549 **development.** Rescue assays with eH3.3 triple AIG mutant. Injections are performed at 2-cell  
550 stage. The morpholino against H3.3 induces gastrulation defects that are not rescued by  
551 eH3.2. In contrast, a triple mutant of H3.3 for the AIG motif, eH3.3 SVM, rescues the



552 phenotype while carrying the same SVM motif than H3.2. Scale bar corresponds to 500 $\mu$ m.  
553 Quantification of properly developed embryos after injection of the eH3.3 triple AIG mutant  
554 form shows similar rescue efficiency than eH3.3 WT after H3.3 depletion. Each experiment  
555 has been reproduced at least 3 times with a minimum of 30 embryos.

556

557 **Figure 4: Swapping the AIG motif to the SVM motif leads to changes in chaperone**  
558 **interactions and histone modes of incorporation into chromatin *in vivo*** **A)**

559 Immunoprecipitation of eH3 variant forms in interphase extract. Recombinant proteins are  
560 produced in rabbit reticulocyte lysates and pulled down by their HA-tag after incubation.  
561 HIRA only recognizes the AIG motif while p60 recognizes solely the SVM motif. \*  
562 corresponds to a non-specific band. **B)** Incorporation of eH3 variant forms into sperm  
563 chromatin in interphase extract. Purified nuclei remodeled in the interphase extracts  
564 supplemented with indicated eH3.3 in the presence or absence of aphidicolin were analyzed  
565 by WB with indicated antibodies. While all eH3 variant forms are incorporated when both  
566 DSC and DSI pathways are available, only the eH3 variant forms with the AIG motif can be  
567 when only the DSI is available.

568

569 **Figure 5: H3.3S31 is critical *Xenopus laevis* early development and is phosphorylated in**  
570 **this model organism.** **A)** Rescue assays with eH3.3 S31A mutant. Injections are performed at

571 2-cell stage. Compared with eH3.3 WT, eH3.3 S31A mutant form cannot rescue the  
572 phenotype. Scale bar corresponds to 500 $\mu$ m. Quantification of properly developed embryos  
573 after injection of the eH3.3 triple AIG mutant form shows similar rescue efficiency than  
574 eH3.3 WT after H3.3 depletion. Each experiment has been reproduced at least 3 times with a  
575 minimum of 30 embryos. **B)** 3D-distribution and timing of H3.3S31 phosphorylation in A6  
576 cell line. H3.3S31p follows the same dynamics than H3S10p, but does not localize to the  
577 same physical places. Scale bar represents 10  $\mu$ m. **C)** Characterization of H3.3S31  
578 phosphorylation in *Xenopus* cell-free extract. Non-remodeled sperm nuclei and nuclei  
579 purified after remodeling in interphase or mitotic extracts, H3 PTMs are analyzed by WB.  
580 H3.3S31p and H3S10p are enriched exclusively in sperm nuclei purified from mitotic  
581 extracts. The marks are prevalently found in the chromatin and not in H3.3 soluble form.  
582 Emi2 (Early mitotic inhibitor) serves as a control to confirm that extracts are in mitosis. CSF  
583 (Cytostatic factor).

584

585 **Figure 6: H3.3S31 negative charge is essential to rescue depletion of H3.3 during**  
586 ***Xenopus laevis* early development.**

587 **A)** Rescue assays with eH3.3 S31D mutant. Injections are performed at 2-cell stage. In  
588 contrast to eH3.3 S31A, the use of an exogenous phosphomimic mutant, eH3.3 S31D,

589 rescues, as the eH3.3 WT, the defects during gastrulation. Scale bar corresponds to 500 $\mu$ m.  
590 Quantification of properly developed embryos after injection of the H3.3 S31D mutant form  
591 shows similar rescue efficiency than eH3.3 WT after H3.3 depletion. In contrast, eH3.3 S31A  
592 mutant form leads to the same proportion of defects than eH3.2 after H3.3 depletion. Each  
593 experiment has been reproduced at least 3 times with a minimum of 30 embryos. **B)**  
594 Immunoprecipitation of eH3 S31 mutant forms in interphase extract. Recombinant proteins  
595 are produced in rabbit reticulocyte lysates and pulled down by their HA-tag after incubation.  
596 Mutations of the residue 31 do not alter histone chaperone interactions. \* corresponds to a  
597 non-specific band. **C)** Incorporation of eH3 S31 mutant forms into sperm chromatin in  
598 interphase extract. Purified nuclei remodeled in the interphase extracts supplemented with  
599 indicated eH3.3 in the presence or absence of aphidicolin were analyzed by WB with  
600 indicated antibodies. Mutations of the residue 31 do not change the mode of incorporation of  
601 these forms.

602

603 **Figure 7: Graphical abstract.** Defects associated with H3.3 depletion can be rescued with  
604 H3 histone variants carrying a potential negatively charge residue regardless of the mode of  
605 incorporation.

606

607 **Film 1: Endogenous H3.3 depletion leads to gastrulation defects and stops *Xenopus***  
608 ***laevis* early development.** Left embryo is injected with eH3.3 WT mRNA while the middle  
609 embryo is injected with 4.6ng of morpholino against H3.3. Right embryo is injected with both  
610 morpholino and eH3.3 WT mRNA. Injection of the morpholino leads to a gastrulation defects  
611 followed by developmental arrest that can be rescued by co-injection of eH3.3 WT mRNA.

612

613 **Film 2: Dose-dependent H3.3 depletion defects.** From left to right, the three embryos have  
614 been injected with 4.6ng, 9.2ng, 18.4ng of morpholino against H3.3. Right embryo has not  
615 been injected. Two replicates for each condition are showed. Injection of increased  
616 morpholino concentration leads to earlier gastrulation defects than previously observed.

617

618 **Supplemental figure 1: Histone chaperone sequence conservation and morpholino**  
619 **strategy and titration.** **A)** Logo for UBN1, DAXX and p60 partial sequences of *Homo*  
620 *sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Xenopus laevis* after performing multiple  
621 sequence alignment with MUSCLE. Complete histone-binding domain HRD (131-171) of  
622 UBN1 is presented, while only a part (from 300 to 339) of the DAXX histone-binding domain  
623 HBD (178-389) is displayed. No crystallographic data are available regarding the CAF1  
624 complex but the overall sequence shows variation as exemplified by the 41 first residues.  
625 Histone chaperones show little conservation between species. Final alignment was made

626 using WebLogo3. **B)** Sequences of the morpholino against endogenous H3.3 and its targeted  
627 sequence. The corresponding sequences matching the targeted sequence are highlighted in  
628 green for the first 10 codons of *Xenopus laevis* H3F3B and eH3 sequences. In contrast,  
629 differences with the targeted sequence are emphasized in red. **C)** Titration of the morpholino  
630 against H3.3. Increased concentrations of morpholino lead to earlier developmental defects.  
631 Morpholino against H3.3 is injected at the 2-cell stage with 3 different doses. While the lower  
632 one previously used in (Szenker et al. 2012) induces a defect at late gastrulation, two and four  
633 times more morpholino induce a defect appearing during early gastrulation. See film 2.

634

635 **Supplemental figure 2: Single mutant forms of eH3.3 AIG motif are all expressed and**  
636 **incorporated in a similar fashion *in vivo*.** **A)** Western blot to control eH3.3 AIG single  
637 mutant expression in embryos. Every eH3.3 AIG single mutants are expressed at similar  
638 levels in whole embryo extracts at stage 12. **B)** Western blots to control eH3.3 AIG single  
639 mutant incorporation in embryos. Fractionation of stage 12 embryos shows that all mutants  
640 are insoluble at 600nm NaCl, and therefore most likely incorporated into chromatin. Note that  
641 both eH3.3 WT and Control conditions are reused in Supplemental Figure 3 B and 6 B since  
642 each condition used comes from the same western blot.

