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2 **Centromere inactivation on a neo-Y fusion chromosome in**
3 **threespine stickleback fish**

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35 **Abstract**

36 Having one and only one centromere per chromosome is essential for proper chromosome
37 segregation during both mitosis and meiosis. Chromosomes containing two centromeres are
38 known as dicentric and often mis-segregate during cell division, resulting in aneuploidy or
39 chromosome breakage. Dicentric chromosome can be stabilized by centromere inactivation, a
40 process which re-establishes monocentric chromosomes. However, little is known about this
41 process in naturally occurring dicentric chromosomes. Using a combination of fluorescence in
42 situ hybridization (FISH) and immunofluorescence combined with FISH (IF-FISH) on
43 metaphase chromosome spreads, we demonstrate that centromere inactivation has evolved on a
44 neo-Y chromosome fusion in the Japan Sea threespine stickleback fish (*Gasterosteus*
45 *nipponicus*). We found that the centromere derived from the ancestral Y chromosome has been
46 inactivated. Our data further suggest that there have been genetic changes to this centromere in
47 the two million years since the formation of the neo-Y chromosome, but it remains unclear
48 whether these genetic changes are a cause or consequence of centromere inactivation.

49

50 **Keywords**

51 Dicentric chromosome fusion

52 Centromere inactivation

53 CENP-A

54 ChIP-seq

55 *Gasterosteus aculeatus*

56 *Gasterosteus nipponicus*

57

58 **Abbreviations**

59 BAC Bacterial artificial chromosome

60 CENP-A Centromere protein A

61 ChIP-seq Chromatin immunoprecipitation sequencing

62 Chr Chromosome

63 DAPI 4',6'-Diamidino-2-phenylindole

64 FISH Fluorescence in situ hybridization

65 GacCEN Threespine stickleback (*Gasterosteus aculeatus*) centromeric repeat sequence

66 IF-FISH Immunofluorescence combined with FISH

67 IP Immunoprecipitation

68 PBS Phosphate-buffered saline

69 PBST Phosphate-buffered saline Tween-20

70

71

72 **Introduction**

73 The centromere of a chromosome was first described by Walther Flemming as the primary
74 constriction observed on condensed chromosomes during both mitosis and meiosis (Flemming
75 1880). This primary constriction is the region of the chromosome where microtubules attach, and
76 it is necessary for the segregation of chromosomes during cell division. Many organisms contain
77 “regional” centromeres, which are found at a single location on each chromosome and comprised
78 of kilobases to megabases of DNA. The sequence of these regional centromeres is often
79 repetitive and AT rich, but the specific sequence varies dramatically among species (Henikoff et
80 al. 2001; Alkan et al. 2011; Melters et al. 2013), and can even vary among centromeres within a
81 species (Nagaki et al. 2004; Piras et al. 2010; Tek et al. 2010; Shang et al. 2010; Gong et al.
82 2012). Centromeres do share a common epigenetic characteristic, which is the presence of a
83 histone variant called centromere protein A (CENP-A) that replaces histone H3 in centromeric
84 nucleosomes (Palmer et al. 1987; Palmer et al. 1989; Palmer et al. 1991; Sullivan et al. 1994a).
85 The presence of CENP-A is considered the hallmark of an active centromere (Warburton et al.
86 1997; Heun et al. 2006; Marshall et al. 2008; Allshire and Karpen 2008; Barnhart et al. 2011;
87 Mendiburo et al. 2011; Sekulic and Black 2012; Fukagawa and Earnshaw 2014; McKinley and
88 Cheeseman 2016).

89 While it is essential that chromosomes have a centromere, in organisms with regional
90 centromeres it is also essential that every chromosome has one and only one active centromere.
91 A normal monocentric chromosome only has a single centromere that can bind microtubules and
92 form a bipolar spindle, allowing normal chromosome segregation. However, a dicentric
93 chromosome has two centromeres that can both bind microtubules, leading to instability during
94 cell division. Barbara McClintock first described this instability and termed it the breakage-

95 fusion-bridge cycle (McClintock 1939; McClintock 1941). If the microtubules from spindle
96 poles on the opposing sides of the cell bind to the two centromeres, the chromosome will be
97 pulled in opposite directions. This leads to merotelic kinetochore attachments where the dicentric
98 chromosome lags in the middle during anaphase as it is being pulled to opposite poles. The
99 physical tension on the chromosome can lead to breakage of the chromosome. When the broken
100 ends are replicated during the next S-phase of the cell cycle, the broken ends can anneal back to
101 each other, re-creating a dicentric. Not only does the breakage-fusion-bridge cycle start again,
102 but the breakage and re-annealing leads to gene loss and gene gain. This is evident in cancer cells
103 where dicentrics are often observed, leading to cells with “increased genetic heterogeneity”
104 (Gisselsson et al. 2000; Gisselsson et al. 2001).

105 Thus, dicentric chromosomes created by chromosome fusions are predicted to be very
106 unstable. However, chromosome fusion (and fission) events are common during evolution as
107 evidenced by the extensive variation in chromosome number among species. For example, in
108 multicellular eukaryotes, chromosome number ranges from a diploid number of two in the Jack
109 jumper ant (Crosland and Crozier 1986) to 1260 in the Adders tongue fern (van der Burg 2004).
110 In eutherian mammals, the diploid chromosome number ranges from 6 in the Indian muntjac to
111 102 in the viscacha rat (Gallardo et al. 1999); this diversity results from many independent fusion
112 and fission events (Ferguson-Smith and Trifonov 2007). Even closely related species can vary in
113 chromosome number; for example, the Chinese muntjac has a diploid chromosome number of
114 46, while the Indian muntjac has a diploid number of 6 in females or 7 in males (Shi et al. 1980;
115 Yang et al. 1995; Wang and Lan 2000). It is speculated that a series of chromosome fusion
116 events between the telocentric chromosomes present in the Chinese muntjac created the much
117 larger Indian muntjac chromosomes (Wang and Lan 2000). Chromosome fusions have also

118 occurred in the primate lineage; human chromosome 2 is a fusion between chimp chromosomes
119 2a and 2b (Lejeune et al. 1973; IJdo et al. 1991; Avarello et al. 1992). Similar chromosome
120 fusion and fission events are common in many taxa and can account for much of the diversity of
121 karyotype number observed today. Many of these chromosome fusion events must have created
122 dicentric chromosomes and therefore been accompanied by mechanisms to stabilize the
123 chromosome fusions.

