Chromosome elimination in the interspecific hybrid medaka between *Oryzias latipes* and *O. hubbsi*

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Abstract

An interspecific hybrid medaka (rice fish) between *Oryzias latipes* and *O. hubbsi* is embryonically lethal. To gain an insight into the cellular and molecular mechanisms that cause the abnormalities occurring in the hybrid medaka, we investigated the behavior of chromosomes and the expression patterns of proteins responsible for the chromosome behavior. The number of chromosomes in the hybrid embryos gradually decreased to nearly half, since abnormal cell division with lagging chromosomes at anaphase eliminated the chromosomes from the cells. The chromosome lagging occurred at the first cleavage and continued throughout embryogenesis even after the midblastula transition. Fluorescent *in situ* hybridization analyses revealed that the chromosomes derived from *O. hubbsi* are preferentially eliminated in both *O. latipes-hubbsi* and *O. hubbsi-latipes* embryos. Whole-mount immunocytochemical analyses using antibodies against α-tubulin, γ-tubulin, inner centromere protein, Cdc20, Mad2, phospho-histone H3 and cohesin subunits (SMC1α, SMC3 and Rad21) showed that the expression patterns of these proteins in the hybrid embryos are similar to those in the wild-type embryos, except for phospho-histone H3. Phospho-histone H3 present on chromosomes at metaphase was lost from normally separated chromosomes at anaphase, whereas it still existed on lagging chromosomes at anaphase, indicating that the lagging chromosomes remain in the metaphase state even when the cell has proceeded to the anaphase state. On the basis of these findings, we discuss the cellular and molecular mechanisms of chromosome elimination in the hybrid medaka.

Introduction

The genus *Oryzias* includes 20 species of medaka (rice fishes) (Roberts 1998, Parenti & Soeroto 2004, Takehana *et al.* 2005), in which gametes of different species can be artificially fertilized to produce interspecific hybrids. However, the resulting hybrids exhibit various abnormalities in reproduction and development, being lethal or sterile according to the combination of parent species (Iwamatsu *et al.* 1984, 1986, Hamaguchi & Sakaizumi 1992, Sakaizumi *et al.* 1993, Iwamatsu *et al.* 1994, Shimizu *et al.* 1997, 2000). Although it is well known that hybrids of many organisms cannot produce offspring, due to various abnormalities occurring during embryogenesis and gametogenesis, as a fundamental mechanism that ensures reproductive isolation for fixing and maintaining the species, the molecular basis and cellular basis of this mechanism are poorly understood. By understanding the cellular and molecular causes of abnormalities occurring in hybrids, we can gain a deeper insight into the mechanisms that properly regulate the progression of embryogenesis and gametogenesis in normal organisms as well as the basic mechanisms of reproductive isolation.

A hybrid medaka between *O. latipes* and *O. hubbsi*[1] shows abnormal embryogenesis, with a short and waved embryonic body, abnormal pectoral fins, microcephaly, retina pigmentation and small eyes, and all embryos die within 9 days after fertilization (Iwamatsu *et al.* 1994, Hori & Iwamatsu 1996). Previous cytological observation of the early cleavage of the hybrid has revealed improper separation of chromosomes, which might lead to chromosome elimination in the hybrid cells (Iwamatsu *et al.* 2003), but details remain to be investigated. Although impaired embryogenesis of hybrid fish is a common occurrence, there have been few investigations of its causative mechanisms. To our knowledge, Fujiwara et al. were the first investigators to reveal the cellular mechanism of abnormality in the hybrid between masu salmon and rainbow trout: they showed that the hybrid embryos are lethal because the paternal rainbow trout chromosomes are preferentially eliminated during early embryogenesis (Fujiwara *et al.* 1997).

Besides hybrid fish, uniparent chromosome elimination in hybrid cells has been demonstrated as a widespread defect in hybrid cells of various organisms, including plants (Bennett *et al.* 1976, Finch 1983, Gernand *et al.* 2005), insects (Breeuwer & Werren 1990, Reed & Warren 1995) and mammalian cultured cells (Weiss & Green 1967, Zelesco &

[1] *O. hubbsi* was formerly included in *O. javanicus*, but it has been established as a new species separately from *O. javanicus* (Roberts 1998).

Graves 1987, 1988, Graves & Barbieri 1992; Matsui *et al.* 2003). It is also known that the origin of chromosomes being eliminated varies depending on the conditions of hybrids. For example, although either paternal or maternal chromosomes are eliminated in a barley hybrid, the decision which of the two chromosomes is eliminated varies from tissue to tissue (Finch 1983). These findings imply that incompatibility between the maternal factor and paternal factor is responsible for the selective loss of chromosomes derived from either parent, but its actual mechanisms, especially those at the molecular level, remain a mystery.

In this study, we examined the behavior of chromosomes in the hybrid medaka between *O. latipes* and *O. hubbsi* as a first step toward understanding the cellular and molecular mechanisms of abnormal cell division that occurs during the embryogenesis of hybrid medaka. Fluorescence *in situ* hybridization (FISH) analyses, i.e., whole chromosome painting (WCP) and comparative genomic hybridization (CGH), have revealed preferential elimination of *hubbsi* chromosomes from the hybrid cells in both *O. latipes-hubbsi* and *O. hubbsi-latipes* embryos through impaired chromosome separation at anaphase with lagging chromosomes. We also performed immunocytochemical analyses using antibodies against several protein candidates for the abnormal cell division, including α-tubulin, γ-tubulin, inner centromere protein (INCENP), Cdc20, Mad2, phospho-histone H3 and cohesin subunits (SMC1α, SMC3 and Rad21). Except for phospho-histone H3, expression patterns of all of the proteins examined in this study were similar in the wild-type medaka and the hybrid medaka. Phospho-histone H3, a general marker for metaphase, remained on lagging chromosomes at anaphase, while it was no longer detected on normally separating chromosomes at anaphase. On the basis of these finding, we discuss the cellular and molecular mechanisms of chromosome elimination in the hybrid medaka.