643

644 **Supplemental figure 3: Triple mutant form of eH3.3 AIG motif is efficiently expressed**  
645 **and incorporated *in vivo*.** **A)** Western blot to control eH3.3 AIG triple mutant expression in  
646 embryos. eH3.3 AIG triple mutant expression compares with the other eH3 conditions in  
647 whole embryo extracts at stage 12. **B)** Western blots to control eH3.3 AIG triple mutant  
648 incorporation in embryos. Fractionation of stage 12 embryos shows that this mutant remains  
649 insoluble at 600nm NaCl, and therefore most likely incorporated into chromatin. Note that  
650 both eH3.3 WT and Control conditions are reused in Supplemental Figure 2 B and 6 B since  
651 each condition used comes from the same western blot.

652

653 **Supplemental figure 4: p60 histone chaperone interaction *in vivo* and mode of**  
654 **incorporation of eH3.3 AIG triple mutant in mitotic extract *in vitro*.** **A)**  
655 Immunoprecipitation of eH3 variant forms from stage 12 embryos. Only the SVM motif is  
656 recognized by p60 *in vivo*. **B)** Incorporation of eH3 variant forms into sperm chromatin in  
657 mitotic extract. Purified nuclei remodeled in the CSF-arrested extracts supplemented with  
658 eH3.3 mutants were analyzed by WB with indicated antibodies. In mitotic extract where the  
659 only pathway available is the DSI, only the eH3 variant form with the AIG motif, *i.e.* eH3.3  
660 WT, is incorporated.

661

662 **Supplemental figure 5: H3.3S31 phosphorylation in human has the same dynamics that**  
663 **in *Xenopus laevis*. A)** Highlights of the histone tail specific residue of H3 variants. H3.3 tail  
664 possesses a serine at position 31 that can be phosphorylated, while the alanine of H3.2 cannot.  
665 Crystal structure adapted from PDB ID codes: 5B0Z (Suzuki et al. 2016) and 3AV2  
666 (Tachiwana et al. 2011). **B)** 3D-distribution and timing of H3.3S31 phosphorylation in HeLa  
667 B cell line. As in the *Xenopus*, H3.3S31p follows the same dynamics than H3S10p, but does  
668 not localize to the same physical places. Scale bar represents 10  $\mu$ m.

669

670 **Supplemental figure 6: Mutant forms of H3.3S31 are all expressed and incorporated in**  
671 **a similar fashion. A)** Western blot to control eH3.3S31 mutant expression in embryos.  
672 eH3.3S31 mutant expression compares with the other eH3 conditions in whole embryo  
673 extracts at stage 12. **B)** Western blots to control eH3.3S31 mutant incorporation in embryos.  
674 Fractionation of stage 12 embryos shows that these mutant remains insoluble at 600nm NaCl,  
675 and therefore most likely incorporated into chromatin. Note that both eH3.3 WT and Control  
676 conditions are reused in Supplemental Figure 2 B and 3 B since each condition used comes  
677 from the same western blot. **C)** Incorporation of eH3.3S31 mutant forms into sperm  
678 chromatin in mitotic extract. Purified nuclei remodeled in the CSF-arrested extracts  
679 supplemented with eH3.3 mutants were analyzed by WB with indicated antibodies. In mitotic  
680 extract where the only pathway available is the DSI, mutations of the residue 31 do not  
681 change the mode of incorporation of these forms.

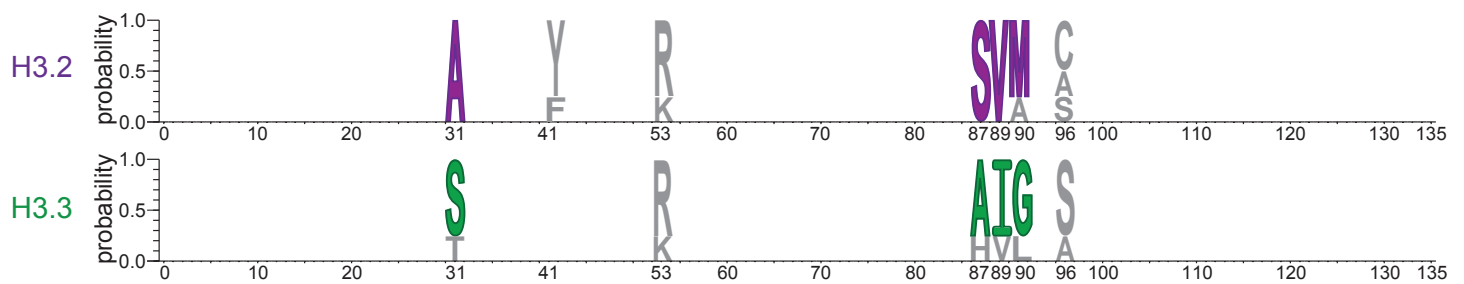
# Figure 1: H3.3 is essential for gastrulation of *Xenopus laevis*

## A Differences of H3 protein sequences

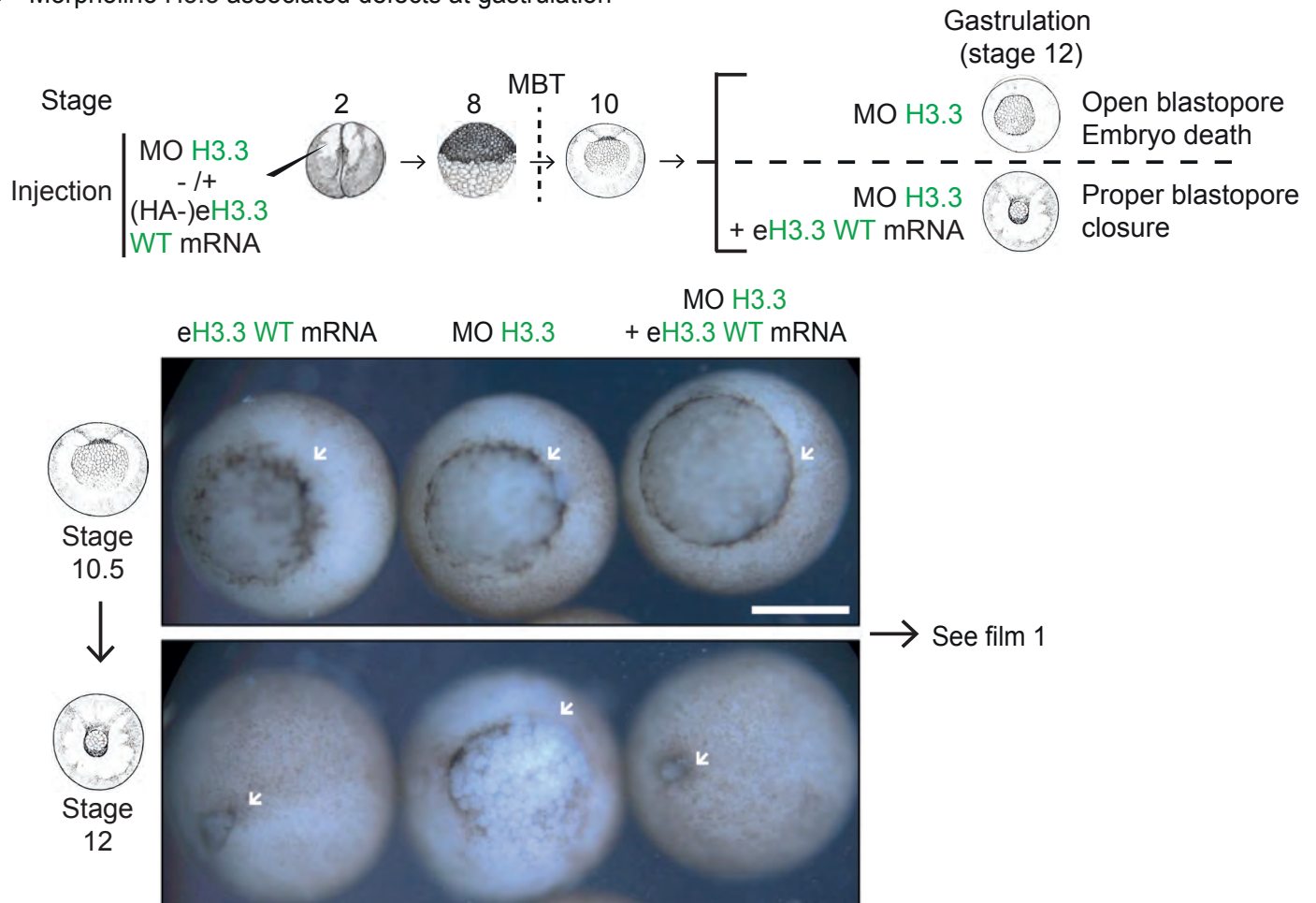
Species	DNA synthesis	Histone fold domain					
		N-terminal tail		loop2	$\alpha$ -2	$\alpha$ -3	
<i>Homo sapiens</i>	coupled	H3.1	...STGGKAPRKQLATKAARKSAP	ATGG	//DLRFQSSAVMALQEA	CLVG	//RIRGERA
	independent	H3.2	...STGGKAPRKQLATKAARKSAP	ATGG	//DLRFQSSAVMALQEA	SLVG	//RIRGERA
<i>Xenopus laevis</i>	coupled	H3.2	...STGGKAPRKQLATKAARKSAP	ATGG	//DLRFQSSAVMALQEA	SLVG	//RIRGERA
	independent	H3.3	...STGGKAPRKQLATKAARKSAP	STGG	//DLRFQSAALIGALQEA	SLVG	//RIRGERA