124 To date, however, relatively little is known about the mechanisms involved in the
125 stabilization of dicentric chromosomes during evolution in natural populations. Most of our
126 current understanding of how dicentrics are stabilized comes from studies of artificially-induced
127 or engineered dicentric chromosomes in yeast (Pobiega and Marcand 2010; Sato et al. 2012),
128 fruit flies (Agudo et al. 2000), and human cell lines (Higgins et al. 1999; Higgins et al. 2005;
129 Stimpson et al. 2010; Stimpson et al. 2012), as well as a few examples of dicentric chromosomes
130 in found in plants (Sears and Câmara 1952; Han et al. 2006; Zhang et al. 2010; Gao et al. 2011;
131 Koo et al. 2011; Liu et al. 2015) and human patients (Therman et al. 1989; Maraschio et al. 1990;
132 Fisher et al. 1997; Page and Schaffer 1998; Sullivan and Willard 1998; Lange et al. 2009;
133 Stimpson et al. 2012). In some engineered systems, the dicentric chromosome can simply break
134 in order to re-establish monocentric chromosomes (Pobiega and Marcand 2010; Sato et al. 2012).
135 In other engineered or patient-derived dicentrics, chromosome fusions can be stably inherited
136 because the two centromeres on a fusion chromosome are close enough to each other to act as a
137 single centromere (Koshland et al. 1987; Page and Shaffer 1998; Sullivan and Willard 1998;
138 Higgins et al. 1999; Lange et al. 2009). However, distance between the centromeres does not
139 fully explain the stable maintenance of dicentric chromosomes, and in many cases, there is
140 inactivation of one of the centromeres to re-establish functionally monocentric chromosomes

141 (Stimpson et al. 2012). Centromere inactivation can either occur by genetic or epigenetic
142 inactivation of one centromere. In genetic inactivation, deleterious mutation of the DNA
143 sequence of one centromere prevents it from binding CENP-A. In epigenetic inactivation, the
144 DNA of the inactivated centromere is still present, but CENP-A is not. In either case, the
145 functional consequence of the loss of CENP-A is that the centromere does not recruit
146 kinetochore components and therefore does not attach to microtubules during cell division.
147 Although both genetic and epigenetic inactivation have been observed in the systems studied to
148 date (Stimpson et al. 2012), almost nothing is known about the relative contribution of these
149 mechanisms to dicentric stabilization during evolution.

150 The stickleback family (Gasterosteidae) provides a unique opportunity to study the
151 mechanisms that stabilize natural dicentric chromosomes during evolution because there have
152 been several chromosome fusion events that have occurred within the past 35 million years
153 (Kitano et al. 2009; Ross et al. 2009). Previous work has identified a chromosome fusion
154 involving the ancestral metacentric Y chromosome present in the Pacific Ocean threespine
155 stickleback (*Gasterosteus aculeatus*). This Y chromosome has fused to the acrocentric
156 chromosome 9 in males of the Japan Sea threespine stickleback (*Gasterosteus nipponicus*)
157 species within the past two million years, creating an X_1X_2Y sex chromosome system in which
158 the ancestral X is X_1 , the unfused chromosome 9 is X_2 , and the fused chromosome is called the
159 neo-Y (Kitano et al. 2009) (Fig. S1). Because this fusion involved the metacentric Y and an
160 acrocentric chromosome, the neo-Y should have been dicentric upon formation. Preliminary data
161 had suggested that there was no loss of a chromosome arm to bring the two centromeres closer
162 on the Japan Sea neo-Y chromosome, or re-breakage of the fusion chromosome. These data
163 suggested that centromere inactivation has occurred on the Japan Sea neo-Y chromosome.

164 In this study, we aimed to first determine if centromere inactivation has occurred on the
165 neo-Y, and to then test whether inactivation is due to a genetic or epigenetic mechanism. Both
166 the genetic and epigenetic mechanisms of centromere inactivation ultimately result in only one
167 functional centromere, as defined by the presence of CENP-A. If centromere inactivation has
168 occurred, we expected to see only one region on the neo-Y with CENP-A staining. If no
169 centromere inactivation has occurred, we expected to see two regions of CENP-A staining on the
170 neo-Y. Using CENP-A antibody staining, we determined that the Japan Sea neo-Y has only one
171 active centromere, as defined by presence of CENP-A. In an extension of our previous study
172 (Cech and Peichel 2015), we then performed chromatin immunoprecipitation followed by high
173 throughput sequencing (ChIP-seq) to determine the centromere sequence in the Japan Sea
174 species. We then used fluorescence in situ hybridization (FISH) to demonstrate that there have
175 likely been genetic changes to the ancestral Y centromere on the Japan Sea neo-Y.

176

177 **Materials and Methods**

178 *Fish use and care*

179 Two species of lab reared threespine stickleback fish were used in this study: Pacific Ocean
180 (*Gasterosteus aculeatus*) and Japan Sea (*Gasterosteus nipponicus*). Lab-reared fish were derived
181 from wild caught fish collected in Akkeshi on Hokkaido Island, Japan (Kitano et al. 2007, 2009).
182 Fish were kept in in 3.5% saltwater (3.5 g/l Instant Ocean salt (Spectrum Brands, USA); 0.4 ml/l
183 sodium bicarbonate) in 29-gal aquarium tanks, at 16 °C and 16 hr light / 8 hr dark. Fish were fed
184 once daily with live *Artemia* nauplii and once daily with frozen *Mysis* shrimp. All institutional
185 and national guidelines for the care and use of laboratory animals were followed, and all
186 procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Animal

187 Care and Use Committee (protocol 1575).
188
189 *CENP-A immunoprecipitation followed by high throughput sequencing (ChIP-seq)*
190 Chromatin immunoprecipitation (IP) with the stickleback specific CENP-A antibody (Cech and
191 Peichel 2015) was performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic
192 Beads; Cell Signaling Technology, USA). 0.1 g of spleen and liver tissue was taken from three
193 Japan Sea females (one single replicate, and one pooled replicate of two individuals) and four
194 Japan Sea male sticklebacks (two pooled replicates of two individuals each). The chromatin
195 immunoprecipitation was performed at the same time and following the same protocol as for the
196 two Pacific Ocean female samples described in Cech and Peichel (2015). To identify the Japan
197 Sea consensus centromere sequence, analysis was performed using the same methods as in Cech
198 and Peichel (2015). All raw fastq sequence files are available in the NCBI Sequence Read
199 Archive (Pacific Ocean female data: SRP063504; Japan Sea male and female data: SRP081499).
200
201 *FISH on metaphase spreads and interphase nuclei*
202 Lab reared Pacific Ocean and Japan Sea fish were injected with 10 µl of 1 % colchicine in
203 phosphate-buffered saline (PBS) for 14-16 hours to arrest metaphase cells. Metaphase and
204 interphase cells were obtained following the same protocol described in Ross and Peichel (2008),
205 with the exception that metaphase slides were dried at 50°C, not 37°C. The threespine
206 stickleback centromeric repeat sequence (GacCEN) probe was made as described in Cech and
207 Peichel (2015). Bacterial artificial chromosome (BAC) probes listed in Table 1 were prepared
208 following the protocol in Urton et al. (2011) and labeled with either Alexa-488 or Alexa-568.

209 Metaphase slides were first washed for 5 min with phosphate-buffered saline Tween-20
210 (PBST), fixed for 10 min in 4% paraformaldehyde in PBS, then washed again in PBST for 5
211 min. Subsequent FISH hybridization with only the GacCEN probe or only BAC probes was
212 performed as described in Cech and Peichel (2015). For metaphase slides hybridized with
213 combined GacCEN and BAC probes, 10 μ l of the GacCEN probe was first lyophilized at 50°C
214 for four hours. The dried GacCEN probe was then resuspended with either 10 μ l of a single BAC
215 probe in hybridization buffer, or 20 μ l of equal parts of two BAC probes in hybridization buffer.

216

217 *Telomere staining*

218 Telomere staining was performed according to manufacturers instructions using the DAKO
219 telomere PNA FISH kit/FITC no. K5325 (Agilent Technologies, USA).

220

221 *Immunofluorescence-FISH (IF-FISH)*

222 For IF-FISH, 15 Pacific Ocean or Japan Sea embryos at 48 hours post fertilization were dounced
223 in 10 ml 0.56 % potassium chloride using a glass dounce. The remainder of the IF-FISH protocol
224 follows Cech and Peichel (2015).