Materials and methods

Fish and artificial fertilization

Sexually mature *Oryzias latipes* fish were obtained from a local fish farm (Yatomi, Aichi, Japan). *Oryzias hubbsi* fish were provided from laboratory stocks maintained at Aichi University of Education, Shinshu University and Niigata University. The fish were cultured in fresh water at 27°C under artificial light conditions (14-hr light and 10-hr dark) to induce and maintain the daily reproductive cycle. Unfertilized eggs were released from the isolated ovary in Iwamatsu's medaka physiological saline (111 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgSO_4$, adjusted to pH 7.3 with 0.1 M $NaHCO_3$) by tearing the ovarian cavity with fine

forceps, and their attaching filaments were cut off with scissors. Sperm suspension was obtained by mincing testis fragments in Iwamatsu's saline. Eggs laid in a plastic dish (3.5 cm in diameter) were artificially inseminated by adding several drops of sperm suspension at room temperature. Embryos were collected at appropriate times and examined by whole-mount immunocytochemistry and FISH analyses with WCP and CGH techniques.

Chromosome spreads

Before preparing chromosome spreads, embryos were cultured in physiological saline containing 30 μM paclitaxel (Sigma-Aldrich, Tokyo) for 1 hr to increase the rate of cells at M-phase. The embryos were then treated with hypotonic solution (27.2 mM trisodium citrate) for 20 min and fixed with acetic alcohol (a 3:1 mixture of methanol and acetic acid) for 30 min at room temperature. The fixed embryos were transferred to phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4), in which the chorion (the egg envelope) was removed from each embryo with fine forceps. The denuded embryos were treated with 50% acetic acid for 5-10 min to separate the blastodisc from the yolk mass. After dissociating the blastodisc cells by pipetting, the cell suspension was put on a slide glass at 50˚C and air-dried. The slides were stained with 2.5% Giemsa for counting the number of chromosomes in each cell or stored at -80˚C until use for FISH analyses as described below.

Whole-mount immunocytochemistry

Embryos treated with 30 μM paclitaxel for 1 hr were fixed overnight with 4% paraformaldehyde at room temperature. The fixed embryos were washed three times with 0.1% Triton X-100 in PBS (PBST), during which time the chorion was removed and the blastodisc was freed from the underlying yolk mass with fine forceps. The isolated blastodiscs were treated with PBS containing 1 mg/ml RNase A (Sigma-Aldrich) for 1.5 hr at 37˚C, washed three times in PBST for 30 min each time at 4˚C, and blocked overnight in a blocking buffer (PBST containing 2% bovine serum albumin (BSA) and 0.1% NaN_3) at 4˚C. The blastodiscs were then treated with one of the following primary antibodies at a 1:200 dilution in a blocking buffer for 24 hr at 4˚C: anti-α-tubulin mouse monoclonal antibody (Sigma-Aldrich, Clone DM1A), anti-γ-tubulin mouse monoclonal antibody (Sigma-Aldrich, Clone GTU-88), anti-medaka INCENP mouse polyclonal antibody (C. Sakai & M. Yamashita, unpublished), anti-phospho-histone H3 rabbit antibody (Upstate Biotechnology, Lake Placid,

NY), anti-medaka Rad21 mouse polyclonal antibody, anti-medaka SMC1α mouse polyclonal antibody, anti-medaka SMC3 rabbit antibody (Iwai *et al.* 2004), anti-medaka Cdc20 mouse polyclonal antibody (T. Yokota & M. Yamashita, unpublished) and anti-medaka Mad2 rabbit antibody (J. Lee & M. Yamashita, unpublished). After washing three times in PBS for 6 hr, the samples were treated with Alexa Fluor 488-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) at a 1:200 dilution in a blocking buffer for 24 hr at 4˚C in the dark (thereafter, all procedures being carried out in the dark). After washing three times in PBS for 6 hr, the embryos were stained with 100 μg/ml propidium iodide (PI) in PBS for 20 min to visualize chromosomes, followed by washing three times in PBS for 30 min at 4˚C. Finally, the samples were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a Bio-Rad MicroRadiance confocal microscope.

FISH

FISH was performed according to the procedures described previously (Fujiwara *et al.* 1997), with some modifications.

WCP: Total genomic DNA was extracted from visceral organs (excluding the digestive tract) of *O. latipes* and *O. hubbsi* by the conventional phenol-chloroform method. A part of the genomic DNA was used for labeling as a probe and the remainder was used for suppression after fragmentation (300-500 bp in length as confirmed by agarose electrophoresis) by sonication. The DNA extracted from one parent was labeled with biotin-16-dUTP by nick translation (Roche, Tokyo, Japan) and mixed with 25-fold the amount of unlabeled DNA from the other parent to suppress non-specific hybridization due to the sequences common to both parents. The probes were dissolved in deionized 100% formamide, denatured for 10 min at 75˚C, and stored at 4˚C until use. Chromosome spreads stored at -80˚C were incubated for 3 hr at 65˚C for hardening. They were then denatured at 70˚C in 70% formamide in 2 x SSC (20 x SSC consisting of 1.5 M NaCl and 0.15M sodium citrate) for 2 min, dehydrated in ethanol, and air-dried. Hybridization was performed at 37˚C for 20 hr in a moist chamber using 250 ng of the biotin-labeled probe per slide of chromosome spreads. The hybridized probes were incubated with 5 μg/ml of avidin-fluorescein isothiocyanate (FITC) (Roche) in 4 x SCC containing 1% BSA for 1 hr at 37˚C. The samples were stained with 1.25 μg/ml of PI, mounted in glycerol containing two anti-fade reagents, 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) and *p*-phenylendiamine

(Wako, Tokyo, Japan), and observed under a Zeiss Axioscope or Leica DMRA microscope.