31
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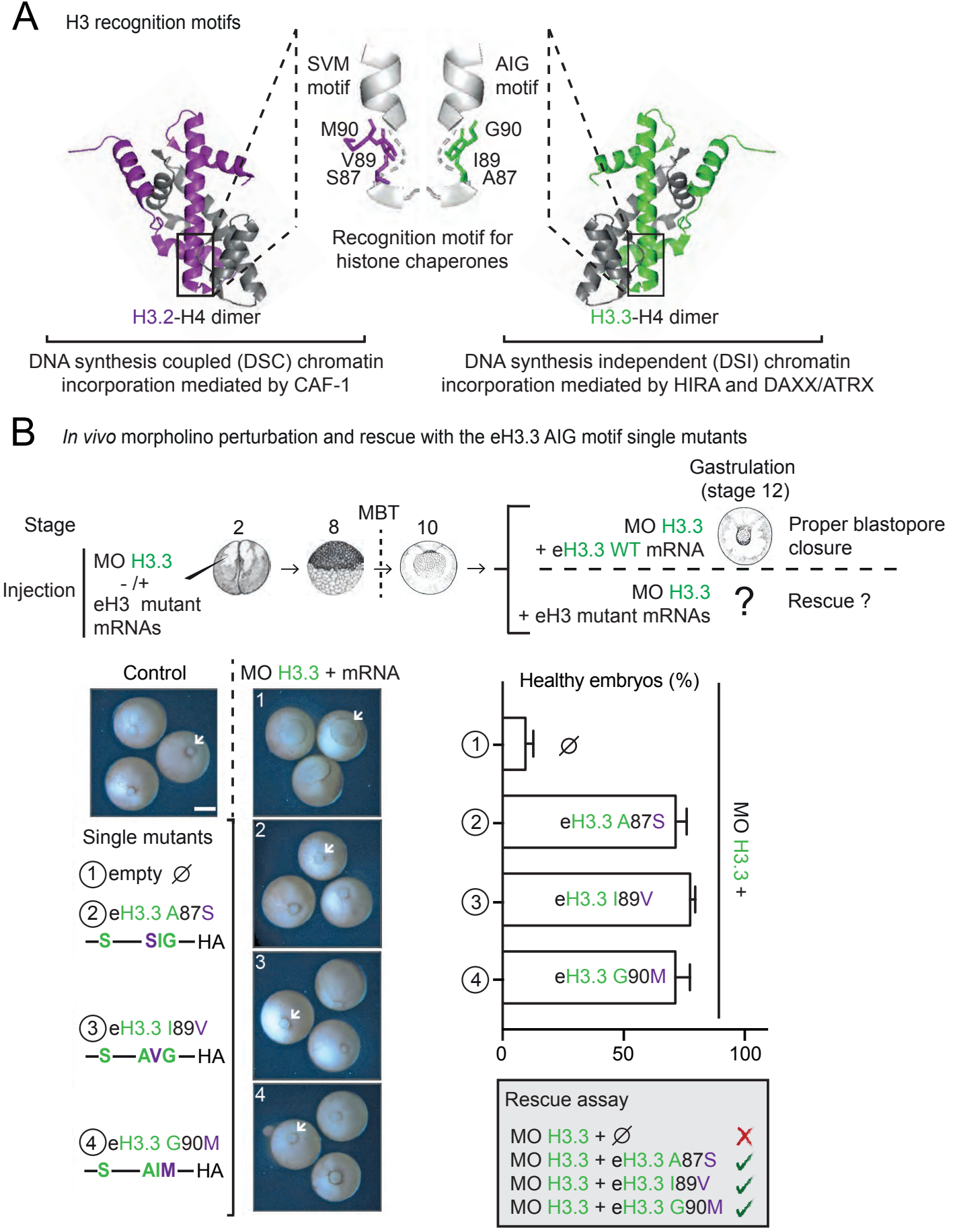
## B Differences in sequence alignments of H3 variant proteins