225

226 *Microscopy*

227 Images were taken using a Nikon Eclipse 80i microscope (Nikon, Japan) with an automated filter
228 turret (Chroma filters 31000v2 (DAPI), 41001 (FITC), and 41004 (Texas Red); Chroma, USA)
229 using the 100x objective. Following Cech and Peichel (2015), images were pseudo-colored using
230 NIS Elements imaging software (BR 3.00, SP7, Hotfix8, Build 548, Nikon, Japan).

231

232 **Results**

233 *No loss of chromosome arms on the Japan Sea neo-Y fusion chromosome*

234 To determine if the neo-Y fusion was a complete fusion between both chromosomes, or if large
235 portions of each or one of the respective p-arms was lost, we performed two types of FISH

236 experiments. First, we conducted hybridization with BAC probes homologous to the ends of

237 chromosome 9 (44L12) and the Y chromosome (101E8) that are near the fusion site, as well as

238 the end of the Y chromosome (188J19) opposite to the fusion site (Table 1). These probes all

239 hybridize to the neo-Y on Japan Sea male metaphase chromosomes, demonstrating that loss of

240 an entire chromosome arm has not occurred on the neo-Y fusion chromosome (Fig. 1).

241 Hybridization of these same BAC probes on Pacific Ocean male metaphase chromosomes further

242 demonstrates that there have not been gross rearrangements between the Japan Sea and Pacific

243 Ocean sticklebacks on either chromosome 9 or the Y chromosome (Fig. S2).

244 Second, to test if there was truly no loss of any genetic material, we also used a FISH

245 probe to the 6bp telomere sequence (TTAGGG) found in all vertebrates (Meyne et al. 1989).

246 There is no internal telomere signal on the neo-Y chromosome, indicating that some loss of

247 genetic material encompassing at least the telomeres has occurred on the neo-Y chromosome

248 (Fig. S3). Interestingly, telomere loss can induce chromosome fusions (Gisselsson et al. 2001;

249 Maser and DePinho 2002; Bailey 2006; Pobiega and Marcand 2010; Murnane 2010; Stimpson et

250 al. 2010). However, we do not know whether loss of telomeres is a cause or consequence of the

251 neo-Y fusion. Taken together, these data do suggest that the neo-Y chromosome resulted from a

252 fusion between two nearly complete monocentric chromosomes, creating a dicentric neo-Y

253 chromosome. The neo-Y chromosome is present in all Japan Sea males examined to date, and we

254 have no evidence for dicentric chromosome segregation associated aneuploidy in the Japan Sea

255 males (i.e. no sex ratio bias in the offspring of Japan Sea males; data not shown). Furthermore,
256 there is evidence of only one constriction on the neo-Y in metaphase chromosome spreads (Fig.
257 S3). Thus, we hypothesized that the neo-Y chromosome has been stabilized by centromere
258 inactivation via either genetic or epigenetic inactivation.

259

260 *Evidence for centromere inactivation on the Japan Sea neo-Y chromosome*

261 Using CENP-A antibody staining in combination with FISH, we determined that only one region
262 of CENP-A staining is found on the neo-Y in multiple independent metaphase spreads from
263 Japan Sea males (Fig. 2). The CENP-A staining was flanked by BAC probes present at the end
264 of the Y near the fusion breakpoint and at the distal end of chromosome 9, indicating that the
265 active centromere is on chromosome 9 (Fig. 2). The CENP-A staining on the neo-Y is in a
266 similar location to the CENP-A staining found on the unfused chromosome 9 (X₂), further
267 suggesting that the active centromere on the neo-Y is retained from the ancestral acrocentric
268 chromosome 9 (Fig. 2f).

269 To confirm that the ancestral Y chromosome centromere was active before the fusion that
270 created the neo-Y occurred, we performed CENP-A staining in combination with FISH using a
271 BAC probe (91G03; Table 1) that specifically labels the Y chromosome on Pacific Ocean male
272 chromosomes (Fig. S4). We found normal CENP-A staining on the ancestral Y centromere (Fig.
273 S5). This is evidence that the ancestral Y chromosome had an active centromere, and that the Y
274 chromosome centromere was inactivated after the fusion to chromosome 9 in Japan Sea males.

275

276 *Evidence for genetic inactivation on the Japan Sea neo-Y chromosome*

277 Because we found evidence for only a single active centromere on the Japan Sea neo-Y
278 chromosome, we sought to determine whether there has been genetic inactivation (i.e. deletion or
279 alteration) of the ancestral Y chromosome centromere. We had previously identified the
280 centromere sequence (GacCEN) in the Pacific Ocean threespine stickleback (Cech and Peichel
281 2015). Although this centromere sequence appears to be present on all autosomes, including
282 chromosome 9 and the X chromosome, we observed very weak hybridization of the centromere
283 sequence to the Y chromosome in the ancestral Pacific Ocean population (Cech and Peichel
284 2015). It was unclear whether the centromere on the Y chromosome has a completely different
285 sequence, a highly divergent sequence, or a reduced number of repeats. Here, we further
286 explored these possibilities in order to analyze the fate of the ancestral Y chromosome
287 centromere on the Japan Sea neo-Y. We performed additional FISH experiments on Pacific
288 Ocean males with the centromere probe and confirmed that centromere hybridization on the
289 ancestral Y is weak and variable. Over 11 independent FISH experiments, we counted 29
290 metaphase spreads with positive GacCEN staining, and 21 with negative GacCEN staining
291 (Table 2). This variability is demonstrated by both positive and negative staining on metaphase
292 spreads from the same male (Fig. 3). These data suggest that the Y centromere is likely not a
293 completely different sequence, but instead might represent a divergent repeat sequence and/or a
294 reduced number of the same repeat sequence.

295 To determine whether the centromere sequence in the Japan Sea species is the same as in
296 the Pacific Ocean species, we also performed CENP-A ChIP-seq in Japan Sea males and females
297 at the same time we performed the Pacific Ocean CENP-A ChIP-seq (Cech and Peichel 2015).
298 We had previously demonstrated that the Pacific Ocean and Japan Sea CENP-A genes only
299 differ by a single amino acid (Cech and Peichel 2015), which is not in the region of the protein

300 used for the antibody design (Fig. S6). Following previously described methods (Cech and
301 Peichel 2015), we first identified the 500 most abundant sequence clusters in each IP sample and
302 then analyzed the clusters with a fold enrichment of greater than one in the IP relative to the
303 input (Table 3). Using these enriched sequence clusters, we identified a consensus CENP-A
304 associated putative centromere sequence in both Japan Sea males and females. As we found
305 previously, most of the sequence clusters enriched in the IP relative to the input aligned to this
306 consensus sequence (Table 3). The enriched sequence clusters that did not align to the consensus
307 sequence did not align to each other and were among the clusters with the lowest enrichment in
308 each sample (Table 3). Thus, these experiments identified one major CENP-A associated repeat
309 in the Japan Sea males and females; this Japan Sea putative centromere sequence is 98.7%
310 similar to the Pacific Ocean GacCEN and is also 186 bp and 61.2 % AT rich (Fig. S7). Similar to
311 the Pacific Ocean population, the Japan Sea GacCEN probe shows hybridization to the
312 constriction on metaphase spreads (Fig. S8) as well as co-localization with CENP-A (Fig. S9).