CGH: Total genomic DNAs extracted from *O. latipes* and *O. hubbsi* were labeled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively, by nick translation (Roche). Hybridization was performed as described above for WCP. The biotin and digoxigenin signals were detected with avidin-FITC and anti-digoxigenin-rhodamine, respectively, and observed under a fluorescent microscope after DNA-staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). In some experiments, the *O. latipes* and *O. hubbsi* DNAs were labeled with Alexa 488-dUTP (Molecular Probes) and Cy3-dUTP (Amersham, Tokyo, Japan), respectively. The obtained results using this system were essentially the same as those using the biotin and digoxigenin labeling system.

Results

Loss of chromosomes from the hybrid medaka

The fertilization rate was very low (less than 10%) when *O. hubbsi* eggs were inseminated with *O. latipes* sperm, but the rate was almost 100% when *O. latipes* eggs were inseminated with *O. hubbsi* sperm, similar to the rate in the case of insemination of gametes in the same species. The resulting hybrid embryos in both crosses underwent normal cleavage (Figure 1E, F) as the wild-type embryos did (Figure 1A-D), but they died before hatching (Figure 2), presenting abnormal embryogenesis, including delayed body formation with a wavy body and small head and eyes (Figure 1G, H). Since the abnormal conditions observed in the hybrid resemble those found in medaka carrying haploid genome, so-called haploid syndrome (Araki *et al*. 2001), we examined the number of chromosomes in the hybrid (Figure 3A-D, 4). Both *O. latipes* and *O. hubbsi* have 48 chromosomes (2n=48) (Iwamatsu *et al*. 1993). Consistent with this, the majority (91-99%) of cells in the wild-type embryo had 48 chromosomes throughout embryogenesis (Figure 4). In contrast, the hybrids exhibited a significantly decreased number of chromosomes. At the morula stage 5 hr after fertilization, only 21% of the cells were equipped with 48 chromosomes and the remainder showed aneuploidy. In 3-day embryos, which seemed normal in appearance, most (95%) of the cells showed aneuploidy with 18-24 chromosomes, and those with 24 chromosomes were predominant (40%) (Figure 4). To determine when the loss of chromosomes begins, we observed the behavior of chromosomes by whole-mount immunocytochemistry with anti-α-tubulin antibody and a DNA-staining dye, PI (Figure 3E-N). Abnormal behavior of chromosomes was observed from the first cleavage (Figure 3J). Some chromosomes lagged in motion

toward the spindle poles at anaphase, being trapped at the metaphase plate. Probably, these lagging chromosomes failed to be delivered evenly to daughter cells and were eliminated from the cells via formation of micronuclei as reported in a salmonid hybrid (Fujiwara *et al.* 1997). Abnormal mitosis with lagging chromosomes continued to be observed thereafter (Figure 3K-N), even after the midblastula transition (MBT: 9-10 hr after fertilization, Aizawa *et al.* 2003). Thus, the chromosome elimination does not occur simultaneously at a specific stage but occurs gradually during embryogenesis, although its frequency seems higher in earlier stages.

Preferential elimination of hubbsi *chromosomes from the hybrid medaka*

The finding that the chromosome number decreases to nearly half in the hybrid cells suggests that the chromosomes derived from one parent tend to be preferentially eliminated from the cells. To verify this possibility, we distinguished *hubbsi* chromosomes from *latipes* chromosomes by FISH analyses using two different techniques, WCP and CGH, and examined their fate during embryogenesis (Figure 5). Essentially the same results were obtained by using both techniques. The lagging chromosomes, which are probably eliminated from cells, were derived from *O. hubbsi* (Figure 5B-E). FISH analyses also showed that the *hubbsi* chromosomes were frequently aggregated at the equatorial plate in the hybrid cells at metaphase (Figure 5A).

Similar to the *latipes-hubbsi* hybrid, the *hubbsi-latipes* hybrid was also inviable due to the loss of chromosomes (data not shown). We then examined which chromosomes are eliminated in this hybrid. Although the number of samples was limited because of the low fertilization rate in this cross, both WCP and CGH revealed that the lagging chromosomes in the *O. hubbsi-latipes* hybrid were originated from *hubbsi* (Figure 5F), demonstrating that the *hubbsi* chromosomes always tend to be eliminated from the hybrid cells irrespective of the combination of parents.

FISH analyses demonstrated preferential elimination of *hubbsi* chromosomes from the hybrid medaka; however, it is notable that the proportion of cells having less than 24 chromosomes reached more than 40% at the morula stage in the hybrid (Figure 4). This implies that some *latipes* chromosomes are also eliminated from the hybrid cells, in addition to *hubbsi* chromosomes.

Molecular characteristics of chromosomes being eliminated in hybrid medaka embryos

To gain an insight into the mechanisms causing chromosome abnormality in the hybrid medaka at the molecular level, we examined expression patterns of several proteins, including γ-tubulin, INCENP, Cdc20, Mad2, phospho-histone H3 and cohesin subunits (SMC1α, SMC3 and Rad21). γ-Tubulin is a component of centrosomes (Oakley 2000). INCENP is thought to play a key role in correcting wrong kinetochore-microtubule attachment (Hauf *et al.* 2003, Vader *et al.* 2006). Cdc20 and Mad2 are involved in the spindle checkpoint (May & Hardwick 2006). Phospho-histone H3 is generally used as a metaphase marker (Hendzel *et al.* 1997). Cohesin is responsible for the cohesion and separation of sister chromatids (Uhlmann 2004).