## C Morpholino H3.3 associated defects at gastrulation

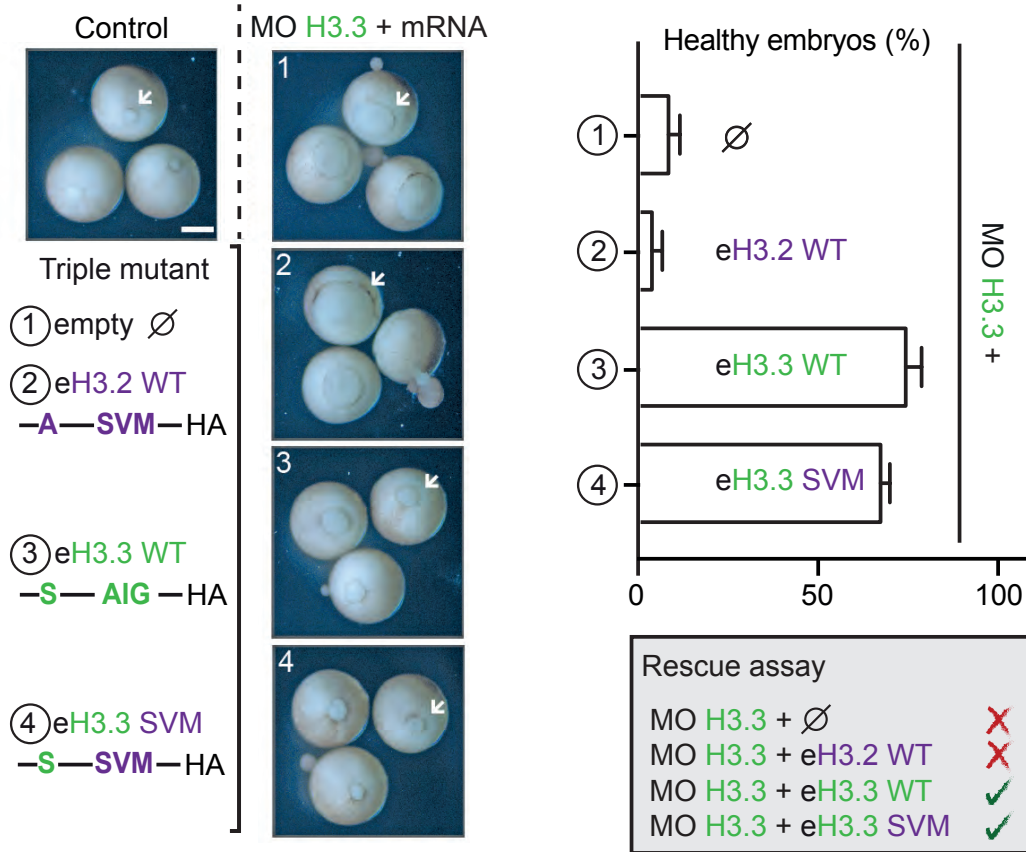


# Figure 2: eH3.3 AIG single mutants rescue depletion of H3.3 during *Xenopus laevis* early development



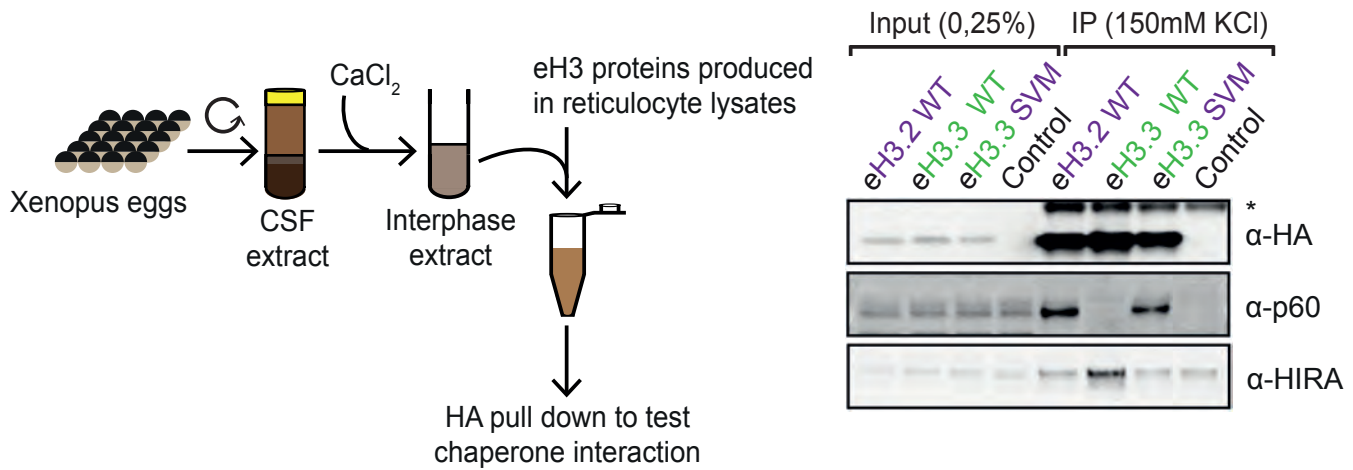
# Figure 3: eH3.3 AIG triple mutant rescues depletion of H3.3 during *Xenopus laevis* early development

*In vivo* morpholino perturbation and rescue with the eH3.3 AIG triple mutant

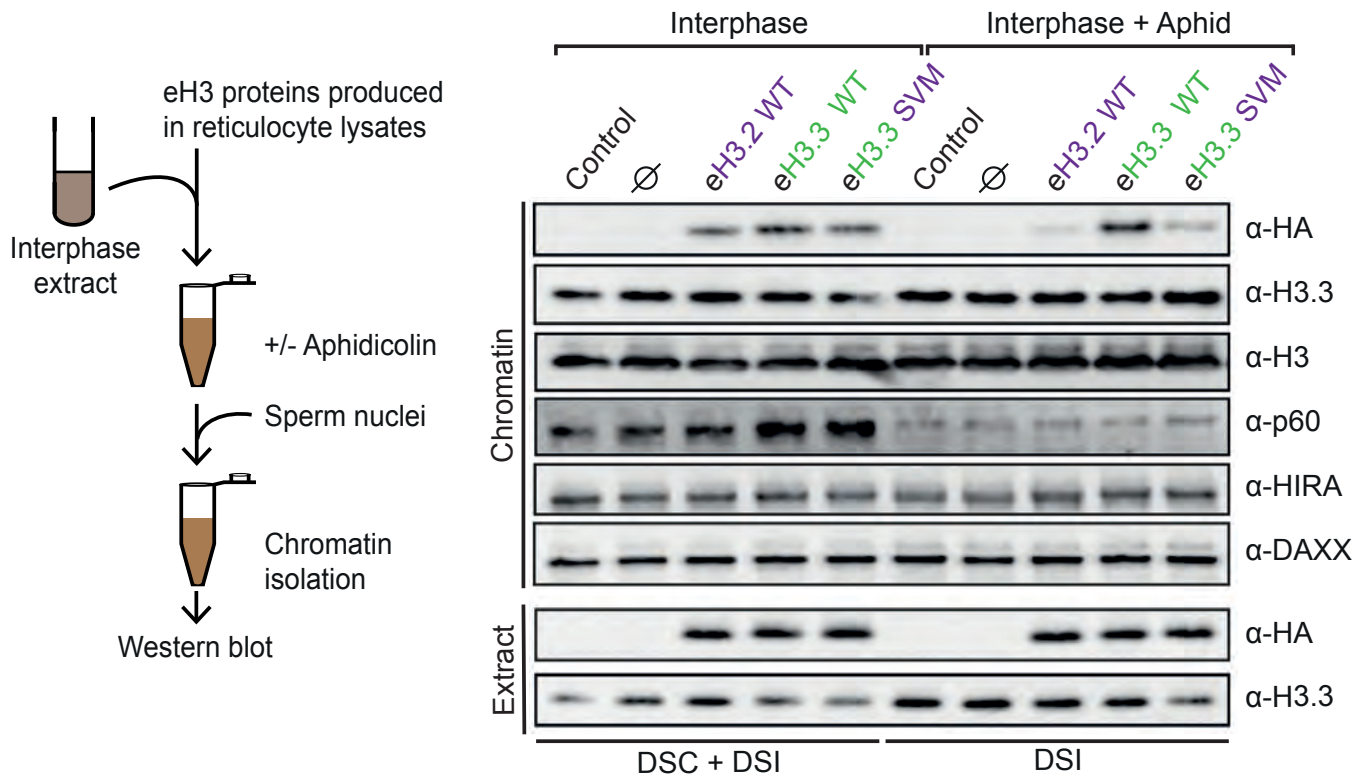


# Figure 4: Swapping the AIG motif to the SVM motif leads to changes in chaperone interactions and histone modes of incorporation *in vivo*