313 We performed FISH using the GacCEN probe in combination with BAC probes on Japan
314 Sea metaphase spreads to determine whether it present on the neo-Y. There is strong
315 hybridization of the GacCEN probe to the ancestral chromosome 9 centromere, providing
316 evidence that this centromere has not been deleted on the neo-Y (Fig. 4). Although we also
317 observed strong GacCEN staining on both X chromosomes in Japan Sea females (Fig. S10),
318 there was no hybridization of the GacCEN probe to the ancestral Y centromere on the neo-Y
319 (Fig. 4). In contrast to the variable hybridization of the GacCEN probe to the Y chromosome in
320 Pacific Ocean males (Fig. 3), the lack of GacCEN hybridization to the Y chromosome was
321 consistently observed on 26 different metaphase spreads from 6 independent Japan Sea males
322 over four different experiments (Table 2). Because we do not know the specific sequence of the

323 Y centromere, we cannot determine whether the lack of staining to the Y centromere on the neo-
324 Y is due to deletion of the centromeric DNA on the neo-Y or further divergence of the
325 centromeric DNA on the neo-Y. However, these data do suggest that there has been a genetic
326 alteration to the ancestral Y chromosome centromere on the Japan Sea neo-Y.

327 To further assess whether there has been a deletion of the ancestral Y centromere on the
328 neo-Y, we examined the hybridization pattern of BAC probes flanking the ancestral Y
329 centromere on the neo-Y. Previous work had identified two BAC probes (171H24 and 180J08;
330 Table 1) flanking the centromeric constriction on both the X and Y chromosomes in the Pacific
331 Ocean species (Ross and Peichel 2008). Note that the order of these probes is different between
332 the X and Y chromosomes due to the presence of several inversions on the Y chromosome
333 relative to the X (Ross and Peichel 2008). There is no loss of hybridization with either probe on
334 the Japan Sea neo-Y (Fig. 5), indicating that these large regions flanking the centromere are still
335 present. However, when compared to hybridization on the Japan Sea X, these probes do appear
336 to be closer together on the neo-Y (Fig. 5). Additionally, hybridization with these two probes and
337 the GacCEN probe shows that there is also a slight distance between these two probes on the
338 ancestral Pacific Ocean Y chromosome, along with weak internal centromere staining (Fig. 6b).
339 By contrast, there is no internal GacCEN signal between these two BACs on the Japan Sea neo-
340 Y (Fig. 6e) although there is clear separation between these BACs and internal GacCEN signal
341 on both X chromosomes in Japan Sea females (Fig. S11). These data suggest, but do not
342 definitively prove, that there has been a deletion of the ancestral Y chromosome centromere on
343 the Japan Sea neo-Y. However, the absence of GacCEN hybridization strongly supports a model
344 in which there has been genetic inactivation of the ancestral Y centromere on the Japan Sea neo-
345 Y. Still, it is important to note that we cannot determine whether genetic changes to this

346 centromere led to its inactivation, or whether genetic changes occurred after an initial phase of
347 centromere inactivation that was mediated by an epigenetic mechanism.

348

349 **Discussion**

350 Overall, we have shown that the Japan Sea neo-Y chromosome fusion has been stabilized by
351 centromere inactivation. The ancestral Y centromere has been inactivated as evidenced by loss of
352 CENP-A staining, and the active neo-Y centromere is derived from chromosome 9. While we
353 currently cannot conclusively determine whether the ancestral Y centromere sequence has been
354 deleted or has changed in some other way on the neo-Y, our experiments suggest there have been
355 genetic changes to the Y centromere on the Japan Sea neo-Y. Our evidence for a partial deletion
356 or loss of centromeric DNA is consistent with genetic deletions observed in engineered
357 dicentrics in yeast and humans (Stimpson et al. 2010; Sato et al. 2012) and in human patients
358 (Maraschio et al. 1990; Fisher et al. 1997). However, further experiments are required to identify
359 the specific mechanism of genetic inactivation of the Y centromere on the neo-Y. These
360 experiments are hindered because there is currently not a Y chromosome sequence assembly in
361 threespine stickleback, as is the case in most organisms. However, we are currently working to
362 obtain an assembled sequence of the Pacific Ocean threespine stickleback Y chromosome, which
363 will allow us to test for genetic deletion by identifying the unique sequences at the centromere
364 boundaries. Combining this assembly with CENP-A ChIP-seq experiments in Pacific Ocean
365 males will also allow us to determine whether there are Y-chromosome specific centromeric
366 repeats in the ancestral Pacific Ocean sticklebacks, and whether there have been genetic changes
367 to these repeats on the Japan Sea neo-Y.

368 It would also be interesting to determine whether additional epigenetic changes have
369 occurred on the inactive centromere. For example, inactive centromeres in wheat show different
370 patterns of histone methylation than those found on active centromeres (Zhang et al. 2010), while
371 the DNA at inactivated centromeres in maize is hypermethylated (Koo et al. 2011). Currently
372 nothing is known about histone modifications or DNA methylation at either active or inactive
373 centromeres in threespine sticklebacks.

374 Interestingly, the ancestral Y chromosome has independently fused to the acrocentric
375 chromosome 12 in males of the blackspotted stickleback (*G. wheatlandi*) species within the past
376 15 million years, creating an independent neo-Y chromosome (Ross et al. 2009). From an
377 evolutionary perspective, it is intriguing that of the seven known species of stickleback, two have
378 undergone fixation of a Y-autosome fusion. It will be particularly interesting to test whether the
379 Y chromosome centromere is also inactivated on the blackspotted neo-Y because previous
380 studies in plants and humans have suggested that there may be differences in centromere size or
381 strength that lead to the preferential inactivation of one centromere on dicentric chromosomes
382 (Sullivan et al. 1994b; Han et al. 2009; Zhang et al. 2010; Liu et al. 2015). Although we do not
383 know if the ancestral Y chromosome centromere in the threespine stickleback is smaller or
384 weaker, we do know that it differs from the centromeres on the rest of the chromosomes (Cech
385 and Peichel 2015), as is also true in humans and mice (Wolfe et al. 1985; Pertile et al. 2009;
386 Miga et al. 2014). Taken together, these data suggest the intriguing possibility Y chromosomes
387 might be better able to tolerate fusions than other chromosomes. Interestingly, Y-autosome
388 fusions are more common than X-autosome fusions in fish and squamate reptiles (Pennell et al.
389 2015). However, the contribution of centromere strength or centromere inactivation to the
390 establishment and fixation of chromosome fusions during evolution remains to be explored.

391

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564

565

566 **Figure Legends**

567 **Fig. 1** The Japan Sea neo-Y is a fusion between two complete chromosomes. FISH with the
568 ancestral X and Y chromosome BACs 188J19 (*purple*) and 101E08 (*green*) and the chromosome
569 9 BAC 44L12 (*purple*) on a Japan Sea male metaphase spread shows (a) three chromosomes
570 with BAC hybridization each highlighted by square boxes. Higher magnification of the boxed
571 regions in panel (a) shows the neo-Y fusion (b), the X₁ chromosome, which is derived from the
572 ancestral X chromosome (c) and the X₂ chromosome, which is derived from the ancestral
573 chromosome 9 (d). *Scale bar, 5 μm*