The specificity of antibodies used in this study was verified by immunoblotting, as described previously (Iwai *et al.* 2004). The immunoblots of crude extracts from medaka embryos clearly showed protein bands of expected molecular mass, demonstrating that the antibodies specifically recognize each target protein (Figure 6).

Whole-mount immunocytochemical analyses using specific antibodies against the above-mentioned proteins showed that, except for phospho-histone H3, there were no remarkable differences in the protein expression patterns between the wild-type and the hybrid medaka (Figure 7A, B, E, F, only the data for INCENP are shown). In contrast, anomalous expression of phospho-histone H3 was observed in anaphase cells of the hybrid medaka. Both in the wild-type and hybrid medaka, phospho-histone H3 was present on chromosomes at metaphase (Figure 7C, G) and disappeared from the chromosomes that moved toward the spindle pole at anaphase (Figure 7D, H). However, phospho-histone H3 was still expressed on the lagging chromosomes at anaphase in the hybrid cells (Figure 7H).

Discussion

Cellular mechanisms of chromosome elimination in the hybrid medaka

We have shown in this study that *O. hubbsi* chromosomes are preferentially eliminated during the embryogenesis of reciprocal hybrids between *O. latipes* and *O. hubbsi*, through abnormal mitosis including impaired chromosome segregation at anaphase (Figure 3, 5). Uniparental chromosome elimination due to a similar mechanism has been reported in salmonid hybrids, and it has been suggested that an incompatibility between the maternal cytoplasm and paternal genome causes the abnormal mitosis, on the basis of the following results (Fujiwara *et al.* 1997): 1) the loss of chromosomes occurs mainly during the period from just after

fertilization until the MBT, when the zygotic genomes, including the paternal ones, begin to be expressed and 2) similar mitotic abnormalities occur in the androgenetic hybrid lacking maternal genomes and in the hypotriploid hybrid having extra maternal genomes. We found in this study that the elimination continues to occur even after the MBT. Therefore, the incompatibility between the maternal cytoplasm and paternal genome is probably not a main cause of the abnormality in the hybrid medaka.

A previous observation that a *hubbsi* pronucleus undergoes chromosome condensation more slowly than does a *latipes* pronucleus during the fertilization process of an *O. latipes-hubbsi* hybrid (Iwamatsu *et al.* 2003) provides a hint concerning the cellular mechanism of preferential elimination of *hubbsi* chromosomes in the hybrid. The timing of chromosome condensation should be tightly coupled to the timing of cell cycle progression to guarantee the accurate delivery of sister chromatids to daughter cells. In the hybrid cells, however, the difference in the timing of chromosome condensation between two parents might disturb the coordination of nuclear (chromosomal) dynamics and cytoplasmic (spindle) dynamics. For example, if the cell cycle progression is conducted by the timing of condensation of *latipes* chromosome, then the slowly condensing *hubbsi* chromatins would fail to catch the cell cycle. Our finding that the lagging (*hubbsi*) chromosomes still express the metaphase marker phospho-histone H3 at anaphase strongly suggests that they remain in the metaphase state. We therefore propose that the *hubbsi* chromosomes have a marked tendency to be left behind in cell cycle progression to anaphase because of their intrinsic slow speed of chromatin condensation. The following scenario leading to the preferential elimination of *hubbsi* chromosomes in the hybrid cells is possible: 1) At prophase, *hubbsi* chromatins tend to condense more slowly than *latipes* chromatins do, 2) at metaphase, some of the *hubbsi* chromosomes are late for accurate alignment at the equatorial plate of the spindle (The aggregation of *hubbsi* chromosomes at the equatorial plate (Figure 5A) might relate to this scene.), 3) these chromosomes can only interact incorrectly with spindle fibers, such as the case of merotelic kinetochore orientation reported for nocodazole-treated mammalian cultured cells (Cimini *et al*. 2001), and 4) they are left at the metaphase plate or improperly separated at anaphase, thereby being eliminated from the cell.

In this study, we noticed that 40% of cells have less than 24 chromosomes at the morula stage in the hybrid (Figure 4), indicating that not only *hubbsi* chromosomes but also some *latipes* chromosomes are eliminated in the hybrid. In addition, all of the *hubbsi* chromosomes are not simultaneously eliminated in the hybrid cells (Figure 3). These events are not explainable by the above-mentioned scenario, suggesting the involvement of plural

mechanisms in the hybrid abnormalities. Identification of eliminated chromosomes, i.e., whether certain chromosomes have a tendency toward preferential elimination or any chromosome is eliminated accidentally, might provide some clues to understanding the cellular mechanisms of chromosome elimination in the hybrid. To this end, we produced chromosome-specific painting probes by amplifying DNA sequences of medaka chromosomes that were singly sorted with fluorescence-activated cell sorter technology, using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) (for technical references, see Yang *et al.* 1995, 1997). We found that at least 9 chromosomes identified with the painting probes underwent various separation patterns, including lagging, during cell divisions, a finding that supports the notion of non-selective elimination (F. Konno, A. Kuroiwa, F. Kasai, M.A. Ferguson-Smith, Y. Matsuda, H. Mitani & M. Yamashita, unpublished). The random and gradual elimination of chromosomes during the cleavage of hybrid embryos implies that the elimination is dependent on factors acting by chance in response to microenvironments in the hybrid cells.