## A Interaction of histone chaperones with eH3.3 AIG triple mutant

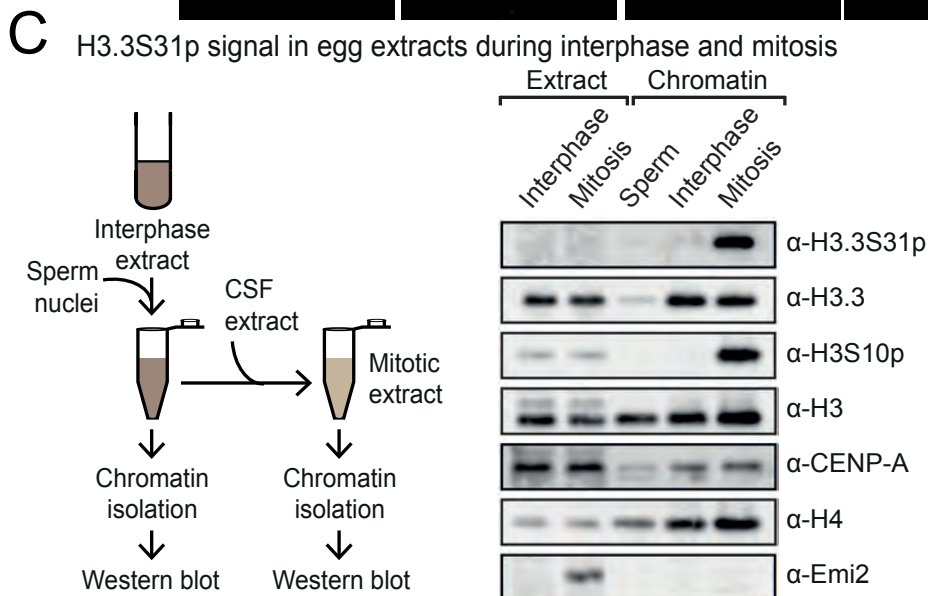
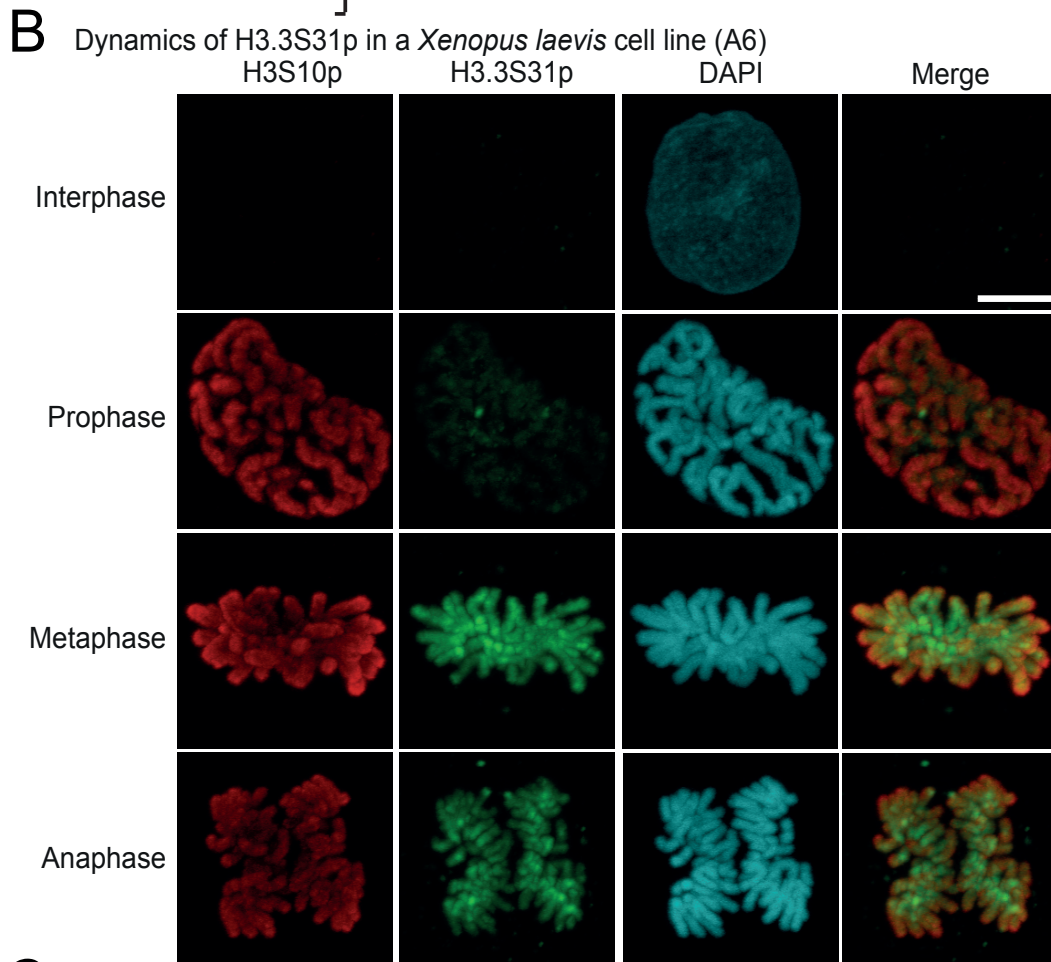
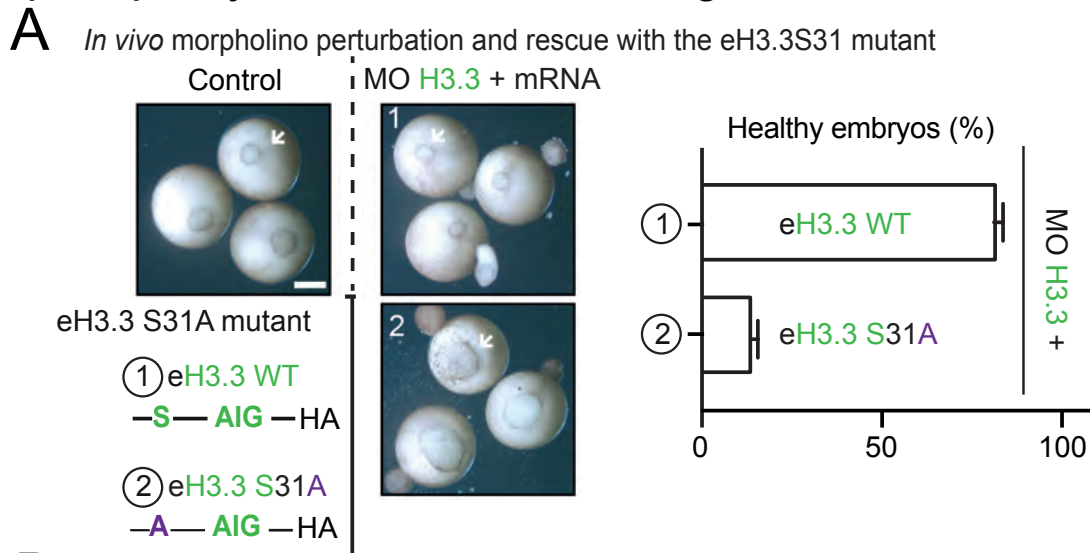