574

575 **Fig. 2** The CENP-A antibody only localizes to one region on the Japan Sea neo-Y chromosome.
576 Panels (a) and (c) show two independent metaphase spreads from Japan Sea embryos stained
577 with the CENPA antibody (*green*) as well as the X and Y chromosome BAC 101E08 (*purple*)
578 and the chromosome 9 BAC 35K20 (*purple*). Panels (b) and (d) are higher magnifications of the
579 boxed regions in panels (a) and (c), highlighting the neo-Y chromosome with the two regions of
580 BAC staining (*purple arrowheads*) flanking two distinct CENP-A puncta (*white arrowhead*).
581 The CENP-A staining is located on the chromosome 9-derived part of the neo-Y chromosome.
582 Panel (e) is a higher magnification of the boxed region in (a), highlighting the ancestral X₁
583 chromosome; 101E08 hybridizes to the middle of the long arm of X₁ (*purple arrowhead*), and
584 there is one region of two CENP-A puncta (*white arrowhead*). Panel (f) is a higher magnification
585 of the boxed region in (c), highlighting the unfused chromosome 9 (X₂); 35K20 hybridizes to the
586 end of the long arm of X₂ (*purple arrowhead*), and there is one region of two CENP-A puncta
587 (*white arrowhead*). *Scale bar, 5 μm*

588

589

590 **Fig. 3** GacCEN staining is weak and variable on the ancestral Pacific Ocean male Y
591 chromosome centromere. FISH with the GacGEN probe (*green*) and Y chromosome BAC
592 91G03 (*purple*) on four different metaphase spreads from the same Pacific Ocean male. Two
593 metaphase spreads show GacCEN staining on the Y chromosome (**a-d**) while two metaphase
594 spreads lack GacCEN staining on the Y chromosome (**e-h**). Panels (**b, d**) show higher
595 magnification of the boxed regions in (**a, c**) highlighting Y chromosomes with weak GacCEN
596 staining (*green arrowhead*), and panels (**f, h**) show higher magnification of the of the boxed
597 regions in (**e, g**) highlighting Y chromosomes with no GacCEN staining. *Scale bar, 5 μm*

598

599 **Fig. 4** GacCEN hybridizes to the centromere of chromosome 9 on the Japan Sea neo-Y
600 chromosome. FISH with the GacCEN probe (*green*), Y chromosome BAC probe 188J19
601 (*purple*), and a chromosome 9 BAC probe 44L12 (*purple*) on a metaphase spread from a Japan
602 Sea male is shown in panel (**a**). Panel (**b**) is a magnification of the neo-Y chromosome from the
603 boxed region in panel (**a**). The GacGEN probe only localizes to the region of the ancestral
604 chromosome 9 centromere on the neo-Y. *Scale bar, 5 μm*

605

606 **Fig. 5** Mapping the regions flanking the ancestral Y centromere on the neo-Y fusion. FISH with
607 the X and Y BACs 180J08 (*green*) and 171H24 (*purple*) on Japan Sea male interphase nuclei (**a**)
608 and metaphase spreads (**b**). Panels (**c**) and (**d**) show higher magnifications of the boxed regions
609 in panel (**b**). Both probes are present on the neo-Y (**c**) and the ancestral X₁ (**d**), although the
610 probes appear to be closer together on the neo-Y (**c**) as compared to the ancestral X₁ (**d**). *Scale*
611 *bar, 5 μm*

612

613 **Fig. 6** GacCEN and centromere flanking regions on the Pacific Ocean X and Y, and Japan Sea
614 neo-Y chromosomes. FISH with the X and Y BACs 180J08 and 171H24 (*purple*) and GacCEN
615 (*green*) on a metaphase spread from a Pacific Ocean male is shown in panel (a) and a metaphase
616 spread from a Japan Sea male in (d). Panels (b) and (c) are magnifications of the boxed regions
617 in panel (a) showing two distinct regions of staining (*purple arrowheads*) flanking weak
618 GacCEN staining (*green arrowhead*) on the unfused Y (b), and two distinct regions of staining
619 (*purple arrowheads*) flanking strong GacCEN staining (*green arrowhead*) on the X (c). Panel (e)
620 is a magnification of the boxed regions in panel (d) showing the two centromere flanking probes
621 (*purple arrowheads*) are very close together on the neo-Y with no GacCEN staining in between.
622 The strong GacCEN staining (*green arrowhead*) is from the chromosome 9 centromere on the
623 neo-Y. *Scale bar, 5 μm*

624

625 **Fig. S1** Pacific Ocean (ancestral) and Japan Sea (derived) chromosomes used in this study. A
626 fusion between the acrocentric chromosome 9 and the metacentric Y chromosome from the
627 ancestral Pacific Ocean species gave rise to the neo-Y chromosome in the Japan Sea species
628 around two million years ago. The Japan Sea sticklebacks still retain the ancestral submetacentric
629 X chromosome (now termed X₁), and the unfused acrocentric chromosome 9 (now termed X₂).

630

631 **Fig. S2** The ancestral state of chromosome 9, and the X and Y in Pacific Ocean stickleback fish.
632 FISH with the ancestral X and Y chromosome BACs 188J19 (*purple*) and 101E08 (*green*) and
633 the chromosome 9 BAC 44L12 (*purple*) on a Pacific Ocean male metaphase spread (a) shows
634 the two unfused chromosome 9s, the X chromosome, and the unfused Y chromosome each

635 highlighted with a square box. Higher magnification of the boxed regions in panel (a) shows the
636 X chromosome with two regions of BAC hybridization (b), the unfused Y chromosome with two
637 regions of BAC hybridization (c), and the two unfused chromosome 9s (d, e). *Scale bar, 5 μm*

638

639 **Fig. S3** Telomere staining in Japan Sea male and female metaphase spreads. Telomere staining is
640 seen on the ends of chromosomes in (a) Japan Sea female metaphase chromosomes and (b)
641 Japan sea male metaphase chromosomes. The neo-Y is the largest chromosome and is
642 highlighted by a box in panel (b). Panel (c) shows a higher magnification view of the neo-Y with
643 no internal telomere signal. The primary centromeric constriction on the neo-Y is indicated by
644 the white arrowhead. *Scale bar, 5 μm*

645

646 **Fig. S4** The BAC clone 91G03 is a Y specific BAC. (a) FISH was performed on a Pacific Ocean
647 male metaphase spread with BACs 101E08 (*green*), and 91G03 (*purple*). 101E08 hybridizes to
648 the X and Y chromosome, while 91G03 only hybridizes to the Y. Panel (b) is a magnification of
649 the Y chromosome from (a) showing hybridization of the known sex chromosome BAC 101E08
650 and BAC 91G03 to the very end of the Y chromosome. Panel (c) is a magnification of the X
651 chromosome from (a), with hybridization of BAC 101E08 to the middle of the long arm, and no
652 hybridization of BAC 91G03. *Scale bar, 5 μm*

653

654 **Fig. S5** CENP-A antibody staining on the ancestral Pacific Ocean Y chromosome. (a) A
655 metaphase spread from Pacific Ocean embryos was stained with the CENPA antibody (*green*)
656 and the Y chromosome specific BAC 91G03 (*purple*). Panel (b) is a magnification of the boxed
657 region in panel (a), highlighting the Y chromosome with 91G03 staining on the end of the long

658 arm, and two distinct CENP-A puncta hybridizing to the centromere of each sister chromatid on
659 the Y chromosome. *Scale bar, 5 μm*

660

661 **Fig. S6** Comparison of the CENP-A protein amino acid sequence between the Pacific Ocean
662 (PO) and Japan Sea (JS) sticklebacks. There is only one amino acid difference (red asterisk)
663 between the two proteins, which is not in the amino acid sequence targeted by the CENP-A
664 antibody (red letters).