Molecules involved in chromosome elimination in the hybrid medaka

Our finding that phospho-histone H3 remains present on lagging chromosomes even at anaphase in the hybrid between *O. latipes* and *O. hubbsi* (Figure 7) raises the possibility that the chromosome elimination is related to the condensation states of chromosomes. Despite the fact that phosphorylation of histone H3 occurs at M-phase in various species, its actual roles in chromosome condensation have not yet been elucidated. Its crucial role in regulation of chromosome behavior has been indicated by the findings that a *Tetrahymena* strain containing non-phosphorylatable histone H3 exhibits abnormal segregation and loss of chromosomes (Wei *et al.* 1999). In striking contrast to this, no causal relationship between phosphorylation of histone H3 and chromosome dynamics has been observed in *S. cerevisiae* (Hsu *et al.* 2000). Results of further studies aimed at understanding the biological significance of phospho-histone H3 that remains on lagging chromosomes in hybrids will clarify its role in normal mitosis.

Lagging chromosomes at the metaphase/anaphase transition are probably produced by some defects in the separation of sister chromatids and/or the interaction between chromosomes and microtubules. One possible mechanism of chromosome lagging is that sister chromatids cannot separate from each other at anaphase in spite of normal spindle architecture and chromosome-microtubule interaction. We therefore examined the behavior of cohesin, a multi-subunit protein complex that is responsible for the cohesion and separation of

sister chromatids from yeast to humans (for review, see Uhlmann 2004), but no remarkable differences in the expression patterns of medaka cohesin subunits (SMC1α, SMC3, and Rad21) were observed between the wild-type and hybrid medaka. It is thus unlikely that non-disjunction of sister chromatids brings about the abnormalities occurring in the hybrid medaka.

Another possible mechanism is a defect in the interaction between chromosomes and microtubules, such that one kinetochore is simultaneously bound to microtubules extending from two opposite spindle poles (known as merotelic kinetochore orientation). A key molecule of the spindle checkpoint, Mad2 (for review, see May & Hardwick 2006), is lost from the merotelically oriented kinetochore on lagging chromosomes in nocodazole-treated mammalian cultured cells, as in the case of the properly oriented kinetochore on normally separating chromosomes (Cimini *et al.* 2001). It is thus likely that cell division cannot be arrested even if the cell harbors improperly oriented kinetochores, because merotelic orientation does not activate the spindle checkpoint that arrests the cell division. Consequently, it has been proposed that merotelic kinetochore orientation is a major mechanism that causes aneuploidy in mitotic mammalian cells (Cimini *et al.* 2001).

If merotelic kinetochore orientation is also responsible for chromosome lagging in the hybrid medaka, the spindle checkpoint would not be activated as well. In support of this, we found that Mad2 behaves similarly in the wild-type and the hybrid cells, suggesting that the spindle checkpoint remains inactive in hybrid cells containing lagging chromosomes. With respect to the involvement of merotelic kinetochore orientation in chromosome lagging in the hybrid medaka, however, it should be noted that the spindle-destabilizing agent nocodazole, but not the spindle-stabilizer paclitaxel, can activate the spindle checkpoint and arrest the cells at metaphase only after the MBT in *Xenopus* (Clute & Masui 1992) and zebrafish (Ikegami *et al.* 1997) embryos. These findings clearly indicate that the spindle checkpoint does not fully function for abnormal chromosomes and spindles during early embryogenesis. Lagging chromosomes are found even at the first cleavage of the hybrid medaka (Figure 3). It is thus possible that defects in the interaction of kinetochores and microtubules other than merotelic orientation are also involved in chromosome lagging, since they may also induce chromosome lagging owing to the incomplete spindle checkpoint that overlooks the abnormalities in early embryogenesis. Defects other than merotelic kinetochore orientation might include detachment of kinetochore microtubules at anaphase and inactivation of pulling forces at kinetochores in anaphase. Fine structural observations of the

kinetochore-microtubule interaction on lagging chromosomes and time-lapse video-microscopic observations of the chromosome-microtubule dynamics in living cells will provide a deep insight into the mechanisms that cause the chromosomal abnormalities in the hybrid medaka.

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Figure legends

Figure 1. Embryogenesis of the wild-type medaka (*O. latipes*; A-D) and the hybrid medaka (*O. latipes-hubbsi*; E-H). Embryos in early cleavage stage (A, E), morula to blastula stage (B, F), 3 days after fertilization (C, G) and 6 days after fertilization (D, H) are shown. The hybrid embryos exhibit abnormal embryogenesis with a wavy body and small head and eyes (G, H), although no apparent abnormalities are found in the early stages (E, F). Scale bar, 200 μm.

Figure 2. Survival curves of the wild-type medaka and the hybrid medaka. The data were obtained by counting the number of embryos with normal morphology at each developmental stage and expressed as a percentage to the number of fertilized eggs (1-cell stage embryos). The wild-type embryos (*latipes-latipes* and *hubbsi-hubbsi* embryos) develop normally, while the survival of the hybrids (*latipes-hubbsi* and *hubbsi-latipes* embryos) gradually decreases, coincident with the occurrence of abnormal embryogenesis, and all of the hybrids die before hatching.