## B Mode of eH3.3 AIG triple mutant incorporation into sperm chromatin in interphase extract



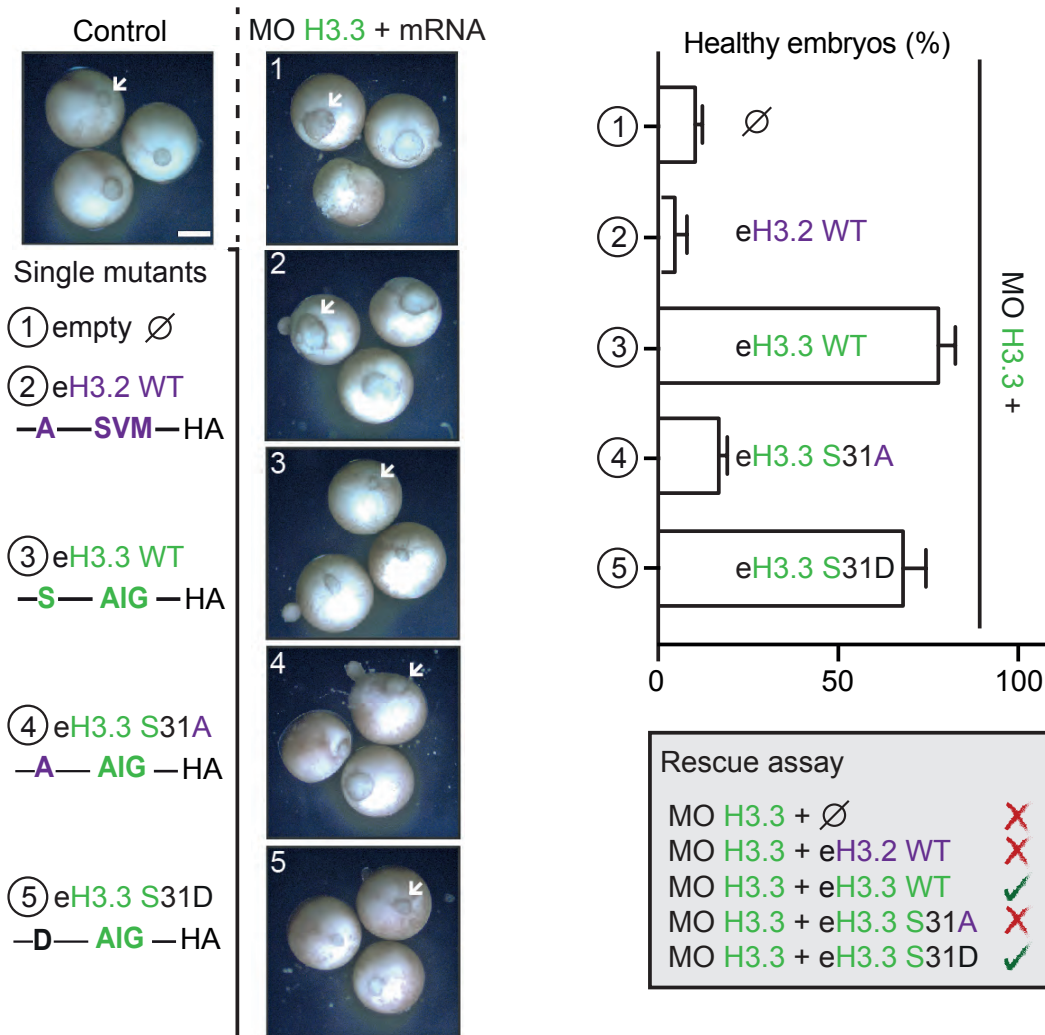


# Figure 5: H3.3S31 is critical for *Xenopus laevis* early development and is phosphorylated in this model organism

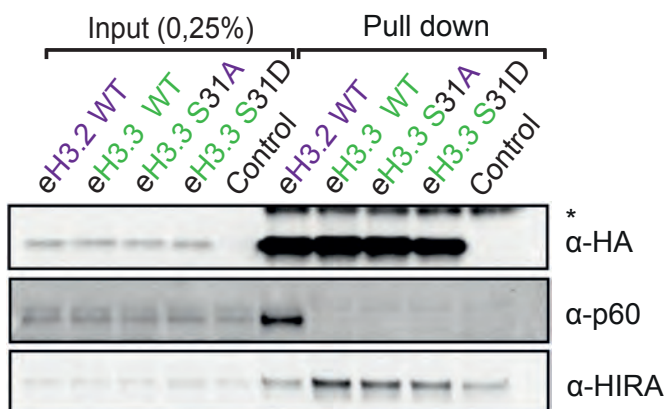


# Figure 6: H3.3S31 negative charge is essential to rescue depletion of H3.3 during *Xenopus laevis* early development

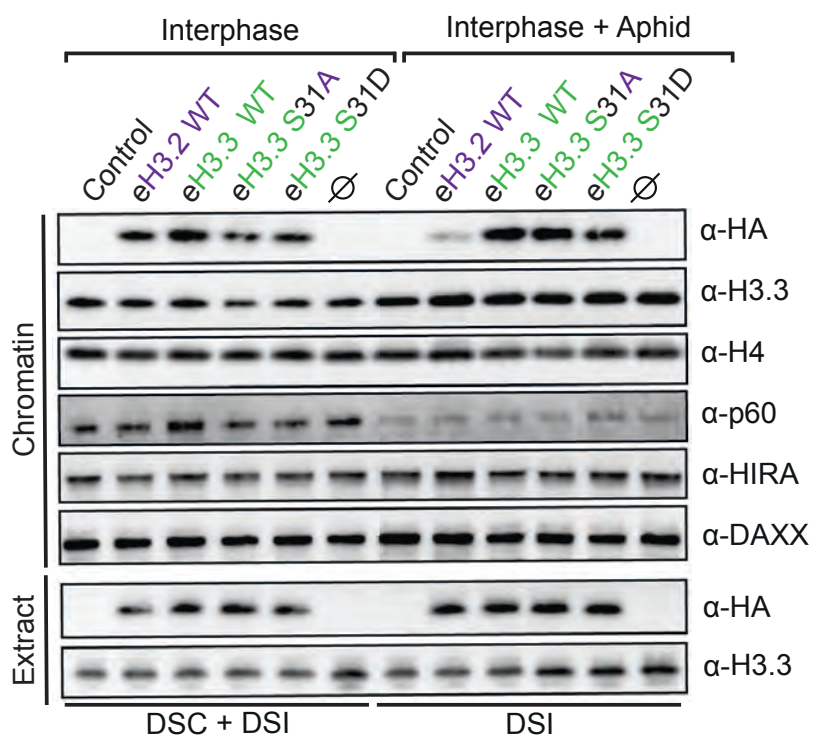
## A *In vivo* morpholino perturbation and rescue with the eH3.3S31 phosphomimic mutant



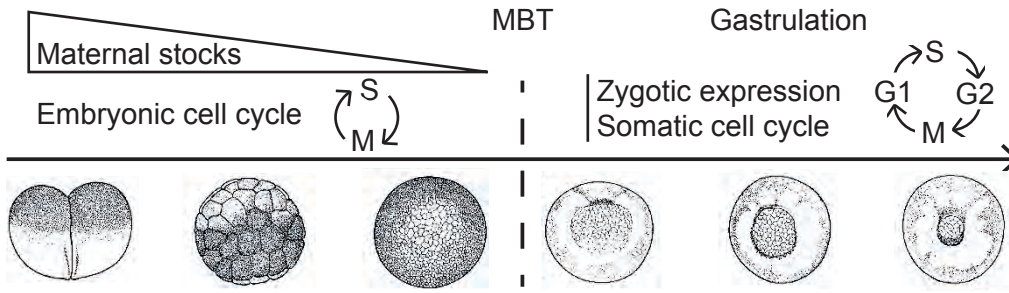
## B Interaction of histone chaperones with the eH3.3S31 mutants