665

666 **Fig. S7** Comparison of the CENP-A associated centromeric sequence between the Pacific Ocean
667 (PO) and Japan Sea (JS) stickleback species. The Japan Sea consensus sequence is 98.9% similar
668 to the GacCEN sequence previously identified in the Pacific Ocean species (Cech and Peichel
669 2015). The red asterisk denotes the only three nucleotide differences between these two
670 consensus sequences. Nucleotide ambiguities: Y = C or T; R = A or G.

671

672 **Fig. S8** The GacCEN probe hybridizes to the centromere on Japan Sea chromosomes. (a) The
673 GacCEN probe hybridizes to a single region on each chromosome in a metaphase spread from a
674 Japan Sea male. Panel (b) shows a magnification of the boxed region in (a), highlighting the
675 hybridization of the GacCEN probe to the primary constriction (*white arrowheads*) on each
676 chromosome. *Scale bar, 5 μm*

677

678 **Fig. S9** The GacCEN probe colocalizes with CENP-A on Japan Sea chromosomes. The GacCEN
679 probe (*green*) colocalizes with the CENP-A antibody (*purple*) at distinct puncta in interphase
680 nuclei (a) as well as to a single region on each chromosome in a metaphase spread from a Japan

681 Sea embryo **(b)**. Panel **(c)** shows a magnification of the metaphase spread shown in **(b)**. *Scale*
682 *bar, 5 μm*

683

684 **Fig. S10** Both submetacentric X₁ chromosomes in the Japan Sea female show strong GacCEN
685 hybridization. FISH with an X chromosome BAC 188J19 (*purple*), and GacCEN (*green*) on a
686 Japan Sea female metaphase spread is shown in panel **(a)**. Panels **(b)** and **(c)** are
687 magnifications of the boxed regions in panel **(a)**, showing the two ancestral X₁ chromosomes,
688 with strong GacCEN staining (*green arrowhead*) consistent with the submetacentric position of
689 the centromere. *Scale bar, 5 μm*

690

691 **Fig. S11** GacCEN and centromere flanking regions on Japan Sea female X₁ chromosomes. FISH
692 with the X and Y BACs 180J08 and 171H24 (*purple*) and GacCEN (*green*) on a metaphase
693 spread from a Japan Sea female is shown in panel **(a)**. Panels **(b)** and **(c)** are magnifications of
694 the boxed regions in **(a)** showing two distinct regions of BAC hybridization (*purple arrowheads*)
695 flanking strong GacCEN staining (*green arrowhead*) on both X₁ chromosomes. *Scale bar, 5 μm*

696

697

698 **Table 1.** List of BACs used in this study

Chr	BAC	Library	Genomic position (bp)	Cytogenetic position*
9	44L12	CHORI-213	497,589 – 729,621	Distal end of short arm
9	35K20	CHORI-215	18,760,858 – 18,914,793	Distal end of long arm
X	188J19	CHORI-213	2,384,200 – 2,563,816	Distal end of short arm
Y	188J19	CHORI-213	unknown	Distal end of short arm
X	180J08	CHORI-213	3,377,138 – unknown	Proximal end of short arm
Y	180J08	CHORI-213	unknown	Proximal end of long arm
X	171H24	CHORI-213	5,465,262 – 5,643,381	Proximal end of long arm
Y	171H24	CHORI-213	unknown	Proximal end of short arm
X	101E08	CHORI-213	11,446,792 – 11,651,387	Middle of long arm
Y	101E08	CHORI-213	unknown	Distal end of long arm
Y	91G03	CHORI-215	unknown	Distal end of long arm

699 *Position is relative to the centromere; proximal is closer to the centromere and distal is further
700 from the centromere. The genomic positions of the BACs on chromosome 9 and the X
701 chromosome were determined by BLASTing the publicly available BAC end sequences
702 (Kingsley and Peichel 2007) to the updated threespine stickleback genome assembly (Glazer et
703 al. 2015). The genomic positions of BACs on the Y chromosome are unknown because a
704 complete genome assembly of the Y chromosome is not available.

705
706

707 **Table 2.** GacCEN staining on Y centromere from Pacific Ocean Y and Japan Sea neo-Y

	Pacific Ocean Y	Japan Sea neo-Y
GacCEN positive staining	29	0
GacCEN negative staining	21	26
Number of males	5	6
Number of experiments	11	4

708 The number of metaphase spreads with GacCEN positive and negative staining, the number of
709 males used to generate the metaphase spreads, and the number of independent FISH experiments
710 are indicated.

711 **Table 3.** Summary of CENP-A ChIP seq data in two Japan Sea male replicates and two Japan Sea female replicates.
 712

	Japan Sea male 1	Japan Sea male 2	Japan Sea female 1	Japan Sea female 2
Number of enriched sequence clusters in 500 most abundant	228	230	175	170
Number of enriched sequence clusters with GacCEN repeat	220	196	166	97
Average fold enrichment of IP/input	10.44	10.19	13.58	3.00
Range of fold enrichment across all sequence clusters	1.00 – 39.29	1.00 – 41.62	1.03 – 49.36	1.00 – 7.24
Range of fold enrichment for clusters without GacCEN	1.00 – 1.17	1.00 – 3.72	1.03 – 2.09	1.00 – 1.29
Total IP reads	34,418,733	28,294,604	23,676,014	32,155,558
Total input reads	32,317,012	26,307,950	24,794,940	38,968,765