Figure 3. Chromosomes in the wild-type medaka (*O. latipes*; A, B, E-I) and the hybrid medaka (*O. latipes-hubbsi*; C, D, J-N). Chromosome spreads prepared from *O. latipes* (A, B) and *O. latipes-hubbsi* (C, D) embryos were stained with Giemsa, showing chromosomes aligned at the metaphase plate (A, C) and those pulled toward the spindle poles at anaphase (B, D). The wild-type medaka embryo retains 48 chromosomes (A) with normally separating sister chromatids (B), whereas the hybrid embryo contains a decreased number of chromosomes (C) with lagging chromosomes at anaphase (arrowheads in D). Confocal microscopic images of chromosomes and spindles at anaphase to telophase in the embryos 1 hr after fertilization (2-cell stage; E, J), 2 hr after fertilization (4 to 8-cell stage; F, K), 3 hr after fertilization (16-cell stage; G, L), 4 hr after fertilization (32-cell stage; H, M), and 5 hr after fertilization (early morula; I, N), as observed by whole-mount immunocytochemistry with anti-α-tubulin antibody and the DNA-staining dye PI. Lagging chromosomes are observed in the hybrid medaka (arrowheads in J-N) but not in the wild-type medaka (E-I). Scale bar, 5 μm (A-D), 10 μm (E-N).

Figure 4. Distribution of the number of chromosomes in the wild-type medaka (*O. latipes*) and the hybrid medaka (*O. latipes-hubbsi*). Embryos exhibiting normal morphology 5-6 hr

after fertilization (morula stage, white bar) and 3 days after fertilization (black bar) were examined. The majority of cells are equipped with 48 chromosomes throughout embryogenesis in the wild-type medaka. In the hybrid medaka, however, 80% and 95% of cells show aneuploidy with 18-24 chromosomes (cells with 24 chromosomes being predominant) at the morula stage and at the 3-day-embryo stage, respectively. The number of cells examined was 72 at the morula stage in *O. latipes*, 55 at the 3-day-embryo stage in *O. latipes*, 533 at the morula stage in *O. latipes-hubbsi* and 374 at the 3-day-embryo stage in *O. latipes-hubbsi*.

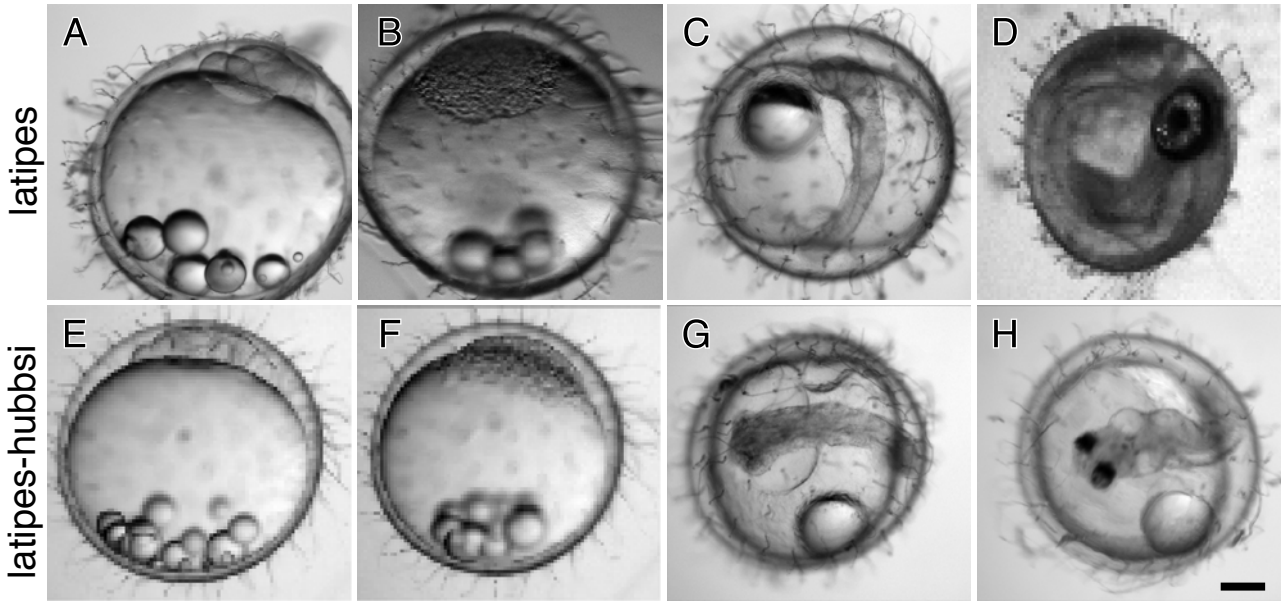
Figure 5. FISH analyses (WCP and CGH) of chromosomes in the hybrid medaka (*O. latipes-hubbsi*, A-E; *O. hubbsi-latipes*, F). Embryos exhibiting normal morphology 5-6 hr after fertilization (morula stage) were examined. In WCP (A, B), the *hubbsi* chromosomes are stained with FITC in green and both the *latipes* and the *hubbsi* chromosomes are stained with PI in red. The *hubbsi* chromosomes (shown in yellow) have aggregated at the metaphase plate (arrow in A) and remained at the equatorial region of the spindle at anaphase as lagging chromosomes (arrow in B). In CGH (C-F), the *latipes* chromosomes are stained with Alexa 488 (C, D) or FITC (E, F) in green and the *hubbsi* chromosomes are stained with Cy3 (C, D) or rhodamine (E, F) in red (DNA is also stained with DAPI in blue.). The lagging chromosomes (shown by arrowheads) are stained reddish, indicating their *hubbsi* origin. Scale bar, 5 μm.

Figure 6. The specificity of antibodies used in this study. Proteins extracted from *O. latipes* embryos (6 hours after fertilization) were immunoblotted with antibodies against α-tubulin (A), γ-tubulin (B), INCENP (C), phospho-histone H3 (D), Rad21 (E), SMC1α (F), SMC3 (G), Cdc20 (H) and Mad2 (I). All of the antibodies specifically recognize the target proteins (arrowheads). The anti-INCENP-reactive three bands probably correspond to isoforms and/or differently phosphorylated forms of medaka INCENP, as described in other species (Cooke *et al*. 1987, Mackay *et al*. 1993, Adams *et al*. 2000).