## C Mode of eH3.3S31 mutants incorporation into sperm chromatin in interphase extract

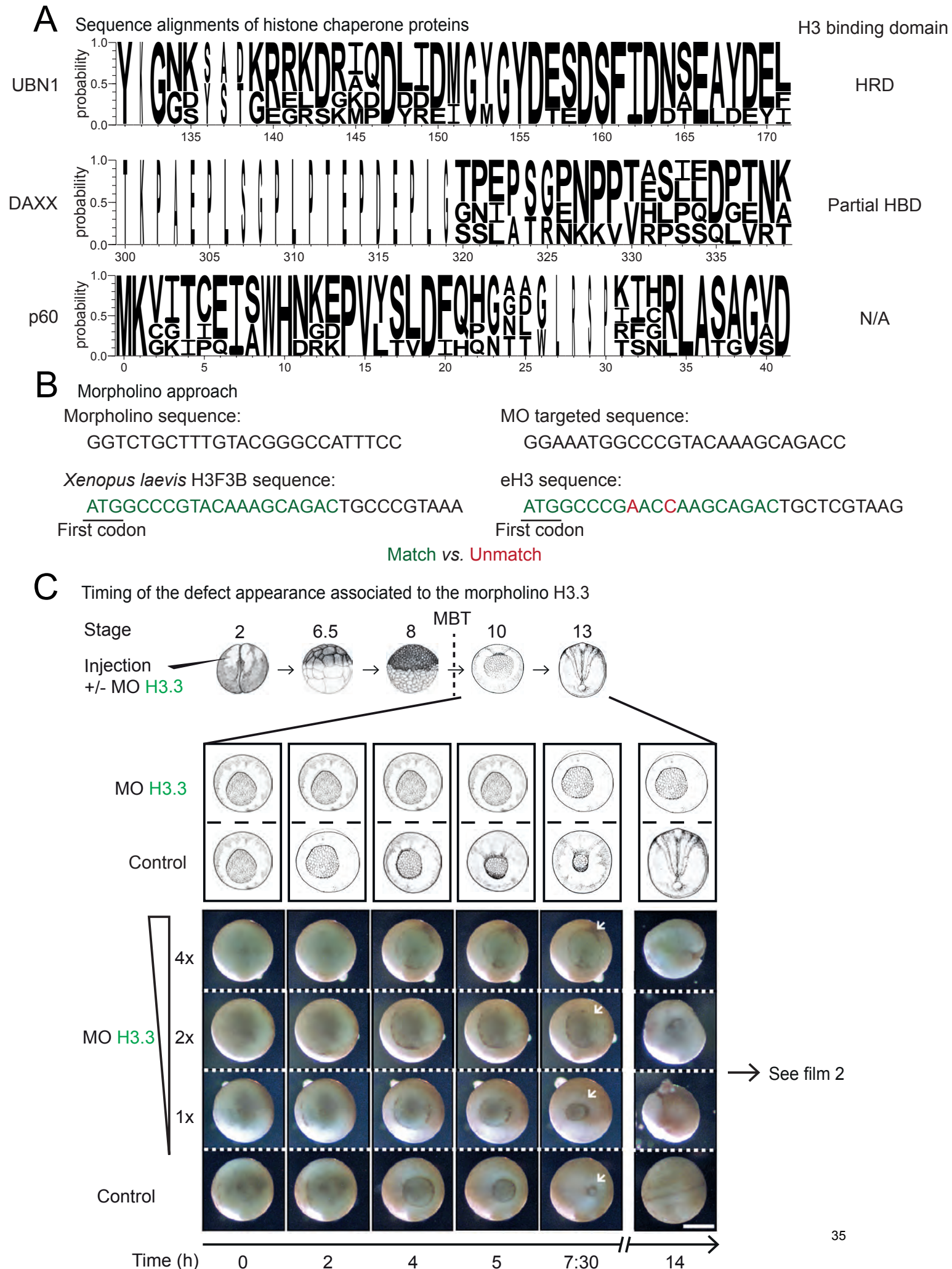


# Figure 7: Graphical abstract



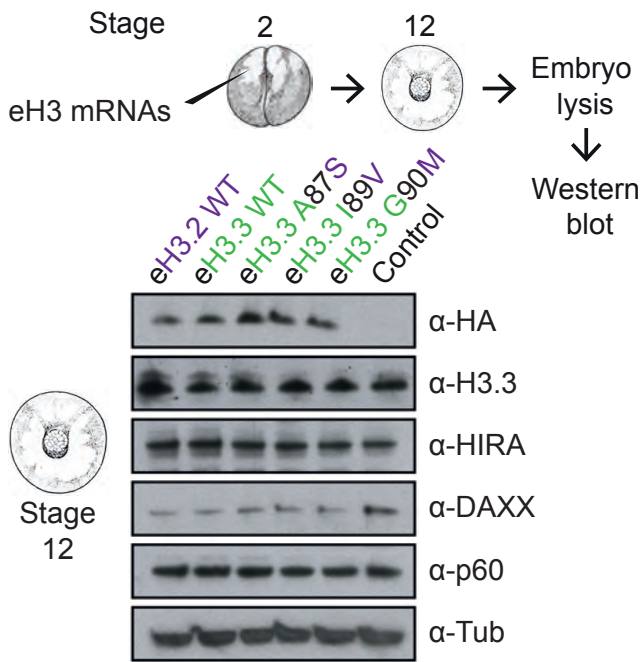
Choice of variant	DNA-synthesis	Outcome
<p>H3.2 WT SVM-A31</p> <p>H3.3 WT AIG-S31</p>	<p>p60, p48, p150 coupled</p> <p>DAXX, ATRX, Cabin1, HIRA, UBN1 independent</p>	Gastrulation
<p>H3.3 depletion</p> <p>H3.2 WT SVM-A31</p> <p>H3.3 WT AIG-S31</p>	<p>p60, p48, p150 coupled</p> <p>DAXX, ATRX, Cabin1, HIRA, UBN1 independent</p>	Gastrulation defects
<p>H3.3 depletion / Rescue</p> <p>H3.2 WT SVM-A31</p> <p>H3.3 SVM hybrid SVM-S31</p> <p>H3.3 WT AIG-S31</p>	<p>p60, p48, p150 coupled</p> <p>DAXX, ATRX, Cabin1, HIRA, UBN1 independent</p>	Gastrulation rescue

# Supplemental figure 1: Histone chaperone sequence conservation and morpholino strategy and titration

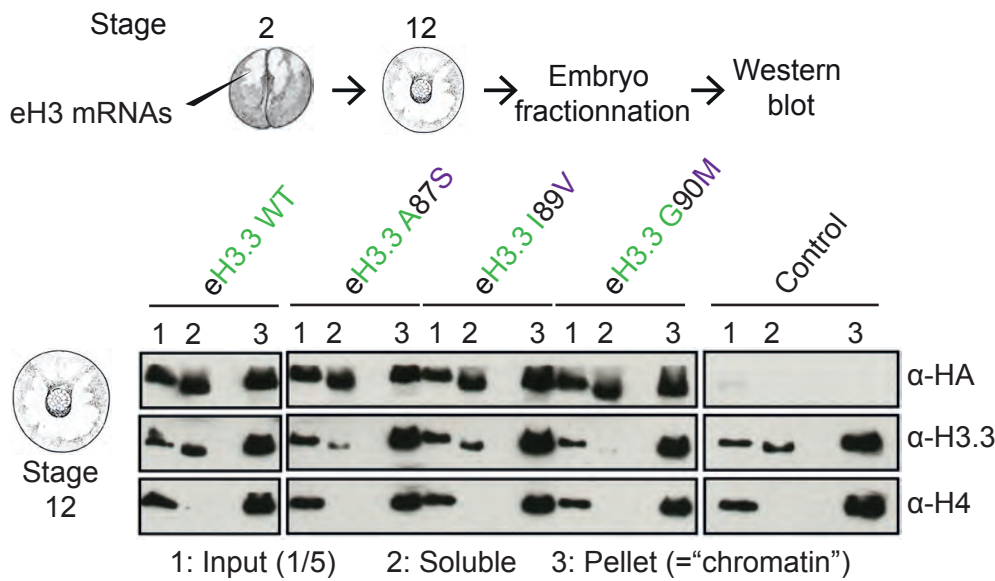


## Supplemental figure 2: Single mutant forms of eH3.3 AIG motif are all expressed and incorporated in a similar fashion *in vivo*