713

Figure 1

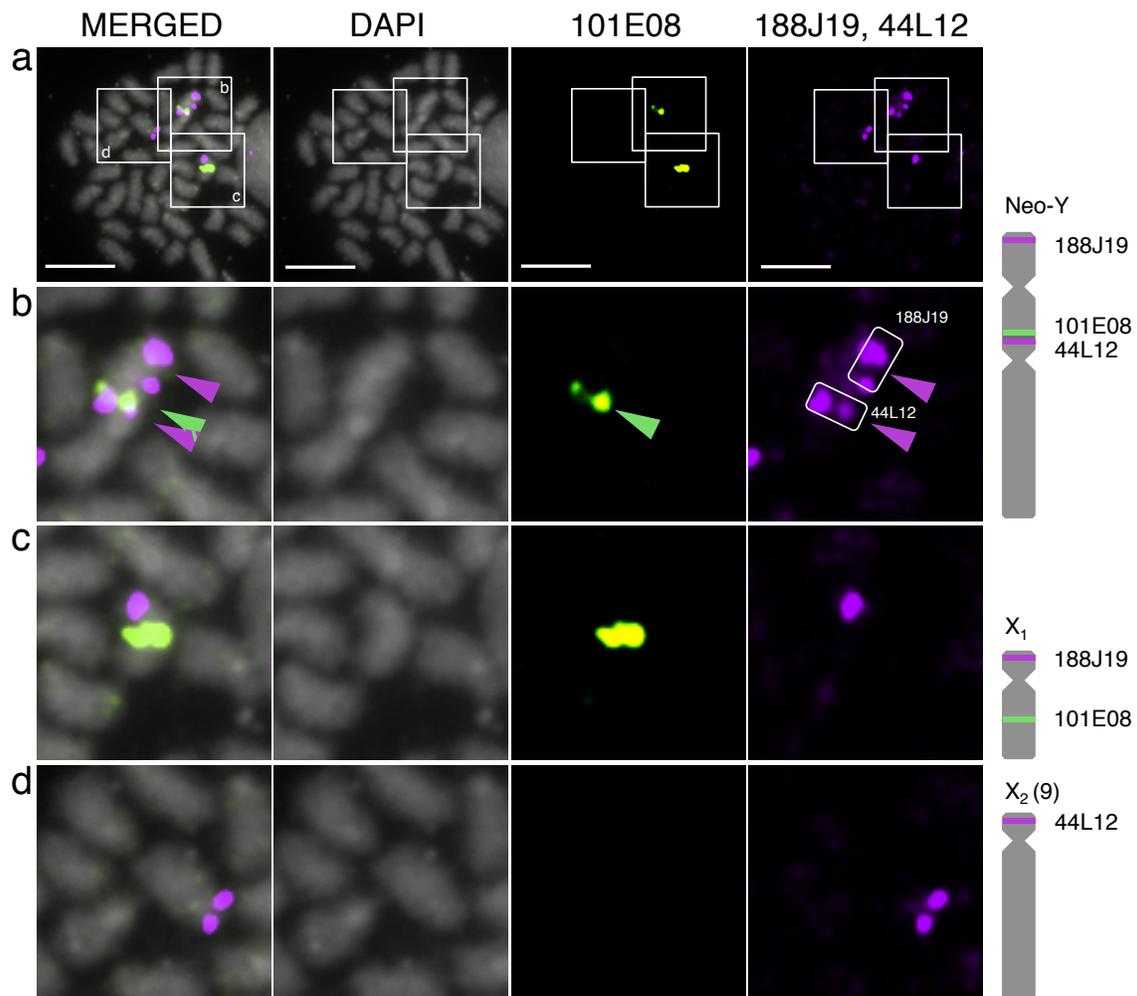


Figure 2

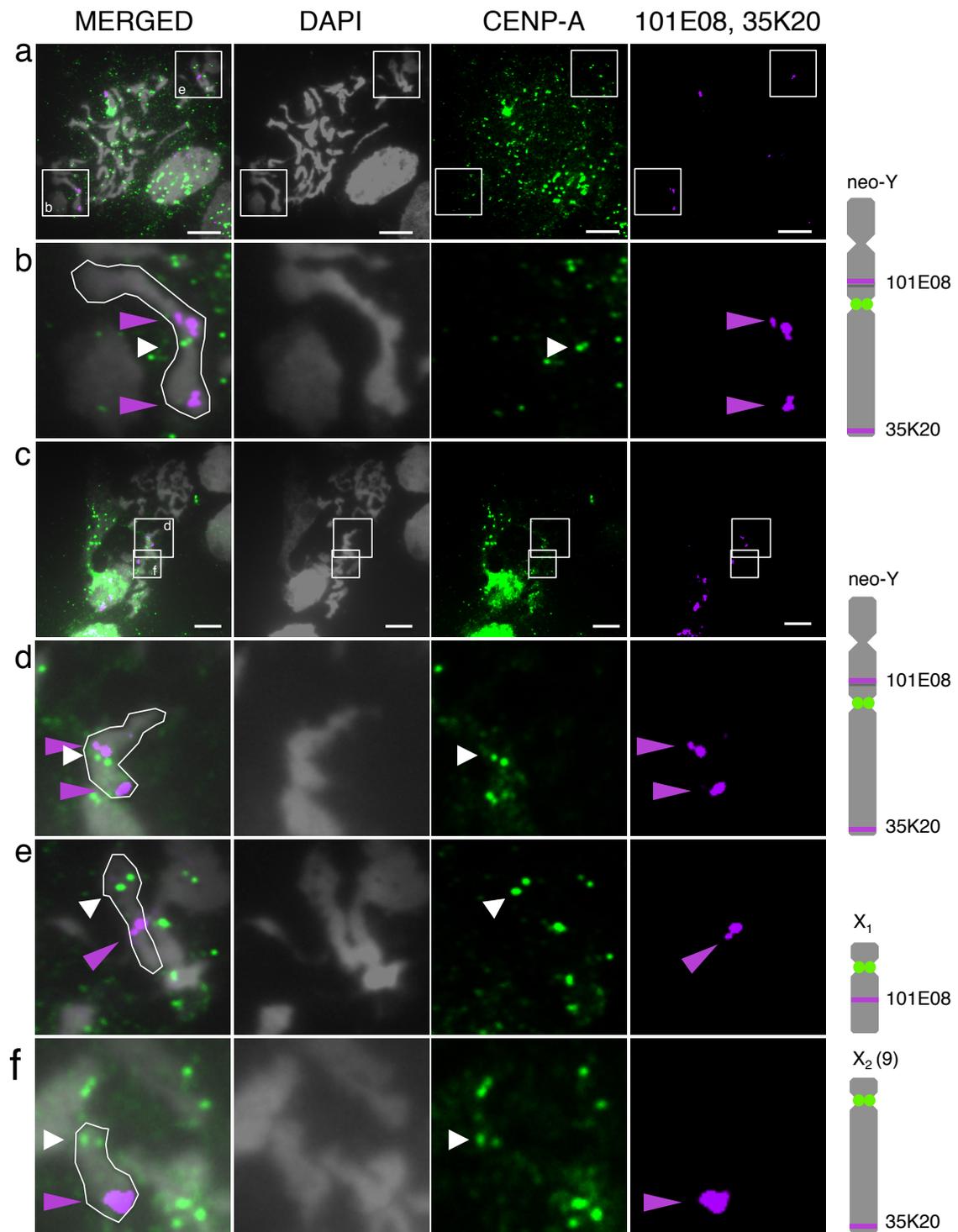


Figure 3