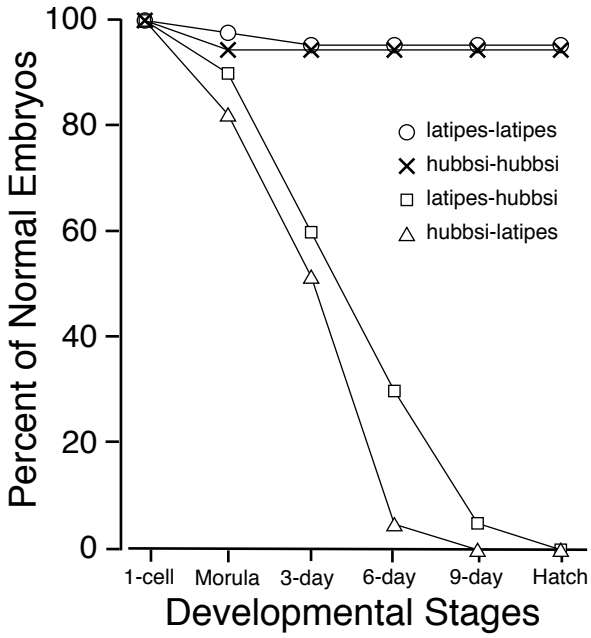
Figure 7. Whole-mount immunocytochemical analyses of chromosomes in the wild-type medaka (*O. latipes*; A-D) and the hybrid medaka (*O. latipes-hubbsi*; E-H) with anti-INCENP antibody (A, B, E, F) and anti-phospho-histone H3 antibody (C, D, G, H). All samples were also stained by the DNA-staining dye PI. Both INCENP and phospho-histone H3 are shown

in green or yellow, since the antibodies and the chromosomes are visualized with Alexa 488 in green and with PI in red, respectively. The expression pattern of INCENP in the hybrid medaka is similar to that in the wild-type medaka. As a member of chromosomal passengers (Adams *et al*. 2001), INCENP localizes to the centromeres of metaphase chromosomes as dot-like signals (A, E) and then moves to the central region of the spindle as fiber-like signals at anaphase (B, F) (for details of the behavior of INCENP during mitosis, see Cooke *et al.* 1987). Note that the INCENP signals on the lagging chromosomes are not dot-like but fiber-like (arrowhead in F), indicating that they do not localize to the centromeres of lagging chromosomes but localize to the central region of the spindle as in the wild-type medaka (B). In both the wild-type and hybrid medaka, phospho-histone H3 is expressed on chromosomes at metaphase (C, G) and is lost to the separated chromosomes at anaphase (D, H), while it continues to exist on lagging chromosomes even when the cell has proceeded to anaphase (arrowhead in H). Scale bar, 10 μm.

Sakai et al. Fig. 1



Sakai et al. Fig. 2



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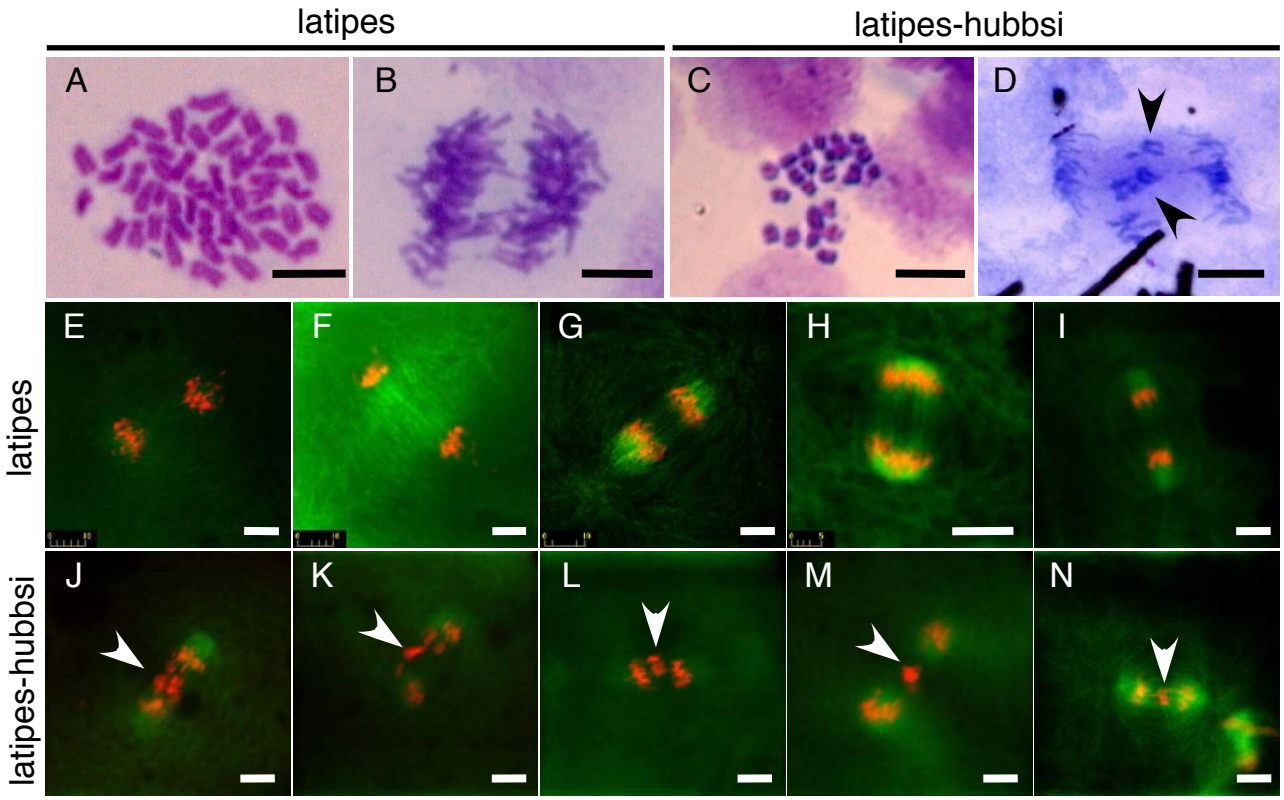