### A Expression of eH3.3 AIG single mutants

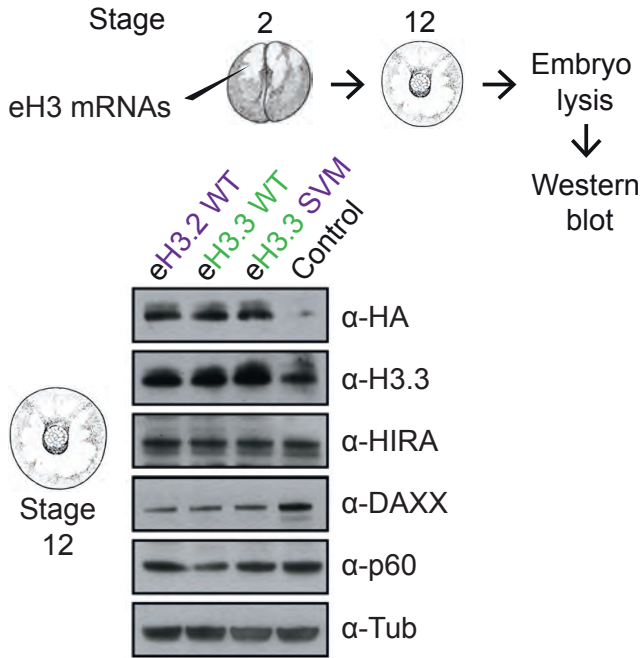


### B Incorporation into chromatin of eH3.3 AIG single mutants

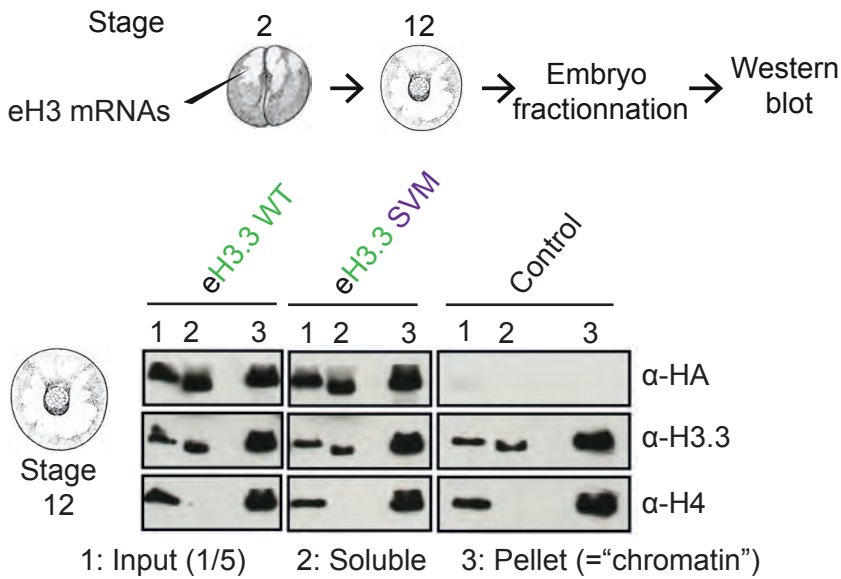


# Supplemental figure 3: Triple mutant form of eH3.3 AIG motif is efficiently expressed and incorporated *in vivo*

## A Expression of eH3.3 AIG triple mutant

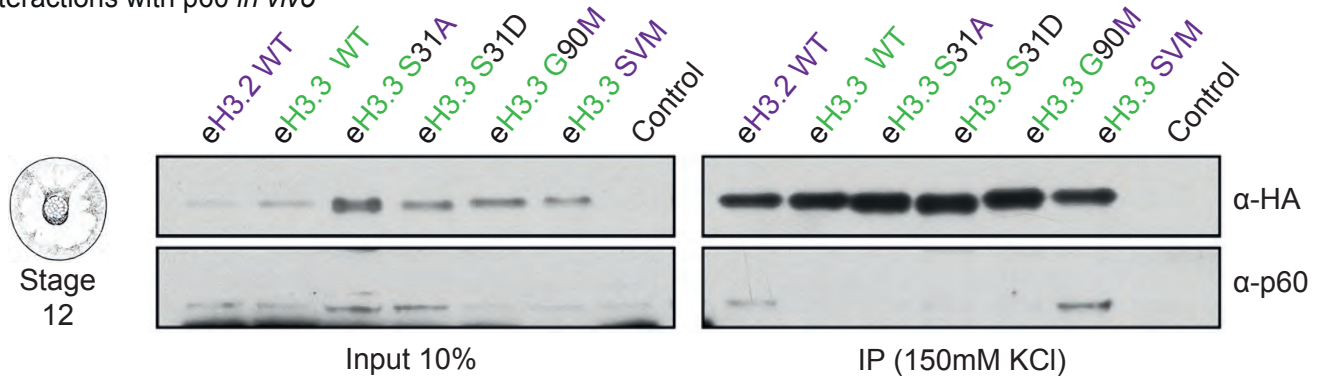


## B Incorporation into chromatin of eH3.3 AIG triple mutant

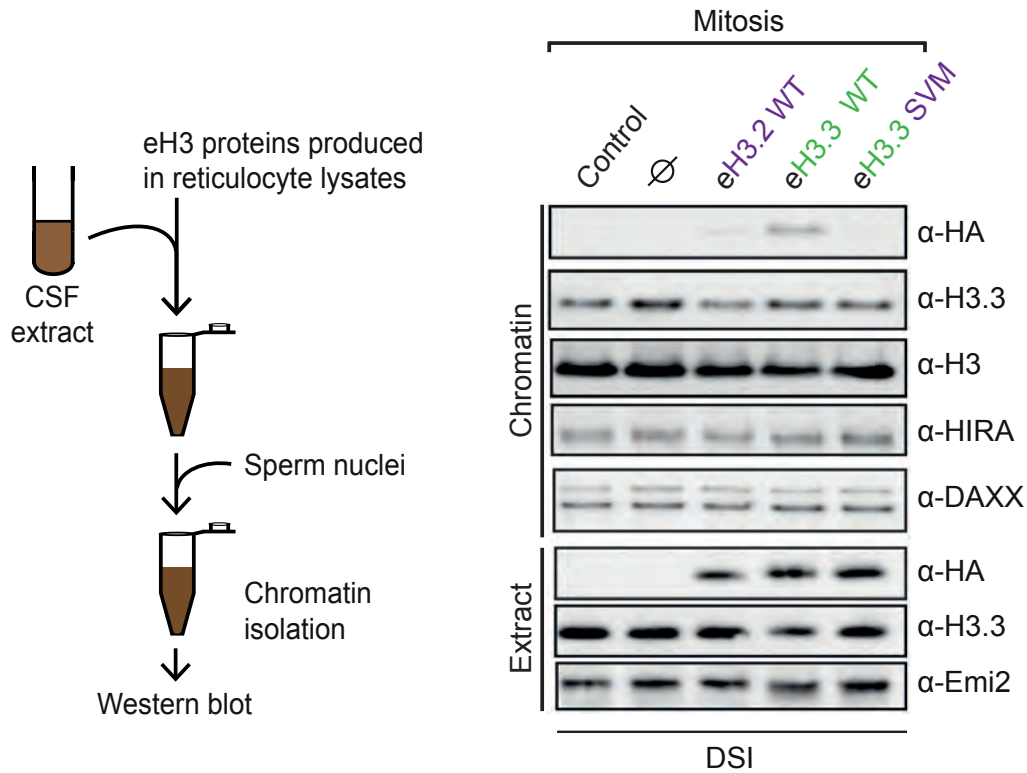


# Supplemental figure 4: p60 histone chaperone interaction *in vivo* and mode of incorporation of eH3.3 AIG triple mutant in mitotic extract *in vitro*

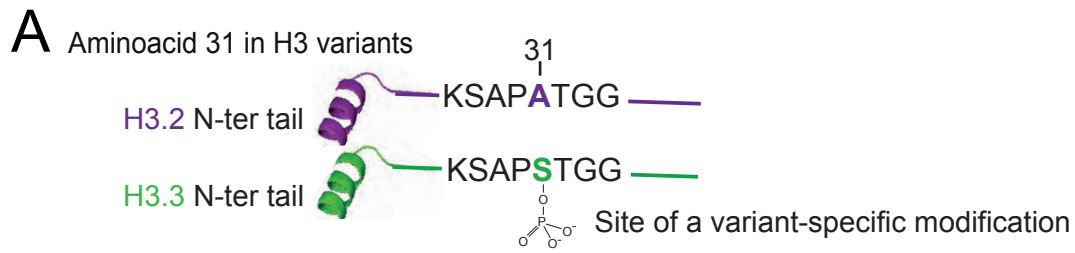
## A Interactions with p60 *in vivo*



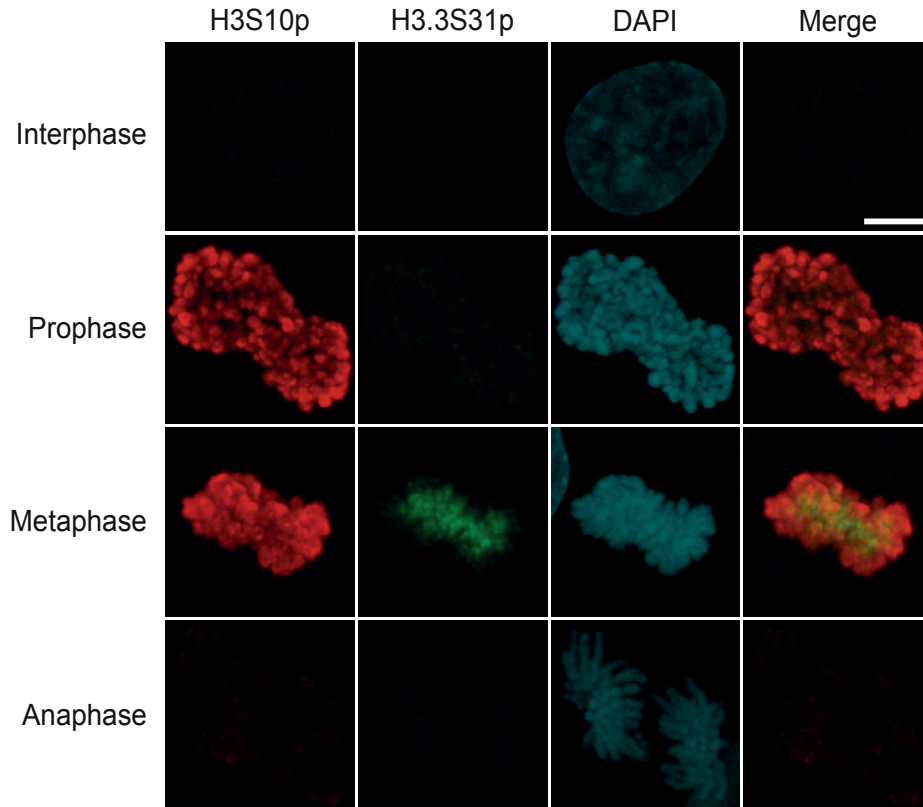
## B Mode of eH3.3 AIG triple mutant incorporation into sperm chromatin in mitotic extract



# Supplemental figure 5: H3.3S31 phosphorylation in human has the same dynamics that in *Xenopus laevis*



**B** Dynamics of H3.3S31p in HeLa B cell line





# Supplemental figure 6: Mutant forms of H3.3S31 are all expressed and incorporated in a similar fashion