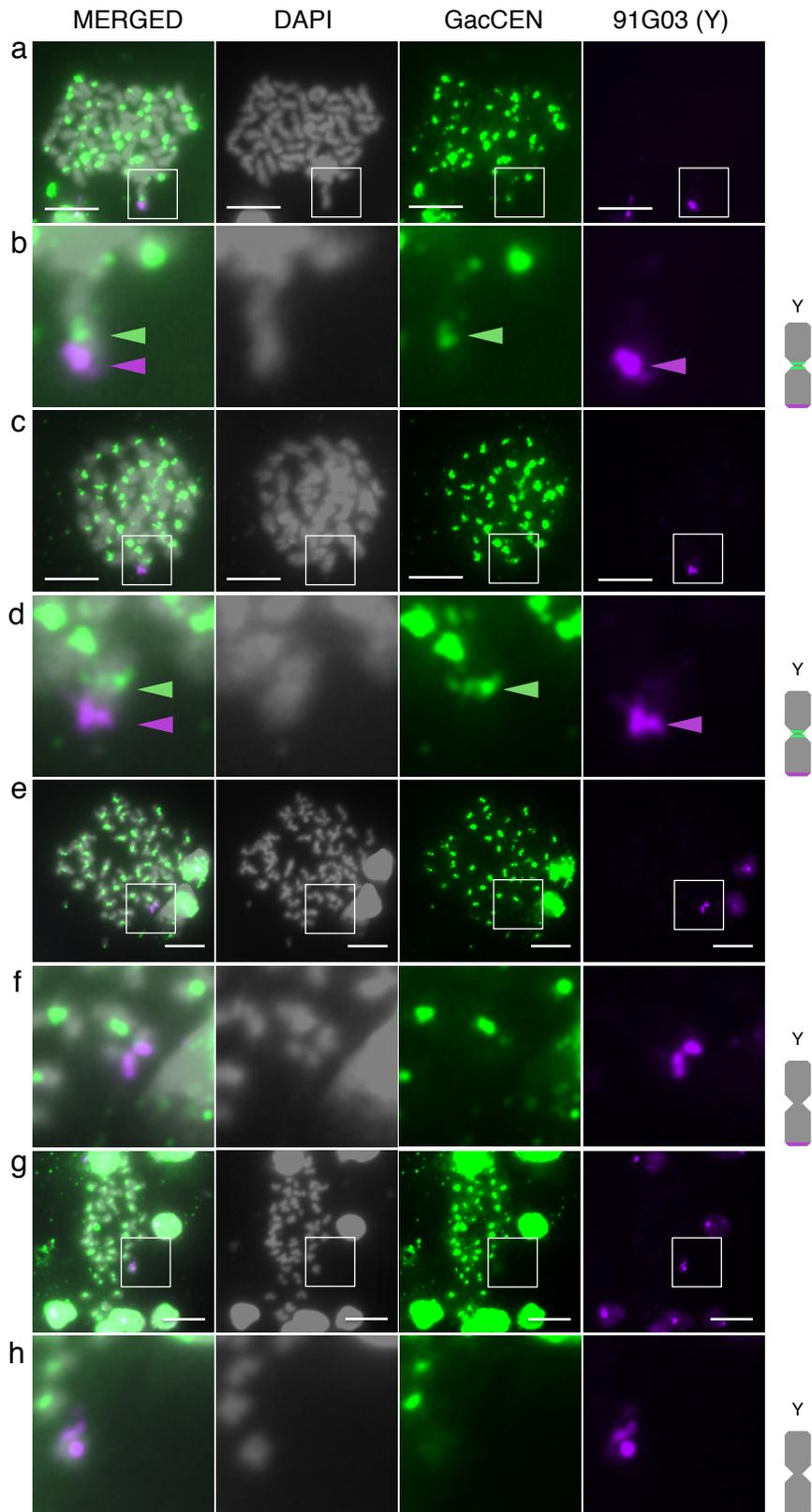


Figure 4

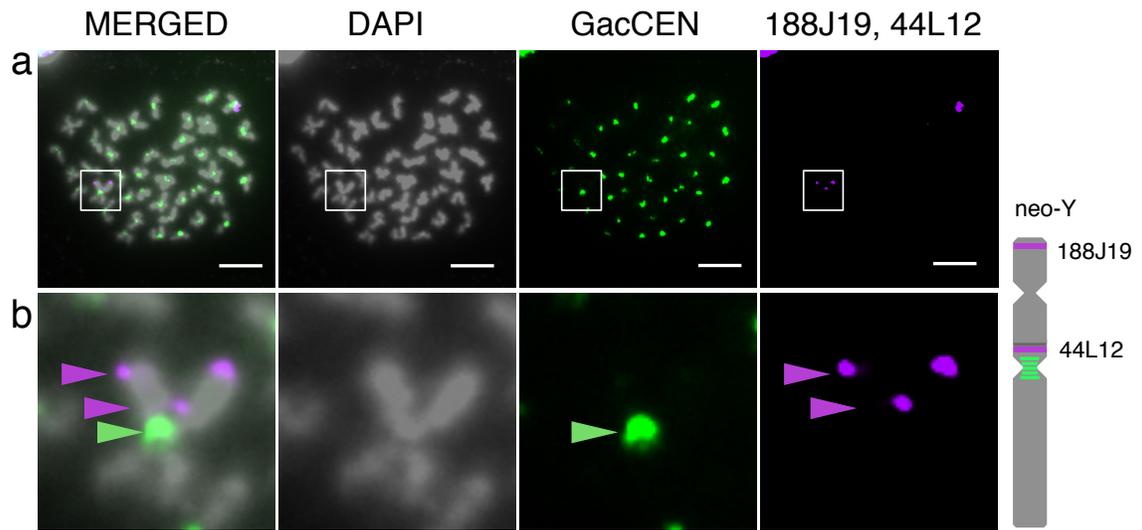


Figure 5

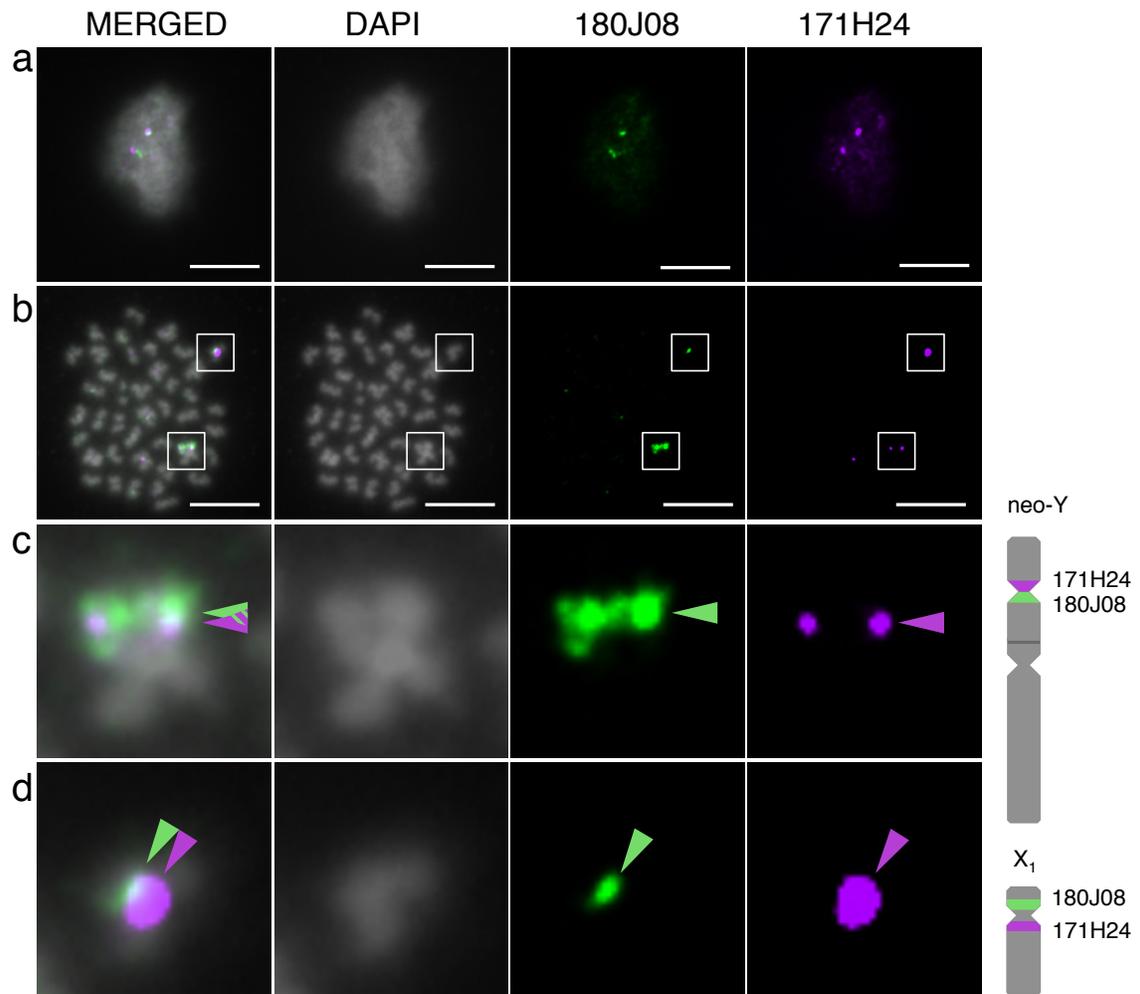


Figure 6