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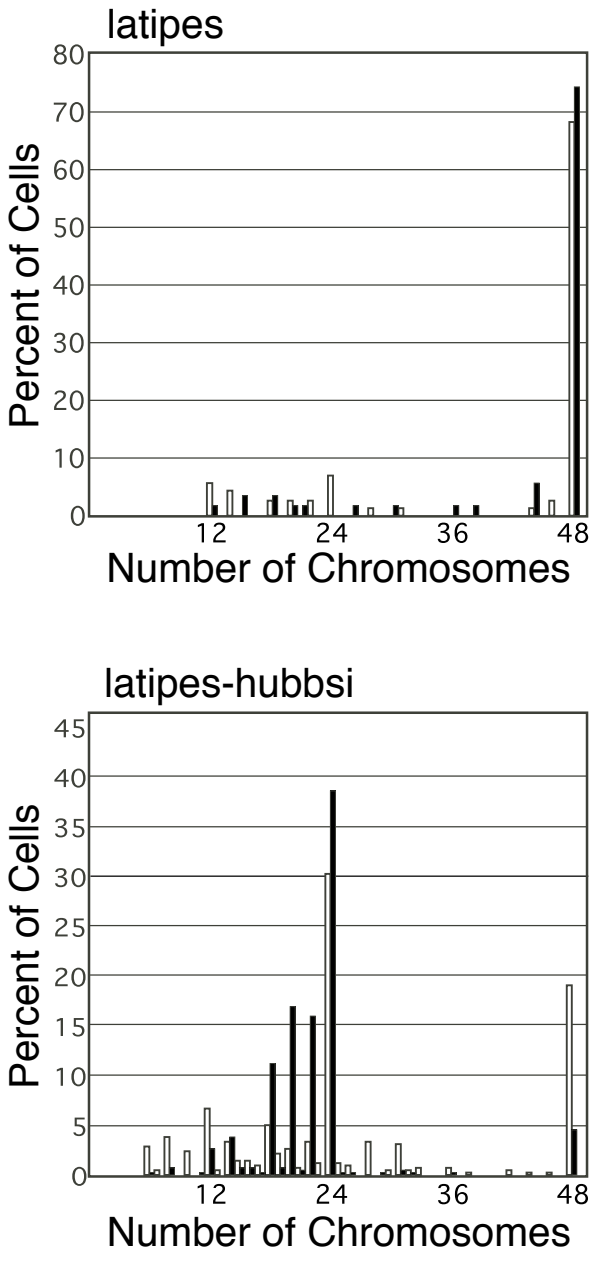

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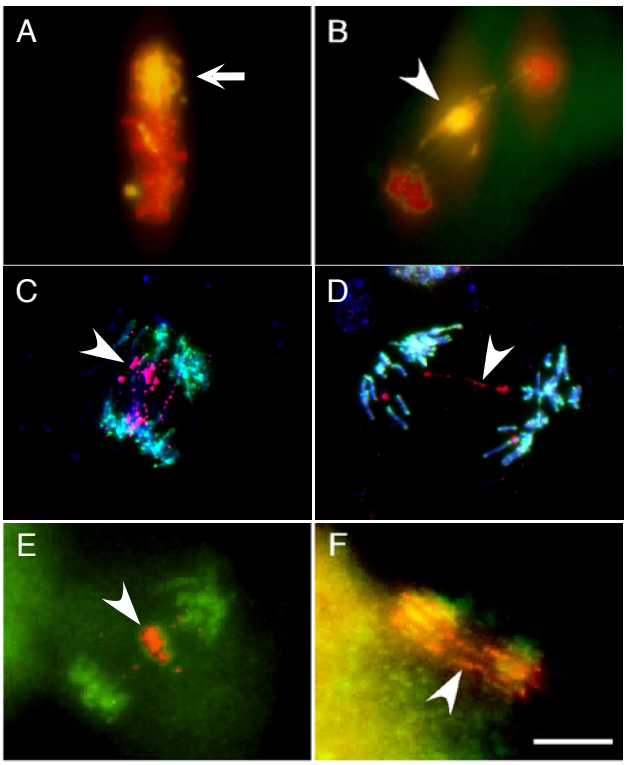

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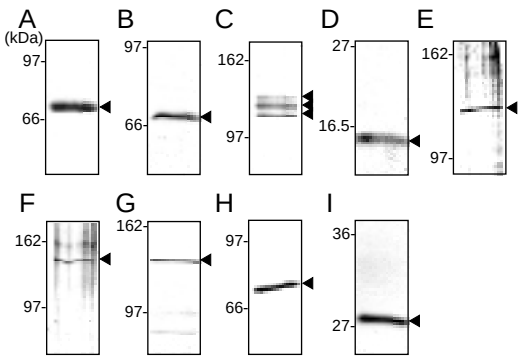

Sakai et al. Fig. 7